Molecular Ecology and Phylogeny of Protistan Algal Symbionts from Corals

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

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STATEMENT OF ORIGINALITY

This thesis contains no materials which have been accepted for award of any other degree or diploma at any other university and, to the best of my knowledge, is original and contains no material previously published or written by another person, except where reference is made in the text.

Robert Bruce Moore 18th April 2006
Dedication and acknowledgments

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Thesis abstract

In the context of global warming, the ability of symbiotic dinoflagellates (Dinophyceae: Alveolata) to adapt by sexual recombination or speciation may arguably be crucial to the survival of many coral reefs. Using molecular markers, this thesis aimed to address the question of sexuality in Symbiodinium ('zooxanthellae'), and also examined phylogeny of the constituent species of the genus, and its order Suessiales. De novo culturing of zooxanthellae was undertaken to augment available pure materials for marker development, and to search for Symbiodinium lifecycle stages that may not have come to attention in previous studies. Fusions between two motile cells were often observed even though cell division does not occur in the motile stage in this genus. The fused forms were therefore interpreted as gamete fusion events. Serendipitous culturing of photosynthetic colpodellids (Alveolata) from corals, occurred as these were mistaken for sexual stages of Symbiodinium. Five molecular markers were investigated in Symbiodinium cultures, and from unculurable in hospite zooxanthellae: nuclear - actin, ITS and large subunit ribosomal DNA (lsu rDNA); chloroplast - psbA; and mitochondria - lsu rDNA. Characterisation of all but the mitochondrial rDNA locus was successful. Dinoflagellates probably possess a fragmented mitochondrial lsu rDNA. In the chloroplast, the psbA locus is on a unigenic minicircle. The non-coding region of the psbA minicircle was hypervariable and contained many double hairpin elements that have putative roles as regulatory elements in psbA expression and as recombination-directed replication origins. Two markers - actin-pseudogene intron, and psbA non-coding region - were used to examine whether allele reassociation may occur within a wild population of Symbiodinium goreau, which is the dominant symbiont species occurring in Great Barrier Reef corals. The amount of variability obtained using these markers over a population of conspecific Symbiodinium goreau in the One Tree Island Lagoon (off Gladstone Queensland Australia), was too low to address the question of sexuality in this species, therefore future studies will adopt a wider sampling geography. A literature review of the biological species concept in protists (unicells) was undertaken, and was linked to ITS variability in order to determine relevance to Symbiodinium. A model for the evolutionary history of peridinin dinoflagellates is suggested based on integration of pre-existing data on the dinoflagellate paraflagellar rod and RNA-editing, with the new data regarding photosynthetic colpodellids, and minicircle double hairpin elements of dinoflagellates.
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Chapter 1

General Introduction: Molecular ecology and phylogeny of protistan algal symbionts from corals

A large volume of literature attests to the level of scientific interest in the reliance of coral reefs on dinoflagellate algae. Almost all of this literature has accrued since the naming of the algal genus *Symbiodinium* in 1962. The reliance of corals on *Symbiodinium* in nutrient-poor tropical waters is absolute: corals need the dinoflagellate symbionts as a carbon source (Muscatine et al. 1984), a nitrogen recycler (D'Elia and Wiebe 1990; Lewis and Smith 1971), and also as an obligate partner for the process of calcification i.e. reef formation (Barnes and Chalker 1990; Goreau 1959; Goreau and Goreau 1959; Pearse and Muscatine 1971; Vandermeulen and Muscatine 1974). In the 20th century coral reefs began to experience an increased rate of stress evidenced by episodes of coral bleaching (Hoegh-Guldberg 1999), defined as expulsion of the symbiotic algae from their hosts. In many cases the symbionts are expelled because they are no longer viable (Lesser 2004). There is evidence (via genotyping) for finely tuned matching of symbionts to hosts, though the basis of this host-specificity has not been shown. One possible way in which coral-symbiont associations might cope with increasing episodes of bleaching is that *Symbiodinium* strains might adapt to environmental conditions via sexual recombination with each other, to facilitate novel symbiont traits and/or novel host-symbiont pairings.

The genus *Symbiodinium* was named by the taxonomist Freudenthal, who assumed the genus was sexual (like other dinoflagellates) and observed putative gametes, but did not report gametes fusing, zygote occurrence, or cite genetic recombination data (Freudenthal 1962). Freudenthal’s logical inference requires testing (Blank 1987). Two research groups (Baillie et al. 2000a; Baillie et al. 1998; Santos et al. 2003c) have recently sought and gained population-level data indicating that two species of *Symbiodinium* are sexual. The question remains open for the remaining *Symbiodinium* species including those that are dominant symbionts on the Great Barrier Reef. Against this background, the thesis has the following aims:
1) To use molecular population genetics to assess whether sexual recombination occurs in the dominant clade of *Symbiodinium* present in corals on the Great Barrier Reef.

2) To culture *Symbiodinium* apart from its host, as a source of genetically pure samples for marker development work.

3) To review the evolutionary and phylogenetic position of *Symbiodinium* within the dinoflagellates, and to speculate on symbiont adaptation potential.

1. **Introduction**

1.1 **Coral (holobiont) anatomy and taxonomy**

Corals are members of the phylum Cnidaria (Medina et al. 2001), formerly called coelenterates, that are modern day representatives of one of the most primitive animal body plans. The term ‘diploblastic’ refers to the possession of only two major tissue layers in this body plan (Seipel and Schmid 2005), the gastrodermis and the epidermis (Figure 1a). Progenitor, now extinct, diploblasts were the second primitive form of multicellular animals (Metazoa) to evolve after sponges (Glenner et al. 2004; Medina et al. 2001). The phylum Cnidaria (Bischoff et al. Online) comprises the classes Scyphozoa (jellyfish), Anthozoa (incl. corals, anemones, fans and zoanthids), Hydrozoa (hydra) and Cubozoa (sea wasps), which all share a stinging organelle called a cnidocyst that gives the phylum its name. The cnidocyst is used to immobilize prey, and common possession of this organelle exemplifies the fact that all members of the phylum are predatory and heterotrophic. However, in many cnidarians, specifically many anthozoans and scyphozoans, heterotrophy is augmented by facultative association with symbiotic unicellular algae found inside the tissues either intracellularly or extracellularly. The symbiotic algae provide a source of fixed carbon (Muscatine et al. 1984) to the hosts (see section 1.3 below). Anthozoans and scyphozoans include by far the largest range of animal species known to house symbiotic algae (Trench 1987). Small numbers of poriferan (sponge) and molluscan species contain algal symbionts of the same genus that dominates among anthozoans and scyphozoans. This very widespread alga is the genus
Symbiodinium (Freudenthal 1962), also commonly known by the non-taxonomic term zooxanthellae (see terminology section below). Many animal hosts of Symbiodinium do not fare well if deprived of their symbionts (Hoegh-Guldberg 1999; Norton et al. 1995), indicating the associations are ancient. However, many of these hosts can survive for significant fractions of their lifetimes without symbionts (Grant et al. 2004), as long as re-infection by new symbionts or regrowth of depleted symbiont population eventually occurs (Lewis and Coffroth 2004; Rodriguez-Lanetty et al. 2004). A term that encompasses host-symbiont pairings, which involve interdependence or semi-interdependence, is ‘holobiont’, being a descriptive term for the partnership.

Among the Anthozoa the majority of species housing Symbiodinium occur in the subclasses Zoantharia and Alcyonaria. Zoantharia includes the orders Scleractinia (stony corals), Actinaria (anemones), Corallimorpharia (corallimorphs) and Zoanthidae (zoanthids) among others. Alcyonaria includes orders Alcyonacea (soft corals), and Gorgonacea (sea fans) among others. Many (>1000 total) animal species among those orders named are hosts of Symbiodinium (Trench 1987; Veron and Stafford-Smith 2000). Indeed the frequency of Symbiodinium-associated vs -unassociated animals in this set of hosts is striking and indicates the success of the strategy of combining predation and phototrophic symbiosis in a marine invertebrate phylum.
Figure 1.1 (a) Anatomy of a coral polyp. Diagram adapted from: (Solomon et al. 2002). Brown inclusions in the gastrodermal layers represent the soma of neurons. The ‘nerve net’ extends throughout the gastrodermis, mesoglea and gastrodermis (Germain Online; Umbriaco et al. 1990). (b) Location of Symbiodinium (‘zooxanthellae’) in coral tissues. Diagram adapted from: (Mojetta 1995). Also shown are the cnidocysts (stinging cells) from which cnidaria derive their name.
1.2 Coral geographic distribution

This thesis specifically focuses on *Symbiodinium* strains that are resident within the family Scleractinia (stony corals). Scleractinia have formed all the living limestone reefs of the modern day oceans, and these solid reefs support a substantial biodiversity rivalled only by rainforests in terms of numbers of species in the ecosystem (Knowlton 2001; Reaka-Kudla 1997). Only scleractinians build such reefs and this ability has been linked to possession of *Symbiodinium* (Barnes and Chalker 1990; Goreau 1959; Goreau and Goreau 1959; Pearse and Muscatine 1971; Vandermeulen and Muscatine 1974). The distribution of scleractinian species on the globe is shown in Figure 1.2. The Great Barrier Reef, off the coast of Queensland Australia, is not the area of the world with the greatest scleractinian biodiversity but is the largest continuous reef system in physical size (Veron and Stafford-Smith 2000).

1.3 Ecology of the coral-symbiont association

*Nutritional interdependence of corals and symbionts*

Corals provide not only shelter to the zooxanthellae, but they also provide nitrogen in the form of ammonia (Anderson and Burriss 1987; Piniak and Lipschultz 2004; Piniak et al. 2003; Prosser 1950; Trench 1979), and phosphates (D'Elia 1977; Pomeroy and Kuenzler 1969; Yonge and Nicholls 1931), which stimulate growth of the symbiont. In return corals acquire from the zooxanthellae reduced carbon (Falkowski et al. 1984; Muscatine et al. 1984; Trench 1971) and oxygen by day. At nights zooxanthellae are a net sink for oxygen (Kawaguti 1953; Kuhl et al. 1995), and as such the primary nutritional benefit that corals seem to derive from them is carbon. One hundred percent of the reduced carbon needs of a coral that is not shaded, are met by photosynthesis of the symbionts (Falkowski et al. 1984; Muscatine et al. 1984).

The zooxanthellae population in a host coral is constantly replaced (Jones and Yellowlees 1997) and controversy surrounds whether the degraded cells are used as food (Titlyanov et al. 1998; Yonge 1936). Corals do not possess vitamin biosynthesis pathways and so vitamins must be obtained from planktonic prey, from degraded symbionts, or from
bacteria. Some corals ingest and degrade bacteria by endocytosis (Ducklow 1990; Sorokin 1973; Sorokin 1978), although it is not known how widespread or significant is this capability.
Figure 1.2 Distribution of reef building scleractinian corals. Diagram from: (Veron and Stafford-Smith 2000)
Figure 1.3 Two of the three symbiont-transmission strategies adopted by coral hosts. [The third, brooding, is a variant of (b) so is not shown (see text)]. (a) Water-borne or ‘horizontal’ transmission – *Symbiodinium* are free-living in the environment, and the host's eggs are devoid of symbionts. Infection of the host by *Symbiodinium* occurs soon after larval settlement. (b) Maternal or ‘vertical’ transmission – the symbionts are enclosed within the egg. The egg is fertilized (by sperm not shown) and develops into a new coral colony containing symbionts that have been derived clonally from the parent colony. Key: 1- Gametes released, 2- Fertilization, 3- Metamorphosis, 4- Settlement, 5- Calcification, 6- Feeding, 7- Colony formation, D- dinoflagellate *Symbiodinium* (free-living). Diagram adapted from: (Mojetta 1995).
Uptake of symbionts by corals

Host corals adopt three strategies for persistence of symbionts across host generations (Harrison and Wallace 1990). First, is the uptake of free-living *Symbiodinium* by settled coral larvae called ‘spat’ (Figure 1.3b). This mode is termed ‘horizontal transmission’. Second, is the inheritance of zooxanthellae directly from a parent, via inclusion of zooxanthellae in the ova of the parent (Figure 1.3a). This mode is termed ‘vertical transmission’. Third, is the uptake of parent-derived zooxanthellae by planulæ (advanced larvae) that are brooded inside the body cavity of the parent. A fourth possibility, that the sexually produced planulæ of a brooder might adopt *Symbiodinium* from the seawater during planula development inside the body cavity has not been investigated. The determination of which mode of zooxanthellae transmission a host uses is complicated by the ability of some corals to also reproduce asexually by brooding [reviewed in (Harrison and Wallace 1990)].
Figure 1.4 Coral bleaching involves loss of zooxanthellae, by one of several mechanisms. Diagram modified from: (Gates et al. 1992; Lesser 2004; Madl Online 2005). The symbiosome space, between the vacuolar membrane and the zooxanthellar cell wall, is depicted in orange.

Loss of symbionts from corals
There are two dimensions to the egress of Symbiodinium from corals. Firstly it should occur on some scale in well balanced associations that are completely healthy (Jones and Yellowlees 1997). If a subset of the healthy Symbiodinium cells did not exit the nutrient rich tissues of corals they might not attain sufficient numbers in relatively nutrient poor waters to infect new hosts. Mechanisms to account for such environmental seeding have been offered, including survival of zooxanthellae in algal pellets that are extruded from the polyp mouth under relatively non-stressful conditions (Steele 1977), and survival through the digestive passages of reef grazing fishes (Augustine and Muller-Parker 1998) and invertebrates (Fitt 1984).
Secondly, corals undergo ‘bleaching’ defined as loss of zooxanthellae (with consequent whitening of the coral), under conditions of high stress. The various stressors that can affect coral bleaching include sedimentation, microbially-caused disease, chemical pollution, and photoinhibition (Jones and Hoegh-Guldberg 1999; Leggat et al. 2004; Lesser 2004; Rosenberg and Falkovitz 2004). It is the latter cause of bleaching, photoinhibition, that attracts the greatest attention and it is thought to account for most of the mass bleaching events that have been recorded worldwide to date (Hoegh-Guldberg 1999; Hughes et al. 2003; Smith et al. 2005; Warner et al. 1999).

The exact cause of light related bleaching is not precisely known and indeed it may be multi causal (Hoegh-Guldberg 1999; Leggat et al. 2004; Lesser and Farrell 2004; Tchernov et al. 2004). However, one mechanism is thought to involve the buildup of reactive oxygen intermediates due to excess electron availability from water splitting (in high light conditions) by photosystem II, and subsequent lack of correct processing or trafficking of the electron acceptor molecules and/or of the oxygen moieties derived from split H₂O molecules (Hoegh-Guldberg 1999; Smith et al. 2005; Warner et al. 2002; Warner et al. 1999). This occurs when two environmental factors coincide: the tolerated temperature range of the zooxanthella is exceeded, and illumination is high. Accordingly, a useful descriptive term for the phenomenon is photothermal bleaching. A similar but not equivalent phenomenon known in other photosynthetic organisms is ‘photoinhibition’ (Singh 2000). Another mechanism of photothermal bleaching that has been suggested is failure of the dark reactions of Symbiodinium photosynthesis (Leggat et al. 2004). A factor that has been shown to protect zooxanthellae from thermal bleaching in high light is lipid content of the thylakoid membrane (Tchernov et al. 2004), though the relationship of this to variation in light conditions has not yet been tested.

The response of a coral to an occurrence of photothermal bleaching in its zooxanthellae is that it proceeds to expel symbionts that are unhealthy, but also symbionts that are healthy (Gates et al. 1992; Glynn 1991; Glynn 1993; Lesser 2004; Ralph et al. 2001). This is done at considerable risk of host non-survival, but the phenomenon is widespread among all coral hosts of Symbiodinium. Mechanisms of expulsion are varied and are depicted in Figure 1.4. Many of the mechanisms involve shedding of symbiont cells and this always takes place internally as zooxanthellae have no access to the ocean through the external
surface of the polyp (Figure 1.1b). The general process of loss of symbionts resembles the process of uptake of symbionts in this respect, as it is apparent that considerable movement of symbiotic gastrodermal cells from the inner gastrodermis of the mesentery (gut) to the outer gastrodermis ('endoderm') occurs during infection and development, while vice versa under bleaching stress.

1.4 Coral ecosystem prognosis under anthropogenic change

Human-induced change of the earth's atmosphere is now an established scientific fact (Crowley 2000; IPCC 1992; Tett et al. 1999; UNFCC online). The burning of fossil fuels has proceeded at pace over a mere two hundred or so years and has released, as CO₂, a volume of carbon that took hundreds of millions of years to lay down (Vitousek 1994). The molecule CO₂ causes global warming by efficiently absorbing heat (Atkins and de Paula 2005; Fleagle and Businger 1980), as do the other major greenhouse gases – methane, water, and sulfate aerosols (Charlson et al. 1992; Delgenio et al. 1991; Held and Soden 2000; Lelieveld et al. 1993; Lelieveld et al. 1998; Mitchell et al. 1995). The prime sinks for atmospheric heat are the oceans (Barnett et al. 2005), as the climate and oceans are a tightly coupled system (Cox et al. 2000; Manabe et al. 1991; Mitchell et al. 1995). One of the major early warning systems of global warming is rapid decline of coral reef ecosystems (Hughes et al. 2003; Walther et al. 2002).

Over evolutionary time corals must have been subject to thermal increases many times before the present day global warming episode, albeit the increases may not have been so rapid (Hoegh-Guldberg and Hoegh-Guldberg 2004), and as such bleaching may have been evolved by hosts as a way to deal with the stress, and to afford themselves the opportunity of gaining new symbionts better adapted to the stress (Douglas 2003). The Adaptive Bleaching Hypothesis (ABH) states that present day corals may gain benefits by bleaching, in allowing them to swap the genotype of their dominant strain of zooxanthellae (Buddemeier and Fautin 1993; Fautin and Buddemeier 2004; Kinzie et al. 2001). Tests of the ABH have generally supported its assumptions, which are two fold: (i) that bleached adult hosts can obtain new Symbiodinium from the seawater (Kinzie et al. 2001; Lewis and Coffroth 2004), and (ii) that they can form functional symbioses with strains that they formerly did not house at that geographic location (Lewis and Coffroth 2004). A qualification on assumption (i) is that ABH tests have used hosts that transmit their symbionts horizontally and have a native ability to adopt zooxanthellae, while the
applicability of the ABH to vertical transmitters is less clear. Additionally, a major unknown or even unknowable in regard to assumption (ii) is whether the hosts may have had associations with the replacement-zooxanthella in recent geological time, and have some memory of the long-lost symbiont to aid recognition and successful establishment of symbiosis.

The ecological significance of the ABH is quite a different matter than its validity on a case by case basis. Factors that enter consideration in predicting whether particular coral ecosystems might survive the current episode of global warming are (i) rate of reestablishment of symbiosis in a range of interdependent hosts composing a functional wave-resistant reef, and (ii) rate of reproduction of recovering corals. Fecundity is severely hampered by bleaching (Mitchelmore et al. 2002), and two to three seasons must pass before a bleached host is ready to undergo fruitful sexual reproduction (Baird and Marshall 2002; Michalek-Wagner and Willis 2001; Rinkevich 1996) which is their main dispersive route (Harrison and Wallace 1990). The outlook for most of the world’s corals appears bleak. On the Great Barrier Reef (GBR), the relevant example for this study, corals reefs are predicted to become extremely impoverished by the year 2050 (Hoegh-Guldberg and Hoegh-Guldberg 2004). Bleaching events have been recorded when corals are exposed to one degree greater temperature than their tolerance, and under sea temperature models published by O. Hoegh-Guldberg, such temperatures will be routinely surpassed every year in the near future (Figure 1.5) leading to permanent loss of fecundity for most of the resident coral colonies (Hoegh-Guldberg and Hoegh-Guldberg 2004; Hoegh-Guldberg 1999) and subsequent failure of succession and renewal. Analyses of sea temperature predictions for reefs across the Pacific and the Caribbean tell a similar story (Hoegh-Guldberg and Hoegh-Guldberg 2004; Hoegh-Guldberg 1999).
Figure 1.5 (a). Sea surface temperature data generated by the global coupled atmosphere-ocean-ice model [ECHAM4/OPYC3, (Roeckner et al. 1996)]. Temperatures were generated for each month from 1860 to 2100, and were forced by greenhouse gas concentrations that conform to the IPCC scenario IS92a (IPCC 1992). Effects of El Niño–Southern Oscillation (ENSO) events are included. Horizontal lines indicate the thermal thresholds of corals at the southern, central and northern sites (28.2°C; 29.2°C and 30°C respectively). Date were generated for three sectors of the Great Barrier Reef: Southern (23.5°S,149.5°E), Central (18°S,147.5°E) and Northern (11°S,143°E). (b). Number of times per decade that predicted temperatures (see part A) exceed coral threshold levels (bleaching events) for (A) Southern, (B) Central and (C) Northern regions of the Great Barrier Reef. Reference for additional model CSIRO DAR GCM: (Gordon and Ofarrell 1997). Both figures are from: (Hoegh-Guldberg 1999).
1.5 *Symbiodinium* (Freudenthal): evolutionary origin

The division Dinoflagellata [the dinoflagellates, sensu (Fensome et al. 1993)] is a monophyletic group of protists often possessing a characteristic flagellar structure and arrangement (van den Hoek et al. 1995) in the ‘zoospore’ (= motile lifestage). This arrangement is composed of a transverse flagellum that is ribbon-like and lies in a groove called the girdle, and a longitudinal flagellum that is relatively straight and lies in a groove called the sulcus (Figure 1.6). The entire half of the cell below the girdle, in the direction of the longitudinal flagellum is called the hypocone, while the half of the cell above the girdle is the epicone. Possession of a rounded hypocone and rounded epicone by a dinoflagellate is referred to as a ‘gymnodinioid’ morphology, which is not a taxonomic assignment (Pfister 1984; Pfister 1989; Pfister and Anderson 1987; Spector 1984; van den Hoek et al. 1995). A common feature of dinoflagellates is that their chromosomes are condensed in interphase (van den Hoek et al. 1995).

![Diagram of Symbiodinium zoospore](image)

**Figure 1.6** Line drawing of *Symbiodinium* zoospore (McLaughlin and Zahl 1959).

Within the dinophyceae, *Symbiodinium* (Freudenthal 1962) belongs in the family Symbiodiniaceae, which has been recently included in the order Suessiales (Fensome et al. 1993) based on ultrastructural data from a free-living isolate (Loeblich and Sherley 1979)
that was said to resemble the symbiont of the scyphozoan *Cassiopea* (clade A *Symbiodinium*). The assignment to Suessiales was first hinted at by Loeblich [Figure 1 in (Loeblich 1984)] presumably on the basis of his own work on the free-living strain (Loeblich and Sherley 1979). Loeblich’s study of ‘thecal plate tabulation’, being polygonal cell wall patterning of the zoospore (Loeblich and Sherley 1979) remains unique.

Suessiales are named according to tabulation similarities with a Triassic fossil dinoflagellate genus Suessia (Morbey 1975). The order Suessiales also contains another fossil genus *Umbriadinium*, family Umbriadinioideae (Palliani and Riding 2003). The living sister of the family Symbiodiniaceae within the order Suessiales is the family Suessiaceae, which has one extant genus *Polarella* (Montresor et al. 1999). The divergence of family Symbiodiniaceae from family Suessiaceae has been estimated at ~200 mya (Montresor et al. 1999).

The origin of the genus *Symbiodinium* is estimated by molecular clocks at ~150 mya (T. LaJeunesse, Florida International Univ. pers. comm.). Two taxa cluster between *Polarella* and *Symbiodinium* on phylogenetic trees, these are *Gymnodinium simplex* and *G. beij* (Carlos et al. 1999; Gast and Caron 1996; Montresor et al. 2003; Rowan and Powers 1992; Takishita et al. 2003b; Takishita et al. 2003a). These may therefore also be Suessiales but they have not been formally assigned as such because of the lack of ultrastructural information (Dodge 1974; Spero 1987) about their thecal plates: the primary character in all inter-order dinoflagellate taxonomies. *G. beij* is symbiotic with the protistan marine group foraminifera (Spero 1987), while *G. simplex* is free living (Dodge 1974).

Ironically, while the original justification for inclusion of *Symbiodinium* in the Suessiales was thecal plate tabulation for a free-living strain (Loeblich and Sherley 1979), the generality of *Symbiodinium* tabulation has been questioned since a *Symbiodinium* strain from a foraminiferan has recently been mooted to have quite different tabulation than that observed by Loeblich (Lee et al. 2003). By contrast, no doubts have been cast on the molecular justification for grouping of *Symbiodinium* with Suessiales which has rather become stronger with each analysis (Kremp et al. 2005; Takishita et al. 2003b; Takishita et al. 2003a).
The specific relationship of Suessiales to other dinoflagellate orders is elusive. Recent published trees lack bootstrap support for placement of Suessiales as sister to any particular order. *Symbiodinium* was formerly classified as a Gymnodiniale (Freudenthal 1959; Freudenthal 1962; Kawaguti 1944; Kevin et al. 1969; Taylor 1969) implying Suessiales may be cousin to the genus *Gymnodinium*. However, the genus *Gymnodinium* has recently been divided into *Karenia*, *Karlodinium*, *Akashiwo*, and *Gymnodinium* [reviewed in: (Daugbjerg et al. 2000)]. The redefined genus *Gymnodinium* now largely contains species that do not harm fish and do not produce toxins (Daugbjerg et al. 2000). A solid finding of late is that Suessiales and the genus *Woloszynskia*, a Gymnodiniale (Fensome et al. 1993), are specifically related in DNA sequence (Kremp et al. 2005; Saldarriaga et al. 2004).

Investigation by Kremp et al. (2005) of morphological differences in plate tabulature between *Woloszynskia* and Suessiales indicated that *Woloszynskia* may be outside the Suessiales *sensu strictu* and that the taxonomic position of *Woloszynskia* may need to be revised to indicate it may be sister to the Suessiales. This was considered by Kremp et al. (2005) to be more preferable than redefining *Woloszynskia* as a Suessiale, as suggested by Saldarriaga et al. (2004) which would alter the definition of Suessiales.

The genus *Symbiodinium* is monophyletic but its name is sometimes wrongly interpreted by protistologists in general (anecdotally) to require that the included organisms all be symbiotic. Rather the genus is united by morphology, and the name merely describes the most dominant habit of most of the constituent species. As such it is proposed here that *Gymnodinium simplex* should be adopted into the genus *Symbiodinium* as it has fully compatible morphology (Loeblich and Sherley 1979), is the closest free-living dinoflagellate to *Symbiodinium*, and was possibly the organism characterized under SEM by Loeblich and Sherley (1979) which prompted Fensome et al. (1993) to induct *Symbiodinium* into the Suessiales. Similarly *Gymnodinium bei*, is phylogenetically proximal to *G. simplex* in numerous published studies, and lies phylogenetically between the two non-fossil genera, *Symbiodinium* and *Polarella*, of the order Suessiales. It is fortunate, in this context, that *G. simplex* and *G. bei* have never been specifically inducted into any dinoflagellate order. Furthermore since the genus *Gymnodinium* is widely
regarded as being polyphyletic, and has recently undergone fragmentation into several new genera, the genus name Gymnodinium currently applying to G. bei and G. simplex does not carry with it a priori membership within the order Gymnodiniales. Accordingly it is proposed here that G. bei be renamed to a novel genus name, and that both it and G./S. simplex be inducted into Suessiales.

This thesis does not seek to formerly describe new species nor to make formal taxonomic changes to nomenclature of existing species. Such descriptions and/or changes to existing nomenclature should occur in subsequent publication of the work.

1.6 Relationships among dinoflagellate orders
The division Dinoflagellata (Fensome et al. 1993) contains many orders with dinokaryons and one order without a dinokaryon (Syndiniales). An approximate phylogenetic tree of dinoflagellates derived from a set of reviews (Fensome et al. 1993; Leander and Keeling 2004; Leander et al. 2003; Litaker et al. 1999; Saldarriaga et al. 2003b) is depicted in Figure 1.7. Dinoflagellata excludes the genus Oxyrrhis, which is nevertheless closely related to it on DNA grounds (Saldarriaga et al. 2003b).

A megagroup the ‘Gymnodiniales-Perediniales-Prorocentrales complex’ (GPP complex) within the dinoflagellata, contains most of the photosynthetic members of the dinoflagellata (hence – ‘dinophycean’). The GPP complex (Fensome et al. 1999; Gunderson et al. 1999; Rehnstam-Holm et al. 2002; Saldarriaga et al. 2003a; Saunders et al. 1997) also includes the Gonyaulacales (Fensome et al. 1993), a major group of dinoflagellates that are present in the Jurassic fossil record as well as in modern oceans (Fensome et al. 1999). A lack of reliable bootstrap resolution for any published dinoflagellate DNA phylogeny beyond the scale of order has been generally noted (Daugbjerg et al. 2000; Fensome et al. 1999; Zardoya et al. 1995), and so the branching orders within the dinophyceae on Figure 1.7 represent ultrastructurally based schemes (Fensome et al. 1993). The current state of dinoflagellate taxonomy is that many genera are not monophyletic when DNA sequences are employed, e.g Peridinium (Daugbjerg et al. 2000). Either the ultrastructure-based taxonomies or the DNA phylogenies (or both) may be hampered by the fast rate of evolution in the division Dinoflagellata (Saldarriaga et al. 2004). A notable feature of the lifecycles of many dinoflagellate genera is that an
interim lifestage (e.g. a gamete often) is of a shape and size resembling a gymnodiniale and is hence referred to as 'gymnodinioid' (Pfiester 1984; Pfiester 1989; Pfiester and Anderson 1987; Spector 1984; van den Hoek et al. 1995). It can be speculated that while Gymnodinium [sensu (Daughjerg et al. 2000)] and Suessiales have retained the early evolving gymnodinioid phenotype in the vegetative (adult) stage and gamete stages, other genera developed novel vegetative phenotypes and regress only during gametogenesis. This does not necessarily imply an early branching position for Gymnodiniales and Suessiales within the dinoflagellates [as in Figure 5 of (Saldarriaga et al. 2004)], but rather a phenotypic stasis in this particular branch.
Figure 1.7 Approximate taxonomic relationships between dinoflagellate orders. The Gymnodiniales-Peridiniales-Prorocentrales (GPP) complex is a classical grouping that has remained valid in recent literature (Fensome et al. 1999; Gunderson et al. 1999). Placement of Suessiales as sisters to the Gymnodiniales is qualified by the finding that Gymnodiniales are polyphyletic (Daughjerg et al. 2000). The Gymnodiniales that allow this grouping are Woloszynskia, G. beii, and G. simplex (Gast and Caron 1996; Kremp et al. 2005; Wilcox 1998). The Perkinsozoa are included as an outgroup (see Figure 6.16 chapter 6, and section 2.5 of this chapter).
1.7 Coevolution of metazoans with dinoflagellates and related taxa.

The genus *Symbiodinium*, or an organism with the same ecology, is indicated to have been present in Scleractinian (reef-forming) corals since the late Triassic and early Jurassic 220-180 mya ago, (Leinfelder 2001; Muscatine et al. 2005; Stanley 2003; Stanley and Fautin 2001; Stanley and Swart 1995) leading to the speculation by Stanley and Swart that scleractinians and zooxanthellae may have coevolved (Stanley and Swart 1995). Dating an origination of *Symbiodinium* during that period, requires the inference that scleractinian associations with some members of the genus *Symbiodinium* have persisted since then, but does not require that specific partner combinations have remained fixed.

A still earlier origin for the general phenomenon of metazoans symbiotic with algae is likely. The now extinct rugose and tabulate corals that existed in the Ordovician, Silurian and Devonian periods, were cnidarian (Stanley 2003) and so of necessity must have been subject to the same calcification rules of Goreau (Barnes and Chalker 1990; Goreau and Goreau 1959) as are *Symbiodinium*-coral associations. This dogma states that coral cannot create calcified skeletons of any significant size without the presence of photosynthetic symbions. The taxonomic affinities of the symbionts of rugose and tabulate corals have not been put forward.

A basic discovery was made very recently: that all dinoflagellates form a sister group to the apicomplexans (formerly sporozoans) and the ciliates (Cavalier-Smith 1991; Gajadhar et al. 1991; Wolters 1991). These taxonomic insights were made possible by the use of molecular methods, in particular PCR, and appropriate algorithms for the derivation of phylogenetic trees. Molecular phylogenies affirmed the outcomes of TEM studies that displayed a common phenotypic trait for dinoflagellates, apicomplexans and ciliates, - subsurface alveoli - which are membrane sacs that are filled with cell wall material in some dinoflagellates but are empty and lie below the plasma membrane in apicomplexans and ciliates (Cavalier-Smith 1991). The Alveolata (Cavalier-Smith 1991) as a group is very ecologically successful, rivalling the rhodophytes, haptophytes and the stramenopiles (major algal divisions) respectively for biomass and biodiversity (Andersen 2004; Corliss 2002; Falkowski et al. 2004; Irigoien et al. 2004; Saunders and Hommersand 2004). The ciliates are mainly scavengers and predators of other protists and bacteria (Hansen et al.
1997; Pernthaler 2005), the dinoflagellates are mainly autotrophic or parasitic (Saldarriaga et al. 2004; Spector 1984), while the apicomplexans are obligate parasites of metazoans (Levine 1978; Levine 1988; Perkins et al. 2002). Much interest centres on the evolutionary nexus of the three major alveolate groups (see Figure 6.16, chapter 6).

A hypothesis similar to the dinoflagellate-Scleractinian coevolution hypothesis of Stanley and Swart (1995), was put forward by Levine (1978, 1988) – that apicomplexans and metazoans coevolved. These authors each came to their conclusions before it had been shown that the dinoflagellates were related to the apicomplexans. In Levine’s case the data are more striking regarding the extant forms. The generalisation Levine noticed was that early-branching apicomplexans parasitize early-branching metazoans, and late-branching apicomplexans parasitize late-branching metazoans, with the intermediate associations occurring in a graded fashion. While Levine’s hypothesis concerned the taxonomic level of family and higher (both of host and of parasite), it must be noted that specificity of apicomplexans to hosts is homoplastic at the levels of: strain-to-host, species-to-host, and genus to host (Escalante and Ayala 1995; Sulaiman et al. 2002).

Apicomplexans are now known to house a remnant plastid, the apicoplast (Gardner et al. 1994; McFadden et al. 1997) that has lost photosynthetic ability, but is still required for viability of the organism. The idea that the ancestor of apicomplexans was possibly a dinoflagellate-like organism was recognised early (Levine 1978). Timelines for the origin of dinoflagellates, apicomplexans and metazoans converge on a common date range of approximately 700-1000 mya (Blair-Hedges 2002; Douzery et al. 2004; Escalante and Ayala 1995; Feng et al. 1997; Nikoh et al. 1997) depending on stringency of the molecular clock.

1.8 Terminology: zooxanthellae c.f. Symbiodinium
A term encompassing Symbiodinium that are resident within a eukaryotic host is ‘zooxanthellae’ (Lesser 2004). This term refers to the yellow-brown colour of the algal protist (root, -xanth) and indicates that the protist is found inside animal tissues (root, zoo-). Indeed, the former name for the genus Symbiodinium was genus ‘Zooxanthella‘ instated by Brandt (Brandt 1881) to refer to the dominant symbionts of reef corals, but this name was informally adopted in the few decades thereafter for any brownish or yellow symbiont (as opposed to both chlorophytic ‘green’ protists, and cyanobacteria) (reviewed in (Trench
This broad usage was retained only until it was formally recognised that the most common zooxanthellae belong to the division dinoflagellates (Chatton 1923; Hovasse 1924), which required a stricter redefinition of zooxanthellae and led at first to the temporary induction of these coral symbionts into the genus Gymnodinium (Kawaguti 1944), and then eventually to the formalisation of the genus Symbiodinium (Freudenthal 1959; Freudenthal 1962; Kevin et al. 1969), named according to its phenotypic trait, with a name that resembles the meaning of the phenotypic term zooxanthellae. A regression from usage 'Symbiodinium' to 'Zooxanthella', as genus name, was briefly attempted by Loeblich and Sherley (1979) but the regression of nomenclature was not widely adopted even though the paper's content of data (thecal plate tabulation) was of high impact (Fensome et al. 1993; Loeblich and Sherley 1979).

Current usage, in symbiosis literature, is that 'zooxanthella' (not italicized, singular of zooxanthellae) is a non-taxonomic description that is reserved almost solely for Symbiodinium spp. Researchers do not adopt this term to describe other (relatively rare) symbiotic dinoflagellates: Amphidinium spp. (Banaszak et al. 1993; Taylor 1971), Scrippsia spp., Gloeodinium sp. (Banaszak et al. 1993), Prorocentrum sp (Yamasu 1988), Pyrocystis sp. (Allredge and Jones 1973), Aureodinium sp. (Anderson and Be 1976), Gyrodinium sp. (Spindler and Hemleben 1980); nor to describe equally rare symbiotic diatoms (Lee et al. 1995) and rhodophytes (Lee 1980) [each of these symbionts reviewed in (Trench 1993)]. The root -zoo as a part of the term zooxanthellae usage is separate from the continued use of 'zoa' ('motile') as a suffix for groups of protists, e.g. Amoebozoa, Cabozoan, Euglenozoa (Cavaler-Smith 2003b), and is also separate from the term zoospore to describe the motile forms of dinoflagellates and other protists.

An essential attraction of the term zooxanthellae is that it implies colour and therefore photosynthesis. The name Symbiodinium does not explicitly invoke colour (pigmentation/chromatophores) and is accordingly a 'drier' and more technical term. Another utility of the term zooxanthellae over Symbiodinium, is that the root "symbio" does not specify the nature of the host, whereas the root "zoo" acknowledges the ecological significance of metazoans as the most widespread hosts of Symbiodinium. Additional utility of the term is that the roots "zoo" and "xanth", when juxtaposed, inherently imply a symbiosis since many marine animals are not otherwise
pigmented/photosynthetic. A recent case where the term zooxanthellae was co-opted to refer to *Symbiodinium* resident inside a ciliate (Lobban et al. 2002), indicates the persistence of the term as an ecological or functional label, regardless of host. It is to be noted that many current publications use the terms zooxanthellae and *Symbiodinium* concurrently [e.g. (Baker 2003; Coffroth and Santos 2005; LaJeunesse et al. 2004b; Lobban et al. 2002; Rowan et al. 1996; Santos et al. 2002b; Savage et al. 2002b; Smith et al. 2005; Tchernov et al. 2004; Van Oppen et al. 2005)], indicating that each term has its use in particular contexts, and that they are not mutually exclusive.

Terminology used in this thesis is as follows. *'Symbiodinium' is the name appropriate for cultures of this genus, and is used in taxonomic discussions. 'Zooxanthellae' is an appropriate term for referring to the in hospite organism and for ecological discussions. In hospite means resident inside a metazoan host. 'Symbiont' is a general term interchangeable with zooxanthellae when in context, but otherwise refers to any of the full range of symbiotic organisms in accordance with the dictionary definition of 'symbiont'. 'Zooxanthellate' is an adjective (in current use in literature), describing any host that contains zooxanthellae (Pochon 2005, Van Oppen 2005). Similarly 'azooxanthellate' means lacking zooxanthellae. Zooxanthellar is an adjective that is widely and correctly used, and cannot be substituted by zooxanthella which is not an adjective, but the latter may be used as an adjectival noun e.g. 'zooxanthella ecology'. It is not used as an adjectival noun in this thesis as it might tend to encourage its use as an adjective.

1.9 Breadth of the genus *Symbiodinium*, and distribution among hosts

*Symbiodinium* isolates in culture display radically different organellar arrangements, cell surface phenotypes, and DNA base compositions (Blank 1986; Blank and Huss 1989; Blank et al. 1988; Blank and Trench 1985a; Blank and Trench 1985b; Trench and Blank 1987). Based mainly on these characters, five species have been formally described (Freudenthal 1962; Trench 2000; Trench and Blank 1987; Trench and Thinh 1995). Five others have been named but not validly published with latin descriptions (LaJeunesse and Trench 2000; McNally et al. 1994), and in some cases are synonymous with the formally named species according to molecular data (LaJeunesse 2001).
Molecular phylogenetic analyses indicate that the genus *Symbiodinium* consists of at least 8 genotypic clades (Figure 1.8), clades A to H ([LaJeunesse 2001; Lesser 2004; Pochon et al. 2004; Rowan and Powers 1992]), which are of a taxonomic level roughly equivalent to species or species groups. *Symbiodinium* culturing history closely parallels the history of *Symbiodinium* taxonomy. Ultrastructural work, especially by Blank and Trench (1987) succeeded in predicting the genotypic clade groups that were established later (LaJeunesse 2001): *Symbiodinium pilosum* and *S. linucheae* (clade A), 'S. pulchrorum' (clade B), *S. goreau* (clade C), 'S. californium' (clade E) and *S. kawagutii* (clade F).

Clade C is by far the dominant clade in scleractinians on the GBR (Carter et al. 2000; LaJeunesse et al. 2004b; LaJeunesse et al. 2003; Loh et al. 1998; Loh et al. In preparation; Rodriguez-Lanetty et al. 2001; van Oppen et al. 2001), with the next most highly represented clade being clade D (LaJeunesse et al. 2004b; van Oppen et al. 2001). Clades A and B that are common in scleractinians of the Carribean (LaJeunesse et al. 2003; Rowan and Powers 1991) are very scarce on the GBR (LaJeunesse et al. 2004b; LaJeunesse et al. 2003; Loh et al. 2001; van Oppen et al. 2001).
Figure 1.8 In-hospite distribution of *Symbiodinium* clades. Adapted from: (Lesser 2004). Numbers above branches indicate bootstrap support. Where a clade occupies multiple hosts, asterisks and/or larger letters indicate the hosts in which that clade is most commonly found.

1.10 Species estimation within the genus *Symbiodinium*

A major question of relevance to ecological studies of adaptive potential within this genus is whether each clade contains one species or multiple species. A biological species is defined by its sexual ability/self-compatibility, and by its sexual incompatibility with taxonomically neighbouring species. The concept of a biological species (defined as a population of sexually compatible individuals) inherently limits the speed of
environmental adaptation, but not necessarily the amount of environmental adaptation, that a species can achieve. Put another way, adaptation is theoretically faster in biological species that occupy more limited niches than in biological species that occupy more extended niches. So regarding potential adaptation by *Symbiodinium* within the predicted century-long period of anthropogenic pressure, the quantity of change required in any case might hypothetically exceed the speed of sharing that is possible across the ecological range. The number of events of adaptive trait sharing that would need to occur among a whole population, in some cases a global population, to allow occupation of new hosts by existing thermostolerant *Symbiodinium*, potentially limits the amount of change that is possible in a short time.

Until the biological species boundaries can be defined in *Symbiodinium* via analysis of sexual recombination in wild populations, the number of biological species within any particular *Symbiodinium* clade cannot be fully estimated. A biological species boundary consists of three physiological boundaries, each of which is comprised of interactions at the protein-protein recognition level (including glycoproteins), and/or protein –DNA recognition level. The three components are: i) barriers to gamete recognition between species, ii) barriers to nuclear recombination between species, and iii) barriers to stable organellar inheritance between species. These three physiological barriers may evolve at similar rates to each other, and at a similar rate as subtle morphological characters evolve, therefore the morphological characters examined by Blank and Trench (1987) for instance may constitute good markers for ‘species’ in this genus.

A study by Rodriguez-Lanetty using variability in the Internal Transcribed Spacer (ITS) region of the nuclear rDNA operon, demonstrated a convenient bioinformatic method of judging “phylotypes” (defined as “independently evolving lineages“ and which may represent biological species boundaries, section 2.2 this chapter). The method employs a single genetic locus, ITS, and is not dependent on the number of isolates sampled for each phylotype, nor is it dependent on reference to phenotypic criteria (Rodriguez-Lanetty 2003a). The keys to the phylotype technique, also called ‘nested-clade’ analysis, are that phylotypes are grouped according to a logarithmic distance scale, and are assessed for potential recombination history amongst each other. Each successive subgroup (‘nest’) of isolates in the hierarchy is twice as many bases away from the neighbouring group as from
the members of its own group (Figure 1.9). Formation of each new subgroup is a step. The greater the number of logarithmic steps by which two isolates diverge from each other, the much more unlikely they belong together in a single phylotype. Meanwhile statistical comparisons between subgroups are performed to determine whether base identities at variable sites are shared between the subgroups. At a limit of taxon nesting where bases are found to be unshared between two subgroups, it is judged that sexual recombination has not occurred. The subgroups that are evolving independently are retained and designated as phylotypes (Figure 1.10) because no further nesting is justified. The ITS recombination analysis by Rodriguez-Lanetty (2003) was performed under two different algorithms. In some clades such as clade A, the ITS sequences were very different across subclades, and so were unalignable in some sections. This immediately established the subclades as phylotypes, in both of the analysis algorithms used.

Rodriguez-Lanetty’s analysis (2003) did not assume that “independently evolving lineages” (also called cohesion-species, Templeton 2001) are sexual in character. However, the analysis is consistent with such a model. Given that the tool used, ITS on the rDNA operon, is a multicopy locus in eukaryotes, and given that rDNA contains many palindromes, the operon is particularly recombinogenic (Feler et al. 2004; Hughes and Petersen 2001; Lovlie et al. 1988; Zhu and Schiestl 2004). Therefore the base sequence of ITS ought to be a relative constant in a sexual species.

The purpose of the ITS recombination test in the nested clade analysis (Rodriguez-Lanetty 2003) was to enable discernment of species boundaries, its purpose was not to prove recombination. Quite the converse, it assumed that recombination maintains the ITS, and so cohesion-species boundaries (Templeton 2001) were able to be assigned accordingly. To summarise, what nested clade analysis is aimed at, is to collect evidence demonstrating lack of recombination across particular nest boundaries, - as part of the analysis those boundaries are sought and assigned on the grounds of genetic distance between nests (cohesion species) and monophyly within each nest.
Figure 1.9 Nested clade analysis of clade C *Symbiodinium* ITS sequences (figure from Rodriguez-Lanetty 2003). Non redundant Genbank accessions were collected and arranged according to number of mutations that separated them. Each branch is a single mutation step. Zeros represent missing haplotypes. Clusters are assigned on the basis of shared characters and single step distances of radial separation. Clusters are then successively grouped with other clusters. The probability that sequences in a given group are conspecific with those in the neighbouring group, decreases as log base 2 with each new merger. In the analysis of clade C, three levels of merger resulted in assignment of two phylogenies, nest 3-1 (C1) and nest 3-2 (Clade C2), between which there was no evidence of recombination.
To put the nested clade analysis of Rodriguez-Lanetty in context, it is desirable to compare it against the other valid method of estimating *Symbiodinium* species numbers, ultrastructural work. Ultrastructure studies have given a basic measure of phenotypic distance (holotypes) that represent species separation within a single clade of this genus, eg ultrastructurally defined *S. microadriaticum* (clade A1) versus ultrastructurally defined *S. pilosum* (clade A2), they are morphologically different yet are in the same genotypic clade, A. The genetic distances separating pairs of phenotypic species within a given clade are known (LaJeunesse 2001). Each phenotypic species forms a subclade, which can be considered to correlate to published species names because the phenotypically characterized (published) species do correlate reasonably well with the nested clade analysis (Figure 1.10), especially within clade A, where there are sufficient subclades and species to make the comparison.

<table>
<thead>
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<tr>
<td>G</td>
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<tr>
<td>Total</td>
<td>23</td>
</tr>
</tbody>
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(clade H not analysed by Rodriguez-Lanetty)

*Figure 1.10* Outcomes of nested-clade analysis of *Symbiodinium* ITS (Rodriguez-Lanetty 2003a) compared to ITS subclades and assigned species names (LaJeunesse 2001). Twenty three 'independently evolving lineages' were indicated as phylotypes (phytotype = non-recombining with any other phylotype). This analysis was current in 2003, and more ITS samples have since become available, so the number of potential 'species' in genus *Symbiodinium* will continue to grow. Species names in brackets are ~ synonymous with those outside the brackets (LaJeunesse 2001).
Proof that recombination occurs within a particular phylotype is a more challenging task. It requires the development and use of multiple markers that are each lower in copy-number than ITS, and less strongly conserved. An example of these marker types are microsatellites. These vary in sequence within a phylotype, depending on which isolates of the phylotype are sampled (Santos et al. 2003c).

1.11 IS THERE ONE *Symbiodinium* STRAIN FOR EACH HOST CORAL ECOTYPE?

Host specificity is a major field of study among *Symbiodinium* researchers. Model strains in culture offer a means of testing Koch’s postulates, by ‘re-infection’ of aposymbiotic (± post-symbiotic, or artificially non-symbiotic) hosts, and subsequent attribution of the infected host phenotype to the presence of the infectious agent (by re-isolation of the agent). However, the more common means of establishing whether host-specificity exists is to genotype zooxanthellae from a given host species over a wide geographic range and to compare the symbiont genotypes obtained to the symbiont genotypes present in other hosts species over the same range (Carter et al. 2000; Fabricius et al. 2004; LaJeunesse 2002; LaJeunesse et al. 2004; LaJeunesse et al. 2003; Loh et al. 1998; Loh et al. 2002; Loh et al. In preparation; Loh et al. 2001; Rodriguez-Lanetty et al. 2001; Rowan 1998; Ulstrup and Van Oppen 2003; van Oppen et al. 2001). In such studies the general finding is that a given hosts species prefers a single symbiont within a limited geographic area, approximately on the scale of <1000 km. However, the symbiont phenotype can differ in the same host species in a different hemisphere or major ocean (Loh et al. 2001). In some studies symbiont genotype in a single host species can differ markedly over a latitudinal gradient (Rodriguez-Lanetty et al. 2001). Possession of two symbionts is uncommon, but occurs (Loh et al. In preparation; Rowan and Knowlton 1995; Rowan et al. 1997; Ulstrup and Van Oppen 2003).

Numerous groups have established the utility of diverse *Symbiodinium* cultures for host reinfection studies (Belda-Baillie et al. 1999; Coffroth and Santos 1997; Schoenberg and Trench 1980c) while other groups have used Freshly Isolated Zooxanthellae (‘FIZ’, i.e. uncultured zooxanthellae) for this purpose (Belda-Baillie et al. 1999; Colley and Trench 1985; Davy et al. 1997; Kinzie 1974; Rodriguez-Lanetty et al. 2004; Schoenberg and Trench 1980c; Weis et al. 2001). The most common practice has been to test the preference of a given host for one strain of *Symbiodinium* over another. In all cases hosts
prefer their native strain of *Symbiodinium* to any other strain offered. In a unique study, Kinzie et al (Kinzie 1974) showed that cnidarians prefer genus *Symbiodinium* over other dinoflagellates offered.

At a finer level, it has become feasible to investigate the molecular basis of the selectivity of host-zooxanthella combinations via analysis of host and dinoflagellate surface molecules (Costas and Lopez-Rodas 1994; Jimbo et al. 2000; Kremp and Anderson 2004; Lin et al. 2000; Smith 1979; Trench 1997; Wakefield and Kempf 2001; Weis et al. 2001). At least two studies have documented instances of gradual change in symbiont genotype over a coral’s lifecycle (Little et al. 2004; Toller et al. 2001). The public availability of established type cultures of *Symbiodinium* in future should facilitate the repetition and extension of host infection studies across labs.

### 1.12 *In vitro* lifecycle of *Symbiodinium*

The *in vitro* lifecycle of *Symbiodinium* has not been pictured consistently in the literature; indeed one recent review (Fensome et al 1993) omitted the sexual part of the lifecycle (Figure 1.11b) because that aspect of the lifecycle has not been conclusively documented *in vitro*. The observation by Freudenthal of ‘microgametes’ (Figure 1.11a) was never confirmed with hard data. However, subsequent population genetic studies have indicated that recombination does occur within some clades of *Symbiodinium* (Baillie et al. 2000a; Baillie et al. 1998; Santos et al. 2003c).
Figure 1.11 Two published versions of the lifecycle of free-living *Symbiodinium*. A sexual stage was proposed in (a) and rejected in (b) for lack of data. White ovoids (labelled AB) represent brown pigmented accumulation bodies that are of unknown function but are characteristic of many members of the genus *Symbiodinium*. Grey ovoids represent chloroplasts (CP) that, while drawn in (b) but not in (a), are present in greater or lesser number in every member of the genus *Symbiodinium*. Dappled regions represent vacuoles (VA), and nucleus (NU) respectively. The zoospore (ZS) is released intermittently from one of the immotile stages.

1.13 The environmental lifecycle of *Symbiodinium* and its potential relevance to coral bleaching

The way in which bleach-prone zooxanthellae might adapt to their environment in the era of global warming (Chapter 1), is that the zooxanthellae may recombine to generate new host-symbiont pairings, or to generate new zooxanthellae traits. It is likely that if either partner is able to adapt, it may be the zooxanthella, and not the coral, because of the comparative timescales of sexual reproduction of *Symbiodinium* versus corals. Most hard corals spawn only once per year, in spring (Harrison and Wallace 1990). If the clade C symbionts of corals are sexual, like their clade A and clade B cousins (Baillie et al. 2000a; Baillie et al. 1998; Santos et al. 2003c), they will likely have the opportunity for sexual reproduction in the open water many times between a single escape event and the following infection event. Therefore
with regard to the potential frequency of recombination events, *Symbiodinium* ought to be more adaptable than the coral hosts. If on the other hand *Symbiodinium* strains are largely asexual then adaptation would be slow. Recombination has been shown to have occurred in populations of clade A and clade B *Symbiodinium* (Baillie et al. 2000a; Baillie et al. 1998; Santos et al. 2003c), though those studies were not explicitly done in the context of adaptation to bleaching.

In what stage of the environmental lifecycle (*in hospite* versus free-living) does recombination occur? The open water is the most likely site of gametogenesis and sexual recombination in *Symbiodinium* that occupy coral hosts intracellularly. Open-water solves the problem of gametes finding each other through the barrier of host symbiosome membranes. There is perhaps only one eukaryote has been documented undergoing sexual recombination within the cytoplasm of another eukaryote: the stramenopile endosymbiont of *Peridinium balticum* and *P. foliaceum* (Chesnick and Cox 1987; Chesnick et al. 1997; Chesnick et al. 1996), but this is perhaps an inappropriate analogue for the *Symbiodinium*-coral relationship because the endosymbiont of *P. balticum* has coevolved with the host, as demonstrated by the maintenance of the symbiont in two separate hosts species of the host genus, implying its presence as a symbiont in the forbear of both and direct transmission across the speciation event/s. By contrast, in the *Symbiodinium*-coral relationship, symbionts are not known to be maintained across host species boundaries. Coevolution of certain *Symbiodinium*-coral associations within a host species nevertheless occurs (D. Carter, University of Sydney, unpublished data).

### 1.14 The importance of meiosis to living organisms

Meiosis is the process by which eukaryotes efficiently recombine genetic traits within a population or 'species' (Barton and Charlesworth 1998; Burt 2000; Goddard et al. 2005). It is nearly universal among multicellular eukaryotes (Barton and Charlesworth 1998; Villeneuve and Hillers 2001), but is in question for many protists (= unicellular eukaryotes). A recent review by Tibayrenc and Ayala (2002) outlines the current state of knowledge for the question of recombination in human parasitic protozoa (Tibayrenc and Ayala 2002), which are predominantly clonal though sample sets used are often too small (Tibayrenc 1999).
Sexuality of many of these protozoan organisms may be epidemic (predominantly clonal but with infrequent recombination) judging from the model species of each genus that have been examined with large data sets (Maynard-Smith et al. 1993; Tibayrenc and Ayala 2002). In non-parasitic protozoa, the question is less urgent, but the presence of meiosis-specific genes in non-pathogenic algal and amoeboid protists (Ramesh et al. 2005), that have not been proven to have a sexual cycle in vitro, indicates that the meiotic process evolved early, amongst the first eukaryotes, and is probably maintained in the vast majority of eukaryotes (Ramesh et al. 2005).

Why organisms need sexual transmission of traits has been the subject of much theoretical discussion. The need is comprised of two forms of evolutionary selection: (i) organisms recombine in order to create novel genetic combinations, i.e. to generate diversity; (ii) organisms recombine to prevent mutations accumulating, i.e. to maintain unity and prevent diversity. These two hypotheses, seemingly contradictory, have both obtained support (Goddard et al. 2005; Rice 2002; West et al. 1999) and are unified by the phenomenon of selection for occupation of multiple niches simultaneously (Hadany 2003; Hadany et al. 2004; West et al. 1999), thus diversity must be both generated and controlled. Conversely, even organisms that occupy a homogenous niche throughout their range require recombination to prevent the accumulation of deleterious mutations in individuals possessing rare advantageous mutations (Hadany and Feldman 2005).

1.15 Use of molecular markers to assess sex in a population

Analysis of molecular markers at multiple loci has allowed assessment of natural lifecycles in various eukaryotes and prokaryotes. Organisms are collected in the field and can be cultivated or not, but are analysed either by extracting genomic DNA and then typing each clonal isolate at several loci, or by extracting proteins and performing a multi-locus enzyme electrophoresis (MEE) study. If markers have sufficient resolution and are informative, then multiple alleles exist in the population at each genetic locus, due to mutation. Different loci evolve independently in a sexual population, but in a clonal (asexual) population loci appear linked (see section 2.3, Figure 1.12). An early application of MEE as a tool to assess recombination in populations was a study of gene-flow in the prokaryotes *Neisseria gonorrhoeae, N. meningioidis, Rhizobium meliloti, Haemophilus influenzae*, and *Salmonella*
spp. (Maynard-Smith et al. 1993), and a concurrent reanalysis of gene-flow within populations of the protists Plasmodium falciparum, Trypanosoma brucei and T. cruzi. All these organisms except Salmonella spp. and T. cruzi were found to show frequent recombination. Since that time DNA sequence analysis has become the more widely used tool for such studies, owing to less susceptibility to homoplasly (Burt et al. 1996; Carter et al. 1996; Koufopanou et al. 1997; Tibayrenc and Ayala 2002).

2. Thesis overview: methods in context

2.1 Molecular marker development in Symbiodinium

Application of a population genetic analyses in Symbiodinium is hampered by the lack of available markers. The current study sought to address this by exploring within the ‘black box’ of Symbiodinium genetics and lifecycle. Much knowledge has been accumulated about Symbiodinium physiology and ultrastructure compared to many other protists. By contrast, lack of an historical focus on Symbiodinium genetic research goals has been conditioned by the lack of a defined sex-cycle in vitro. Thus while it was one of the first dinoflagellates to be cultured (Kawaguti 1944), accumulation of knowledge about its sexual potential has lagged behind that obtained for other dinoflagellates (Beam and Himes 1974; Blackburn et al. 1989; Destombe and Cembella 1990; Figueroa and Bravo 2005a; Figueroa and Bravo 2005b; Himes and Beam 1975; Sako et al. 1992; Silva and Faust 1995) (Coats 2002; Giacobbe and Yang 1999; Kennaway and Lewis 2004; Montresor 1995; Nagai et al. 2003; Parrow and Burkholder 2003; Probert et al. 2002; Steidinger et al. 1995)

Successful and reproducible karyotyping of many Symbiodinium strains has been achieved using serial sectioning TEM (Blank and Huss 1989; Blank and Trench 1985), though chromosome counts of other zooxanthellae were not reproducible (Udy et al. 1993). Chromosome shape was hard to define in the study of Udy et al. (1993) because of the permanent state of condensation of dinoflagellate chromosomes, and possession of fine tendrils of DNA linking two otherwise separate condensed chromosomes. Pulsed Field Gel Electrophoresis (PFGE) of whole chromosomes could address the shortfall of microscopic karyotyping, and will be an exciting development when it occurs because it will presumably be feasible to use washed FIZ as the subject material, avoiding the need to culture. Ploidy of
Symbiodinium is known only for one strain (Santos and Coffroth 2003a), a haploid in subclade B1. Dinoflagellates, in general, are predominantly haploid (Beam and Himes 1984; Pfiester 1984; Pfiester 1989; Pfiester and Anderson 1987). The number of extant Symbiodinium strains is at least in the hundreds (Baker 2003). It is quite probable that different strains will contain different levels of ploidy, and that even in haploids the copy-number of a given gene may be variable. Differences of ploidy across species boundaries within dinoflagellate genera has been documented (Beam and Himes 1984; Hayhome et al. 1987; Loeblich et al. 1981; Parrow and Burkholder 2002), other protists (Green et al. 1996; Sansome et al. 1991), and other eukaryotes (Dufresne and Hebert 1995; Otto and Whitton 2000; Petit et al. 1999).

The difficulty of bringing a desired strain of Symbiodinium into culture has slowed research regarding the in vitro lifecycle. It has also restricted marker development to: (i) those markers that have been sampled from hosts and symbiont alike: e.g. nuclear rDNA, mitochondrial protein coding genes, and (ii) markers that do not occur in the host: chloroplast genes. Studies of nuclear rDNA (Rowan 1991; Rowan and Powers 1991; Rowan and Powers 1992) brought about the renaissance in zooxanthellae studies that is now enjoyed. Extension of DNA-based work to finer taxonomic scales is hampered by lack of available cultures and by contamination of FIZ with host cells and bacterial cells. Two research groups (Maruyama lab and Coffroth Lab) working with field populations have been able to culture their subject zooxanthella strains, and have gone on to develop finescale molecular markers that were applied at the population genetic level for studies of recombination (Baillie et al. 2000a; Baillie et al. 1998; Santos et al. 2003c).

It is desirable to characterise single-copy nuclear genes as markers in Symbiodinium, owing to potential such markers would bring to examination of the ecological differences between strains. Its genomic structure has been studied before the advent of PCR, and was shown to be similar to other dinoflagellates (Blank and Huss 1989; Blank et al. 1988). Published Symbiodinium gene sequences since that time have mainly been multicopy genes (LaJeunesse 2001; Norris and Miller 1994; Reichman et al. 2003; Rowan and Powers 1992; Sadler et al. 1992; Weis et al. 2002), and polyproteins (Rowan et al. 1996; Wray and DeSalle 1994). These constitutively expressed housekeeping genes make products that are needed in high
quantity. However, the existence of potentially low copy-number, regulatory genes in dinoflagellates, has been evidenced (Leveson and Wong 1999; Snyder et al. 2005) (Guillebault et al. 2002). In the current study the introns of a low copy-number gene (actin) are sought, as variable sequence markers that will be useful for studies of heredity. Current EST projects (O. Hoegh-Guldberg University of Queensland; C. Woodley, National Ocean Service, South Carolina; M. Coffroth State University of New York; personal communications) should address this gap. *Symbiodinium* introns sequences in multi-copy genes are available in Genbank, but only for two clades, A and E (Genbank accessions: AF020781 U43532 AAO49353). Organellar markers for *Symbiodinium* hold considerable promise (Santos et al. 2002b; Takabayashi et al. 2004; Takishita et al. 2003a). Variable regions in organellar genes can evolve at a faster rate than nuclear markers (Santos et al. 2002b) and are thus useful for population genetic studies at fine scales.

2.2 Definitions of levels of *Symbiodinium* genetic variability as relating to the questions of genus boundaries, biological species boundaries, and host specificity

The genus *Symbiodinium* is anecdotally regarded by some phycologists as polyphyletic, but this is a misunderstanding based on ignorance concerning the lack of study done on the only remaining interloper *Gymnodinium varians* (Maskell 1887). A gradual process of change of genus name away from *Gymnodinium* is evident in publications on the *Symbiodinium* lineage, the first case being the change by Freudenthal and the latest being the change of nomenclature of *Gymnodinium linucheae* (Trench and Thinh 1995) to *Symbiodinium linucheae* (LaJeunesse 2001) subclade A4. This process reflects the molecular monophyly that exists (e.g. monophyly evident on Figures 3.1 and 3.2 of chapter 3, and Figure 7.1 of chapter 7). *G. varians* is thus overdue to be renamed, has already been informally renamed “*Symbiodinium* sp. CCMP421” in the literature (Saldarriaga et al. 2003b) and the formal change is accordingly made here (*Symbiodinium varians* comb. nov., type material CCMP421). This thesis documents the current state of the genus *Symbiodinium* (sections 1.9 - 1.10 of chapter 1, section 2.4 of chapter 7, and Figure 7.3 of chapter 7). At the subgenus level, the *Symbiodinium* and coral reef research community has accepted the use of the term ‘clade’ to refer to monoplyetic groupings within *Symbiodinium*. The biological meaning of these clades is that an animal host of a given order tends to be capable of housing a limited
number of alternative \textit{Symbiodinium} clades, and apparently excludes many more clades (Figure 1.8, and section 1.8 of this chapter). There is much interest in the definition of ‘species’ as relevant to protists, and as relevant to the genus \textit{Symbiodinium}. To define the various terms that describe hierarchical levels of population variability within this genus, Table 1.1 contains a list of terms that are used throughout the thesis.
<table>
<thead>
<tr>
<th>Hierarchical level</th>
<th>Term</th>
<th>Synonyms</th>
<th>Definition</th>
<th>Comments</th>
<th>Possible example</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Strain‡</td>
<td>‘Cell line’, ‘Clone’</td>
<td>• Definition A. A clonal organism, maintained as an <em>in vitro</em> culture, and as far as known, derived asexually from a single starting cell. <em>Definition B</em>. Potentially clonal organism (cell line) such as characterized from a single nubbfin or host individual (though a single nubbfin may in some cases contain more than one strain). Both definitions rely on use of the most variable DNA marker available at the time, or on knowledge that a strain is derived from a single cell.</td>
<td>The same strain may or may not be found in another host nubbfin/individual. If a strain is found in only one host species, that strain is an ‘Ecotype’</td>
<td>Sym-Gt17 (chapter 5)</td>
</tr>
<tr>
<td>2.</td>
<td>Subspecies</td>
<td>‘Phylogenetic species’</td>
<td>• A set of strains sharing phylogenetic monophyly (DNA sequence similarity to the exclusion of other phylogenetic species). A phylogenetic species may <em>potentially</em> recombine with sister phylogenetic species, but may rarely have contact that might facilitate this. A phylogenetic species X can form in the absence of sexual recombination, but retain sexual ability. A phylogenetic species Y may form in the presence of sexual recombination, but may subsequently lose sexual ability or opportunity. A phylogenetic species Z may form in the presence of sexual recombination, and may then subsequently live apart from other phylogenetic species, but it may retain the ability for sexual recombination with itself and with other phylogenetic species.</td>
<td>May correlate to host preference, but not necessarily restricted to occupying a single host species. Affected by tendency of a host to select for a small number of interrelated strains, or tendency of host to select for strains possessing a character similar to the preferred strain/s. Also affected by physical barriers to symbiont dispersal.</td>
<td>all Sym-Gt (chapter 5)</td>
</tr>
<tr>
<td>3.</td>
<td>Species</td>
<td>‘Biological species’, ‘Subclade*’, ‘Phylyotype’ ‘Independent evolving lineage’ ‘Cohesion species**’</td>
<td>• Definition A. A biological species is potentially able to recombine with self (that is with ranks 1,2,3 except 2Y), but is completely unable to recombine with other species (of rank 3) and unable to recombine with non-self (ranks 4,5,6) above the rank of species. <em>Alternative definition B</em>: possesses a fixed morphology that is clearly different in some consistent way from the morphology of neighbouring species. In the alternative definition B, it is assumed that the diagnostic traits of the species have been distributed between the constituent subspecies by means of sexual recombination between the subspecies. Definition B does <em>not</em> require that recombination is <em>currently</em> occurring between the constituent subspecies, nor that it will continue to occur between them in future. <em>The two definitions 3A and 3B are fully incompatible</em></td>
<td>The recombination definition A, means: not able to recombine with other phylyotypes</td>
<td>• Subclade C1 (LaJeunesse 2001) S. goreaui  • Subclade B1 (Santos et al. 2003c) S. pulchrorum  • Subclade A3 (Baillie et al. 2000a; Baillie et al. 1998) unnamed</td>
</tr>
</tbody>
</table>

40
4. Clade

- Essentially undefined biologically, but in wide use in *Symbiodinium* literature.
- Originally the term ‘clade’, for *Symbiodinium*, referred to presence of particular restriction sites at particular positions in nuclear small subunit rDNA (refs). Its meaning is now essentially ‘a monophyly consisting of multiple species’.

Some clades that are currently undersampled as yet contain only one species, but are predicted to contain more than one species, due to the large degree of sequence difference between each clade and its neighbouring clades.

Sections 1.9 and 1.10 this chapter

5. Group

- Monophyletic assemblage of clades.
- *Symbiodinium* contains two large groups: the clade A group, versus grouped clades B-H. These each consist of many smaller groups. In phylogenetics a group is defined as any monophyletic assemblage supported by bootstrap analysis, morphological synapomorphy, or statistical similarity, to the exclusion of other groups.

Clade A has been used as the default clade name for a set of *Symbiodinium* species that tend to have an ability to live extracellularly. This very large clade may deserve to be broken into more than one clade, but new clade letters, or agreeable bounds for such, have yet to be proposed.

Sections 1.9 and 1.10 this chapter

6. Genus

- Currently includes all *Symbiodinium* plus *Gymnodinium varians*** [clade E. *Symbiodinium*, (Wilcox 1998)], and *Symbiodinium linucheae* [clade A. *Symbiodinium*, (Laeunesse 2001)] formerly *Gymnodinium linucheae*.

Arguably *Symbiodinium* could also include *Gymnodinium simplex* (Kofoid and Swezy 1921) as evolutionary forebearer of *Symbiodinium* with compatible morphology by light microscopy, see Chapter 7.

Section 1.9 this chapter

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See Chapter 7 and Appendix 8 for full definitions of the terms used in this table.

* The term “subclade” has been variously used in the literature to refer to strains ecotypes and species. Even the criteria of monophyly (which should apply to taxa in all ranks) has often been ignored. It is therefore proposed here that the term subclade be standardized to the rank of species or putative species, that it require monophyly. Accordingly, since clade A includes many species it should be subdivided into several new clades (that action not done here as it is within the terms of reference of this thesis).

***Cohesion-species” was defined by Templeton (2001) as “an evolutionary lineage of or set of lineages with genetic exchangeability and/or ecological interchangeability” but here is excluded from rank 2 ‘phylogentic species’ and elevated to rank 3 ‘Species’ because the ‘phylotypes’ of Rodriguez-Lanetty (2003) were identified using the ‘Cohesion-species’ concept and because phylotypes correlate well (Figure 1.10) to the formally named species of Blank and Trench (1987) and others.

*** *Symbiodinium varians* (comb nov.) formerly *Gymnodinium varians* (Maskell 1887), synonym *Gymnodinium minimum* (Klebs 1912; Popovsky and Pfeifer 1990).

Note: “cryptic species” under current eukaryotic microbiological usage, is of a rank midway between rank 1 and rank 2, and most closely resembles phylogenetic species Y in rank 2.
2.3 Do clade C *Symbiodinium* undergo sexual recombination?

The dominance of clade C *Symbiodinium* in the middle and southern GBR is striking, and the adaptive potential of these in the face of global warming is of interest. This study sought to culture clade C *Symbiodinium*, develop markers from it, and then use the markers to assess whether sexual recombination exists in the clade. An overview of the overall rationale adopted is presented in Figure 1.12. A marker was sought in each of three DNA-bearing organelles (nucleus, chloroplast, and mitochondrion). Recombination of isolates from a single population would be detectable as reassortment of organellar alleles, whereas clonality would be evident if each organellar allele partitioned strictly with each *Symbiodinium* strain.
Figure 1.12 Using organellar genes to investigate allele reassortment. Protist strains are represented as blue. Alleles at specific loci are represented by colours, and the alleles in organelles of each parental strain are assigned a common colour. Key to letters signifying organelles; n-nucleus, c-chloroplast, m-mitochondrion. Each allele (colour-letter) is a unique DNA sequence. In asexual organisms alleles appear linked, while in sexual organisms alleles appear unlinked. Only some of the possible combinations from sexual reassortment are shown.
2.4 Symbiosis on two levels: ecological partnerships versus heritable organelles.

The symbiotic associations of unicellular algae need to be understood on two levels: i) the endosymbiosis of an alga inside a multicellular eukaryotic host (for example, a dinoflagellate inside a coral, or a chlorophyte inside a lichen) which is temporary and does not involve genetic merger; ii) the endosymbiosis of one protist inside another, which is permanent, involves genetic merger and precludes free-living by either partner. Process (ii) is introduced in the next section. Briefly: the chloroplast and nucleus of a protist can be obtained by another protist over evolutionary time. During stabilization of the new chloroplast, genetic material can be transferred from chloroplast to nucleus, or nucleus to nucleus (Bachvaroff et al. 2004; Cavalier-Smith 2003a; Doolittle et al. 2003; Hackett et al. 2004a; Palmer 2003; Patron et al. 2006).

2.5 Protistan algae are taxonomically varied and their plastids are interrelated

In examining the dinoflagellate symbionts of coral, and particularly in studying their chloroplasts (plastids), a general background on the evolution of protistan algae is important. Figure 1.13 (Keeling 2004) summarises the current state of the consensus model for the interrelatedness of protistan algae and their plastids. Stable uptake of photosynthetic primary ‘endosymbionts’, which are cyanobacteria, is the rarest of these events that have occurred over evolutionary time. A single primary endosymbiosis resulted in the green plant lineage, the rhodophyte lineage, and the glaucophyte lineage, collectively called the Archaeplastida (Adl et al. 2005), while an independent primary endosymbiosis occurred in the Cercozoan genus Paulinella (Bhattacharya et al. 1995; Marin et al. 2005; Melkonian and Mollenhauer 2005). Not much more frequent is the establishment of a secondary endosymbiosis, involving the uptake of a eukaryotic alga by another eukaryote, and the persistence of the plastid as a heritable element in the new host (Cavalier-Smith 1999; Durnford et al. 1999; Ludwig and Gibbs 1989; McFadden 1999; Schwartzbach et al. 1998; Van de Peer et al. 1996; Watanabe et al. 1990; Watanabe et al. 1987). A particularly biodiverse group that arose from one such secondary endosymbiosis event is the chromalveolates (Cavalier-Smith 1999; Harper and Keeling 2003; Takishita and Uchida 1999a) descended from the topmost light-blue eukaryote in Figure 1.13. The most frequent events to occur in later eras have been tertiary endosymbioses, involving the uptake of an alga already bearing a secondary plastid, and the persistence of the plastid inside a new eukaryotic host (Chesnick and Cox 1987; Chesnick et al. 1997; Hackett et al. 2004b; Hackett et al. 2003; Yoon et al. 2002a; Yoon et al. 2005).
The relevance of the evolutionary history of plastids to this thesis is twofold. Firstly, the plastids of *Symbiodinium* contain a characteristic pigment called peridinin, and plastids of this type are known to house unigenic minicircles of DNA (Barbrook and Howe 2000; Barbrook et al. 2001; Green 2004; Howe et al. 2003; Zhang et al. 2002; Zhang et al. 1999; Zhang et al. 2000) instead of a large circular or large linear genome composed of many genes in series (Douglas and Penny 1999; Glockner et al. 2000; Hallick et al. 1993; Kowallik et al. 1995; Reith 1995; Shivji et al. 1992). Speculatively, this fragmentation of the genetic information, that is normally unfragmented in a chloroplast, may be due to the influence of a predatory lifestyle on the evolutionary history of the peridinin dinoflagellate plastid (section 2.5 and 2.6 of chapter 7). Also, it should be noted that a photosynthesis gene of one peridinin dinoflagellate, *Ceratium horridum* was found to be encoded in an electrophoretically-determined high molecular weight (HMW) DNA fraction (migrating at >40 kb) as well as on minicircles (Laatsch et al. 2004). The HMW DNA is yet to be sequenced and it could be argued that it may simply consist of concatenated minicircles. The authors of the *Ceratium* work (Laatsch et al. 2004) also showed evidence that the HMW DNA fraction containing the photosynthesis gene was in the nucleus. By contrast, mRNA of a photosynthetic gene of *Symbiodinium* was shown by Takishita et al. (Takishita et al. 2003a) to be present only in the plastid, and not in the cytoplasm (implying the minicircle DNAs are present in-, and transcribed in-, the plastid), and this distinction was clear on a single cell-section clearly labeled by immunogold so is unequivocal. Wang and Morse (2006b) have shown that the psbA gene of another peridinin dinoflagellate *Lingulodinium polyedra* is encoded by AT-rich DNA of between 50 – 100 kb, and they suggested that the location of this DNA molecule may be in the plastid. Thus, indications are that peridinin dinoflagellates transfer genes to the nucleus over evolutionary time, perhaps via transfer of the minicircles, but various peridinin dinoflagellates may differ in the degree to which this has occurred (Hackett et al. 2004a; Koumandou et al. 2004; Laatsch et al. 2004; Wang and Morse 2006b).

Secondly, the putative symbionts that are culturable from corals are not limited to *Symbiodinium*. This will become clear in chapter 6, in which Apicomplexa (Levine 1978) were cultured from corals. As introduced in section 1.7 of this chapter, the Apicomplexa (Figure 1.13) are a sister group to the peridinin dinoflagellates (Fast et al. 2001; Gajadhar et al. 1991; Wolters 1991). The remnant plastid in apicomplexans is called the ‘apicoplast’ because it is one of an apical complex of organelles (McFadden et al. 1997). The apicoplast bears a 35 kb genome containing functional genes (Foth and McFadden 2003; Ralph et al. 2004; Williamson et al. 1994). It is no longer photosynthetic but remains essential to the life
of the cell, due to its role in fatty acid metabolism (Gardner et al. 2002; Ralph et al. 2004). Algae in the red-derived chromalveolate lineage maintain secondary and tertiary plastids that possess chlorophyll $a$ and in many cases chlorophyll $c$ as well, whilst primary and secondary plastids in the green-derived lineages possess chlorophyll $a$ and chlorophyll $b$ (Bhattacharya et al. 2004; Durnford et al. 1999; Palmer and Delwiche 1998; Wolfe et al. 1994). Apicomplexans possess no chlorophyll at all. Accordingly there has been some debate in the literature about whether apicomplexans derived their apicoplast from the red or the green lineage (reviewed in Chapter 6), but this has resolved in favour of the red lineage based on molecular phylogenetic evidence (Fast et al. 2001; Zhang et al. 2000), and the resolution is parsimonious with the tree in Figure 1.13.

Ultrastructural evidence also supports the model that the apicoplast and the peridinin dinoflagellate plastids are sister organelles vertically inherited from a common ancestor (Fast et al. 2001; Zhang et al. 2000). Chiefly, the number of membranes bounding the plastid is similar, three membranes in peridinin dinoflagellates, and four membranes in apicomplexans (Cavalier-Smith 2000; Obornik et al. 2002b). Two other orders (arguably ‘classes’) exist that phylogenetically link the nuclear lineages of dinoflagellates and apicomplexans. Curiously, neither is photosynthetic. These are the perkinsids and the colpodellids (Adl et al. 2005), which emerged prior to the dinoflagellate and apicomplexan clades respectively (see Figure 6.16, chapter 6).

The lifestyle of colpodellids is to siphon the contents from protistan prey (Brugerolle 2002a; Cavalier-Smith and Chao 2004; Simpson and Patterson 1996), a process known as myzocytosis. As regards nutrition, the lifestyle of genus *Perkinsus* contrasts with that of colpodellids, since it is parasitic on multicellular animals, resembling the lifestyle of apicomplexans (Perkins 1996). However, at least two other perkinsids are predators of unicells (Brugerolle 2002b) (Noren et al. 1999), just as colpodellids are. This predatory ability is also shared by numerous dinoflagellates (Noren et al. 1999), and on that account a systematic grouping, the Myzoozoa, was instated (Cavalier-Smith and Chao 2004). Myzoozoa includes all dinoflagellates, perkinsids, *Oxyrrhis*, colpodellids and euapicomplexans. The taxon Myzoozoa has been ignored in a recently published system for nomenclature of protozoa (Adl et al. 2005), but this thesis decisively finds in favour of the reality of the concept of Myzoozoa (chapters 6 and 7), and therefore the term should be accepted by the protist community.
One problem with the acceptance of the term Myzooza until now, has been an apparent contradiction when compared against the same author’s concept of Chroma-lveolata (Cavalier-Smith and Chao 2004; Cavalier-Smith 1999). Could the ancestral myzoozan have really been a siphon feeder, wouldn’t that imply it had no plastid? This thesis finds in favour of the idea that the ancestral myzoozan may have been “mixotrophic” (hereditary phototrophy supplemented with occasional predation). It is widely taken for granted that the plastid group consisting of the apicoplasts and the peridinin dinoflagellate plastids is monophyletic (Fast et al. 2001). Accordingly, the basal relationship of Perkinsids to peridinin dinoflagellates (Goggin and Barker 1993; Leander and Keeling 2004; Reece et al. 1997; Saldarriaga et al. 2003b; Siddall et al. 1997), and the basal relationship of colpodellids to apicomplexans (Kuvardina et al. 2002; Leander and Keeling 2004; Leander et al. 2003), imply these two basal lineages may have lost photosynthesis secondarily. The close ultrastructural, functional, and phylogenetic relationship between the plastids of apicomplexans and peridinin dinoflagellates, is therefore not contradicted by the existence of the heterotrophic Perkinsids and colpodellids. Nor is the concept of Chroma-lveolata contradicted by that of Myzooza.
Figure 1.13 The evolutionary tree of protistan algae and their plastids. Lineages represent major divisions, except Dinophysis, Kryptoperidinium, Lepidodinium and Paulinella which are single genera (Keeling 2004). In primary (1°) endosymbiosis events a cyanobacterium is engulfed and becomes a heritable plastid. In secondary (2°) and tertiary (3°) endosymbiosis events, the pre-existing plastid is usually replaced by the incoming plastid, except in Kryptoperidinium in which the pre-existing plastid became an eyespot (Dodge 1969), the latter derived organelle not known to possess a genome. Where present, nuclei remnant from the engulfed alga (Archibald et al. 2001; Keeling et al. 1999; Ludwig and Gibbs 1989; Van der Auwera et al. 1998) have become nucleomorphs (shown within the endosymbiont membrane in Cryptophytes, Chlorarachniophytes and Kryptoperidinium) with the caveat that the nucleomorph of Kryptoperidinium is perhaps best described as a second nucleus, since that guest retains sexual ability and thus a degree of self-determination (Chesnick and Cox 1987; Chesnick et al. 1997; Chesnick et al. 1996; McEwan and Keeling 2004). Colours of eukaryotic host cells represent nuclear monophilies (green Archaeplastida; purple euglenid; yellow Cercozoa; blue chromalveolates) but note that Dinophysis, fucoxanthin dinoflagellates, Kryptoperidinium, and Lepidodinium are all derived from the peridinin dinoflagellate nuclear lineage. The correct terminology for the event that yielded Lepidodinium is a ‘serial secondary endosymbiosis’
(Keeling 2004). Phylogenetic relationships among the nuclear lineages; Archaeplastida, Euglenozoa, Cercozoa, and Chromalveolata are regarded as unknown, in relevant literature, i.e. each is a separate ‘kingdom’. The figure is from: (Keeling 2004).

2.6 Models of plastid macroevolution involving portable plastids and replaceable plastids

A published model competing against the tentatively established monophyly of the chromalveolates (Cavalier-Smith 1999; Harper and Keeling 2003; Takishita and Uchida 1999a; Yoon et al. 2002b), is based on the observation that secondary plastids of the red lineage have greater gene content (on the plastid genome) than green plastids do, and that the plastid was therefore more “portable” over evolutionary time (Grzebyk et al. 2003). The authors postulated, and indeed showed, that plastids of the red algal lineage contain “a set of genes that potentially confer more capacity to autonomously express proteins regulating oxygenic photosynthetic and energy transduction pathways” than the green lineage (Grzebyk et al. 2003). This was a qualitative point that implicitly assumed that some proteins which are imported into the red-lineage plastids of cryptophytes, haptophytes and stramenopiles, were already coded in the nucleus before ingress of the portable red plastids, and had to have come originally from another plastid that predated these endosymbioses. The source of the predating plastid genes was not discussed.

The portable plastid hypothesis was not originally developed as an alternative to the chromalveolate hypothesis, though it was later claimed to be so (Falkowski et al. 2004; Grzebyk et al. 2004), rather it was originally intended to address why red-line protistan algae (which dominate in today’s pelagic food webs) seem to be more adaptable than green-line protistan algae (Grzebyk et al. 2003). To be of value in determining whether or not today’s chromalveolate plastid endosymbioses are monophyletic for the plastid gain event, the hypothesis would need to address whether a set of eukaryotic hosts had developed which already had red plastid genes transferred to their nuclei, and so were pre-adapted hosts for accepting secondary symbioses of the red-line. Circular logic is apparent in any attempt to use the “portable plastid” hypothesis to invoke polyphyly of chromalveolate plastid gains, because the cohort of the pre-adapted nuclear line would most parsimoniously have been related to each other, and therefore the cohort is effectively the chromalveolate lineage anyway (Keeling et al. 2004). That there was a cohort of pre-adapted nuclear lines at all, gains support from the timing of the origins of the cryptophyte, haptophyte, stramenopile, and alveolate groups, which in each case followed the end Permian extinction, as Grzebyk et
al. (2003) pointed out. This coincidence of timing invites the systematics of parsimony with regard to plastid gain, rather than the systematics of ‘splitting’ (independent gains).

In contrast to the concept of a portable plastid, the plastid of peridinin dinoflagellates has been easily ‘replaceable’ over evolutionary time (Figure 1.13). A simplistic explanation might be that because many genes were transferred from the peridinin plastid to the peridinin nucleus, the location of the genes might allow them to be reused to target to any new plastid. Cavalier-Smith (2003a) offered a slightly different explanation. Surprisingly, replaceability of the peridinin plastid was not attributed to the nuclear location of plastid genes, but rather to the adaptability of the plastid targeting machinery for these nuclear-encoded proteins in dinoflagellates (Cavalier-Smith 2003a). Two studies (Patron et al. 2006; Yoon et al. 2005), of the genetic impact of the replacement of a peridinin plastid by a tertiary plastid, have gathered data that can be used to address Cavalier-Smith’s (2003a) concept that plastid-protein targeting is adaptable in the dinoflagellate lineage. One of the studies found that some plastid proteins from the peridinin dinoflagellate nucleus had been reused and targeted to a haptophyte-derived tertiary plastid (Patron et al. 2006), but in the other study it was reported that very few plastid proteins from the peridinin dinoflagellate nucleus had been reused and targeted to a haptophyte-derived tertiary plastid in Karenia brevis (Yoon et al. 2005). On the basis of these data, the reusability of peridinin plastid-targetted genes that reside in the nucleus may not be a large factor affecting the replaceability of the peridinin plastid. As noted by Patron et al. (2006), another factor affecting the success of the Karlodinium micrum tertiary endosymbiosis (haptophyte adopted by a dinoflagellate) is that there appears to have been efficient transfer of genes from the nucleus of the donor to the nucleus of the acceptor (Patron et al. 2006). This efficiency of transfer implies that nuclear recombinogenicity is one of the defining features which separates dinoflagellates from other protistan algae in terms of relative ability to accept tertiary plastids (see chapter 7, section 2.6).

The ‘portable plastid hypothesis’ states that plastid gene ‘losses’ (translocations to the nucleus) reduce a plastid’s portability for subsequent symbiotic associations. In the dinoflagellate case, an extremely reduced plastid genome is not very portable, but ironically is extremely replaceable. By contrast, replaceability is not a character possessed by the reduced plastid genomes of the green lineage. The distinction by Grzebyk et al. (2003) of an ‘enslaved’ (green) plastid would seem to apply to the peridinin plastid as well as to the green plastids, in fact moreso, because there are four cases of portability of a green plastid, but no cases of portability of the peridinin plastid. To keep score then: rhodophyte plastid-
extremely portable; chromist and green plastids- quite portable; alveolate plastids- not at all portable. Indeed a correlation does therefore exist between portability and genome reduction. Similarly to keep score on the count of replaceable, it is apparent that replaceability is not an adjective that should be applied at all to a plastid, rather it invokes the qualities of a host- its ability to adopt new genes, and sometimes its prior possession of another plastid. Host adaptability: high in chromalveolates, moderate in Cercozoa and Euglenozoa, absent in Archaeplastida.

Whether the plastid genome of Symbiodinium is reduced (and fragmented) similarly to those of other peridinin dinoflagellates is a question raised and partially addressed in this thesis, in the context of the search for a plastid DNA marker for Symbiodinium populations. During the process of this thesis research, two other laboratories have also used single-gene sequences from the Symbiodinium plastid as phylogenetic markers (Santos et al. 2002b; Takishita et al. 2003a). In an analysis of intra-Suessiales phylogeny using a plastid-encoded gene, Takishita et al. (2003a) considered it necessary to empirically address an evolutionary issue, “where is the plastid gene encoded?” While the answer was ‘in the plastid’ for genus Symbiodinium, the conclusion may have been different in a distantly related lineage of peridinin dinoflagellate, Ceratium in which that gene may be nucleus encoded (Laatsch et al. 2004). Broad coverage has been provided here (chapter 1) concerning the evolution of plastid genomes, to allow correct assessment of location of genes used in the current study. The new data of this study are integrated into models of the evolution of the algal symbionts of corals, in chapters 6 and 7.

2.7 Synopsis
Documented in the main three results chapters of this thesis, are aspects of research aimed at the goal represented in Figure 1.12:

chapter 3 – culturing to obtain pure material intended for genetic marker development;
chapter 4 – genetic marker development, from organellar DNA and nuclear DNA;
chapter 5 – genetic marker application to a Symbiodinium population.

In the remaining results chapter - chapter 6, an unexpected offshoot of the work is documented, which was the detection of two new taxa of putative symbionts (not Symbiodinium) while culturing.
In Chapter 7, ways to define ‘species’ in Symbiodinium and other alveolates, are discussed, because of relevance to the question of adaptability of Symbiodinium in an era of global warming. The phylogeny and evolution of dinoflagellates is also addressed in chapter 7, in order to provide a framework for comparative studies between Symbiodinium and other alveolates and to contribute to that larger literature.

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Chapter 2

General Materials and Methods

To address the question of sexual recombination in *Symbiodinium*, organisms were cultured *de novo*, and the set of cultures was augmented with extant cultures purchased from the CSIRO (http://www.marine.csiro.au/microalgae/supply.html). PCR markers were subsequently developed from the cultures and used in analysis of *in hospite* zooxanthellae.

This chapter has aims to:

- **a)** Supply the rationale for choice of model hosts
- **b)** Document methods of sample collection and DNA extraction
- **c)** Describe PCR, sequencing, editing, and phylogenetic methods

A flowchart of the general experimental procedure that was used is:

1. Obtain and/or make *Symbiodinium* cultures, observe and document these
2. Extract DNA and begin PCR with broad-specificity primers
3. Sequence amplicons, and BLAST sequences against Genbank database
4. Demonstrate phylogenetically that the markers are specific to *Symbiodinium*
5. Design unique primers based on sequences that were obtained from cultures
6. Use the primers to amplify markers from *in hospite* zooxanthellae
7. Sequence the markers from a population, to assess potential history of recombination
1. Field site and geographic region in context

The field site chosen for the study was One Tree Island (OTI) reef, which is a research zone maintained by The University of Sydney. OTI reef is located off Gladstone at the southern end of the Great Barrier Reef (GBR), in Queensland Australia (Figure 1.1).

Figure 2.1 Position of One Tree Island (OTI) in the Great Barrier Reef. Map source: www.reef.hq.com.au. The inset shows the Capricorn-Bunker group of Islands and is adapted from; (Kingsford 2001)
2. Host choice

In selecting host-symbiont assemblages (together the “holobiont”) were chosen based on preliminary large subunit (lsh) ribosomal DNA (rDNA) phylogeny of the symbionts that indicated they were candidates for having sexual recombination histories. The hosts of these chosen Symbiodinium strains were the scleractinian corals Goniopora tenuidens, Heliofungia actiniformis and Leptastrea purpurea (Figure 1.2) from OTI. Terminology used is that Sym-Gt represents the symbiont of G. tenuidens, Sym-Ha represents the symbiont of H. actiniformis, etc. These three Symbiodinium strains were closely related to one another: Sym-Ha and Sym-Lp are identical along ~600 bp of the nuclear lsh rDNA, covering the D1 region and part of D2; Sym-Ha and Sym-Gt are separated by only a single base in this ~600bp of lsh rDNA (Loi 1998).

![Goniopora tenuidens (Gt)](image)

![Heliofungia actiniformis (Ha)](image)

![Leptastrea purpurea (Lp)](image)

**Figure 2.2** Model Symbiodinium strains. Clade C Symbiodinium from three host coral species G. tenuidens, H. actiniformis, and L. purpurea were used as model organisms for population genetic marker development, and culturing. These symbionts are termed Sym-Gt, Sym-Ha, and Sym-Lp.

In addition, each of these symbiont rDNA genotypes was invariant within a host and they were basal to other members of the OTI clade C population (Figure 1.3). It was reasoned that their rDNA homogeneity might be constrained in variation due to recombination at
the rDNA operon, under the same logic used by Rodriguez-Lanetty (2003a) to propose biological species complexes, as described in chapter 1.

As a control for culturing studies, a fourth coral host *Pocillopora damicornis* of OTI was used as a source of *Symbiodinium*. *P. damicornis* had previously been used as a source of *Symbiodinium* for successful culturing by York (York 1986). The genotypes of its symbionts were all within clade C yet were unusually variable (Gaya 1999) compared to the symbionts of other hosts at OTI which are more restricted in diversity.

*G. tenuidens*, *L. purpurea* and *H. actiniformis* are all horizontal transmitters: zooxanthellae are acquired by their offspring via the water column (R. Babcock, CSIRO Marine Research pers. comm.; Selina Ward, University of Queensland; pers. comm. via Dee Carter University of Sydney). *H. actiniformis* can also transmit zooxanthellae through asexually brooded planulae. [R. Babcock, pers. comm., also: (Abe 1937 )]. Similarly, *P. damicornis* uses both transmission routes (Ward 1992). As part of this study it was of interest to choose horizontal transmitters to allow for the possibilities that the transmission routes of symbionts might be correlated with culturability, the ability to live freely, or the opportunity for recombination outside the host (Figure 1.3). The final coral used as a source of putative symbionts reported in this thesis was *Plesiastrea versipora*, which occurs naturally in Sydney Harbour, NSW (latitude 34° 0' S, longitude 151° 0' E) and so was a convenient source host. The *Symbiodinium* resident in this coral at this latitutde are of clade B (Rodriguez-Lanetty et al. 2001), and so are arguably of little relevance to the aims of this study, but were considered a challenge to culturing, as they are likely to be maternally transmitted, having been documented closely adhering to ova excised from a gravid parent (Withers 2000). Culturing of zooxanthellae from Tridacid clams of OTI (plus an anemone of unknown geographic origin) is detailed only in chapter 3, since their culture was not a main aim of the thesis, though it had been so in a much earlier draft of the aims.
**Figure 2.3** Preliminary 16S rDNA survey by Loi 1999, of zooxanthellae from selected scleractinian corals of OTI reef. Nucleotide distance neighbour-joining tree obtained using PHYLIP (Felsenstein, 1989; Cladistics 5, 164-166). Coloured labels denote zooxanthellae sequences from the selected host species: *H. actiniformis* (Ha), *L. purpurea* (Lp), *G. tenuidens* (Gt), *Stylophora pistillata* (Sp), *Seriatopora hystrix* (Sh), *Acropora millepora* (Am) and *Porites cylindrica* (Pc). Yellow boxes contain clusters of zooxanthellae (on relatively long phylogenetic branches) that are putatively under selection for asexual reproduction, perhaps due to maternal transmission in host ova. Blue boxes contain zooxanthellae that are putatively shared among hosts, and have a frequent free-living phase during which sexual reproduction might be possible. The phylogeny of the symbiont of *Pocillopora damicornis* is given in Appendix1.
3. DNA extraction

The *Symbiodinium* cell wall is difficult to penetrate by SDS (at 55°C), proteinase K (at 37°C) and phenol:chloroform (at room temperature) as evidenced by the frequent inability of this chemical combination to efficiently extract DNA for use as a PCR template (personal observations). Efficiency of lysis was judged by amount of DNA yield, and by the level of DNA degradation evidenced by smearing of genomic DNA when run on a 1% agarose 0.5X TBE gel (see electrophoresis, section 4). Observations of unacceptable DNA yield and quality led to the development, in this thesis, of a novel DNA extraction method for *Symbiodinium*. It is a high-yield protocol employing the positively charged detergent N-lauryl sarcosine (Sarkosyl), and is applicable equally to small amounts of cultured cells and large amounts of host tissue-slurry containing zooxanthellae.

Holobiont tissue stored at -20°C was pelleted by centrifugation at 12000 rpm, from 0.4 ml of slurry in a DMSO;EDTA;NaCl base preservative (Loh et al. 2001; Seutin et al. 1991), or from 0.4 ml of a 50% EtOH: 20% tissue: 30% seawater preservative. Cultured *Symbiodinium* cells were harvested at stationary phase, and stored in 70% EtOH, 30% sterile seawater at -20°C, until the day of extraction.

Stocks made prior to preparation of extraction buffer were 5M NaCl, 1M TrisHCl pH 7.7, 0.5M EDTA pH 8.0, 10% w/v SDS, and 10% w/v n-lauryl sarcosine ("sarcosyl"). NaCl, TrisHCl, EDTA disodium, SDS, and n-lauroyl sarcosine were each obtained from Sigma-Aldrich Corporation, Saint Louis Missouri. Extraction buffer (EB) was made freshly on each day that DNA extraction was done. EB consists of NaCl 0.5M, TrisHCl 0.1M, EDTA 50mM, SDS 1%, Sarcosyl 2%, made by diluting the stocks and combining them. Enough EB was made to allow 0.6 ml of EB per sample. The 0.6 ml of EB was placed into a 1.5-2.0 ml eppendorf tube, containing either a 100-200 µl pellet of holobiont tissue material (prepared as above) or a 20-200 µl pellet of cultured algal cells (from 0.2 -2ml of culture), pipetted up and down to break the pellet and then vortexed thoroughly to resuspend the pellet. The pipette tip used for assisted-resuspension was a 200 µl plugged pipette tip. Stock Proteinase-K was kept at a concentration of 10 mg/ml at -20°C. A heatblock or water bath was pre-heated to 55°C. 50 µl of Proteinase K stock was added to each tube of EB/cells mix, vortexed, and incubated overnight at 55°C.
The EB/cells/Proteinase K mixture was cooled to room temperature. An equal volume of Tris-HCl equilibrated phenol, pH 8.0 (Sigma-Aldrich Corporation, St Louis Missouri) was then added and vortexed well. After 5 minutes centrifugation at 12,000 rpm at 4°C, the aqueous (top) phase was decanted (using a pipette) to a sterile microfuge tube that had been labelled. Need for fresh tube applies also to the subsequent three steps. The aqueous phase was always at the top because DMSO or other amphiphilic solvents, had been removed in the pelleting of holobiont from preservative. Care was taken to avoid the interface between the two phases when harvesting the top phase. The extraction was repeated, again with phenol, pH 8.0, centrifuged and decanted as before. A third extraction was performed with phenol:chloroform:isoamyl-alcohol that had been pre-mixed in ratio 25:24:1 (Sigma, St Louis Missouri). A fourth extraction was performed with chloroform:isoamyl alcohol that had been premixed in ratio 24:1 (Sigma-Aldrich Corporation, St Louis Missouri).

DNA was precipitated by adding an equal volume of isopropanol, vortexing and then centrifuging at 12,000 rpm 4°C for 10 minutes. To wash the pellet, all the liquid was carefully discarded without disturbing the pellet, and 1ml of cold 70% ethanol (kept at -20°C) was added but not vortexed, then centrifuged for 5 minutes at 12,000 rpm and 4°C. To dry the washed pellet, all the liquid was removed using a pipette tip without disturbing the pellet. The last remnants of ethanol were extracted by brief centrifugation and then collection again by pipette. This eliminated the need for vacuum drying of pellets, as drying inhibits DNA resuspension. 100 μl of autoclaved mQ water was added to the DNA pellet and placed at 4°C O/N, or room temp. for 1 hour, to dissolve the DNA. The suspension was then vortexed and stored at -20°C.

4. PCR and Electrophoresis

Oligonucleotide primers were designed manually using DNA and/or protein alignments, and were synthesized by Sigma Genosys, Castle Hill NSW. PCR reactions were carried out using Amplitaq (Applied Biosystems, Foster City, California) or Expand Long Template PCR system (Roche Diagnostics Corporation, Indianapolis, Indiana), on a Perkin Elmer 2400 GeneAmp thermocycler (Perkin-Elmer, Shelton, Connecticut), using the cycling conditions detailed in each chapter. Electrophoresis was done in 0.5x TBE (45
mM TrisHCl, 45 mM boric acid, 1 mM EDTA) using apparatus from BioRad (Hercules, California). TrisHCl, boric acid and EDTA disodium salt were obtained from Sigma-Aldrich Corporation, Saint Louis Missouri.

5. PCR strategy to amplify genes from *Symbiodinium*

In order to amplify non-coding DNA from an organism in a targeted way, an approach that can be taken is to first amplify the conserved coding regions that flank the non-coding DNA, sequence them, and use the consequent knowledge of the sequenced coding regions to better enable targeting to the non-coding regions. This was the approach taken in this thesis. In the cases of the plastid minicircle *psbA* gene and the nuclear actin gene, degenerate primers were used. A single degenerate primer is actually a mix of tens of alternate primers, also called a degenerate set. Each of primers in the degenerate set differs from the other by a few base substitutions but all are of the same length and target the same stretch of template DNA. To perform degenerate PCR primers homologous to a conserved part of the gene were designed to amplify the coding region. When an amplicon representing a fragment of the gene was obtained, it was sequenced directly (as detailed in next section), the sequenced was used as a query against the NCBI genbank database to confirm gene identity, and then the sequence was used to design a more specific (less degenerate) pair of primers, so that PCR would not result in the amplification of non-specific ('background' or 'noise') bands. Such an approach often allows a final PCR product to be sequenced without electrophoretic separation of bands. However, prior to that, a fraction of all sequenced products was visualized on an electrophoresis gel and if only a single band was present, only then was the remainder purified of salts, nucleotides, primers, and enzymes (using a GFX kit, next section) before sequencing.

After a coding region had been obtained and sequenced, new primers were designed at positions in the gene that would allow non-coding regions to be amplified. In the case of *psbA* this was not trivial, as the gene contains no introns, and instead the felicitous occurrence of the gene on a unigenic minicircle allowed amplification of the entire non-coding region by pointing primers outward away from the gene but still towards each other (by virtue of the circular template), as shown in Figure 2.4.
Figure 2.4 Strategy for amplifying the non-coding region of the *psbA* minicircle from *Symbiodinium*. I. (a) Amplification of coding region, and subsequent amplification of non-coding region from available pure cultures of clade A and B *Symbiodinium* (CSIRO microalgal culture collection). (b) Resulting gels. II. (a) Amplification of non-coding region from *in hospite* clade C *zoanthellae*, using low degeneracy primers that had been designed based on the full-length clade A coding sequence obtained in I. (b) Resulting gels. Note: Gels are shown only to aid clarity. For a fuller explanation of the results portrayed on the gels see *psbA* sections of methods and results Chapter 4.
6. DNA sequencing, sequence editing and alignment

Amplicons were purified with a GFX PCR and gel band purification kit (Amersham Biosciences, Piscataway New Jersey), including cutting bands from the gel on rare occasions when more than one band was present per lane. All DNA sequencing was carried out commercially at the Australian Genome Research Facility, Brisbane. Sequencing was usually achieved by directly adding one PCR primer to a GFX purified PCR product, and PCR sequencing with Big Dye Terminator. Some DNAs detailed in chapter 6 were cloned using a TOPO TA Cloning Kit (Invitrogen Corporation, Carlsbad, California), and these were sequenced with commercial primers T3 and T7 that came with the TOPO TA Cloning Kit. Base calls in sequence chromatograms were edited using Chromas (Technelysium, Tewantin Queensland, http://www.technelysium.com.au), later re-edited using Sequencher (Gene Codes Corporation, Ann Arbor Michigan), and then sequences were aligned using ClustalX (Thompson et al. 1997). Editing of the alignments was done in Bioedit (Hall 1999), MacClade (Sinauer Associates Inc., Sunderland Massachusetts) and QuickAlign (Muller 2003).

7. BLAST queries and phylogenetic analysis

Preliminary taxonomic assignment and gene homology searches were performed using the BLAST program (Altschul et al. 1990) on the webserver of the US National Center for Biological Information (ncbi.nlm.nih.gov/BLAST).

Phylogenetic analysis was undertaken using Mr Bayes v3.0 or v3.1 (Huelsenbeck and Ronquist 2001). Full and partial gene sequences were combined in alignments, and analysed via the program Mr Bayes without truncation of any of the available sequences, as this program is not sensitive to end gaps, nor to the attraction of gapped taxa to other gapped taxa. This benefit is due to the fact that Mr Bayes does not consider gaps as characters, but equally does not ignore columns containing some gaps if a subset of taxa have a real character in that position and the if shared bases are informative within that subset or across subsets (Mr Bayes 3.1 manual and http://mrbayes.csit.fsu.edu/wiki/index.php/FAQ).
In Mr Bayes, analyses of DNA sequence alignments were done using the General Time Reversible (GTR) model of bases substitutions (Felsenstein 1981) with gamma (γ) correction for variable sites (Swolford et al. 1996) in eight variable categories and one invariant category. In some DNA analyses, a codon based model NY98 was additionally employed (Nielsen and Yang 1998), that weights phylogenies according to positively selected codons. Such instances are noted where they were used. Analyses of protein sequences were done using the WAG model of amino-acid substitutions (Whelan and Goldman 2001) with gamma correction of variable sites in eight variable categories and one invariant category (Swolford et al. 1996). Parameters specified within Mr Bayes for all DNA and protein analyses were: four markov chains (one hot), temperature 0.5, ten perturbations and a random starting tree, number of runs was one, and all other settings used were left at their default values for Mr Bayes v3.0 and v3.1. Output was sampled at every 1000th tree. The point at which burn-in occurred was judged in Microsoft Excel (Microsoft, Redmond Washington) by plotting the log likelihood against number of trees. Trees computed before the burn-in point were discarded and the consensus of the remaining trees was generated using the sumt command in Mr Bayes, and then viewed using Treeview (Page 1996). The sumt command in Mr Bayes was used to obtain the value of alpha for DNA and protein analyses, or to compute the number of positively selected codons in the case of the codon-triplet based DNA substitution model NY98.

TreePuzzle version 5.0 (Schmidt et al. 2002; Strimmer and vonHaeaseler 1997) was used in chapter 3 to perform likelihood mapping analyses, to test information content of an alignment of nuclear lsu rDNA sequences.

Phylogenetic analyses were performed on a Macintosh 16-cpu parallel cluster operated by the Roy J. Carver Centre for Comparative Genomics, University of Iowa USA, and running an ‘iNquiry Bioinformatics Portal’ environment (BioTeam, Cambridge Massachusetts).

8. Graphics, text and text-data manipulation

Diagrams were drawn using materials detailed in the respective figure legends and manipulated using Adobe software: Illustrator, Photoshop and Acrobat (Adobe Systems
Incorporated, San Jose California). Text and data manipulations were carried out in BBedit Lite v6.1 (Bare Bones Software, Bedford Massachusetts) and Microsoft Word (Microsoft, Redmond Washington). Programs in sections 5 to 7 were installed and run either on a Macintosh Powerbook G4 supplied by Dr. John Logsdon Jr., University of Iowa, or on a Pentium V PC supplied by Dr. Dee Carter University of Sydney

References

Abe N (1937) Post-larval development of the coral *Fungia actiniformis* var. *palauensis* Doderlien. Palao Trop Biol Stat Stud 1


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Chapter 3

Culturing of *Symbiodinium* from corals of the Great Barrier Reef

The development of molecular markers is hampered by the difficulty of obtaining pure *Symbiodinium* DNA that is not contaminated by DNA of the coral host, or DNA of resident or trapped microorganisms. The initial step in the current thesis study was therefore to purchase pure *Symbiodinium* cultures from a public collection. The second step was to augment this set of cultures by:

A) adapting existing methods and developing new methods to culture *Symbiodinium* from scleractinian corals.

B) genotyping the *Symbiodinium* cultures produced in (A)

1. Introduction

1.1 The challenges and obstacles to culturing representative *Symbiodinium*

*Symbiodinium* are the dominant genus of symbiotic algae in corals and are the keystone primary producer of coral reefs (Baker 2003). The *Symbiodinium* community has an ongoing challenge to generate relevant cultures for studying the interactive physiology of cnidarian hosts and their symbionts. Of particular use for symbiont speciation and host-specificity studies would be an understanding of the lifecycle of *Symbiodinium*, with emphasis on (a) whether the vast majority of *Symbiodinium* strains have a free-living stage in their lifecycle outside the metazoan host, (b) whether all *Symbiodinium* are sexual, (c) whether sex between *Symbiodinium* occurs in the water or in the host, and (d) whether symbiont recombination adapts them to new hosts.

To obtain ecologically relevant cultures, ideally one would first assess the range of symbiont genotypes that occur in each host, by detecting all of numerically major (dominant) and minor symbionts that live in each host, using a methodology such as
quantitative PCR and genotyping. Minor symbionts, by definition, may be outnumbered by major symbionts. Symbiont-host specificity is evident in many cases (Baker 2003), and so the ratio of minor to major symbionts in a host can be expected to tend towards infinite rarity. This expectation has led to the common research device: the default but potentially flawed assumption that the majority of hosts are effectively unialgal.

Assuming that the identities of major and minor symbionts are ascertainable, then the ability of each symbiont to adapt to the free-living aspect of their lifecycle might be questioned by the test of culturability, which might roughly correlate to ability for free-living, but this idea ignores unpredictable factors like culture-medium preference, temperature preference and light preference of each strain. In practice, only major symbionts are targeted for culturing, whilst minor symbionts are not targeted because a) they potentially have less ecological relevance, and b) the idea that minor symbionts are present in a single coral colony has only rarely been supported by hard evidence (Rowan and Knowlton 1995; Rowan et al. 1997; Santos et al. 2001). That is, the culturing of the major symbiont in any given host has been the objective in all published culturing attempts to date. The suitability of the culture medium for major symbionts has been assumed. Nowadays, molecular genotyping methods afford an opportunity to compare in hospite zooxanthellae with the cultures isolated from them, with the caveat that PCR can potentially bias what is amplified and thus detected.

Some symbiont strains, e.g. in coral hosts that pass zooxanthellae from host parent to host larvae directly, may be expected to engage in free-living less frequently than others, as a limitation on their natural lifecycle, and thus some strains should be more culturable than others. It follows that cultures of minor symbionts could result from attempts to culture major symbionts (Santos et al. 2001), if the major symbiont is well adapted to life in hospite rather than free living, and the minor symbiont is more adapted to free-living. The converse also applies: one reason why a symbiont with strong free-living ability might be minor in ratio within a host is that it might not be well adapted to the in hospite life.

In addition to the inherent presence of minor symbionts, a more diverse range of eukaryotes and prokaryotes can inhabit corals along with Symbiodinium (Knowlton and Rohwer 2003). These include endolithic algae, which are filamentous green algae living within the skeleton beneath coral tissue (Fine and Loya 2002), apicomplexans (Toller et al.
2002; Upton and Peters 1986), cyanobacteria (Lesser et al. 2004) and other bacteria (Knowlton and Rohwer 2003; Rohwer et al. 2001; Rohwer et al. 2002). Many of these could pose an obstruction to the de novo culturing of Symbiodinium, by competing as 'contaminants' in culture. Some of these are integral to holosymbioses. Endolithic algae, cyanobacteria and apicomplexans are found in corals at high frequency (Fine and Loya 2002; Lesser et al. 2004; Toller et al. 2002). Of these, the photosynthetic minor symbionts are likely to contribute to holobiont nitrogen and carbon metabolism (Fine and Loya 2002; Lesser et al. 2004).

1.2 A brief history of Symbiodinium culturing

Symbiodinium spp, at first classified as a symbiotic Gymnodiniales, were originally cultured by Kawaguti from Acropora corymbosa (Kawaguti 1944), as well as being earlier 'isolated' (but not 'grown') by Brandt from radiolarians (Brandt 1885). The free-living bivalveellate organisms were in neither case submitted to a collection. Kawaguti's zooxanthellae did divide in vitro and proliferated persistently because he provided them with enriched seawater medium (supplements were nitrate, phosphate and iron) and subcultured them every four to eight weeks (Kawaguti 1944). McLaughlin and Zahl subsequently expanded upon Kawaguti's success (Kevin et al. 1969; McLaughlin and Zahl 1957; McLaughlin and Zahl 1959; McLaughlin and Zahl 1962a; McLaughlin and Zahl 1962b; Zahl and McLaughlin 1957; Zahl and McLaughlin 1959), used more complex media (McLaughlin and Zahl 1959) and sourced Freshly Isolated Zooxanthellae (FIZ) from a wider range of hosts. This led to the establishment of the new genus Symbiodinium by Freudenthal (Freudenthal 1959; Freudenthal 1962) who used McLaughlin and Zahl's culture from the scyphozoan (jellyfish) Cassiopeia xamachana, as type material. Many labs subsequently achieved de novo culturing of Symbiodinium, using alterations of suitable seawater media (Ahles 1967a; Baillie et al. 2000; Baillie et al. 1998; Carlos et al. 1999; Chang and Trench 1982; Coffroth and Santos 1997; Colley and Trench 1983; Deane and O'Brien 1978; Kawaguti 1944; Polne-Fuller 1987; Polne-Fuller 1991; Provasoli 1968; Rogerson et al. 1989; Santos et al. 2001; Schoenberg and Trench 1980; Schoenberg and Trench 1980a; Trench and Thinh 1995). In each case, minimal medium with sparingly added supplements gave the desired results. Cultured members of the genus Symbiodinium are fully autotrophic. While the vitamins thiamine, biotin and cobalamin are usually included in dinoflagellate culture media, e.g. f2 (Guillard and Ryther 1962; Guillard and
Keller 1984) the absolute necessity of these vitamins for *Symbiodinium* viability is debatable, as Kawaguti did not include them in the growth medium (Kawaguti 1944).

Santos et al. (Santos et al. 2001), working with the cultures of Santos, Kinzie and Coffroth, showed that *Symbiodinium* cultures often do not represent the major symbiont of the host individual from which the culture was taken. The authors first showed that a host in the wild can house mixed populations, for example two clades can be present. Given an initially mixed-clade inoculum at the time of culturing, it was found that inadvertant purification of one clade over another could take place. Factors that contributed to a gradual change in clade mix over time included differential motility and phototaxis, leading to uneven distribution in a culture flask and subsequent founder effects on subculturing. The presence of antibiotics was suggested to favour the persistence of clade B over clade A in mixed cultures.

### 1.3 Culture collections, culture conditions and relevance to *in hospite* conditions

Many public collections of microalgae contain isolates of *Symbiodinium*, (e.g CCMP USA, CSIRO Australia, NQAIF Australia, CCAP UK, CCAC Germany). At the time of the experiments in this study, most cultures in public collections were of clade A, which could be because clade A *Symbiodinium* are more easily brought into culture compared to other clades, or could be historical in that the type culture of the genus was clade A. Clades B and F were the other represented *Symbiodinium* clades in the culture collections. The publicly accessible set of cultures was not representative of the ecologically relevant zooxanthellae, given that clade C are the dominant clade in corals of the Indo-Pacific (LaJeunesse et al. 2003). Ecologically relevant work with Pacific corals was therefore problematic using the then-available cultures. Strain CS-156 of the Australian CSIRO collection, also known as *Symbiodinium kawagutii* (Trench and Blank 1987) from the scleractinian coral host *Montipora verrucosa* (Kaneohe Bay Hawaii) was available, and had been assigned to clade C by Loh et al (Loh et al. 1998) based on 18S rDNA RFLP analysis (Rowan and Powers 1991; Rowan and Powers 1992). Carlos et al (Carlos et al. 1999; Carlos et al. 2000) and Baillie et al. (Baillie et al. 2000a; Belda-Baillie et al. 2002) after Loh, had also been using this culture as their type material for clade C genotyping. However, this assignment became outdated when LaJeunesse (LaJeunesse 2001) created the new clade F (a close sister to clade C), because *S. kawagutii* was assigned as the type
strain for clade F. Culturing of clade C *Symbiodinium* from Australian corals has been attempted (M. tenLohuis & D. Miller, James Cook University), but is unpublished so it is unclear whether the particular protocol used had not resulted in clade C being brought into culture. Thus at the beginning of this study, no public culture collection contained any clade C culture. T. LaJeunesse has since deposited a number of *Symbiodinium* strains into CCMP (Culture Collection of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, Maine), and this includes a clade C organism, *Symbiodinium goreau* from host anemone *Discosoma sancti-thomae* from Jamaican waters.

Ideally representatives of each clade should be present in public collections so that labs with the time and resources to perform comparative culturing experiments, such as tests of medium preference, may do so. Whilst it has only once been demonstrated that different *Symbiodinium* clades respond differently to different growth medium or additives (Santos et al. 2001), it is possible that such preferences across genera, clades and strains could form the basis of selective growth media. Little is known of the physiological condition inside the symbiosome space, which is the space formed between the hosts endosome membrane and the zooxanthellae cell wall, and across which all metabolic interaction between host and symbiont must take place (Grant et al. 2004; Rands et al. 1993; Trautman et al. 2002), but it is likely that the chemistry of this space varies across classes of metazoan hosts. Those *Symbiodinium* that live intracellularly inside cnidian cells experience a sodium shock upon leaving the host and entering seawater (Goiran et al. 1997), implying the symbiosome space is low in sodium, as is the algal cytosol (Goiran et al. 1997). Indeed, Freudenthal noted that *Symbiodinium* cells can survive in fresh water for an unexpectedly long period (Freudenthal 1959). The potential relevance of *in-vivo* conditions to the design of optimal *de novo* culture medium has not been tested.

1.4 *Symbiodinium* lifecycle in-vitro

The *Symbiodinium* lifecycle includes three diagnostic traits. Firstly, the division of the cell only happens while in a non-motile state (Crafts and Tuliszewski 1995; Deane et al. 1979; Fitt et al. 1981; Fitt and Trench 1983; Freudenthal 1959; Freudenthal 1962; Kevin et al. 1969; Lerch and Cook 1984; Loeblich and Sherley 1979; McLaughlin and Zahl 1957; McLaughlin and Zahl 1959; Schoenberg and Trench 1980b; Taylor 1983). Secondly, the lifecycle includes a characteristic motion (Freudenthal 1959; Kawaguti 1944; Loeblich and Sherley 1979; McLaughlin and Zahl 1957), which is the attachment of one flagellum to the
substrate, with consequent stirring of the water layer by the cell’s continuous unidirectional motion around that fixed point, presumably to accelerate gas exchange (Rosalind Hinde, University of Sydney, pers. comm.). The third prescriptive lifecycle trait is the presence of a single brown-ochre accumulation body within the cell at most phases of the lifecycle (Freudenthal 1959; Freudenthal 1962; Kevin et al. 1969; McLaughlin and Zahl 1959; Schoenberg and Trench 1980b; Trench and Blank 1987). As is the case for the motility traits described, the accumulation body is easily detectable with a light microscope under bright field conditions with an objective lens in the range 10x-40x.

An early assertion by Freudenthal that gametes of clade A are smaller than the adults, a phenomenon known as ‘anisogamy’, has been questioned by Blank (Blank 1987), even though Taylor (Taylor 1974) and Kevin et al. (Kevin et al. 1969) claimed, citing unpublished data, that they had repeated Freudenthal’s observation. The report of Kevin et al. (1969) involved a written description alluding to Freudenthal’s diagram (Freudenthal 1962) of multiple zoospores emerging from a cyst. In general there is a lack of hard evidence describing confirmed gametes of Symbiodinium, because no one has claimed to observe two motile individuals (gametes) fusing and resulting in a zygote. Freudenthal (Freudenthal 1959; Freudenthal 1962), as well as Taylor (Taylor 1974) and Kevin et al. (Kevin et al. 1969) hypothesised on the basis of repeated microscopic observations that intercompatible gametes of Symbiodinium may be of equal size (isogamous), and each of a diameter around half that of the parent cell. Among these three studies, only Freudenthal’s detailed the fate of the small cells. He observed that they were produced intermittently within a clonal culture, and on one occasion he observed two of these small cells becoming appressed but he noted that they did not fuse or develop further. Freudenthal attributed the instance of apparent aborted fusion to the union of genetically identical, thus incompatible, gametes of a putatively heterothallic (self-incompatible) strain.

Many dinoflagellate genera contain species with isogametes (Burkholder 2000; Elbrachter 2003; Silva and Faust 1995), and these may either be each smaller than adult zoospores, or they may each be of the same size as adult zoospores, thus indistinguishable microscopically from adults. In strains of Symbiodinium other than the Scyphozoan symbionts studied by Freudenthal, Kevin et al. and Taylor (1959, 1969, 1974), gametes might not have been noticed if they were morphologically identical to somatic cells, a
phenomenon called hologamy and known in dinoflagellates (Elbrachter 2003). This could explain why no other Symbiodinium culturists have reported observing gametes.

Exemplifying the phenomenon of hologamy, cells of the model haploid protist Chlamydomonas reinhardtii transmute into haploid gametes upon expressing new cell surface proteins but without altering cell size or shape, nor undergoing reductive division (Adair et al. 1980; Ferris and Goodenough 1997; Ferris et al. 1996). These gametes are of two mating types + and -, indistinguishable to the eye (Harris 2001). By analogy in Symbiodinium, cell size is not expected to be halved at gametogenesis, because ploidy is not halved. Rather the transmutation of Symbiodinium cells into gametes might perhaps be modelled by analogy to the hologamy of Chlamydomonas reinhardtii.

A way to overcome doubts about the details of the lifecycle is to make and observe fresh cultures of zooxanthellae, that have not had time to lose sexual competency in vitro, and have not been selected for clonality, which would preclude sexual activity if the strain happens to be heterothallic. Sexual potential has been found to be highly variable in cultures of microalgae on account of continual passive selection for cells that efficiently propagate asexually (Ursula Goodenough, University of Washington St Louis, pers. comm.). The way to overcome this limitation is to observe sexuality happening among some cells of a culture, isolate those cells from the rest of the culture and then continue this process of artificial selection until ‘supermating’ strains are obtained. Such methods were adopted by Chlamydomonas researchers in order to obtain the strains that are in use in the Chlamydomonas research community today (Goodenough, pers. comm.). For Symbiodinium, artificial selection for ‘supermaters’ has never been attempted, and research motivation to attempt it has been hampered by the lack of confirmed sightings of gametes, and complete absence of sightings of zygotes.

1.5 Symbiodinium lifecycle in hospite and in the environment
The lifecycle of Symbiodinium in hospite is as well characterised as it is in culture. The main observable difference is that flagella only rarely appear while the zooxanthellae reside in hospite (Kevin et al. 1969; Schoenberg and Trench 1980b). The obligate trait of division only while in the non-motile state, may be an adaptation to the in hospite state, as cultured cells throw off their flagellae at the slightest contact with viscosity (Freudenthal
1959; Taylor 1983). *In hospite* zooxanthellae undergo normal ecdysis with shedding of thecal plates (Wakefield et al. 2000). They bear thinner cell walls than when in culture (Fensome et al. 1993) and are maintained in G1 arrest by the host/s so as to control their numbers (Smith and Muscatine 1999). Passage though the digestive tracts of vertebrates (Augustine and Muller-Parker 1998) and invertebrates (G. Muller-Parker pers. comm.) that graze on corals and anemones has been proposed as a vector for the spread of zooxanthellae. Excretion through the digestive systems of cnidarian hosts themselves is another means of environmental replenishment, and has been proposed as a stimulant for zoospore formation (Steele 1977). Zooxanthellae survive in clam excreta (Trench et al. 1981), inside the digestive systems of brine shrimp (Fitt 1984), and of amoebae (Polne-Fuller 1991; Rogerson et al. 1989), all of which are potential vectors for the spread of *Symbiodinium* in the environment, though they can also survive in the marine environment even without a refuge or carrier (Taylor 1983). Their proliferation to large numbers in low salinity high temperature tide pools bearing no metazoan hosts (Taylor 1983), raises the question of how they obtained nutrients. Taylor’s is one of only a few reports of free-living *Symbiodinium* and the others did not report such large numbers (Carlos et al. 1999; Loeblich and Sherley 1979; Santos 2004b), so there was presumably an unusual nutritive environment in the tide pools examined by Taylor. Zooxanthellae do not reside on the mucosal surfaces of corals (Coles and Strathmann 1973), while other algae and protozoa do (Coles and Strathmann 1973).

Baillie et al. (1998) asked whether a zooxanthella sexual cycle could occur parallel with the alternating cycle of *in hospite* state to free-living state. While this has not been investigated it is a reasonable hypothesis in view of the life strategy of many other dinoflagellate genera, that involves germination/blooming at the time of nutrient availability, and sexual encystation at time of nutrient dearth (Burkholder 2000; Costas et al. 1993; Pfiester 1984; Pfiester 1989; Pfiester and Anderson 1987). In the case of *Symbiodinium* the analogy that can be proposed is that the *in hospite* state may represent the nutrient-rich state, and life in tropical seawater or sand the nutrient-deprived state (Baillie et al. 1998). The richness of fecal matter is a complicating factor, but the overall oligotrophy of tropical sediments (Birkeland 1997; Sorokin 1995) weighs in favour of the quick dissipation of fecal nutrients, accordingly zooxanthellae could indeed become nutrient deprived in the absence of a new host. If the extraction of FIZ and inoculation to minimally-enriched seawater were to generate gametes by default, then that could explain
why it is so difficult to culture zooxanthellae. As hosts are often unialgal for a single clonal strain of zooxanthella (Chapter 1), the production of gametes in culture and the terminal nature of a gamete’s development (Pfiester 1984; Pfiester 1989; Pfiester and Anderson 1987) could mean that the culture is non-viable unless a compatible mating type can be found.

1.6 Establishing a greater range of *Symbiodinium* cultures is desirable for studies of recombination

A reliable culturing method for the zooxanthellae of GBR corals, could in future facilitate analysis of isolates from many separate colonies of a single coral host at a single location, such as OTI. This would allow the analysis of recombination history in a set of interrelated but unialgal cultures. Using a culturing approach combined with analysis of molecular markers, Baillie et al. (Baillie et al. 2000a; Baillie et al. 1998) showed that recombination occurs within the clade A symbionts of clams. This was shown for multiple pure cultures derived from a single clam, and for multiple pure cultures derived from several separate clams. Similarly, though less directly, hypervariable DNA markers can be developed using cultures, and then population data regarding recombination history can subsequently be obtained directly from *in hospite* zooxanthellae of the same hosts at the same geographical location from which the cultures were derived. By the latter method, recombination was evidenced in clade B (Santos et al. 2003b). The available precedents therefore suggest that culturing is a highly desirable precursor to population recombination studies on this genus.

The recombination (sexual) potential of *Symbiodinium* clade C is of interest. The current global warming trend is placing selection pressure on zooxanthellae to adapt, and the presence or absence, and difficulty or ease, of sexual recombination may be a critical factor in such adaptation. Clade C was proposed by LaJeunesse (2001) to have a life history involving recombination, but empirical proof is lacking.

1.7 Chapter summary

In this chapter *Symbiodinium* cells were brought into culture by several methods, in some cases by development of an initial-growth medium that mimics the conditions present in host’s digestive cells or digestive organs. Genetic typing was performed and demonstrated that at least four novel strains within clades A and F have been cultured, as well as
organisms from the existing subclades/strains A2, F1, C1, B1, A3 and A5. Also documented in this chapter are instances of fusion between two motile zooxanthellae cells. The cells that fused were no smaller than the parent cells, arguing in favour of isogamy and a gametogenesis program that conserves adult size. The cells that were observed fusing (three instances), were all of clade A.

2. Materials and Methods

2.1 Choice of hosts
The rationale for choice of hosts *Goniopora tenuidens*, *Heliofungia actiniformis*, *Leptastrea purpurea*, and *Pocillopora damicornis* (Table 3.1) was as described in the General Materials and Methods chapter of this thesis. Additionally, the coral *Plesiastrea versipora* was chosen as it was locally available (Sydney Harbour), and contains zooxanthellae that associate with the maternal ova (Withers 2000). If the zooxanthellae of temperate *P. versipora* are maternally transmitted, then they may or may not live freely in the ocean, and may represent a challenge to culturing. Finally, two non-coral hosts (Table 3.1), a clam and an anemone, were chosen as sources of easily culturable zooxanthellae, to allow methods development and verification.
Table 3.1 Sources of zooxanthellae for this culturing study: dates obtained, numbers of host individuals used, and expected clade of symbiont.

<table>
<thead>
<tr>
<th>Host species (Order and Family)</th>
<th>Location and collector/s</th>
<th>No. of reps</th>
<th>Symbiont clade in hospite</th>
<th>Reference for in hospite clade designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heliofungia actiniformis (Scleractinia:Fungiidae)</td>
<td>OTI-i,ii</td>
<td>94+10</td>
<td>C</td>
<td>(Carter et al. 2000; Loh et al. 1998; Loi 1998)</td>
</tr>
<tr>
<td>Lepiastrea purpurea (Scleractinia:Faviidae)</td>
<td>OTI-ii,iii</td>
<td>15</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Goniopora tenuidens (Scleractinia:Poritidae)</td>
<td>OTI-ii</td>
<td>3</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Pocillopora damicornis (Scleractinia:Pocilloporidae)</td>
<td>OTI-iii</td>
<td>5</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Pliastraia versipora (Scleractinia:Faviidae)</td>
<td>Sydney Harbour-iv</td>
<td>10</td>
<td>B</td>
<td>(Rodriguez-Lanetey et al. 2001)</td>
</tr>
<tr>
<td>Tridacna maxima (Veneroida:Tridacnidae)</td>
<td>OTI-i</td>
<td>30+10</td>
<td>A</td>
<td>(Baillie et al. 2000b)</td>
</tr>
<tr>
<td>unidentified Aiptasia sp. Actinaria:Aiptasiidae</td>
<td>Sydney-Aquarium, Darling Harbour-i</td>
<td>3</td>
<td>B</td>
<td>(LaJeunesse 2001; Santos et al. 2002b)</td>
</tr>
</tbody>
</table>


2.2 Transport and storage of live host samples

Culturing from live hosts close to their place of habitat

In November 2000, zooxanthellae were harvested from ninety-four individuals of Heliofungia actiniformis at OTI Research Station. As well as being used for culturing on-site, these were also intended to serve for population genetics studies. Similarly, on this occasion culturing of zooxanthellae from thirty adult individuals of Tridacna maxima was also conducted at OTI Research Station. The large number taken was because the aims at the time included assessing population genetic history of Symbiodinium from Tridacnids, which subsequently proved unnecessary as it had been achieved by Baillie et al. (Baillie et al. 2000a; Baillie et al. 1998). However, the Tridacnids were useful practice for axiomising readily-culturable Symbiodinium strains. Three polyps of an anemone sp. resembling Aiptasia were collected from Sydney Aquarium, Darling Harbour (their ‘habitat’), where they were in November 2000 an aquarium pest of unknown geographic origin.
Culturing remote from natural habitat

In November 2000, ten juvenile individuals of *T. maxima* were transported live, via air courier at room temperature, to Sydney Aquarium, Darling Harbour, where they were maintained alive, in case culturing attempts from adult Tridacnids at OTI should fail. Owing to fungal contamination of the thirty agar cultures from adult Tridacnids, the ten juvenile Tridacnids maintained alive did become useful starting material for culturing and were retrieved from Sydney Aquarium after two months.

In July 2001 ten more individuals of *H. actiniformis* were collected and were shipped in the unpressurised cargo hold of a commercial passenger flight from Gladstone Qld to Sydney NSW. These were wrapped in seawater-soaked paper towel inside a tightly-lidded foam box, during the flight and during the two boat-trips to the airport (from OTI) and two car rides. Three individuals of *Goniopora tenuidens* from OTI were transported by the standard mode. Ten individuals of *Leptastrea purpurea* were collected from OTI, and transported by the standard mode.

Five more individuals of *L. purpurea* and five individuals of *Pocillopora damicornis* were transported in November 2001 by car from Gladstone Qld to Sydney NSW. The corals were wrapped in seawater-soaked paper towel and carried in a lidded foam box. Ten individuals of *Plesiastrea versipora* were collected in Sydney Harbour, and transported without dessication to a 20°C holding tank at the University of Sydney. No corals were kept at the Sydney Aquarium, Darling Harbour, because of the possibility that the zooxanthellae population in hosts might change through altered environmental conditions, or through contact with other zooxanthellae in the public reef aquarium water.

2.3 Storage of live corals prior to harvesting zooxanthellae

Upon arrival in Sydney most of the tropical corals i.e. all of the *H. actiniformis*, *L. purpurea* and *G. tenuidens* (abbreviated *Ha, Lp* and *Pd* respectively) were placed directly into unsterilised Sydney seawater (pewarmed to 27°C) in a 27°C light room with L:D period of 14h:10h. In some cases 4 to 5 coral nubbins were stored in this light room in a 30 litre seawater tank with a high surface area to depth ratio, and oxygenation provided by an airstone for a few days. In other cases 3 to 23 corals were stored for a few weeks in this light room in a 100 litre tank with the water filtered and recirculated briskly (~1200 litres
per hour) by an aquarium powerhead (immersible, sterilised). Filter outflow was directed at the water line, where oxygen transfer occurs. Oxygenation of the water to near maximum saturation (at the ambient temperature used) was assumed.

### 2.4 Extraction of Freshly Isolated Zooxanthellae (FlZ) from hosts

**Existing methods**

**Airpicking:** At a convenient harvest time as close as possible to the collection date, tissue from coral skeletons was removed by airpick into a strong plastic bag (method of W. Loh, University of Queensland, pers. comm.). The airpick consisted of a clean pipette tip pushed onto the end of a triggered airgun, attached either to a scuba tank in the field or to a pressurised air outlet in the Sydney lab.

**Mincing and tissue maceration:** Clam mantle slivers of ~1cm x 3cm, were minced into pieces of ~1mm x 1mm using a one-sided razor on a support consisting of a new piece of clean rigid plastic for each sample. The razor was immersed in 70% ethanol between samples.

**Partial decay method for coral holobionts:** A protocol for culturing zooxanthellae was obtained from the laboratory of Dr David Miller, communicated through Dr Madeleine van Oppen. It involved placing coral nubbins into sterile seawater plus antibiotics in sealed 15ml falcon tubes for 12 to 36 hours before shaking the tube by hand to decay host cells and thus loosen symbiont cells for easier release. This protocol was the basis for developing the shaking method to release zooxanthellae from coral cells and symbiosomes, that is detailed below. The 12 to 36 hour decay step of Miller was also varied to include extra additives in addition to antibiotics.

**Modifications for this study**

**HOCl (hypochlorite) pre-treatment:** Hypothetical contaminants such as non-symbiotic algae were stripped from host surfaces, by stringently pretreating with hypochlorite. It was noted that this eliminated ciliates that otherwise consume Symbiodinium. Hypochlorite used was the same as detailed in culture media. The method of surface 'sterilising' corals, was adapted from that of R. H. York Jr. (York 1986). The York protocol entailed exposing
each coral nubbin to dilute (1:10,000 parts) household bleach (active ingredient sodium hypochlorite) for 1 hour. The York protocol was modified in this study to minimize the risk of the high pH harming zooxanthellae, as household bleach contains NaOH. Household bleach (‘White King’ brand, manufacturer Sara Lee, Clayton South, Victoria Australia) was neutralised to pH 7.4 by adding HCl. This procedure also precipitated a detergent present in the bleach. After settling, the supernatant fraction was largely free of detergent, and was carefully removed for use in treating corals. This hypochlorite stock was diluted to pretreat corals before FIZ extraction. A range of hypochlorite dilutions was used in successive instances of coral pretreatment and zooxanthellae culture.

Neither the anemone nor the clam tissue samples were subjected to hypochlorite pretreatment. The majority of coral host surfaces used were subjected to HOCI pretreatment. Among the exceptions was the following control experiment. Each of ten Ha nubbins was broken into two pieces before use, and half was bleach treated (1:10,000 dilution of HOCI stock) while the other half was not bleach treated. Bleached Ha samples were labelled H1 to H10, unbleached Ha samples were labelled H’1 – H’10.

FIZ harvesting: About 5ml of freshly harvested coral-zooxanthellae-bacteria slurry (~50% tissue, 50% seawater) was agitated via vigorous hand shaking in a capped 15ml polystyrene tube for ~2 minutes, followed by centrifugation at ~300 rpm for three minutes and discard of the supernatant, leaving a brown-coloured zooxanthellae pellet. The supernatant was cream coloured and appeared to contain many bacteria. Similarly, the top layer of the zooxanthella pellet was cream coloured and was assumed to also be enriched in bacteria. To remove these bacteria the tube was gently inverted ~20 times without disturbing the pellet, until most of the cream coloured layer was in suspension which was then discarded. The pellet was resuspended in seawater that contained 1x-2x antibiotics (ampicillin and streptomycin at 50-100μg/ml each final conc.). The entire process of centrifuging, purging of bacteria, replacement of seawater-AmpStrep and shaking by hand, was repeated ~8 times to yield a dark coloured zooxanthellae pellet. The vast majority of the coral cells and bacterial cells appeared to be eliminated by this process as evidenced by increasing dark colour after each wash. Washed cells were resuspended in seawater-AmpStrep for serial dilution into various growth media.
The FIZ used to generate cultures BPdN1 and BPdN4 were obtained by natural bleaching of the nubbins during transport. The bleachate (algal cell mass) was collected immediately on arrival by immersing nubbins in a bag of seawater containing dilute HOCl (1:100), and leaving the zooxanthellae to settle overnight. The seawater+HOCl was replaced and the cells left for another night before extracting the zooxanthellae from sloughed coral cells the next day by the shaking method outlined above.

2.5 Culture media

Definitions of media used
The novel medium fEs used in the current study was devised based on existing media (Guillard and Ryther 1962; Guillard and Keller 1984). Medium fEs was prepared using the following basic procedure: unautoclaved seawater was mixed with all pre-autoclaved stocks (Table 3.2), other than NaH₂PO₄.2H₂O, and the mix was autoclaved for 20 minutes at 121°C. NaH₂PO₄.2H₂O was autoclaved in a separate bottle, before 0.5ml of it was added to autoclaved medium, while both were still hot. All individual reagents other than seawater, were autoclaved after each opening and then stored with lid screed on tightly until next use.

Antibiotics were used in growth media at most times. These were Ampicillin sodium (final concentration 50 μg/ml in growth medium) and Streptomycin sulfate (final concentration 50 μg/ml in growth medium). Zooxanthellae from the clam T. maxima were isolated on f/2 agar with antifungal agent Amphotericin B included at 25 μg/ml final concentration.

HOCl was added to some culture media when isolating zooxanthellae from coral hosts. HOCl stock was prepared as in section 2.4 (‘Extraction of freshly isolated zooxanthellae’).
Table 3.2 Composition of growth media

<table>
<thead>
<tr>
<th>Composition</th>
<th>f/2-CSIRO</th>
<th>fEs</th>
<th>NaK-fEs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seawater (SW)</td>
<td>1 litre</td>
<td>1 litre</td>
<td>0.5 litre</td>
</tr>
<tr>
<td>mQ water</td>
<td>-</td>
<td>-</td>
<td>0.5 litre</td>
</tr>
<tr>
<td>Stocks:</td>
<td>Composition of stock (grams/litre)</td>
<td>Amount of stock* per litre of final medium</td>
<td></td>
</tr>
<tr>
<td>Trace metals stock;</td>
<td></td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>20 x 10⁻³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>44 x 10⁻³</td>
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<td></td>
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<tr>
<td>CoCl₂·6H₂O</td>
<td>22 x 10⁻³</td>
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<td></td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>360 x 10⁻³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaMoO₄·2H₂O</td>
<td>13 x 10⁻³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>63</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Na₂SiO₃·5H₂O</td>
<td>22.7</td>
<td>0.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>Fe citrate;</td>
<td></td>
<td>0.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>Fe citrate</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe EDTA</td>
<td>23</td>
<td>-</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Vitamins;</td>
<td></td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>2.0 x 10⁻³</td>
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<td></td>
</tr>
<tr>
<td>Biotin</td>
<td>1.0 x 10⁻⁵</td>
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<td></td>
</tr>
<tr>
<td>Cobalamin</td>
<td>1.0 x 10⁻³</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3 Composite media and nomenclature

<table>
<thead>
<tr>
<th>Medium name</th>
<th>Base is fEs</th>
<th>Base is NaK variant of fEs</th>
<th>Ratio of HOCI stock to base</th>
<th>[PMA] in medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>fEs-PMA</td>
<td>yes</td>
<td>-</td>
<td>-</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td>fEs-HOCI</td>
<td>yes</td>
<td>-</td>
<td>1:10 to 1:250</td>
<td>-</td>
</tr>
<tr>
<td>NaK-fEs-PMA</td>
<td>-</td>
<td>yes</td>
<td>-</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td>NaK-fEs-HOCI</td>
<td>-</td>
<td>yes</td>
<td>1:100</td>
<td>-</td>
</tr>
</tbody>
</table>

2.6 Properties of each medium

b) fEs

Medium fEs (Table 3.2) was devised to maximise the potential for culturing autotrophic, carbon-walled microalgae without competition from other algae and other microbes. It is selective for microalgal growth over bacterial and fungal growth because it does not contain a carbon source. i.e. citrate was avoided as the Fe ion chelation agent, because of
its potential to serve as a carbon source for bacteria. Medium fEs also selects against diatom growth because it does not include silicate, a necessary constituent of the cell wall architecture of some diatoms. Medium fEs thus resembles medium f2-Si (Guillard 1975; Guillard and Keller 1984). Iron is a limiting reagent in the growth of marine algae (Geider and Laroche 1994), so more iron was added in the current study than is used in f2-Si (Guillard and Keller 1984). As a corollary, it was considered important in this study that the chelation of the iron be complete because unchelated iron is toxic to all organisms, including microalgae (Barros et al. 2003).

**ii) NaK-fEs**

The rationale for using potassium chloride enriched seawater as a treatment was the same rationale as that employed by Freudenthal (Freudenthal, 1959) who experimented with *Symbiodinium* tolerance to chemistry that mimicked the high K, low Na, present inside animal cytoplasms. There is currently no evidence in the literature for the ratio of the Na:K balance in the symbiosome space. The high K concentration in the cytoplasms of all animals, does not necessarily hold in the symbiosome space, but it was of interest to test *de novo* culturing potential of this medium.

**iii) fEs-HOCl growth medium**

Washed FIZ from *H. actiniformis* colonies H1 to H10 inclusive and *L. purpurea* colonies L1 to L10 inclusive (Table 3.1, set ii) were each inoculated into fEs containing 1 volume of neutralized hypochlorite (HOCl, section 2.4) per 10 volumes growth medium (shorthand notation 1:10). This was done to test whether the tolerance of zooxanthellae to chlorinating agents was being underestimated, and therefore whether host nubbins could tolerate a far more rigorous treatment during the host surface cleaning step of the FIZ extraction, than was used by York (York 1986). *Symbiodinium* that had been extracted from host *L. purpurea* were successfully cultured *de novo* in 1:10 HOCl (L4 cultures in Table 3.7, cultured in one well of a 96-well culture plate), therefore this additive was subsequently used again, this time to culture the FIZ of Lp11 to Lp15 inclusive. For the latter samples HOCl was used at a concentration of 1:250, which was chosen as a moderate level, based on crude experiments that established relative growth rates of previously cultured *Symbiodinium* (cultures G1 and L4, Table 3.7) in a range of HOCl concentrations (data not shown). In all these experiments in which HOCl was added to fEs, it was assumed that the
exposure of zooxanthellae to HOCl and other potential constituent oxohalides was temporary because Cl₂ gas readily evaporates from aqueous ·OCl or HOCl.

iv) fEs-PMA
To improve the chances of successfully bringing reticent Symbiodinium into culture, an alternate growth medium for Symbiodinium was tried containing Phorbol 12-Myristate 13-Acetate (PMA, Sigma). It had been found by Costas et al. (1993) that mammalian growth factors, and the artificial growth factor Phorbol 12-monoacetate (a phorbol ester), can increase Amphidinium growth rates in f/2 medium by several fold, as can fetal calf serum (an abundant source of mammalian growth factors). PMA was chosen over calf serum for this study, because PMA is a lesser nutrient, and would have less ability to promote bacterial growth. Purified natural growth factors were avoided because of expense, and because they could be equally well mimicked by phorbol esters which have a similarly high level of growth promoting activity for Amphidinium as did Platelet Derived Growth Factor (Costas et al. 1993).

v) NaK-fEs-PMA, and vi) NaK-fEs-HOCl
These composite media are variants of NaK, and also contained PMA or HOCl (additives itemised above).

2.7 Growth conditions
Light levels at the location of the cultures in the 27°C light room was 5-10 µE m⁻² s⁻¹, on a 14/10h day night cycle. The temperature in the light room was stable at 27°C except for summer 2001-2002 when the temperature on a dozen occasions went to 32°C in the daytime and back down to 27-28°C at night. Cultures Tm4.1, Tm8.2, Tm10.2, L4, L9, G1, G2, H9 and HOPLIC, were exposed to those elevated temperatures. All other cultures in this study were isolated in that room after the 2001-2002 summer and were maintained stably at 27°C.

Light and temperature were uncontrolled for the initial 6 months of cultures BPdN1 and BPdN4. These were unexpected cultures that resulted when leftover FIZ were left in the lab in 10 ml of autoclaved seawater without nutrient additives, in sterile 15ml polypropylene tubes with lids screwed on for 8 months (Spring 2001). The lab was at room temperature, but was not air-conditioned nor temperature controlled, and lights were on for
10-24 hrs per day in unpredictable fashion. The light level in the room was less than 3\(\mu\)E m\(^{-2}\) s\(^{-1}\) during the culturing of BPdN1 and BPdN4.

2.8 Culture inoculation

Bulk inoculation

Bulk inoculation is a standard means of obtaining *Symbiodinium* cultures (e.g. Santos et al 2001). The potential requirements of *Symbiodinium* isolates for a quorum have not been methodically tested, although it has been shown that some strains can be grown from single cells (McLaughlin and Zahl 1962b; Schoenberg and Trench 1980a; York 1986). A safe method of avoiding quorum absence is to inoculate a culture at sufficient cell density. The problems with this approach are that if the major symbiont dies en-masse, then the medium will have already been polluted with abundant decaying material which could have unknown consequences. Use of pre-conditioned medium as an additive (50% of total culture volume added to fresh fEs) to overcome potential quorum-sensing requirements, was tried for the symbiont of *P. versipora* (Sydney Harbour) because it consistently resisted being brought into culture. The culture used to prepare pre-conditioned medium was CSIRO culture CS-156. The conditioning period of the medium was 2 months, after which it was sterilised through a 2\(\mu\)m filter.

Mixed inoculation

Studies of marine dinoflagellates in general indicate that periods of low nutrient availability induce gametogenesis and mating, with subsequent zygotic cyst production, while periods of high nutrient availability induce germination of cysts and entry to the proliferative vegetative phase of the life cycle (Pfiester 1984; Pfiester 1989; Pfiester and Anderson 1987). The *in hospite* state of zooxanthellae is the nutrient-replete stage of its ecological cycle, while life in seawater is the nutrient-deprived stage. The hypothesis used in mixing inocula was that extracting FIZ from corals into seawater-based media may resemble the conditions for gametogenesis, and thus result in terminal differentiation of gametes and non-proliferation unless compatible mates are available. Compatible mating types could be missed if FIZ were taken from a single coral individual only, as many corals are thought (Chapter 1) to contain only a single *Symbiodinium* strain (e.g see Chapter 5

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results for *G. tenuidens*), presumably clonally propagated from a single founding cell or single most competitive cell (Goulet and Coffroth 2003a; Goulet and Coffroth 2003b). It is currently unknown whether several mating types exist in each *Symbiodinium* strain, and because of this all pairwise mixtures of Ha1-Ha10, and all pairwise mixtures of Lp1-Lp10 were used as culture starting material. FIZ pairs were mixed together in seawater with antibiotics for four days. The mix was then placed on agar and incubated in the 27°C light room for two months to obtain colonies, one of which was picked.

**Serial dilution**

Serial dilution in tissue culture wells is a standard means of obtaining microbial cultures *in situ* where the desired microbe sought is numerically dominant in the inoculum. Serial dilution is iterated to the point where no cultures grow on account of insufficient inoculum. To obtain the numerically dominant microbe, the tissue culture well in which the inoculum was at the most diluted extreme of a range, yet still yielded a *de novo* culture, was usually chosen for further characterisation.

**2.9 Purification and maintenance of cultures**

**Partial purification**

The unidentified algal colonies and *Symbiodinium* colonies were separately purified, by picking colonies or aggregates from the bottom or sides of liquid-culture wells (48-well plate, ~1 month after serial dilutions) with a 100 μl pipette tip. *Symbiodinium* often formed a ring at the meniscus of a well, adhering to the sides of the well. Colonies/aggregates of *Symbiodinium* and other algae were picked wherever they were discreet, and locally homogeneous, and before any single culture took over the well completely through competitive advantage. Algal colonies so picked were placed into a fresh well of a 48-well tissue culture plate, in medium fEs. This procedure often resulted in purification of cultures to single strains.

**Routine maintenance**

After two weeks to two months, when picked algae had begun proliferating in the secondary well, they were subcultured by transfer to fresh medium. In the case of
Symbiodinium, this was done as soon as a brown ring was present at the meniscus of the tissue culture well. Cells were transferred to a 25 cm² tissue culture flask containing ~20 ml of fEs, and incubated on end with the lid loose. The 20 ml cultures were then subcultured at intervals of between 2 months and 8 months.

Final purification
For a subset of cultures (L.9, L.4, G.1, G.2, Tm4.1, Tm8.2, Tm10.1, Tm10.2, Tm10.3, see Table 3.7 in results section) dilution streaking on fEs agar (1.5% agar) was performed (nomenclature Sc for single colony was used to record these isolates). When culturing clam (Tm) symbionts on agar the antifungal agent Amphotericin B at 0.25 μg/ml final concentration, was also included on the fEs plate.

Submission to culture collections
RM designations (in Table 3.7, results section) are isolator information used for submission of cultures to both of two culture collections: North Queensland Algal culture and Identification Facility (NQAIF) Australia, and Culture Collection Marine Provasoli (CCMP) USA. A small subset of these cultures was also submitted to the Culture Collection of Algae and Protozoa (CCAP) Scottish Association for Marine Science, Argyll Scotland. Each Symbiodinium culture submitted to public collections was a homogeneous culture, i.e. effectively unialgal, as judged by unambiguous chromatograms from DNA sequencing. The submitted cultures were free of fungal and other eukaryotic contaminants, but some may have contained bacteria. Staff of NQAIF report successfully maintaining the cultures in f2 medium at 24°C, with a light intensity of 40 μE m² s⁻¹ and light dark cycle of 12/12h (K. Heimann, James Cook University, pers. comm.).

2.10 DNA manipulation and phylogenetic analysis

DNA extraction
Holobiont tissue was removed by air gun as described in General Materials and Methods, chapter 2. DNA template extraction began with cultured zooxanthellae or holobiont tissue, pelleted at 12000 rpm to remove the growth medium (fEs or similar) or preservation medium (>50% Ethanol in seawater). A combination of detergents (1% SDS, 2% Sarkosyl) was found to achieve the most complete cell-rupture and DNA release (based on nuclear
lysis buffer of Triplett et al. (Triplett et al. 1993). The protocol is detailed in General Materials and Methods.

**PCR primers, PCR conditions, and electrophoresis**

Table 3.4 details the primers and annealing temperatures used for each amplification. Primers are published (Coleman et al. 1994; White et al. 1990b; Zardoay et al. 1995) or pers comm. (William Loh, unpublished data) sources, and anneal at rDNA regions highly conserved across eukaryotes. Pair 1 (Table 3.4) amplify the D1 and D2 regions of the lsu rDNA. Primers ITS4 and ITSa bind in the ssu rDNA gene, while primers dinoITS and ITSb bind in the lsu rDNA gene. Pair 2 (Table 3.4) amplifies ITS1 region, the 5.8S gene, and the ITS2 region, as does pair 3 (Table 3.4). The two alternative pairs of ITS primers (pair 2 and pair 3) were employed, because pair 3 did not always result in an amplicon, and so pair 2 was subsequently adopted for typing of some cultures. The thermocycler machine used was a Perkin Elmer 2400. Cycling conditions on all occasions were 94°C for 2 minutes, then 40 cycles of: 94°C for 30 seconds, 53-65°C for 30 seconds, 72°C for 2 minutes; followed by 72°C for 10 minutes and then a 25-35°C hold. Electrophoresis was carried out in 1% agarose in 1x TBE (90 mM Tris-Borate, 2mm EDTA pH 8.3) with a 1xTBE running buffer (Sambrook 1989).

**Table 3.4 PCR primers used in this chapter**

<table>
<thead>
<tr>
<th>Pair no.</th>
<th>Primer name and direction</th>
<th>Sequence</th>
<th>Target locus</th>
<th>Annealing temp</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ZardF F</td>
<td>5'-CCCGCTGAATTTAAGCATATAAGTAAGCAGG</td>
<td>nuclear, lsu</td>
<td>53°C-65°C</td>
<td>[a]</td>
</tr>
<tr>
<td></td>
<td>ZardR R</td>
<td>5'-GTTAGACTCCTGTTCGTTCAGAAGA</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>2</td>
<td>ITS4 F</td>
<td>5'-TCCTCCGCTTATGATATGC</td>
<td>nuclear, ITS</td>
<td>&quot;</td>
<td>[b]</td>
</tr>
<tr>
<td></td>
<td>dinoITS R</td>
<td>5'-GTGATTATTCGGACTGAGG</td>
<td>&quot;</td>
<td>&quot;</td>
<td>[c]</td>
</tr>
<tr>
<td>3</td>
<td>ITSa F</td>
<td>5'-GTTTCCGTTAGTGAACCTGC</td>
<td>&quot;</td>
<td>&quot;</td>
<td>[d]</td>
</tr>
<tr>
<td></td>
<td>ITSb R</td>
<td>5'-ATATGCTTAAGTCAGCAGGG</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

[a] (Zardoay et al. 1995), [b] (White et al. 1990a) [c] [William Loh, unpublished data], [d] (Coleman et al. 1994)

**Amplicon purification, DNA sequencing, editing and alignment**

Amplicons were purified as in General Materials and Methods (Chapter 2) and in all cases were directly sequenced without cloning. DNA sequencing, sequence editing and alignment techniques were performed as detailed in General Materials and Methods.
Taxon choice for alignments
For phylogenetic placement of the new cultures, the expectation was that all the cultures would lie within the Suessiale lineage. An alignment was generated from 108 lsr rDNA sequences (D1/D2 region) obtained from Genbank. Of the 108 taxa, 75 were Suessiales or sisters to Suessiales (Table 3.5), and were retained for phylogenetic presentation. The other 33 also used in the phylogenetic analysis were dinoflagellates from orders Peridiniales, Prorocentrales and Gymnodiniales, together constituting an outgroup cluster, and these have been omitted from the presented results, by truncating the tree. Within the Suessiales and their kin, taxa were chosen to be representative of all the major branches of genus *Symbodinium*, plus the Suessiale *Polarella glacialis* and two other *incertae sedis* Suessiales *Gymnodinium bei* and *Gymnodinium simplex* (Montresor et al. 2003). *Wołoszynska pseudopalustris* is a sister to Suessiales (Kremp et al. 2005; Saldarriaga et al. 2004), and so was also included. BLAST of the lsr rDNA sequences showed that most of the novel cultures were putatively of *Symbodinium* clades A and F. In order to achieve a fine-scale placement of the new cultures among previously characterised strains, the BLAST output was used as the basis for strain sampling within these two clades. In clades other than A and F, standard strains were selected from genbank, taking care to represent a range of subclades. Before arriving at the set of 75 Suessiales, redundant genotypes of *Symbodinium* were removed in a preliminary alignment. For example, sequence AY074938 from *Agaricia* sp. which was published by Savage et al. (2002b) is identical to sequence AY239384 cited as “clade C1” of various hosts by LaJeunesse et al. (2003). A unique host name was recorded in the former case, but not in the latter. The former sequence is accordingly labelled C1 here and allocated to *S. goreai* after LaJeunesse (2001). A similar transmission of nomenclature was adopted for *S. kawagutii* sequences as follows: sequence AF427462 (Santos et al. 2002b) was from a culture obtained by Kinzie from Trench (T. LaJeunesse pers. comm.). The culture was originally from scleractinian host *Montipora verrucosa* Hawaii (LaJeunesse 2001; Trench and Blank 1987). No lsr rDNA from the latter had been published, so the Santos sequence (Santos et al. 2002b) is used here and is allocated to the species *S. kawagutii* after Trench & Blank (1987). The lsr rDNA of culture 156 of the CSIRO collection (also from *M. verrucosa*, donated to CSIRO by Trench) was sequenced in this study in order to test for sequence identity of this culture with that of Santos et al. (2002b). However, many other redundant sequences were removed, across the genus, and only sequences attached to publications were kept.
### Table 3.5 Genbank accessions and references for LSU rDNA sequences used in phylogenetic analysis of Symbiodinium isolates

<table>
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<th>ref</th>
<th>accession</th>
<th>Host name (and Syn sp. if named)</th>
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<td>H</td>
<td>xi</td>
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The rDNA Internal Transcribed Spacer (ITS) region was amplified in cases where LSU rDNA was not able to be amplified for unknown reasons. ITS reference sequences used are listed in Table 3.6. The ITS regions ITS1 and ITS2 are reported to be more variable than LSU rDNA regions D1 and D2 (LaJeunesse 2001) so the ITS outgroup used was deliberately
limited to only the sister Suessia, G. simplex, on the assumption that the ITS of other dinoflagellates might not align informatively with those of Symbiodinium. Type strains that were available for various clades of Symbiodinium were chosen from LaJeunesse (2001). Additional taxa were obtained by BLAST, using the ITS of the novel cultures as query. Sequences with accession numbers AF427468 and AF180123 are annotated in genbank as both being from the same culture, but were not redundant so both were included in the phylogenetic analysis. The latter sequence contains many apparent deletions and point mutations compared to the former.

Table 3.6 Genbank accessions and references for ITS sequences used in phylogenetic analysis of Symbiodinium isolates.

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<th>clade ref</th>
<th>accession</th>
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[i] (LaJeunesse 2001), [ii] (Savage et al. 2002b), [iii] (Bailie et al. 2000a), [iv] (Santos et al. 2002b), [v] (Hunter et al. 1997), [vi] (Bui et al. 2003). Dashes indicate unpublished sequences from genbank, used with permission of the submitters. * Attribution of AF184948 to clade ‘A1’ by [iii] has been disregarded here in view of strong sequence dissimilarity compared to S. microadiaticum, the type species of A1.

Phylogenetic analyses

For the lsi rDNA analysis, TreePuzzle (Schmidt et al. 2002; Strimmer and vonHaeseler 1997) was used to perform likelihood mapping. This indicated that the data were predicted to comprise suitable input for a well resolved phylogenetic analysis, as 92.8% of quartets
fell in the tree-like regions of the mapping analysis. Mr Bayes (Huelsenbeck and Ronquist 2001) was used for phylogenetic analysis as detailed in General Materials and Methods. The total number of trees computed was 250,000, and burn-in was estimated as occurring at the 135,000th tree stage. Trees computed before the 135,000th tree were discarded and the consensus of the remaining trees was generated.

The same process and parameters as above were employed to analyse ITS sequences using Mr Bayes, and Treepuzzle (mapping analysis showed 91.8% of quartets fell in the tree-like regions). In the Bayesian analysis of ITS, burn-in was reached after 20,000 trees of 250,000. The remaining 230,000 trees were used to generate the consensus tree.

2.11 Microscopic examination
Criteria for visual screening of putative Symbiodinium cultures
Putative Symbiodinium cultures were initially identified in this study by phenotypic criteria. To be picked, cells were required to have any one or more of the following phenotypes: i) gymnodiniod morphology of the zoospore ii) less than 20% of the cell population motile at any one time, with each motile cell required to demonstrate an ability to attach or detach one flagellum to/from substrate instantly, and to gyrate around a fixed point while attached; iii) positive phototaxis towards the microscopic light source and/or a penchant for proximity to air while motile. The product of this behaviour can also manifest as adhesion of cells at the ring where the plastic of the tissue culture vessel joins the meniscus of the growth medium; iv) cell size 5-20 μm and a brownish to ochre colour; v) a single brownish accumulation body within the cell; vi) Symbiodinium–like colony morphology, as determined by reference to clade A and clade B strains obtained from the CSIRO microalgal collection. A Symbiodinium colony morphology consists of close-packed immotile cells rarely more than four cell layers high, tendency of motile cells to stay close to colonies, and most characteristically a tendency for vacated positions or ‘holes’ to be present at the centre of the colony seemingly due to cells having become motile and left the colony.
**Equipment and frequency of observations**

The primary microscope used for observations was an inverted-objective (Leitz), so that tissue culture wells containing de novo cultures could be regularly viewed at 100x to 300x magnification without compromising aseptic conditions. Observations were made weekly during the first two months after de novo culture inoculation, then approximately monthly thereafter. The microscope used for these routine observations was a Leitz Labovert.

Photography of *Symbiodinium* strains was performed on only one occasion, and was under 1000x magnification with phase contrast and Nomarski differential interference contrast. It was kindly performed by Dr Kirsten Heimann (as noted in the captions to the images), James Cook University, Townsville Queensland, using an Olympus BX51 microscope with an Olympus DP70 camera. Dr Heimann photographed the strains as part of the documentation process of adopting them into the culture collection NQAIF – North Queensland Algal Identification and culture Facility.

3. Results

3.1 Establishment of cultures

Novel *Symbiodinium* cultures were obtained from one clam species, one anemone species and four coral species that transmit zooxanthellae horizontally (Table 3.7). Substantial variability was encountered in the culturability of organisms from different hosts. *P. versipora*, a vertically transmitting host of zooxanthellae, consistently failed to yield any culturable *Symbiodinium* (Table 3.8). Efforts to culture the characterised clade B *Symbiodinium* of *P. versipora*, led to the use of an additional chlorination reagent (trichloroisocyanurate) discussed in Chapter 6.

Any cultured cells that satisfied only one criterion for *Symbiodinium*-like phenotype (listed in Methods 2.11 microscopic examination) were kept, but none of those were subsequently confirmed as *Symbiodinium*, and so are assessed within Chapter 6 as putative novel symbionts. Cells that satisfied more than two criteria always subsequently genotyped as *Symbiodinium*.  

110
3.2 Effect of FIZ mixing, and effect of transport mode

Mixing of FIZ form two separate colonies of single host species did not result in greater success of culturing in fEs medium, implying either that transfer from the in hospite condition to enriched seawater medium does not stimulate gametogenesis, suggested by Baillie et al. (1998) or that compatible mating types were not present among the zooxanthellae of the ten colonies of H. actiniformis used. Whilst two cultures H2xH3 and H4xH7 were obtained from the mixing experiment, versus one culture H9 obtained from the control non-mixing experiment, none of these were the dominant symbiont, so the mixing experiment was altogether inconclusive (Table 3.7).

Mode of transport, and proximity of culturing location to the host’s natural environment did not correlate meaningfully. The optimal situation observed regarding successful culture of the dominant symbiont from a host was clearly to culture at the host’s place of origin, or from hosts that were completely healthy, but successful culture of the dominant symbiont was limited to cultures from Tridacnid clams and Aiptasia, which harbor symbionts known to be easily culturable (Baillie et al. 1998; Deane and O’Brien 1978; Fitt et al. 1981; Schoenberg and Trench 1980a). Regarding culturability of zooxanthellae from corals, air transport of the hosts from Gladstone to Sydney did not appear to be any advantage over road transport. Indeed while all the corals transported by road (a three day trip) experienced some degree of bleaching (zooxanthellae expulsion) during the trip, presumably due to darkness and fluctuation of temperature, it was still possible to culture a variety of zooxanthellae from these samples, including the dominant symbiont, (section 3.4).

3.3 Increase in culturing success by using more hypochlorite

A steady increase in the rate of successful culturing from hosts other than P. versipora, occurred as the concentration of hypochlorite was optimised over successive experiments (Table 3.9). Culturing success rate for FIZ from H. actiniformis increased from 0.01 to 0.3 attributed to use of moderate HOCl pretreatment, prior to FIZ extraction (York 1986). Likewise the culturing success rate for FIZ from L. purpurea increased from 0.1 to 0.8, when a high HOCl concentration was used in the pretreatment. The high concentrations of hyperchlorite that were tolerated supported the assumption that pretreatment of coral nubbins, using pH-neutralised hypochlorite, would not fatally harm zooxanthellae.
Table 3.7 Novel *Symbiodinium* cultures obtained in this study.

<table>
<thead>
<tr>
<th>Culture name</th>
<th>Purity</th>
<th>Host species</th>
<th>HOCl pre-treatment</th>
<th>Medium in primary culture well</th>
<th>Antibiotics present</th>
<th>Expected genotype</th>
<th>Culture genotype obtained</th>
<th>Cult. name at NQAIF + CCMP</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Symbiodinium</em> cultures established near host's place of origin, testing canonical culturing methods:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tn 4.1</em></td>
<td>Sc</td>
<td><em>T.</em> maximus 4.1</td>
<td>-</td>
<td>fEs</td>
<td>Y</td>
<td>A</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td><em>Tn 8.2</em></td>
<td>Sc</td>
<td><em>T.</em> maximus 8.2</td>
<td>-</td>
<td>fEs</td>
<td>Y</td>
<td>A</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td><em>Ha OPIC</em></td>
<td>R</td>
<td><em>H. actiniformis</em> OP1</td>
<td>-</td>
<td>fEs</td>
<td>Y</td>
<td>C</td>
<td>A</td>
<td>RM65</td>
</tr>
<tr>
<td><em>Alpasteria</em> 2</td>
<td>R</td>
<td>putative <em>Alpasteria</em> sp.</td>
<td>-</td>
<td>fEs</td>
<td>Y</td>
<td>B</td>
<td>B</td>
<td>-</td>
</tr>
<tr>
<td><em>Alpasteria</em> 3</td>
<td>R</td>
<td>putative <em>Alpasteria</em> sp.</td>
<td>-</td>
<td>fEs</td>
<td>Y</td>
<td>B</td>
<td>B</td>
<td>-</td>
</tr>
<tr>
<td><em>Symbiodinium</em> cultures isolated with or without HOCl, after air transport of host:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 dkhue</td>
<td>Sc</td>
<td><em>G. tenudens 1</em></td>
<td>1:100</td>
<td>fEs-PMA</td>
<td>Y</td>
<td>C</td>
<td>A</td>
<td>RM16</td>
</tr>
<tr>
<td>G1 It hue</td>
<td>Sc</td>
<td><em>G. tenudens 1</em></td>
<td>1:100</td>
<td>fEs-PMA</td>
<td>Y</td>
<td>C</td>
<td>-</td>
<td>RM17</td>
</tr>
<tr>
<td>G2</td>
<td>Sc</td>
<td><em>G. tenudens 2</em></td>
<td>1:100</td>
<td>fEs-PMA</td>
<td>Y</td>
<td>C</td>
<td>A</td>
<td>RM19</td>
</tr>
<tr>
<td>H2xH3</td>
<td>Sc</td>
<td><em>H. actiniformis 2+3</em></td>
<td>1:10,000</td>
<td>fEs-PMA agar</td>
<td>Y</td>
<td>C</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H4xH7</td>
<td>Sc</td>
<td><em>H. actiniformis 4+7</em></td>
<td>1:10,000</td>
<td>fEs agar</td>
<td>Y</td>
<td>C</td>
<td>A</td>
<td>RM21</td>
</tr>
<tr>
<td>H9</td>
<td>R</td>
<td><em>H. actiniformis 9</em></td>
<td>1:10,000</td>
<td>fEs</td>
<td>Y</td>
<td>C</td>
<td>-</td>
<td>RM18</td>
</tr>
<tr>
<td>H10K</td>
<td>R</td>
<td><em>H. actiniformis 10</em></td>
<td>-</td>
<td>fNaK-fEs-PMA</td>
<td>Y</td>
<td>C</td>
<td>A</td>
<td>RM03</td>
</tr>
<tr>
<td>LA dkhue</td>
<td>Sc</td>
<td><em>L. purpurea 4</em></td>
<td>1:10,000</td>
<td>fEs-HOCl 1:10</td>
<td>N</td>
<td>C</td>
<td>-</td>
<td>RM14</td>
</tr>
<tr>
<td>LA It hue</td>
<td>Sc</td>
<td><em>L. purpurea 4</em></td>
<td>1:10,000</td>
<td>fEs-HOCl 1:10</td>
<td>N</td>
<td>C</td>
<td>A</td>
<td>RM15</td>
</tr>
<tr>
<td>L9</td>
<td>Sc</td>
<td><em>L. purpurea 9</em></td>
<td>1:10,000</td>
<td>fEs</td>
<td>Y</td>
<td>C</td>
<td>A</td>
<td>RM20</td>
</tr>
<tr>
<td><em>Tm 10.1</em></td>
<td>Sc</td>
<td><em>T.</em> maxima 10.1</td>
<td>-</td>
<td>fEs</td>
<td>Y</td>
<td>A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Tm 10.2</em></td>
<td>Sc</td>
<td><em>T.</em> maxima 10.2</td>
<td>-</td>
<td>fEs</td>
<td>Y</td>
<td>A</td>
<td>-</td>
<td>RM4</td>
</tr>
<tr>
<td><em>Tm 10.3</em></td>
<td>Sc</td>
<td><em>T.</em> maxima 10.3</td>
<td>-</td>
<td>fEs</td>
<td>Y</td>
<td>A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Symbiodinium</em> cultures isolated with HOCl, after road transport of host:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z1</td>
<td>R</td>
<td><em>L. purpurea 12</em></td>
<td>1:25</td>
<td>fEs</td>
<td>Y</td>
<td>C</td>
<td>A</td>
<td>RM23</td>
</tr>
<tr>
<td>Lp13</td>
<td>R</td>
<td><em>L. purpurea 13</em></td>
<td>1:25</td>
<td>fEs</td>
<td>Y</td>
<td>C</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td>Z3</td>
<td>R</td>
<td><em>L. purpurea 14</em></td>
<td>1:25</td>
<td>fEs-HOCl 1:250</td>
<td>Y</td>
<td>C</td>
<td>F</td>
<td>RM10</td>
</tr>
<tr>
<td>Z6</td>
<td>R</td>
<td><em>L. purpurea 14</em></td>
<td>1:25</td>
<td>fEs-HOCl 1:250</td>
<td>Y</td>
<td>C</td>
<td>F</td>
<td>-</td>
</tr>
<tr>
<td>B29</td>
<td>R</td>
<td><em>L. purpurea 14</em></td>
<td>1:25</td>
<td>not recorded</td>
<td>Y</td>
<td>C</td>
<td>-</td>
<td>RM07</td>
</tr>
<tr>
<td>Z4</td>
<td>R</td>
<td><em>L. purpurea 15</em></td>
<td>1:25</td>
<td>fEs-HOCl 1:250</td>
<td>Y</td>
<td>C</td>
<td>F</td>
<td>-</td>
</tr>
<tr>
<td>BpDN1</td>
<td>R</td>
<td><em>P. damicornus 1</em></td>
<td>1:100, 36 hrs</td>
<td>SW, 8 months</td>
<td>Y</td>
<td>C</td>
<td>A+C</td>
<td>-</td>
</tr>
<tr>
<td>BpDN4</td>
<td>R</td>
<td><em>P. damicornus 4</em></td>
<td>1:100, 36 hrs</td>
<td>SW, 8 months</td>
<td>Y</td>
<td>C</td>
<td>A</td>
<td>RM08</td>
</tr>
</tbody>
</table>

Sc = derived from a single colony on agar, R = raw bulk culture, SW = seawater.
Table 3.8 Comparison of culture rates from different non-target hosts (control experiments testing methods).

<table>
<thead>
<tr>
<th>Host species</th>
<th>Location and date</th>
<th>No. of reps</th>
<th>No of cultures resulting</th>
<th>Cultivation ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. maxima</td>
<td>OTI, November 2000, then held in Sydney Aquarium Darling Harbour for 6 months before isolation of zooxanthellae</td>
<td>45</td>
<td>2</td>
<td>0.04</td>
</tr>
<tr>
<td>unidentified Alptasia sp.</td>
<td>Resident fauna of Sydney Aquarium, Darling Harbour ~ March 2001</td>
<td>5</td>
<td>3</td>
<td>0.6</td>
</tr>
<tr>
<td>P. versipora</td>
<td>Various locations in Sydney Harbour, spanning 2001-2003</td>
<td>3</td>
<td>2</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Table 3.9 Comparison of culturing rates from scleractinians in presence and absence of hypochlorite.

<table>
<thead>
<tr>
<th>Host species (Order and Family)</th>
<th>Location and date</th>
<th>No. of reps</th>
<th>No of cultures Resulting, names</th>
<th>Cultivation ratio</th>
<th>HOCl used?</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. actiniformis</td>
<td>OTI, November 2000</td>
<td>94</td>
<td>1 (HOPIC)</td>
<td>0.01</td>
<td>N</td>
</tr>
<tr>
<td>H. actiniformis</td>
<td>OTI, July 2001</td>
<td>10</td>
<td>3 (H2xH3, H4xH7, H9)</td>
<td>0.3</td>
<td>Y</td>
</tr>
<tr>
<td>L. purpurea</td>
<td>OTI, July 2001</td>
<td>10</td>
<td>2 (L4, L9)</td>
<td>0.1</td>
<td>variable</td>
</tr>
<tr>
<td>L. purpurea</td>
<td>OTI, November 2001</td>
<td>5</td>
<td>4 (Lp13, Z1-3-4-7,B29)</td>
<td>0.8</td>
<td>Y</td>
</tr>
<tr>
<td>G. tenuidens</td>
<td>OTI, July 2001</td>
<td>3</td>
<td>2 (G1, G2)</td>
<td>0.66</td>
<td>Y</td>
</tr>
<tr>
<td>P. damicornis</td>
<td>OTI, November 2001</td>
<td>5</td>
<td>2 (BPdN1, BPdN4)</td>
<td>0.4</td>
<td>Y</td>
</tr>
</tbody>
</table>

3.4 Phylogenetic analyses of cultures

Phylogeny of Symbiodinium cultures

Appendix 2 lists the genbank accession numbers for the sequences of novel Symbiodinium isolates that were cultured in this chapter. Phylogenetic placement of the cultures obtained in this study among the published Suessiales are shown in Figure 3.1 (based on lsu rDNA sequence data) and Figure 3.2 (based on ITS sequence data). The phylogenetic positions of Symbiodinium cultured from clams and anemones in this study concorded with those expected based on previous studies (Table 3.1) and indicated that they are likely to be representative of the strain dominant in each of these hosts. However, among the symbionts cultured from corals, only the clade C symbiont in a co-culture BPdN1 appeared to represent the dominant symbiont found in those corals at our field site (Table 3.1 and Figures 3.1 and 3.2). This culture inoculum was derived from the host P. damicornis (Table 3.1) and contains a clade C isolate together with a clade A isolate (clade A sequence...
incomplete and not shown on these trees). The rest of the isolates apparently were minor symbionts that had cultured preferentially to the dominant symbionts. The cultures obtained from hosts expected to house clade C, *H. actiniformis*, *L. purpurea* and *G. tenuidens* (based on extensive and ongoing sequencing in the laboratory of D. Carter, University of Sydney, Table 3.1) were instead predominantly of clades A and F. The *L. purpurea* tissues sampled in this study and numbered Lp11-Lp15 were obtained within ten metres of each other, and were assumed to house conspecific zooxanthellae. Zooxanthelllar ITS from one holobiont tissue (*L. purpurea* 14) of this set was sequenced and this confirmed that it was identical to that of the genbank entry for *Sym-Lp OTI* (Bui et al. Genbank accession AY237299, Figure 3.2).

The lsr rDNA sequence obtained from novel culture *Sym-Z4* of the current study was identical to a sequence from CSIRO culture CS-156 and Genbank sequence AF427462 also of a cultured *Symbiodinium* sp. (Santos et al. 2002b). This confirms that the latter two cultures are subcultures (derived from host *M. verrucosa*, Hawaii) shared by R. Trench (Trench and Blank 1987) and R. Kinzie (Santos et al. 2002b) as muted (T. LaJeunesse pers. comm.). This putative symbiont of *M. verrucosa* Hawaii is apparently an opportunistic strain not dominant in the host (LaJeunesse et al. 2004a). It had been described as clade C *Symbiodinium* in the literature (Baillie et al. 2000a; Carlos et al. 1999; Loh et al. 1998) prior to the establishment of clade F (LaJeunesse 2001), and although the genbank entry for sequence AF427462 was labelled clade C, the current analysis confirms this symbiont is of clade F (sequence labelled *S. kawagutii*, Figure 3.1).
Figure 3.1 Lsu rDNA phylogeny of novel *Symbiodinium* isolates among clades A, B, C and F. New cultures are bolded and indicated by red stars.
Figure 3.2 ITS phylogeny of novel *Symbiodinium* isolates among clades A and C. New cultures are bolded and indicated by red stars. Asterisked branch discussed in text.
Table 3.10 Synapomorphic base positions within lts rDNA of *Symbiodinium* subclades Fr5*, Fr5 and F1.

<table>
<thead>
<tr>
<th></th>
<th>378</th>
<th>380</th>
<th>382</th>
<th>385</th>
<th>388</th>
<th>410</th>
<th>420</th>
<th>435</th>
<th>436</th>
<th>437</th>
<th>516</th>
<th>532</th>
<th>535</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fr5*</td>
<td>T</td>
<td>T</td>
<td>G</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>A</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>T</td>
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<td>Fr5</td>
<td>T</td>
<td>T</td>
<td>Δ</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>G</td>
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<td>F1</td>
<td>C</td>
<td>C</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>A</td>
<td>T</td>
<td>G</td>
<td>G</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>C</td>
</tr>
</tbody>
</table>

Base positions are numbered relative to an alignment (not shown). The subclade F1 sequence is AF427462.

**Novel strains of Symbiodinium, and variants of some existing phylotypes**

Definition of terms: genotype = strain = an individual; phylotype = a sequence that clusters monophyletically with close relatives (a phylotype may be a species or a subspecies); subclade = a phylotype that has been established by other authors; type = a term encompassing genotype/ phylotype/ subclade.

Lts rDNA sequencing and Bayesian phylogenetic analysis revealed that both extant and novel types had been cultured. Novel strains belonged within clades A and F but did not fit well within previously defined subsections of these clades.

In clade F a novel cluster within Fr5 was identified, comprised of sequence AJ291529 from *Sorites* sp., sequence AJ291535 from *Amphisorus* sp., and the novel sequence from culture Z3 of *L. purpurea*. The novel cluster has here been named Fr5* to enable discussion. Table 3.10 illustrates the informative bases that separated F1, Fr5 and Fr5*. Bases that were synapomorphic within any one of these three clusters are underlined. Criteria for synapomorphic bases was strict, all aligned members of a cluster were required to possess the base, in order for the base to be included in the table. The bases that were distinctive for a particular cluster are underlined (Table 3.10).

Within clade A, distance criteria were used to assess the amount of biodiversity represented in the new cultures (Table 3.11), with synapomorphies again defined strictly (as for Table 3.10). The novel clade A cultures possessed sequences that were dissimilar to existing clade A subclades, and where complete sequence was available, the pairwise distances were comparable to those between selected pairs of named *Symbiodinium* species. Distances between *S. microadriaticum* and *S. linucheae* (subclades A1 and A4) are included for comparative purposes to indicate a minimum distance for putative species-
level or subspecies-level distances. Bold labelled types indicate new isolates each having *S. pilosum* (A2) as their nearest existing named species and subclade, but these differ from it by a substantial distance. Cultures H10K and L9 were assigned to phylotype A2*, the asterisk indicating they represent a distinct variant of phylotype A2 (Figures 3.1 and 3.2 and Table 3.11).

Cultures Lp13 and Z1 could not be assigned to any known clade A phyloplane. As an example of the biodiversity represented in the new cultures, A2* and Lp13 differ from each other and from A2, by a greater number of bases than separates existing *Symbiodinium* species (subclades A1 and A4). Likewise, the novel culture Z1 is distinct from the temperate clade A subclade AT (Savage et al. 2002b), by 49 bases, from A2* by 26 bases, and from Lp13 by at least 12 bases (the Lp13 sequence is partial, but this did not interfere with the Bayesian analysis of Figure 3.1, nor the distance analysis of Table 3.11 as lower limit). The separation of A2*, Lp13 and Z1, from A2 and AT was in each case supported by a Bayesian posterior probability of 95% or greater (Figure 3.1).

In the fragment containing ITS1, ITS2, and 5.8S, only the 5.8S rDNA was completely informative, whereas a mixture of unalignable and informative bases occurred between clades and between strains in the ITS1 and ITS2 regions. Informative positions within ITS1 and ITS2 were carefully identified, and separation of types is given in Table 3.12, demonstrating again that the novel cultures are as distinct as existing 'subclades'.

Two cultures, Z1 and L9 (the latter analysed as A2*), were successfully genotyped for both of the markers (lsu rDNA and ITS) and these provide valuable cross-references. The L9 cross reference allowed the assignment of phylotype A2* in the ITS tree after its assignment in the lsu tree, and the Z1 cross-reference corroborated the distinctiveness of the position of this isolate in both trees. For greater confidence in the phylogenetic placement of isolates, it would be desirable to sequence a large number of taxa sequenced at both loci. Only a small number are so duplicated in genbank, and this exercise was beyond the scope of the chapter which rather aimed to genotype each new culture cursorily, and by use of whatever locus was empirically amplifiable. Lsu rDNA amplification was attempted for all novel cultures but was not successful in every case.
It is noted that two separate genbank entries exist for the clade A2 symbiont of *Zoanthus sociatus* cultured by R. Kinzie. AF427468 (*Z. sociatus* a on Figure 3.2) differs from AF180123 (*Z. sociatus* c on Figure 3.2) in that the latter has many point mutations and indels, which is curious as these two strains were from the same raw culture (R. Kinzie, University of Hawaii pers. comm.). This may document an instance of mutations occurring over several decades of *in vitro* culture of a *Symbiodinium* strain, but is more likely to represent strain-replacement, a founder effect, or erroneous base calls.

**Table 3.11** Total numbers of base substitutions between strains of clade A, using ~600bp of *lsu* rDNA.

<table>
<thead>
<tr>
<th></th>
<th>A1</th>
<th>A4</th>
<th>A2</th>
<th>A2*</th>
<th>Lp13</th>
<th>Z1</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td></td>
<td></td>
<td>5(5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A4</td>
<td></td>
<td></td>
<td>28(5)</td>
<td>25(5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>26(3)</td>
<td>23(3)</td>
<td>7(7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2*</td>
<td>10(2)+</td>
<td>5(2)+</td>
<td>8(5)+</td>
<td>6(3)+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lp13</td>
<td>27(15)</td>
<td>25(15)</td>
<td>29(20)</td>
<td>26(17)</td>
<td>12(9)+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z1</td>
<td>42(31)</td>
<td>41(31)</td>
<td>50(36)</td>
<td>46(33)</td>
<td>26(21)+</td>
<td>49(44)</td>
<td></td>
</tr>
</tbody>
</table>

Unbracketed numbers are the total number of base differences between two types being compared. Bracketed numbers are the subset of bases that are unique to the pair being compared. All numbers are derived from a single alignment, not shown. ‘+’ indicates pairwise cases where numbers are limited by partial data (i.e. partial sequence of culture Lp13), thus distance is underestimated, but valid as a lower limit. All bases in the *lsu* rDNA alignment were informative in this clade. Bolded types are novel cultures. In the distance and TiTv analyses of this study, sequence AF427468 was used instead of AF180123 because the former groups with other clade A2 cultures, while the latter contains probable base-call errors.

**Table 3.12** Relative numbers of base substitutions between strains of clade A, using informative positions in ITS rDNA.

<table>
<thead>
<tr>
<th></th>
<th>A1</th>
<th>A4</th>
<th>BpDn4</th>
<th>Z1</th>
<th>A2</th>
<th>A2*</th>
<th>Lp13</th>
<th>Z1</th>
<th>A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td></td>
<td></td>
<td>13(5)+</td>
<td></td>
<td>14(5)+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A4</td>
<td></td>
<td></td>
<td>32(6)</td>
<td>19(5)</td>
<td>16(7)+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BpDn4</td>
<td>44(0)</td>
<td>45(1)</td>
<td>36(4)+</td>
<td>16(7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z1</td>
<td>48(4)</td>
<td>49(4)</td>
<td>40(7)+</td>
<td>19(10)</td>
<td>3(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Unbracketed numbers are the number of base differences between two types being compared. Bracketed numbers are the subset of those bases that are unique to the pair being compared. All numbers are derived from a single alignment, not shown. ‘+’ indicates pairwise cases where numbers are limited by partial data (i.e. partial sequence of culture BpDn4), thus distance is underestimated, but valid as a lower limit. The same informative positions of the ITS alignment were used for all of the six *Symbiodinium* clade A types analysed. Bolded types are novel cultures.

**TiTv ratios indicate probable lack of HOCl-caused mutations**

Transition/transversion ratios of the sequences were assessed to determine whether HOCl-induced mutation could account for any substantial fraction of the sequence variation observed. The *lsu* rDNA bases from the pairwise comparisons of Table 3.11 were analysed
(Appendix 3), and the Ti/Tv ratios obtained were high e.g.: A1-A4 5/1; A2-A2* 7/0; A2*-Lp13 4/2; Lp13-Z1 9/3; Z1-A2* 16/11; Z1-AT 36/13. Transitions were favoured in all cases as would be expected in a molecule with secondary structure that is under selection at the level of the nucleotide. Ti/Tv ratios in ITS1+ITS2 between related clade A types analysed were low e.g.: A1-A4 3/2; A4-BPdN4 11/3; BPdN4-Z1 6/10; BPdN4-A2 15/20; Z1-A2 9/7; Z1-A2* 9/9; A2-A2* 0/3.

High Ti/Tv ratios obtained among novel cultures, are similar in magnitude to those between a pair of existing subclades (A1-A4 5/1). Ti/Tv ratios were similarly high in the clade F lsu rDNA phylotypes analysed (Fr5*-Fr5 4/1; Fr5*-F1 12/2) when compared to the ratio from a pair of existing subclades (F1-Fr5 8/1).

In summary of the Ti/Tv results: what was being tested as a control was the value of the Ti/Tv ratio between existing cultures (pairwise) such as A1 and A2, A1 and A4, which were cultures not obtained by the author and therefore not obtained using HOCl. What was being tested, as an actual assay, was the Ti/Tv ratios between the control cultures and the novel cultures (or between novel cultures), the latter having been cultured de novo with use of HOCl. If the Ti/Tv values of the control pairs were smaller than the Ti/Tv values of the assay pairs then it could be construed that the HOCl had a mutagenic effect. However, no differences were found when the lsu rDNA locus was used. Ti/Tv differences between control pairs and assay pairs when the ITS was used, were due to natural tendency of ITS to evolve with many transversions (discussion, section 4.8 this chapter).

3.5 Microscopic examination

Photographic documentation of microscopic features

Personnel at two culture collections photographically documented subsets of the cultures that were produced in this study. At NQAIF, pyrenoids and cyst walls were noted and these features have been arrowed in the photoplates (Figure 3.3, photos by K. Heimann, James Cook University, used with permission). Accumulation bodies (containing waste oils) are not apparent under the culture conditions and microscopic conditions used at NQAIF. However, in a subset of these cultures photographed by CCMP (images available permanently on the internet, http://ccmp.bigelow.org, photos by R. Anderson, cited with permission), orange or brown pigmented accumulation bodies are present in cultures
H'10K, Tm10.2, and Z3, presumably reflecting a difference in subculturing frequency or growth medium (Freudenthal 1959; Freudenthal 1962).
Figure 3.3 Photomicrographs of novel *Symbiodinium* cultures obtained in this study, and submitted to NQAIF culture collection. Arrows mark features of interest being in most cases the pyrenoid (P), and the cell wall (C). In some cases a dinokaryon (D) [in L4 light hue, and H4xH7] or a tetrad division wall (T) [in G1 dark hue] are marked. Photos by K. Heimann, NQAIF, used with permission. Scale bars 5μm.
Three independent Symbiodinium cultures contained flagellated cells engaged in cell-cell adhesion

Raw culture L4 (that was subsequently purified into L4 light hue and L4 dark hue), and cultures G2, and Z1 (all clade A) were observed to contain pairs of flagellated cells adhering, as if engaged in the early stages of cell-cell fusion. This has not been reported before for Symbiodinium and is not an expected phenomenon in the vegetative lifecycle, because cytokinesis in this genus occurs strictly in the non-flagellated stage (Crafts and Tuliszewski 1995; Fitt et al. 1981; Fitt and Trench 1983; Freudenthal 1959; Freudenthal 1962; Schoenberg and Trench 1980b). In cultures L4 and G2, the fused cells were free-swimming, tumbling in rapid and chaotic turns, and did not settle to the substrate, so could not be photographed with the apparatus that was available. However, culture Z1 contained fused cells that were still flagellated but had come to rest on the substrate, unmoving, and could be photographed (Figure 3.4). Flagella (when present) are normally ceaselessly moving in the motile cells of Symbiodinium and live flagellated but unmoving cells of Symbiodinium have not been previously reported. The two cells photographed in Figure 3.4 were presumably alive because they spent two days fused in the position shown, but were gone from that site in the tissue culture flask by the third day. The two cells were fused in an apparently significant way, in that the flagellae were symmetrically entwined (Figure 3.4) as if involved in mating.

Curiously the two cells seen attached together in culture Z1 (but not L4 or G2) were larger (~2X) than all of the cells surrounding them, and also larger than all of the cells in their flask, indicating that this particular subculture was either not unialgal, or that the fusing forms are larger than the vegetative forms in this strain. The assumption that the molecular type of the photographed pair was Z1 is therefore tenuous. However, if contamination was present then the contaminants photographed (Figure 3.4) were almost certainly Symbiodinium since no other dinoflagellate genera of gymnodiniod morphology were evidenced in this study. No occurrence of large cells was seen over several months in a separately genotyped aliquot of Z1 (aliquot Z1b, which did not contain large cells) that was not derived linearly from the photographed one (Z1a). Large cells were not again seen in culture Z1a. Their equivalence of sister subcultures Z1a and Z1b was verified by genotyping both cultures (accessions DQ174725 and DQ174724) after sighting and photographing of the cell-cell attachment event in culture Z1a. The sequence of Z1b was
identical to that of Z1a, and both chromatograms were unambiguous. However culture Z1a, in which cells were photographed fusing, may still have contained contaminants that were in the minority and that therefore did not influence the sequence chromatogram. Cultures of G2 and L4 that were also seen fusing, were each homogeneous in size and morphology.

Of the three cultures that contained cells engaged in flagellated cell-adhesion, only culture G2 had been obtained by use of PMA (a known mitogen) in the de novo culture medium. Cultures L4 and Z1 had not been exposed to PMA at any time. Therefore the cells photographed entwined, were not PMA artefacts.
Figure 3.4 Micrograph showing entwined flagella of two fused Symbiodinium cells in culture Z1. Each cell was ~15μm in diameter. The longitudinal flagellum of the left cell passes under that of the right cell and then continues to curve up toward the girdle of the left cell. Assuming this flagellar arrangement is repeated by the right cell, behind the line of sight, then a symmetry of mutual wrapping is suggested.

4. Discussion

Three main findings require discussion:

i) Culturing attempts from scleractinians containing clade C repeatedly result in the culture of minor symbionts that are not of clade C
ii) Culturing has identified some novel zoox genotypes.
iii) Conditions have been found that promote culturing zooxanthellae (albeit maybe only clades A and F)

Major findings are addressed in that order: category (i) sections 4.1- 4.6; category (ii) sections 4.7 and 4.8; category (iii) sections 4.9 and 4.10. A further and minor finding, in the sense that it was accidental, is the sighting of possible gametes in this study, which is discussed in sections 4.11 and 4.12.

4.1 Clade representation in this study: comparison to culturing results from other labs
The low rate of success of culturing the clade C major symbionts of H. actiniformis-, L. purpurea- and G. tenuidens-OT1 in this study could imply that these strains are not
culturable. However, clade C organisms have been cultured repeatedly by two research
groups which can be called the Trench group and the Coffroth and Kinzie group (Santos et
al. 2002b; Schoenberg and Trench 1980a; Trench and Blank 1987) Those cultures are in
the private collections of those groups (S. Santos, Auburn University; T. LaJeunesse
Florida International University; personal communications) and one of those clade C
cultures (CCMP 2466) has during the course of this study been submitted to a public
culture collection by T. LaJeunesse. The clade C type culture S. goreau and the publicly
available clade C culture, both from the Trench group, were isolated from an actinarian
Heteractis lucida and a corallimorph Discosoma sancti-thomae respectively, rather than
from scleractinians. Similarly two clade C cultures that have been sequenced and reported
by the Coffroth and Kinzie group are from a scyphozoan Mastigias sp. and an anemone
tentatively identified as Entacmaea quadricolor (Santos et al. 2002b).

The problem of obtaining any Symbiodinium culture at all from a range of desired starting
materials, goes back to the work of Schoenberg and Trench who obtained cultures (in
minimal medium Asp-8A) from only 17 out of 70 host species from which they tried to
culture de novo (Schoenberg and Trench 1980a). The success ratio obtained in the current
study is not comparable to that obtained by Schoenberg and Trench (1980a) because
isolation techniques were entirely different.

The work of Schoenberg and Trench (1980a) did demonstrate that some hosts are harder to
culture any zooxanthellae from than are other hosts, in particular they obtained cultures
from only 2 of 27 scleractinians. These were the clade A symbiont from Meandrina
meandrites, and clade B symbiont of Oculina diffusa (LaJeunesse 2001; Schoenberg and
Trench 1980a). Given the low rate of symbiont culturing success encountered by
Schoenberg and Trench in a broad survey of scleractinians, techniques such as selective
growth media are desirable developments. Nevertheless, Kinzie et al. (Kinzie et al. 1984),
using medium f/2, targetted for culture the major symbiont from the scleractinian
Montipora verrucosa. The culture obtained did not represent either of this host’s two major
symbionts, which are of clades C and D (LaJeunesse et al. 2004a), but rather is indicated to
have been of clade F (LaJeunesse 2001; LaJeunesse et al. 2004a). Kinzie et al. (1984) did
not report any attempts to culture symbionts from many host coral genera (only M.
verrucosa). The audit of successful cultures of symbionts from scleractinians is completed
by mention of cultures from hosts Pocillopora damicornis and Stylophora pistillata,
reported in LaJeunesse (2001), which are of clade B and clade A respectively. It anecdotally appears that it is difficult to obtain success with culturing the major symbiont from scleractinians that are host specialists (T. LaJeunesse, pers comm.).

The cultures of the Schoenberg and Trench group were established by picking single cells from washed and diluted FIZ (Schoenberg and Trench 1980a). The cultures of the Coffroth and Kinzie group were mostly established from bulk FIZ in single flasks of liquid medium (S. Santos, Auburn University, pers comm.) and often purified by serial dilution (M. Coffroth, SUNY Buffalo, pers. comm.). In the current study the mixed culture BPdN1 contains clade C *Symbiodinium* as well as clade A *Symbiodinium*. Likewise Santos reported that the culturing technique of the Coffroth and Kinzie group produces mixed clade cultures (Santos et al. 2001).

It is enigmatic that the current study produced so many minor symbionts. Potential explanations, comparison-wise, are that the hosts used by Coffroth and Kinzie did not contain as many minor symbionts as the hosts used in this study, or else the serial dilution of this study was not as rigorous as theirs. Another possibility is that the storage conditions used for corals in the current study (1-21 days, average 7 days) may have led to differential survival rates of different clades, prior to FIZ extraction. However, the most likely explanation for the difference in results between this study and the serial dilution culturing work of Coffroth and Kinzie group is that HOCl introduces a strong culturing selection for environmentally tolerant generalist strains. The results of the study of Schoenberg and Trench which attempted culturing from a broad range of hosts (Schoenberg and Trench 1980a), can be interpreted as implying low affinity of some dominant symbiont strains for the *in vitro* culture medium and culture conditions used. It is not possible to say, on the basis of current data, whether this is the case for the dominant symbiont strains from *Sym-Ha*, *-Lp* and *-Gt* OTI.

Since the HOCl medium used selects against ciliates, which are alveolate sisters of dinoflagellates, it may therefore also be expected to select for one symbiont over another in much the same way that antibiotics were found by Santos et al (2001) to favour the proliferation of one clade of *Symbiodinium* over another. In this respect it is noted that while multiple clade C cultures were sought, instead two organisms of clade F and a much greater number of clade A organisms were obtained, with only a single clade C obtained.
While these results could be interpreted in the sense that the chlorine medium favours clade A over clades C and F, this is not supported by all the data because clade A organisms also resulted from the same hosts when no chlorine or extremely low levels of chlorine were used (eg culture H'10).

4.2 Significance for future culturing attempts
The culturing of major symbionts (such as clade C from GBR corals) continues to be desirable, so as to facilitate ecologically relevant work aimed at understanding zooxanthella-host specificity and the adaptive and sexual potential of these major symbionts. Further culturing of clade C might proceed from this work by picking single cells of the numerically dominant symbiont from FIZ and placing them into HOCl-enhanced medium.

4.3 Major versus minor symbionts
In the current study, clade A organisms frequently resulted from attempts to culture Symbiodinium from hosts that appear, by molecular analysis, to house clade C as the dominant symbiont. There are three possible explanations for this result;

(i) clade A dominates but the PCR primers used in the molecular analysis preferentially amplify clade C DNA, thereby overriding the clade A signal
(ii) clade A organisms are present as minor symbionts producing a negligible rDNA signal but are preferentially brought into in vitro culture
(iii) clade A was present in the Sydney Harbour water used to store the corals, or is present in the coral mucous i.e. is not an endosymbiont.

The weight of data favours explanation (ii) over explanation (i) because in this study and in other studies of the same hosts at the same location (Carter et al. 2000; Ferguson 2001; Gava 1999; Loh et al. 1998; Loh et al. unpublished; Loi 1998), all primers used were universal for all Symbiodinium clades (by alignments, data not shown), all sequencing was direct (no amplicons cloned), and primer selectivity has not been shown or indicated. Indeed, some corals genotyped as clade A in the previous studies using the ZardF and ZardR primers (Carter et al. 2000; Ferguson 2001; Gava 1999; Loh et al. 1998; Loh et al. unpublished; Loi 1998) indicating that clade A template is probably not preferentially avoided by these primers. Similar arguments prevail in judging that the clade F cultures obtained from L. purpurea are minor symbionts rather than major symbionts. Clade F has
not been found as a major symbiont in corals of OTI (Carter et al. 2000; Ferguson 2001; Gava 1999; Loh et al. 1998; Loh et al. unpublished; Loi 1998). To test whether the clade F isolates found in this study were potentially the first cases of clade F dominance in an OTI coral, the FIZ of one *L. purpurea* colony (*Lp14* that yielded two clade F cultures) was used as template for PCR of ITS DNA. The sequence obtained was of clade C, identical to that obtained previously from this host at this location (Table 3.7 and Figure 3.2) (Bui et al. 2003).

### 4.4 Explanation iii: Are the minor symbionts cultured in this chapter really symbionts?

That the clade A and F cultures were derived from free-living *Symbiodinium*, is considered to be unlikely as these would need to have been present either on the coral surface or in the Sydney seawater used to store corals. *Symbiodinium* (and the free living syngener *G. varians*) have only rarely been reported in seawater (Gou et al. 2003; Hallegraeff and Reid 1986; Taylor 1983; Wood 1963). While *Symbiodinium* may reside in sediment (Carlos et al. 1999) no Sydney Harbour sediment was used in the storage tanks in this study, so sediment is unlikely to be the direct source of these novel cultured strains. Similarly, *Symbiodinium* are not known to occur in coral mucus (Coles and Strathmann 1973). From this it is assumed that all the clade A and F *Symbiodinium* isolates obtained in this study (other than from clams) were minor symbionts.

Clade A *Symbiodinium*, cultured repeatedly in this study, may be present in hosts as freeloaders and opportunistic minor symbionts, playing no role in the development or maintenance of symbioses. Alternatively, the hardy clade A organisms, which are proficient at occupying a wide range of hosts and an wide range of environments, might pioneer the initial establishment of symbioses in diverse larvae, and persist as a minor symbiont during the adult stage of many coral host’s lifecycles (Coffroth et al. 2001; Toller et al. 2001b). The relative hardness and adaptability of this clade may translate into an increased ability to survive *in vitro*, where it may quickly outcompete other clades.

The ecological relevance of the presence of vanishingly dilute minor symbionts *in hospite* remains unknown. It has been suggested that robust minor symbionts may serve hosts as backup symbionts when the more finely-tuned major symbionts become bleached, but evidence for this idea is limited to the redistribution of equally dominant symbionts of
three-clades within artificially moved hosts according to light and depth (Baker 2001; Rowan et al. 1997).

Molecular work undertaken on major symbionts that have been successfully cultured decades ago and maintained in culture to this day (LaJeunesse 2001; Santos et al. 2001), has shown that in many cases the isolates are genetically identical to those that are detected in hospite in the corresponding hosts at the corresponding locations (reviewed in Santos et al. 2001). As suggested by Santos, the process of back-checking is desirable, given the outcomes of Santos’ study and this culturing study, which have shown that a host can contain more than one symbiont, and that in some cases the non-dominant symbiont is the culturable one. It will likewise be desirable to confirm by PCR that the cultures obtained in the current study were present in the FIZ taken from the respective hosts at the One Tree Island field site.

4.5 Is light level a crucial factor for de novo culturing of clade C?

It is not clear in the literature whether clade C Symbiodinium has a preference for lower or higher light than clade A. This question bears on the outcomes of this culturing work reported here, in terms of methods validation. Rowan and Knowlton (Rowan and Knowlton 1995) and Rowan et al. (Rowan et al. 1997) reported that clade A hosts are found in higher light regimes than clade C in situ in a single host colony of Montastrea sp.. In the current study the conditions which yielded a clade C co-culture (with clade A) were uncontrolled room temperature, and a very low light level of <5 μE m⁻² s⁻¹. By contrast Schoenberg and Trench (1980a) obtained the clade C type culture from Heteractis lucida by incubating the culture 24 cm from a bank of three 40W bulbs, which should yield a photon flux of ~100 μE m⁻² s⁻¹.

The extremes of high and low light requirements tolerated within the genus are also not clear. McLaughlin and Zahl (Zahl and McLaughlin 1959) reported a zooxanthellate scleractinian coral living at 100 fathoms depth, though they did not report successful culturing of the symbiont nor did they establish the genus as equivalent to their other cultures which they grouped into “Gymnodinium adriaticum” the then current name for Symbiodinium (McLaughlin and Zahl 1959). Schlichter and Fricke (Schlichter and Fricke 1991) reported that the zooxanthellate scleractinian Leptoseris fragilis occurs exclusively
at depths between 95 m and 145 m where light is minimal. Similarly several groups have reported that zooxanthellate cnidarians can grow in fully shaded caves (Secord and Muller-Parker 2005).

Work needs to be done to correlate the growth response and stress response of *Symbiodinium* clades, subclades and strains, with light level. A comparative study of this nature has been done rigorously (Iglesias-Prieto and Trench 1994) using cultured *S. microadriaticum* (clade A1), *S. pilosum* (clade A2) and *S. kawagutii* (clade F1). To investigate whether the light level used in the series of experiments reported in this study may have selected against zooxanthellae of particular clades (eg clade C) and selected for other clades such as clade A, a literature review of commonly used irradiance levels in culture was undertaken (not shown). One study in particular was found to be relevant to the aims of the current study: Fitt and Trench (Fitt and Trench 1983) documented that at 20 μE m$^{-2}$ s$^{-1}$, a light level only double that used in this study, clade C *Symbiodinium* grew at four times the rate that clade B and clade A *Symbiodinium* grew. The *S. goreau* culture used by Fitt and Trench (1983) has an identical ITS genotype to the symbionts of *H. actiniformis* and *L. purpurea* that attempts were made to culture in the current study (sequence comparison not shown), and so considerations of strain variability of light requirements should not be relevant, and these symbionts should perform as the archetypal *S. goreau* culture does. Cultured cladeC behave in one way, but in hospite cladeC may behave in another: many groups have documented that the natural preference of clade C is for light higher than 100 μE m$^{-2}$ s$^{-1}$ (Trench 1993).

Tchernov et al. (Tchernov et al. 2004), directly asking a similar phylophysiological question, investigated temperature tolerances of a range of zooxanthellae clades and subclades, and no consistency was seen within any particular clade. If light requirements are as hyperevolvable as temperature requirements, then it may be impossible to predict for any given symbiont strain what level of light should be used for de novo culturing, and rather a range of light levels should be tried.

Light of 10 μE m$^{-2}$ s$^{-1}$ is able to support in hospite growth of *Symbiodinium* as documented by Secord and Muller-Parker (2005) in a dimly lit area of an intertidal cave. The host *Anthopleura elegantissima*, that bore the symbionts in that study, houses 'S. muscatinei' (clade B4) at the northern (temperate) part of its range, and 'S. californium' (clade E) at the
southern (warmer) part of its range (LaJeunesse 2001; LaJeunesse and Trench 2000), as well as alternatively or conjointly housing ‘zoochlorellae’ (chlorophyte symbionts) (LaJeunesse and Trench 2000; Lewis and Muller-Parker 2004). The shade-tolerant zooxanthellae of the Secord and Muller-Parker study (2005), being at the northern latitudes were presumably ‘S. muscatinei’.

Subtleties that must be considered include (a) the fact that the strain of S. goreau used by Fitt and Trench (1983) was an already established culture and may have become adapted to low light in vitro over many years, (b) was obtained from an anenome, not a scleractinian so is only a partial model for the symbiont of H. actiniformis and L. purpurea; and (c) the light level of ~10 \( \mu \text{E m}^{-2} \text{s}^{-1} \) used in this study is half that used in the relevant across-clade comparison by Fitt and Trench (Table 1 of Fitt and Trench 1983), and the light-responses across clades may not hold constant across this range of irradiances. In summary it is unclear whether light level plays a part in aiding culture of particular strains.

4.6 Microecologies in culture

Among the serial dilutions carried out in this study, it was often observed that each individual well of a given dilution series gave a different microecology than its neighbouring wells. Of any three adjacent wells it was not unusual to find the two furthest apart wells contained Symbiodinium while the middle well did not (data not shown). This may indicate that cell density alone does not dictate the culture success probability, and it implies that other microbes in co-culture (bacteria for instance) might influence Symbiodinium in vitro viability. Similarly, genotyping with actin primers revealed that two culture wells of a single dilution series inoculated with FIZ from L purpurea resulted in three different clade F symbionts being cultured (Z3, Z4 and Z6 each genotyped for actin locus in chapter 4). It may be that culturing of algal symbionts may be a complex competitive interplay of symbionts, bacteria, and potentially other eukaryotes and viruses during the initial stages of the culture before any one Symbiodinium clade or strain gains dominance.

As the inoculum becomes increasingly dilute, conditions in each well may become substantially different from each other. Due to this multi-environment aspect of serial dilution, it is a relevant method of culturing in its own right. In this study, as well as selecting cells from the well containing the most extreme dilution of inocula that yielded a
culture, some cells were also picked from wells not containing the most extreme dilution. The numerically dominant symbiont is theoretically the only symbiont present in the well containing the most extreme dilution that yielded a culture, but this principle is confounded if cells are carried over from well to well by adhering to the pipette tip. A new pipette tip was not used for each dilution in this study.

The finding that wells of a single dilution series contained different *Symbiodinium* strains resembles the finding of Santos et al. (2001) that mixed-strain *Symbiodinium* cultures can become stratified into micro-environments, such as when one strain prefers the water-air interface, while another strain accumulates on the vessel floor. Similar effects may account for the low success rate of culturing clade C *Symbiodinium* in this study, because the two methods favoured here were: picking microcolonies from liquid culture, and picking a loopful of material from the meniscus. If Clade C symbionts were to prefer to settle on the vessel floor, and yet were not to form discrete colonies on the vessel floor then they would have been unintentionally selected against by the method of picking a single microcolony from initial culture wells to found a culture. Similarly but inversely, settled cells (not forming foci) that appeared after initial culturing were favoured by subculturing as they were easiest to pipette and transfer. Artificial favouring of some ecotypes over others may therefore have occurred either during *de novo* culturing or during subculturing or both.

The long period between culture initiation and culture genotyping may have played a part in the attainment of unialgal cultures, via repeated founder effects at each periodic subculturing. The purity of the final cultures may also have been influenced via allelopathic interactions over this same time period. In multiple studies, filtrates of growth medium occupied by dinoflagellates have been observed to inhibit the growth of other marine phytoplankton divisions, and this action is attributed to allelopathic chemicals (Arzul et al. 1999; Fistarol et al. 2004a; Fistarol et al. 2004b; Kubanek et al. 2005; Legrand et al. 2003; Rengefors and Legrand 2001; Smayda 1997; Windust et al. 1996). In some studies allelopathy (antagonism through species-specific antimicrobial chemicals) between dinoflagellate genera has been evidenced, such as between Harmful Algal Bloom (HAB) genera (Arzul et al. 1999; Fistarol et al. 2004a; Kubanek et al. 2005). HAB dinoflagellates possess toxins, and *Symbiodinium* likewise possesses zooxanthellatoxins (Murata and Yasumoto 2000; Nakamura et al. 1995; Nakamura et al. 1993; Rein and Borrone 1999) though the biological/ecological functions of these have not been established. Allelopathy
can be proposed as one alternative among many potential functions. Since allelopathy is equally as evident at the between division level as at the between genera level it may be interpreted that allelopathy is not a function of taxonomic distance but rather of the frequency with which the two organisms meet and compete with each other in the environment (Gross 2003; Kubanek et al. 2005; Legrand et al. 2003; Rengefors and Legrand 2001; Smayda 1997; Vardi et al. 2002). By inference, *in hospite* and environmental competition might be a positive selector for allelopathy between strains within the genus *Symbiodinium*, an idea expanded on in the general Discussion (Chapter 7) including presentation of testable hypotheses.

4.7 Phylogenetic analysis of *Symbiodinium* cultures reveals novel diversity within clades A and F

The sequence diversity observed within the rDNA operon across isolates documents three diverse new genotypes and one novel phylotype within clade A, and one new phylotype within clade F (Figures 3.1 and 3.2, and Tables 3.10, 3.11 and 3.12). The distances between the novel genotypes in clade A are equal to or greater than the distances between some existing clade A subclades. A novel clade F phylotype assignment is within existing subclade Fr5 (Pawlowski et al. 2001; Pochon et al. 2001) on the basis that it is unknown whether this new phylotype is equivalent to F2 or not. These will be discussed individually.

**Novel cluster within clades A and F**

It was not possible to confidently assign culture Z3 to either F1 or Fr5 unaltered, rather it belongs in some subset of Fr5, here dubbed Fr5*. Previous authors (Pawlowski et al. 2001; Pochon et al. 2001) did not include sequences from F1 or F2 on their trees containing Fr5, perhaps initially because lsl rDNA sequences were not available for F1 or F2 at that time. This may be why Z3 subgroup within Fr5 has not been previously recognised.

Two former members of subclade A2, the environmental genotypes from *Amphiscolops* sp. (B. Baillie unpublished sequence AF201746, used with permission) and *Sarcophyton glaucum* (Carlos et al. 1999) are here transferred to novel subclade A2* (asterisk indicates divergence from A2), which includes cultures H10K and L9 (Figures 3.1 and 3.2). The symbiont of *S. glaucum* was basal to other members of subclade A2 in the study of Carlos
et al. (1999). The novel culture Z1 is assigned to a novel genotype. All such novel genotypes in clade A that are represented by single sequences remain unnamed, pending the culturing of an appropriately larger environmental sample set. While precedents exist in which novel phylotypes were established on the basis of single sequences: F1, F2 (LaJeunesse 2001), D1 and D2 (Pochon et al. 2001); and are probably each valid subclades in these particular cases, nevertheless this practice is accompanied by a risk of oversegregating, so is avoided in this study. It is apparent that many authors have not intended to instate a subclade, but are merely assigning a unique epithet to their new phylotypes to enable discussion. Authors of subsequent publications have tended to slide into non-monophyletic subclade-naming based on such precedents, so it is here suggested that in future the criterion of monophyly be adhered to at the taxonomic level of subclade before a subclade letter–number allocation is made. Such a system would be consistent with practice of the National Centre for Biological Information USA (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=taxonomy), which recognises monophyly of groups (rather than single strains) as the justifiable basis for acceptance of newly published taxonomic revisions.

4.8 Sequence diversity is unlikely to be due to HOCl-catalysed base mutations

In this study, zooxanthellae of four clades were cultured (A, B, C, F). The clade A and F cultures (the bulk of cultures obtained) were argued above as not representing the dominant symbiont. Detailed phylogenetic investigation of these clade A and F cultures (Figures 3.1 and 3.2), based on comprehensive taxon selection from BLAST results, sorted some apparently novel strains of clades A and F, from some less novel ones (section 4.7).

As a prior condition to acceptance of the additional diversity in these clades, an assessment was undertaken of the validity of the sequences from cultures obtained using HOCl, in view of the possibility of mutation of DNA bases by the HOCl during de novo culturing (Jiang et al. 2003; Kawai et al. 2004; Masuda et al. 2001; Whiteman et al. 1999; Zavodnik et al. 2004). Base changes in variable and conserved regions of the LSU rDNA and ITS loci were assessed. This was done by analysis of the relative transition/transversion ratios at these loci, as this was the only available measure of potential damage.
Ti/Tv ratios observed in pairwise ITS sequence comparisons, including the comparator strains, were much lower than those observed for lsu rDNA within this clade, presumably because ITS is not under as strong a selection for secondary structure as lsu rDNA. A similar contrast has been documented (Roalson et al. 2001) in a comparison of chloroplast tRNA (Ti/Tv 1/0.76) and nuclear ITS (Ti/Tv 1/2.34) of a set of congeneric plants. A low Ti/Tv ratio of 1.668521/1 has also been documented in the ITS of lichens and their algal symbionts (Piercey-Normore and Depriest 2001). Therefore the low ratio of Ti/Tv in the ITS of the novel Symbiodinium cultures does not necessarily point to a mutagenic effect of HOCl treatment. Furthermore, the ITS based analysis of Ti/Tv ratios was undertaken on the same cultures as were used in the lsu rDNA based analysis of Ti/Tv ratios, and the latter had contraindicated a mutagenic effect, by virtue of bias for transversions toward the variable regions of the molecule. Just as in the lsu rDNA analysis, transversions in ITS sequences were biased towards the variable regions (alignment not shown), since the 5.8S sequences are invariable across all isolates of a given clade, and yet these form part of the same amplicons respectively as yielded the variable ITS sequences.

The high Ti/Tv values in lsu rDNA comparisons argue against the possibility that hypochlorite exposure was mutagenic, because chemical alteration of bases by hyperchlorite and its degradation products would be expected to have the following effects: (a) to target purines and pyrimidines equally (Henderson et al. 1999; Kawai et al. 2004; Masuda et al. 2001), (b) to thus negate any positively-selected bias to transitions, (c) to therefore yield an apparent increase in transversions above that seen in the comparator pairs A1-A4 and F1-Fr5 which are sequences not suspected of chemical-induced mutation.

Lsu rDNA sequences of cultures L9, L4 and H4xH7 used in the Ti/Tv analysis were from unialgal material, as were ITS sequences of G1-darkhue and G2. In other analysed cases: Z1, Lp13, Z3, Z4, BPdN4 and H'10K (cases ‘R’ in Table 3.7 column 2), cultures from corals were derived from a population of cells in which clonality was implied, because chromatograms were unambiguous. It is unlikely that a mutation sweep that could produce the quantity of base changes in a clonal cell line in each of the lsu rDNA sequences of this study would not also result in fatal harm to each of these protists. Accordingly it is likely that the sequences documented here from HOCl-induced cultures are reflective of real sequence diversity in the field, and that the base changes have been gradually obtained in the field rather than suddenly obtained in culture.
HOCl degrades to O\textsuperscript{•}, O\textsubscript{2}H\textsuperscript{−} (aqueous) and Cl\textsubscript{2} (gas) (Wang and Margerum 1994) and that the Cl\textsubscript{2} escapes the liquid within a few days in the absence of a chlorine buffer such as trichloroisocyanurate (pool chlorine buffer)(Debowski and Gerber 1993; Solastiouk and Degliis 1988). As such, the HOCl molecule was not present in the growth media of these *Symbiodinium* cultures for more than a few generations (*Symbiodinium* cell cycles are >1 day long), and not for the number of generations that would be required to accumulate transversions in only the variable regions of the rDNA operon while maintaining the conserved regions.

After Cl\textsubscript{2} has escaped the medium, the degradation products O\textsuperscript{•} and OH\textsuperscript{−}, themselves potential mutagens, should combine with free H\textsuperscript{+} ions in the medium because buffering of the HOCl stock and of the f2 and fEs media was in the neutral pH range 7-8. This again favours a temporary oxidative effect of the HOCl additive rather than a long term one.

Analysis of Ti/Tv ratio across cultures was useful. In the absence of any evidence for a mutagenic effect of HOCl it is noted that damage to DNA bases is normally alleviated by base-excision repair (Krokan et al. 1997; Tuteja et al. 2001; Wood 1996). Finally, whilst HOCl can damage nucleic acids (Jiang et al. 2003; Kawai et al. 2004; Masuda et al. 2001; Whiteman et al. 1999; Zavodnik et al. 2004), its biocidal action is more a result of protein and lipid peroxidation (Abid et al. 2004; Gutteridge 1995; Hawkins et al. 2003; Khor et al. 2004; Spickett et al. 2000; Zavodnik et al. 2004). Its use as a temporary selective culturing agent may be feasible in organisms such as dinoflagellates that possess oxidation protection compounds (expanded on in General Discussion, chapter 7) which may prevent cell death otherwise occurring via DNA oxidation, or protein and lipid peroxidation.

### 4.9 Hypochlorite as a selective agent for culturing *Symbiodinium*?

Pre-filtering FIZ through glasswool (McLaughlin and Zahl 1957; McLaughlin and Zahl 1959; Schoenberg and Trench 1980a) or 20μm gauze is an option for culturing of *Symbiodinium* free of ciliates and macroalgae, but this was not tried in the current study. The chlorine enhanced medium used here is an alternative to filtering for removal of such predators and competitors.
The initial impetus for treating corals to a greater concentration of household bleach than was employed by York (York 1986), was because in this study two Gt nubbins, Gt1 and Gt2 had been stored alive for approximately two to three weeks in the light room in seawater with no filtration and only a gentle aeration, and they appeared alive but very bloated. A third Gt had already died of this bloat condition. To overcome the effect of this presumably septic condition on zooxanthellae, the live Gt nubbins were exposed to a 1:100 dilution of neutralized hypochlorite for a long period: overnight.

This study documents a gradual improvement in the capacity of HOCl-enriched fEs medium and HOCl pre-treatment of nubbins to aid the generation of cultures from host extracts (Table 3.7, Table 3.9). Two hypotheses can be made for why HOCl improved the culturing success rate and these hypotheses are not mutually exclusive:

1) HOCl may select for growth of intracellular symbionts. This may be due to biocidal effects against the growth of other protists such as ciliates, bacteria and fungi which would otherwise compete with *Symbiodinium* or harm it. The biocidal effect of HOCl is hypothesised to be less pronounced on *Symbiodinium*.

2) HOCl may stimulate zooxanthellae growth or development directly.

*Support for hypothesis 1*: It has been documented that *Symbiodinium* from grazed corals, can pass undigested and alive through the alimentary tract of a pufferfish (Augustine and Muller-Parker 1998), implying resistance to stomach chemicals, as well as resistance to digestive enzymes and bile. Stomach chemistry in vertebrates is chloride ion based (Bhaskar et al. 1992; Soybel et al. 1995; Trischitta et al. 1998), raising the possibility that the HOCl medium documented in this study may mimic these fluids, which could help explain the success of the medium. While it is unknown whether small bites of anenome tissue would generate significant amounts of HCl in the pufferfish stomach, it can be assumed that some increased level of reactive chloride species are encountered there by *Symbiodinium*, relative to the chloride level in seawater.

*Support for hypothesis 2*: Steele (Steele 1977) has proposed that motile *Symbiodinium* cells which are seen swimming from anenome excreta, may have been stimulated to flagellate by some chemical element in the excreta. In the current study, free-swimming *Symbiodinium* were seen in the aquarium water when *H. actiniformis* corals were stressed by substandard temperature and oxygen levels, and were extruding large pellets of many
millions of immotile zooxanthellae per pellet. The number of motiles in the aquarium at such times was ~10 cells per drop of seawater (data not shown).

**4.10 Prospects for a selective growth medium for *Symbiodinium***

The fEs-HOCl medium tested here may provide a tool for the design of a selective medium for growth of *Symbiodinium* and other intracellular symbiotic algae. Selectivity shown here was limited to exclusion of ciliates, and possibly some bacteria, so the hypothesis of selectivity against non-symbiotic microalgae has not been directly tested, merely raised. Of relevance, two novel taxa from the tissues of corals were also cultured with the fEs-HOCl medium. Those taxa are documented in chapter 6, and at least one of the taxa is indicated to be a photosynthetic apicomplexan, which may be of ecological significance and may demonstrate that the chlorine enhanced media used to culture F1Z are selective for symbiotic phototrophs.

An alternative medium that could be tried, in order to positively boost growth of all clades of *Symbiodinium*, would include the agent mimosine that has been shown to confer positive growth increase on a dinoflagellate while separately inhibiting the growth of a stramenopile, a cryptophyte and a haptophyte (Yeung et al. 2002). Mimosine interferes with deoxyribonucleotide metabolism (Gilbert et al. 1995; Oppenheim et al. 2000), and subsequently halts cell cycle progression (Mikhailov et al. 2000; Tsai and Ling 1971). The selective action of mimosine for the growth of dinoflagellates was shown for several dinoflagellate genera (Yeung et al. 2002), and was attributed by the authors to the unusual chromatin structure and DNA metabolism of the group, compared to all other eukaryotes including the other algal divisions studied.

The medium Asp-8A (Ahles 1967b) which differs from fEs, successfully cultured the major symbiont in most pioneering studies (Fitt et al. 1981; Schoenberg and Trench 1980a) and may be the preferred medium for future attempts to culture *Symbiodinium* from scleractinians. Projects that deliberately seek to culture dominant symbionts and minor symbionts, might consider using Asp-8A-HOCl in parallel with Asp-8A. In studies that attempt to *de novo* culture the major symbiont from scleractinians, the media used here, fEs (a close variant of f/2) and fEs-HOCl, may be used with caution, given the outcomes of this study (possible selective culturing of clades A and F). Optimistically, the exact
formulation of the medium (f/2 versus Asp-8A) may not matter since no systematically broad survey of the efficacy of f/2 in culturing major symbionts (like the Asp-8A response survey of Schoenberg and Trench 1980a) has been reported.

4.11 Motile Symbiodinium cell-cell attachment observed

Fusion of motile cells was observed in freshly made cultures of clade A organisms (L4, G2, Z1, this study). These observations could indicate that ready adaptation to culture may correlate to sexual potential, or could indicate that gametogenesis may occur preferentially in freshly isolated cultures as opposed to old cultures. An observation of motile cell-cell fusion has also been made by Dr Kirsten Heimann (James Cook University, Queensland Australia, pers. comm.): the organism was a freshly isolated symbiont of a scleractinian host from the GBR. Dr Heimann's observation was that a motile cell-cell fusion resulted in the formation of a zygote bearing two longitudinal flagella, a typical phenotype of dinoflagellate zygotes (Pfiester 1984; Pfiester 1989; Pfiester and Anderson 1987). Dr Heimann noted the similarity of the Symbiodinium zygote to the zygote of Woloszynska in terms of number of flagella, but the organism was sufficiently different from Woloszynska to determine that it was not Woloszynska (K. Heimann, unpublished data, cited with permission). Dr Heimann's is the first overt observation of gamete fusion and zygote formation in Symbiodinium. That encounter was not photographed. Such a report should provide strong motivation for researchers to make fresh de novo cultures and observe these for in vitro sexuality, with a view to photographic documentation. Since the motile cell-cell attachment events observed in this study and by Dr Heimann have not been reported before, it can be speculated that cells observed by other workers were adapted to asexual propagation in culture, and that the competency of cells to undergo gametogenesis may be dependent on the length of time since initial culturing. Additionally, if gametogenesis could be reproducibly obtained in artificial medium, then it would begin to suggest that seawater, as opposed to the in hospite environment, is the site of gametogenesis.

In order to eventually understand the sexual cycle of Symbiodinium, mating competent cell lines will be required. Since media such as fEs and f2 resemble seawater, and may induce gametogenesis in young cultures (this study), cells that are potentially gametogenesis-competent ought to be picked individually from young cultures, so as to enrich for efficient maters (U. Goodenough, Washington University St Louis, pers. comm.). Otherwise, mating competent cells are likely to be swamped by cells that are more competent at
proliferating asexually, given that mitosis is typically faster than meiosis, requires less stimuli, and cultures are often deliberately made clonal thus inadvertently precluding the option of a sexual cycle.

In this study, each of the cell lines that was observed to undergo attachment while motile (L4, G2, Z1) had been cultivated via a HOCl step, raising the question of whether this agent might aid induction of gametogenesis. Cell observations on all cultures were made weekly during the first few months of their cultivation and observation frequency was equal across all cultures, so the occurrence of motile cell-cell attachment events only in zooxanthellae from strongly HOCl-exposed corals and strongly HOCl-exposed FIZ, may potentially be significant. However, gamete fusing was not seen in all HOCl exposed cultures.

4.12 Does the transition to free-living involve a transition from high to low nutrient conditions, and is this the cue for a sexual cycle?

Host coral individuals can contain a single strain of *Symbiodinium*, as shown by unambiguous sequences obtained from hypervariable loci (e.g. *psbA* non-coding region chapters 4 and 5). Attempts to culture *Symbiodinium* from unialgal coral hosts may thus rely on the strain dividing asexually. Unialgal versus polyclonal host status has not been correlated to culturing success, perhaps because of the similarity of phenotypes across *Symbiodinium* strains, and because hypervariable marker loci have not been characterized until recently (Goulet and Coffroth 2003a; Santos and Coffroth 2003a; Santos et al. 2003c; Santos et al. 2002b). Potential linkage of the *Symbiodinium* environmental- and sexual-lifecycles has likewise not been investigated, but if present could help explain the canonical low success rate of culturing (Schoenberg and Trench 1980a).

A logical corollary to the hypothesis that low nutrient conditions may favour gametogenesis in *Symbiodinium* (Baillie et al. 1998), is that culturing of single strains from unialgal corals would be difficult, because induced gametes would be unable to find a fusion partner. Mixing inocula of FIZ from *H. actiniformis* did not yield a greater culturing success rate than the control experiment that employed unmixed inocula of FIZ from this host. Two cultures (H2xH3, and H4xH7) resulted from mixing inocula, but one of these (H4xH7) was established on an agar plate and so was of doubtful significance given that
dessicating surfaces are not common in the organism’s natural environment, and that immobility may prevent mixing. Two cultures also resulted from unmixed inocula (H9, and H10K, though H10K was from a separate experiment). Considering the approximate equivalence of the culturing success rate from mixed and unmixed inocula, no support was gained for the hypothesis that transfer of FIZ from in hospite to a seawater medium induces gametogenesis, zygote formation and re-entry to the asexual cycle with associated proliferation. However, it is not known whether the ten H. actiniformis colonies used contained intercompatible strains. Accordingly, FIZ of H. actiniformis and the closely related FIZ of L. purpurea were also mixed but no cultures resulted (data not shown).

In reply to the hypothesis of Baillie et al. (1998) that nutrient-limitation might be a gametogenesis cue, the results of this chapter raises an alternative hypothesis. All three of the sightings of putative fusing gametes in this chapter were in young cultures that had been obtained with the aid of HOCl, that is they had recently experienced exposure to HOCl. It is hypothesised here that HOCl may be an analogue of digestive fluids (of hosts and of grazers) and that digestive fluids may be a natural cue for Symbiodinium gametogenesis. This could be a qualitative rather than an absolute requirement since the putative appressed gametes observed by Freudenthal (Freudenthal 1959) were not exposed to HOCl.

Even giving benefit of doubt to the idea that digestive fluids may induce gametogenesis, the utility of very low nutrient media for Symbiodinium growth is noted. The lowest nutrient regime used in this study that resulted in a Symbiodinium culture being generated was incubation in autoclaved seawater for 8 months and it was interesting that this was the treatment that resulted in clade C being cultured (mixed clade C / clade A culture, BPdN1, from P. damicornis). These cells were presumably living off degradation products from dying algae. The possibility that extremely low nutrient levels may be beneficial to de novo culturing of Symbiodinium may warrant further investigation.

4.13 Chapter summary in the context of the thesis aims
The finding of several new basal members of clade A in this study indicates that deep-branching members of the genus Symbiodinium might be obtained by surveying a range of hosts for minor symbionts. The chlorine-rich medium developed in this chapter may
eventually have an impact on attempts to culture the major symbionts from clade C-bearing scleractinians, based on the precedent of a weakly growing co-cultured clade C isolate gained in this chapter.

Some of the cultures isolated in this chapter proved useful in defining markers for use other chapters: In chapter 4, culture Tm4.1 was used to isolate the \textit{psbA} ORF, and cultures Z3, Z4 and Z6 were used to trace actin intron evolution. The clade C isolate remains in co-culture with a clade A isolate because the growth rate of both was insufficient to allow separation by serial dilution to be attempted.

The observations of motile cell-cell fusion in this study, should lead to purification of mating-competent cells (by picking of fused cells) from young cultures, so that mating competency of lab strains can be maintained, enhanced, and eventually stabilised which would allow the sexual lifecycle of \textit{Symbiodinium} to be followed \textit{in vitro}.

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Chapter 4

Development and analysis of novel molecular markers from *Symbiodinium*

Worked described in this chapter formed the basis of the following manuscripts:


The major objective of the current chapter was to develop three unlinked hypervariable markers. These would then be applied to a study of recombination among isolates of a population of clade C *Symbiodinium* (in chapter 5). In view of the lack of sequence information available for the genome of *Symbiodinium*, and the predominance of multicopy nuclear gene sequences of this organism in Genbank, it was decided to target loci from the chloroplast and mitochondrion, as well as the nucleus. The aims of this chapter were therefore:

1) To characterise a molecular marker from each DNA-bearing organelle of *Symbiodinium*. Candidate loci were:
   i) Nuclear genome: actin gene
   ii) Chloroplast genome: *psbA* gene
   iii) Mitochondrial genome: *lsu* rDNA gene

2) To establish, using phylogenetic analyses, that the three markers were derived from *Symbiodinium* and not from contaminants.
1. Introduction

1.1 Actins as nuclear markers for population genetics

Dinoflagellate actins were first sequenced as comparators to elucidate the phylogeny of the genus *Perkinsus*, which was consequently shown to be related to dinoflagellates (Reece et al. 1997). The use of the coding region of the actin gene as a population genetic marker within a dinoflagellate genus has not been preceded, but actin nucleotides have been used for this purpose in the genus *Cryptosporidium* of the related division Apicomplexa (Sulaiman et al. 2002), and in plants (Bouget et al. 1995; deSa and Drouin 1996). Sulaiman et al. (2002) showed that distinct genetic variations exist between *Cryptosporidium* species, as well as between host-specific genotypes of *C. parvum* (hosts: human, ferret, monkey, mouse, marsupial, pig, bear and dog). The nucleotide distance between *C. parvum* genotypes (1.1–13.9%) resembled that between *Cryptosporidium* species (2.5-13.6%). Intra-genotype variations were very low to absent. A precedent therefore exists to show that actin is a useful strain marker for an intracellular eukaryote with host-specificity.

It has been asserted by Fukuda et al. (Fukuda et al. 2002) that *Symbiodinium* actins and scleractinian actins have been sequenced. Further, an additional actin has been published from zooxanthellate scleractinian *Stylophora pistillata* (Fukuda et al. 2002; Unger and Roesijadi 1993; Zoccola et al. 2004). These data require corroboration if actin is to become a phylogenetic or population genetic marker either in corals or in *Symbiodinium*. Investigation of the origin of actin sequences from both organisms comprising a eukaryote-eukaryote symbiosis, should proceed in tandem via phylogenetic analysis. Omission of this step erodes confidence in the data and is a disincentive to further work using such markers (S. Dove, Univ. of Queensland, pers. comm.). The standard way to develop a new genetic marker from *Symbiodinium* that are associated with coral is to characterise the markers first from pure *Symbiodinium* cultures in the absence of host tissue. This was done during the development of the nuclear rDNA operon, and a mitochondrial housekeeping gene, as markers (Baillie et al. 2000a; Hunter et al. 1997; LaJeunesse 2001; Rowan 1991; Rowan and Powers 1991; Santos et al. 2002b; Takabayashi et al. 2004). By contrast, a standard way to characterise host markers that are
free of symbiont contamination is by amplifying them from sperm DNA (Takabayashi et al. 1998) which is zooxanthella-free, or if that is not possible then by sequencing both the host and symbiont ortholog of the marker and undertaking phylogenetic analyses (Takabayashi et al. 1998).

1.2 Dinoflagellate chloroplast genes

As markers for *Symbiodinium* phylogeny, the potential utility of genes encoding photosynthesis-related functions is that the marker is by definition specific to the symbiont and not the host. An attraction of using a chloroplast marker together with a nuclear-coded marker, is that the two loci would be by definition unlinked. This would make the pair of markers suited to studies of gene reassortment across a population for the purpose of assessing recent history of sexual recombination, or clonality.

Peridinin dinoflagellates have a reduced complement of genes encoded in the chloroplast, by comparison to the chloroplast genomes of other algae and plants (Bachvaroff et al. 2004; Hackett et al. 2004b; Hackett et al. 2004a). While other algal plastid genomes contain tens of genes (Martin et al. 2002; Martin et al. 1998), the peridinin-type dinoflagellate plastid contains but a handful of genes (Koumandou et al. 2004), the other genes having been transferred to the nucleus with protein reimportation to the plastid (Cavalier-Smith 2003; Foth et al. 2003). One gene confirmed as coded within the *Symbiodinium* plastid is the *psbA* gene (Takishita et al. 2003a). Takishita et al. (2003a) proved this localisation by labelling a probe specific for *psbA* mRNA and showing that the probe hybridised only to the chloroplast lumen and not to the *Symbiodinium* cytoplasm.

The *psbA* gene encodes the D1 protein of photosystem II, a quinone-binding protein and regulator of photosynthetic flux, that is present in all algal phyla (Pakrasi 1995; Singh 2000; Warner et al. 1999). This gene has been widely used for phylogeny in unicellular algae (Inagaki et al. 2004; Morden et al. 1992; Takishita et al. 2003a; Takishita et al. 1999b; Takishita et al. 2000; Takishita and Uchida 1999a; Zhang et al. 2000), although not for population studies within a species, owing to the highly conserved amino acid sequence of the protein.

The *psbA* gene is encoded on a single gene minicircle in a number of different peridinin-containing dinoflagellates, including species of *Heterocapsa* (Zhang et al. 2002; Zhang et
al. 1999), *Amphidinium operculatum* (Barbrook and Howe 2000; Barbrook et al. 2001; Ellen et al. 2004; Nisbet et al. 2004), *Amphidinium carterae* (Hiller 2001; Zhang et al. 2002; Zhang et al. 2000) and *Protoceratium reticulatum* (Zhang et al. 2002). Each of these species contains several minicircles, and most minicircles contain a single gene. Less frequently, two genes are encoded (Barbrook et al. 2001; Hiller 2001) and in one instance a three gene minicircle was found (Nisbet et al. 2004). The non-coding regions of the minicircles are highly divergent across dinoflagellate genera, except that short stretches of a single nucleotide are frequent. A common format across dinoflagellate genera and species is that the non-coding regions contain conserved core regions, usually of between two and four in number, separated by variable regions (Howe et al. 2003; Zhang et al. 2002). In a given species, two or more cores are often identical to each other, seemingly duplicated or triplicated within the same minicircle, such as the two 9G regions of *Heterocapsa triquetra* (Zhang et al. 2002; Zhang et al. 1999), and the three 5G regions of *Heterocapsa pygmeae* (Zhang et al. 2002).

It has been shown that conserved core regions are shared between all the minicircles present in one culture of *Heterocapsa triquetra*, implying that recombination maintains the sequences of the core regions (Zhang et al. 2002; Zhang et al. 1999). Likewise, core regions are identifiable across all the minicircles from *Amphidinium operculatum*, again implying intra-circle recombination (Barbrook and Howe 2000; Howe et al. 2003) but the sequence of these cores is not alignable with those from *Heterocapsa triquetra*. A hypothesis of species-specific conserved cores has been tested on two minicircles, psbA and 23S rDNA, from three further species of *Heterocapsa* and from the distantly related *A. carterae*, and was supported in each case (Zhang et al. 2002). While common cores were apparent within the two sequenced minicircles of each culture, there was a lack of core homology between species of *Heterocapsa* (Zhang et al. 2002). Only cores that contain inverted repeats or have a high A+T content have been assigned putative roles, the former as replication origins, recombination sites, integron-like sites, or DNA segregation loci, and the latter as replication origins (Barbrook and Howe 2000; Howe et al. 2003; Zhang et al. 2002; Zhang et al. 1999). Inverted repeats have not been found in cores that are present in duplicate or triplicate (Zhang et al. 2002).
1.3 Dinoflagellate mitochondrial genes

Dinoflagellate mitochondrial genes have been studied in the case of the three protein-coding genes that are known to be encoded there: cytochrome oxidase subunit 1 (cox1), cytochrome oxidase subunit III (coxIII) and cytochrome b (cob) (Chaput et al. 2002; Norman and Gray 1997; Norman and Gray 2001; Takabayashi et al. 2004; Zhang et al. 2005; Zhang and Lin 2002). The dinoflagellate cox1 and cob genes have been shown to be edited at the level of RNA (Lin et al. 2002) and have recently been used for phylogenetic analyses (Takabayashi et al. 2004; Zhang et al. 2005). Cox1 nucleotide sequence provided less variability than nuclear rDNA (Takabayashi et al. 2004) for distinguishing strains of *Symbiodinium* within clade B, but was useful in determination of clades. Cob amino acid sequence was indicated to be informative for distinguishing dinoflagellate orders and constituent genera (Zhang et al. 2005).

High-variability intergenic sequences, from the mitochondrial genomes of red algae, have been used as population genetic markers (Provan et al. 2005), though this has not been achieved with dinoflagellates or apicomplexans. Mitochondrial Lsu (lsu) rDNA makes a reasonable alternative target to intergenic regions for obtaining variable sequence, as it has been used to demonstrate species and cryptic species in various vertebrates, invertebrates and fungi (Bastrop et al. 1998; Bond and Sierwald 2002; Demanche et al. 2001; Etter et al. 1999; Govindarajan et al. 2005; Kinzie and Chee 1979; Lee et al. 2003).

Lsu rDNA from the dinoflagellate *Cryptophycodinium cohnii* has been characterised by J. Norman (Norman 2000). The gene was found to be fragmented (M. Gray, Dalhousie University, pers. comm.). A similar situation exists in mitochondrial genomes of apicomplexans (Feagin et al. 1991; Kairo et al. 1994), where the rDNAs are fragmented and rearranged, but maintained on a single DNA element (Feagin et al. 1991; Feagin et al. 1992). It has been shown that these fragments of rDNA and the neighbouring cytochrome ORFs are polycistronically transcribed (Ji et al. 1996; Rehkopf et al. 2000), and that the concatemer is cleaved to produce rRNAs and mRNAs (Feagin et al. 1997; Gillespie et al. 1999; Rehkopf et al. 2000). It is assumed that the rRNA fragments assemble into functional ribosomes (Feagin et al. 1997). Parsimony might suggest that the *C. cohnii* and apicomplexan mitochondrial genomes shared a fragmented common ancestor. However, no information is yet available for the mitochondrial genome of *Perkinsus*, which represents a common ancestor of dinoflagellates and apicomplexans (Leander and Keeling
2003; Leander and Keeling 2004; Saldarriaga et al. 2003), and is the subject of a genome sequencing project (www.tigr.org). It is therefore unknown whether the fragmentation of the mitochondrial rDNAs of *C. cohnii* and apicomplexans may have occurred independently. The next closest exemplars relevant to dinoflagellate mitochondrial evolution are the ciliates, their sister group in the Alveolata (Baroin et al. 1988; Gajadhar et al. 1991; Wolters 1991). Two ciliate mitochondrial genomes have been sequenced and the encoded LSU rDNAs are fragmented at the ends, but a long sequence from the middle of the rDNA aligns with that of stramenopiles, which are in turn the next closest eukaryotic group to alveolates (Baldauf et al. 2000; Van de Peer and De Wachter 1997).

1.4 Chapter 4 overview

The work outlined in this chapter resulted in the development of two novel molecular markers for *Symbiodinium*: actin from the nuclear genome, and a minicircle non-coding region from the chloroplast. In addition sequencing revealed a number of interesting and unique features, including: (i) the first systematic analysis of spliceosomal intron evolution in a nuclear gene of a dinoflagellate, and (ii) an analysis of double hairpin structures and inferred recombination in the non-coding region of the *psbA* minicircle from *Symbiodinium*.

2. Materials and methods

DNA sequencing, identification of gene-coding regions, generation of sequence alignments, and phylogenetic analysis are as detailed in General Materials and Methods (Chapter 2)

2.1 Cultured and *in hospite* dinoflagellates

Actin, *psbA*, cox1 and mitochondrial LSU rDNA analyses were undertaken on the cultured and *in hospite* dinoflagellates specified in Tables 4.1 and 4.2. Clade designations for cultured *Symbiodinium* strains are from chapter 3 and from Baillie et al 2000b, and Lajeunesse 2001. Clade designations for *in hospite Symbiodinium* are from the General Introduction and chapter 3. Clade C *Symbiodinium* was a desirable subject for ecological reasons but was not available in culture so was sourced directly from *in hospite*
Symbiodinium of coral hosts (Table 4.1). Cultures Tm8.2, CS-152, CS-153, and CS-158 were each made clonal (by dilution streaking a loopful of raw culture onto an agar plate, incubating for two months in a light room at 27°C on 14 hr light-10 hr dark cycle, then picking a single colony and growing the organism further in liquid medium) prior to being used as sources of DNA.
Table 4.1 *Symbiodinium* strains used for marker development

<table>
<thead>
<tr>
<th>Original host of cultured zooxanthella</th>
<th>Clade of culture</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tridacna maxima,</em></td>
<td>A</td>
<td>OTI (novel culture Tm8.2, chapter 3)</td>
</tr>
<tr>
<td><em>T. crocea</em></td>
<td>A</td>
<td>Public culture CSIRO-158, GBR</td>
</tr>
<tr>
<td><em>T. maxima</em></td>
<td>A</td>
<td>Public culture CSIRO-152, Enewetak</td>
</tr>
<tr>
<td><em>Cassiopea xamachana</em></td>
<td>A</td>
<td>Public culture CSIRO-153, Florida</td>
</tr>
<tr>
<td><em>Aiptasia sp.</em></td>
<td>B</td>
<td>Public culture CSIRO-164, Bermuda</td>
</tr>
<tr>
<td>minor symbiont of <em>Leptastrea purpurea</em></td>
<td>F</td>
<td>OTI (novel culture Z3, chapter 3)</td>
</tr>
<tr>
<td>minor symbiont of <em>Leptastrea purpurea</em></td>
<td>F</td>
<td>OTI (novel culture Z4, chapter 3)</td>
</tr>
<tr>
<td>minor symbiont of <em>Leptastrea purpurea</em></td>
<td>F</td>
<td>OTI (novel culture Z6, chapter 3)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>In hospite zooxanthella from host</th>
<th>Clade of FIZ</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Heliofungia actiniformis</em></td>
<td>C</td>
<td>OTI; coral tissue</td>
</tr>
<tr>
<td><em>Goniopora tenuiders</em></td>
<td>C</td>
<td>OTI; coral tissue</td>
</tr>
<tr>
<td><em>Leptastrea purpurea</em></td>
<td>C</td>
<td>OTI; coral tissue</td>
</tr>
<tr>
<td><em>Pocillopora damicornis</em></td>
<td>C</td>
<td>OTI; coral tissue</td>
</tr>
</tbody>
</table>

Table 4.2 Other dinoflagellate species used for genomic PCR in this chapter

<table>
<thead>
<tr>
<th>Culture</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Polarella glacialis</em> CCMP1383</td>
<td>Chris Bolch, University of Tasmania</td>
</tr>
<tr>
<td><em>Amphidinium carterae</em></td>
<td>Roger Hiller, Macquarie University, NSW</td>
</tr>
<tr>
<td><em>Heterocapsa pygmeae</em></td>
<td>&quot;</td>
</tr>
</tbody>
</table>

2.2 Reference sequences and strains

AGfact-p and AGcact-p are publicly available actin sequences from the scleractinian corals *Galaxea fascicularis* and *Favites chinensis* respectively (Fukuda et al. 2002), that both harbour zooxanthellae. Syact-p is a sequence from the same study and attributed to a *Symbiodinium* culture HG39, that was reported as clonal and was characterised by a high level of ssu rDNA identity with the clade A symbiont of the foraminiferan *Amphisorus h่มprichii*. The genbank accessions for those sequences are AB094432, AB086827 and AB086828. *Gymnodinium varians* actin (CCMP421, genbank accession AF482423) is regarded as clade E *Symbiodinium* actin as rDNA of this culture has consistently
segregated with this clade in several published studies (Pochon et al. 2005; Pochon et al. 2001; Santos et al. 2002b; Takabayashi et al. 2004). Sequences Tridactpart and Tridactfull putatively encode actins from symbionts or host cells of Tridacna gigas (unpublished) and were kindly provided by the Yellowlees Lab, James Cook University.

2.3 DNA templates and PCR reactions

DNA was extracted from cultures of Symbiodinium, Amphidinium and Heterocapsa, using buffer containing sarkosyl and SDS as detailed in Chapter 3. Genomic DNA of a Polarella sp. strain equivalent to CCMP1383 was kindly donated by C. Bolch, University of Tasmania.

Primers developed in this study are listed in Tables 4.3 and 4.4 and in section 2.6. The PCR conditions for all amplifications were: 94°C 2 min; then 35 cycles of 94°C 10 sec, 48-63°C 30 sec (depending on locus), and 72°C 2-5 min; followed by a single incubation at 72°C for 10 min, using a Perkin Elmer Gene Amp 2400 thermocycler. Reactants in each 100 μl reaction were 20 pmol of each primer, combined with 1-10 ng of genomic template DNA and a mix consisting of 1 μl AmpliTaq (Perkin Elmer), 80mg bovine serum albumin, 0.1 mM (final conc.) of each of dATP, dCTP, dTTP and dGTP, and 10 μl of 10X PCR buffer (1M Tris.HCl pH 8.3, 5M KCl, 150 mM MgCl2, Gelatin 1.0% w/v: (Sambrook et al. 1989) For the long range PCR of minicircles other than psbA, Expand Long Template PCR System (Roche Diagnostics, Indianapolis Indiana) was used in the place of AmpliTaq, and the reaction done using the buffer provided by Roche, and according to the temperature and time conditions above.

Amplicons were purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech Inc. Uppsala, Sweden), and directly sequenced without cloning, using the amplification primers (Table 4.3) and Big Dye Terminator. All sequencing was performed commercially at the Australian Genomic Research Facility, Brisbane.
2.4 Actin methods

Primer design, PCR and sequencing

Primers dinoactForw and dinoactRev are novel to this study, and were designed from a published alignment (Reece et al. 1997), placing primers as near to the ends of the alignment as possible, and keeping nucleotide degeneracy as low as possible at each base position, the limiting factor being variation among dinoflagellates at each base position. The 3’ ends of these primers were placed on G or C bases. They were used to amplify genomic actin fragments from four clades of *Symbiodinium*. The gene was also amplified from the dinoflagellates *Polarella* sp., *Amphidinium carterae* and *Heterocapsa pygmeae* to assess the conservation of introns, or otherwise. With the exception of dinoactForw and dinoactRev, all the remaining actin primers in this study (Table 4.3) were designed from *Symbiodinium* actin sequences gained during the study, and so are in many cases presumably *Symbiodinium*-specific. Nucleotide degeneracy at each base position was kept as low as possible and in most cases an attempt was made to include multiple clades of *Symbiodinium* in any alignment used for primer design. This aim relied on actin sequences being available and so was cumulative. Additionally, internal sequencing primers were often required due to large size (900-1100bp) of actin amplicons obtained e.g. with dinoactForw and dinoactRev. Ambiguous chromatograms resulted at putative intronic regions of clade A and C actin gene fragments that had been amplified e.g. with primers dinoactForw and dinoactRev. Further amplifications with a greater range of primers were undertaken, and direct sequencing used again, in order to overcome the ambiguities.

To verify that several copies of actin exist in the clade C symbiont of *G. tenuidens* (*Sym-Gi*), fourteen separate amplifications were performed encompassing pairwise combinations of five forward and four reverse primers in different positions of the coding sequence. Only two of these primers, CactForw1 and CactForw2, were in a similar position, and these possessed different 3’ ends.
Table 4.3 Primers used for amplification and sequencing of actin loci in this study

<table>
<thead>
<tr>
<th>Primer and direction</th>
<th>Primer sequence</th>
<th>Anneal temp</th>
<th>Specificity in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin (primers used for both amplification and sequencing)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dinoactForw</td>
<td>5'-GAGACNTTCAYTYGCNGCNATGTAYG</td>
<td>55-63°C</td>
<td>dinoflagellates and corals</td>
</tr>
<tr>
<td>dinoactRev</td>
<td>5'-CCCTRACTTCATNGTGANGGGCC</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>BActForw</td>
<td>5'-CGAACCACSGGAATGGATG</td>
<td>55°C</td>
<td>clades B, C, F Symbiodinium</td>
</tr>
<tr>
<td>zooactRev</td>
<td>5'-AGYCTCTGTCATNGCCGCTAC</td>
<td>&quot;</td>
<td>Symbiodinium</td>
</tr>
<tr>
<td>GActForw</td>
<td>5'-GTGACTCTYACGGAGTCATGAAG</td>
<td>60°C</td>
<td>clade C Symbiodinium</td>
</tr>
<tr>
<td>GActRev</td>
<td>5'-GTCCTTGGGCTCTCAGAAAG</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>AActForw</td>
<td>5'-CGGMACCACSGGNATGTSATGGAC</td>
<td>55°C</td>
<td>clade A Symbiodinium</td>
</tr>
<tr>
<td>AActRev</td>
<td>5'-GACTTYGAYATGAGATGAAGGC</td>
<td>&quot;</td>
<td>clades A, B Symbiodinium</td>
</tr>
<tr>
<td>GActRev2</td>
<td>5'-GTCCTTGGGCTCTCAGAAAG</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>AActRev2</td>
<td>5'-CAGCAGTTGAGRTGCCTTTCG</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>FactRev</td>
<td>5'-CCTCTTNCCACRAAGC</td>
<td>&quot;</td>
<td>clade F Symbiodinium</td>
</tr>
</tbody>
</table>

Actin (primers used for electrophoretic examination of clade C actin copy number in conjunction with above primers)

<table>
<thead>
<tr>
<th>Primer and direction</th>
<th>Primer sequence</th>
<th>Anneal temp</th>
<th>Specificity in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>CActForw</td>
<td>5'-CGGGGATGAATGATGAAG</td>
<td>58°C</td>
<td>clade C Symbiodinium</td>
</tr>
<tr>
<td>CActForw3</td>
<td>5'-GAGCAGYAGATGGGTGACAG</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>CActRev</td>
<td>5'-CGGTGATGATGTTCRTCAGGC</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

Identification of coding regions

All dinoflagellate actin contigs reported in this study consisted of unambiguous sequences obtained by direct sequencing of bands excised from gels. In cases where more than one sequence composed a contig, the overlaps were fully alignable and of more than 50bp.

Symbiodinium actin gene fragments were amplified from genomic DNA. Actin coding regions were identified initially by alignment with the actin cDNAs of other dinoflagellates (Reece et al. 1997), then later with the actin cDNA of Gymnodinium varians (Saldarriaga et al. 2003) and finally with the full length clade C actin from a Symbiodinium EST sequencing project (kindly provided by W. Leggat and O. Hoegh-Guldberg, University of Queensland). These comparisons allowed assignment of exon-intron boundaries. Actin fragments that were initially obtained with primers dinoactForw and dinoactRev were subjected to direct end-sequencing and direct internal-sequencing, to allow the characterization of the coding and non-coding regions of this actin fragment for all clades analysed.

Alignments and phylogenetic analyses

Taxa chosen for examination of actin nucleotide phylogeny were the set of all dinoflagellate actins available in Genbank, excluding the basal non-photosynthetic
dinoflagellates *Perkinsus* and *Oxystis*. Nucleotide sequences of two actins, Tridactpart and Tridactfull, obtained from D. Yellowlees, James Cook University, were included to determine whether they were derived from the zooxanthellae of Tridacnid clams.

An alignment of protein sequences for all the available dinoflagellate actin sequences was generated and to this was added to actin sequences for the infrakingdoms Apicomplexa, Stramenopila, Cryptophyta, Haptophyta, Rhodophyta, and kingdom Metazoa. These represented algae and animals that were selected on the basis of potential affinity with actin sequences Tridactpart, Tridactfull, Syactp, and *Heliofungia* actin examined in this study.

### 2.5 PsbA methods

#### Primer design, PCR and sequencing

Degenerate primers *psbAF6-Forw* and *psbAL1-Rev* (Table 4.4) were designed to anneal to conserved regions in an alignment of: dinoflagellate (*Heterocapsa triquetra* AF130033), stramenopile, Cryptophyta, Rhodophyta, Glaucoctophyceae, Euglenozoa, Viridiplantae and cyanobacterial *psbA* gene sequences. Primers were placed in regions of high amino acid sequence conservation, to allow amplification of a *psbA* gene fragment from any dinoflagellate. These primers also proved capable of amplifying *psbA* from non-dinoflagellate algae (chapter 6).

Primers *psbAF6-Forw* and *psbAL1-Rev* were used to amplify DNA from cultured clade A *Symbiodinium* strains Tm8.2, (*Tridarca maxima* OTI, see chapter 3), CS-153.S2, and CS-152.L1. Each of these is a clonal culture derived as described in Actin methods. The sequence of the partial *psbA* gene was used to design primers 4.6Forw and 4.3Rev (Table 4.4) that orient out from the coding region, on the assumption that the *psbA* gene of *Symbiodinium* would lie on a 2-3kb DNA minicircle as had been published for *Heterocapsa* (Zhang et al. 1999). The coding region of the clade A *Symbiodinium psbA* gene was deduced by alignment with the *psbA* gene of *H. triquetra*.

Primers 7.4Forw and 7.8Rev were designed by reference to the full length ORF of clade A *psbA*. The goal was to place them at the extremes of the 5' and 3' ends of the ORF, yet still at conserved regions, and this was done by reference to a protein alignment of
*Symbiodinium* and *Heterocapsa psbA*. This primer pair proved capable of amplifying the non-coding region of the *psbA* minicircle from clade C *Symbiodinium* spp. as well as clade A *Symbiodinium* spp. Sequencing was performed using the amplification primers and augmented using internal primers (Table 4.4) based on information from the 400-600 bp end sequences. Primers (C3Forw and C3Rev) for amplification of minicircles other than *psbA* (Table 4.4), were designed by reference to the non-coding region of the *psbA* minicircle. For placement of these primers, conserved (C) regions were targetted, but regions that contain hairpins and Inverted Repeats (IRs) were avoided. Region C3 was the optimal position for primers under these criteria, because C1, C2 and C4 all contain IRs.
Table 4.4 Primers used to characterise plastid minicircles of *Symbiodinium*.

<table>
<thead>
<tr>
<th>Primer and direction</th>
<th>Primer sequence</th>
<th><em>minicircle region</em></th>
<th>base position</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>PshA</em>: PCR amplification primers for <em>Sym-Tm8.2</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>psbAF6Forw</td>
<td>5'-GARCAACAACATHYTNATGCAVCC</td>
<td><em>psbA</em> gene</td>
<td>565-587 #</td>
</tr>
<tr>
<td>psbAL1Rev</td>
<td>5'-CRTGCATWACTTCATWCC</td>
<td>&quot;</td>
<td>978-997 #</td>
</tr>
<tr>
<td>4.6Forw</td>
<td>5'-GTTTTAAAACCTCAACCCATCCC</td>
<td>&quot;</td>
<td>895-919 #</td>
</tr>
<tr>
<td>4.3Rev</td>
<td>5'-CTCCTGCAACTTCTCAGAAG</td>
<td>&quot;</td>
<td>667-688 #</td>
</tr>
<tr>
<td><em>PshA</em>: PCR amplification primers for <em>Sym-Ha, -Gl, -Lp, -Pd</em> non-coding region</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.4Forw</td>
<td>5'-GATGAAAGAGAATACTCACACACCTCCC</td>
<td><em>psbA</em> gene</td>
<td>993-1019 #</td>
</tr>
<tr>
<td>7.8Rev</td>
<td>5'-GTTCTCTTTATATCCATATATCTACTG</td>
<td>&quot;</td>
<td>170-197 #</td>
</tr>
<tr>
<td><em>PshA</em>: Internal forward sequencing primer for <em>Sym-Gi</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.11Forw</td>
<td>5'-ACGAAGTGTGTRACGGAGAAAG</td>
<td>C1e</td>
<td>286-319 @</td>
</tr>
<tr>
<td><em>PshA</em>: Internal forward sequencing primers for <em>Sym-Ha, -Lp</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.7Forw</td>
<td>5'-ACGAAGTGTGTRACCG</td>
<td>C1e</td>
<td>286-299 @</td>
</tr>
<tr>
<td>18.1Forw</td>
<td>5'-TTONAGCGGGGAAACKGAC</td>
<td>C3i</td>
<td>543-560 @</td>
</tr>
<tr>
<td><em>PshA</em>: Internal reverse sequencing primer for <em>Sym-Ha, -Gl, -Lp</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.20Rev</td>
<td>5'-TAGGCCAAAATATGCGCC</td>
<td>C1f</td>
<td>322-340 @</td>
</tr>
<tr>
<td>Any <em>Sym-Gi</em> minicircle: Amplification primers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3Forw</td>
<td>CGAGTGAGCGTGCGGACC</td>
<td>C3g</td>
<td></td>
</tr>
<tr>
<td>C3Rev</td>
<td>CCGCGCGCTGCGTTMCC</td>
<td>C3g</td>
<td></td>
</tr>
</tbody>
</table>

*Nomenclature of *Symbiodinium* strains is as specified in Materials and Methods. *Nomenclature of minicircle regions is as specified in Figure 4.13. # Base numbering for these primers refers to the clade A *psbA* gene sequence, Genbank accession AY160884.

@ Base numbering for these primers refers to Figure 4.13.

Alignments and phylogenetic analyses

Taxa initially selected for the *psbA* nucleotide alignment were the complete set of dinoflagellate *psbA* sequences in Genbank. However, the nucleotide analysis failed to resolve monophyly of Suessiales. This was attributed to homoplasy prevalent in nucleotide-based analyses of protein coding sequences. Therefore a Suessiales-only alignment was used, consisting of all Suessiale *psbA* sequences available in Genbank plus those unique to this study.

DNA secondary structure and DNA tertiary structure analysis

Inverted repeats in DNA sequences were identified by visual inspection. Individual DNA hairpin structures (secondary structures) were investigated using the Mfold server (Zuker
2003) [http://www.bioinfo.rpi.edu/applications/mfold]. DNA Double Hairpin Elements (tertiary structures) were modelled with copper wire (as single-strand DNA backbones), fishing swivels (as between-strand H-bond surrogates) and coloured beads (as bases).

2.6 Putative mitochondrial locus methods

Primer design, PCR and sequencing

Mitochondrial lsu rDNA primers were designed from an alignment of mitochondrial lsu sequences from stramenopiles and ciliates. Apicomplexan sequences were not included in the alignment because their mitocondrial rDNAs are fragmented across the mitochondrial genomes (Feagin et al. 1997; Gardner et al. 1993; Gillespie et al. 1999; Kairo et al. 1994; Ossorio et al. 1991). Primers 11.3Rev (5’GGRTGAAAGGCYWATCAAAC) and 11.6Forw (5’CCGNSAAGGRATTTYGCTACC) were annealed at 55°C for amplifications and were also used for direct sequencing of the ends of the fragments obtained.
Cultured and in hospite sources of Symbiodinium

A range of cultured and in hospite Symbiodinium samples were used as DNA templates for amplified of putative mitochondrial lsu rDNA sequences (Table 4.5). Holobiont tissue DNA preparations were used in the case of the in hospite samples.

Table 4.5 Cultured and in hospite Symbiodinium used for amplification of putative mitochondrial sequences

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Host species (if in hospite)</th>
<th>dinoflagellate</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT7</td>
<td>H. actiniformis</td>
<td>Symbiodinium clade C</td>
<td>OTI</td>
</tr>
<tr>
<td>Gt3</td>
<td>G. tenuidens</td>
<td>&quot;</td>
<td>OTI</td>
</tr>
<tr>
<td>HT9</td>
<td>H. actiniformis</td>
<td>&quot;</td>
<td>OTI</td>
</tr>
<tr>
<td>H3tiss</td>
<td>H. actiniformis</td>
<td>&quot;</td>
<td>OTI</td>
</tr>
<tr>
<td>Lp15tiss</td>
<td>L. purpurea</td>
<td>&quot;</td>
<td>Southwest sandflats, OTI</td>
</tr>
<tr>
<td>CS164</td>
<td>(cultured)</td>
<td>Symbiodinium clade B</td>
<td>-</td>
</tr>
<tr>
<td>HaSW2</td>
<td>H. actiniformis</td>
<td>Symbiodinium clade C</td>
<td>Southwest sandflats, OTI</td>
</tr>
<tr>
<td>CS158</td>
<td>(cultured)</td>
<td>Symbiodinium clade A</td>
<td>-</td>
</tr>
<tr>
<td>HT6</td>
<td>H. actiniformis</td>
<td>Symbiodinium clade C</td>
<td>OTI</td>
</tr>
<tr>
<td>HaTB8</td>
<td>H. actiniformis</td>
<td>&quot;</td>
<td>Turtle Bay, OTI</td>
</tr>
<tr>
<td>HaHB8</td>
<td>H. actiniformis</td>
<td>&quot;</td>
<td>Home Bommie, OTI</td>
</tr>
<tr>
<td>Amphupper</td>
<td>(cultured)</td>
<td>Amphidinium carterae</td>
<td>-</td>
</tr>
<tr>
<td>Amphlower</td>
<td>&quot;</td>
<td>&quot;</td>
<td>-</td>
</tr>
<tr>
<td>L9</td>
<td>L. purpurea</td>
<td>Symbiodinium clade C</td>
<td>OTI</td>
</tr>
</tbody>
</table>

Alignments and phylogenetic analyses

159 taxa were selected for the lsu rDNA alignment by multiple NCBI BLAST searches using each of the novel putative mitochondrial sequences as baits. No BLAST hits were obtained to beta-proteobacteria, chloroplasts or apicoplasts, but these were included to serve as outgroups. No BLAST hits were obtained to Archea. A single BLAST hit was obtained to a chromalveolate mitochondrion, that of the stramenopile Laminaria digitata (Phaeophyta). Redundant accessions were removed. After initial alignment in ClustalX, environmental sequences were removed. Preliminary trees were generated using Mr Bayes and viewed with Treeview (as described in Chapter 2). Long branch taxa were removed and Bayesian analyses were rerun.

The lsu rDNA trees were derived from nucleotide alignments in which uninformative or ‘loop’ regions were deleted (by hand using the program QuickAlign), so that artifactual groupings would be minimised. The large tree was generated as a 158 taxon analysis. In parallel the full 158 taxon set was divided into three constituent subsets of 61, 52, and 57
taxa respectively with selected overlapping taxa included to serve as outgroups, and these three subsets were each used to generate separate trees.

Taxon L9 was analysed separately. BLASTn hits to this sequence were added to a representative set of the alpha-proteobacterial sequences used in the large tree, giving 22 taxa, and loop regions were removed, before Bayesian analysis.

3. Results

Genbank accessions for sequences obtained in this chapter are contained in Appendix 2.

3.1 Actin markers

_Actin gene amplification, sequencing and generation of sequence contigs_

Putative actin fragments obtained from clades A and B of cultured _Symbiodinium_ were longer than the expected 608 bp that would be amplified from an intron-less dinoflagellate actin gene using primers dinoactForw and dinoactRev (Figures 4.1 and 4.3). Similarly, primers BCactForw and zooactRev amplified fragments from three strains of cultured clade F _Symbiodinium_, that were each longer than the expected size of 506 bp (Figures 4.2 and 4.3). Amplicons were sequenced directly, using internal and end primers, and the sequences were used as BLAST queries against the full Genbank non-redundant nucleotide database. Sequences of all these putative _Symbiodinium_ actin amplicons retrieved published dinoflagellate actin sequences as the highest scoring BLAST hits (data not shown). Whole DNA extracted from _H. actiniformis_ tissue contained zooxanthellae and host cells, and was used as a source of putative clade C _Symbiodinium_ actin, in the absence of a culture of this clade (Figure 4.1). Two well-separated actin bands were obtained from the holobiont template using a single pair of primers (dinoactForw and dinoactRev). The upper band of ~1100 bp was sequenced and subsequently used as an NCBI BLAST query. This search retrieved dinoflagellate actins as best scoring matches (not shown). The lower band of ~600 bp was also sequenced and a BLAST search with it retrieved highest hits to cnidarian actins (not shown). Sequences were aligned against a retrospectively obtained putative clade C _Symbiodinium_ actin cDNA (Leggat et al. 2005)(Appendix 4a).

Figure 4.2 Actin amplicons from Clade F *Symbiodinium* cultures obtained with primers BCact_Forw and zooact_rev (850-950 bp). Lanes [1] strain Z3; [2] strain Z6; C [3] strain Z4; [M] pGEM. DNA markers

**Symbiodinium specificity of the new introns**

Assembly of raw sequence data into contigs indicated the presence of one or more intron/s in all four clades (Figure 4.3 and Appendix 4a). Additionally, genomic actin fragments were amplified and sequenced from: *Polarella* sp., a taxonomically close genus (Montresor et al. 1999); and *A. carterae*, a representative of a genus that includes species symbiotic with metazoans (Murray et al. 2004; Trench and Winsor 1987). This was done to assess whether introns were unique to *Symbiodinium*, and thus reliable markers for fieldwork, or whether the introns also occurred in the actin gene of relevant dinoflagellates other than *Symbiodinium*. The *Polarella* and *A. carterae* actin amplicons did not contain any introns, and the chromatograms were unambiguous, implying a single gene copy or heterogeneity of multiple copies. A putative actin fragment of *H. pygmeae* was also amplified and was of equal size to those of *Polarella* and *A. carterae* (indicating probable lack of introns) but was not sequenced.

**Assessment of actin copy number in clade C**

Sequences of three distinct actin genes were obtained from the assembled actin sequences of *Sym-Gt*. One of these ('copy-2') contained a 7 bp deletion in the actin coding region,
followed closely by a stop codon that was 400 bases premature, identifying it as a pseudogene. The other two actin genes were incomplete ORFS (no frameshift deletions detectable), that is they were presumed to be parts of genes that encode full actin proteins. These were clearly coded at more than one locus, evident from two differing intron sequences (which are then most distinguishing features of ‘copy-1’ and ‘copy-3’, Appendix 4a) bounded by nearly identical partial exon sequences.

A pairwise analysis by PCR with 14 primer combinations supported the sequence assembly results, by showing that multiple copies of actin exist in Sym-Gt, and that the sizes of the electrophoretically visualised bands were in accordance with the results of sequence assembly (Figures 4.3 and 4.4, Table 4.6, and Appendix 4a).

At least three, and up to five “copies” or loci of actin-like sequences were discerned in Sym-Gt by consistent amplification of bands using a range of primers in conserved positions (Figures 4.3 and 4.4 and Table 4.6). Only three of these actin loci of Sym-Gt were confirmed by assembled contigs sequences (Appendix 4a) and were accordingly named copy-1, -2, and -3. The other two actin loci of Sym-Gt are putative, and are denoted as copy “L” (large) and copy “S” (small). There were eight observations of the unsequenced copy L (band observed eight times, each time employing a different PCR primer combination), and two observations of unsequenced copy S.

The primers dinoactForw and dinoactRev, amplified both the host and the symbiont bands (Figure 4.1), indicating that these primers may be universal. Nevertheless the range of fragment sizes that would be expected from Sym-Gt (Figures 4.3 and 4.4, Table 4.6, and Appendix 4a) was not observed when using that particular pair of primers. Instead the primers dinoactForw and dinoactRev amplified a single band of ~1100 bp (Figure 4.1). This may be interpreted in either of two ways; (i) that dinoactForw and dinoactRev are selective for non-pseudogenes; or (ii) that the PCR process selectively amplified the gene of highest copy number. In regard to (i), it is possible that more than one of the actin copies are pseudogenes and that the sequences at the places of primer annealing may be unconserved in some pseudogenes.
Figure 4.3 (a) Schematic of intron locations, intron sizes, and primer locations in a partial fragment of the *Symbiodinium* actin gene. The fragment in blue is ~600 bp in length and represents the cDNA of clade C *Symbiodinium* actin accession DQ174766 (Leggat et al. 2005, used with permission). Introns of a single colour are at identical positions in the coding sequence, i.e. are derived from the same intron-creation event. Distances (bp) between introns and cDNA ends are shown on the cDNA. (b) Positions of primers that were used in amplifications of clade C actin (*Sym-Gt*) to obtain Figure 4.4 and Table 4.6. Scale divisions are at every 100 bases.

Figure 4.4 Amplification with multiple primer combinations, showing that a number of copies of actin occur in Sym-Gt. Primers were combined pairwise as detailed in the matrix below the gels. Band sizes (listed in Table 4.6) were deduced by comparison to the standards indicated on each gel.
Table 4.6 Fragment sizes of five predicted actin copies from Sym-Gt, using a range of primers in conserved positions. Numbers on the left side of each cell are band sizes observed electrophoretically, while bolded numbers on the right side of each cell are the matching fragment sizes calculated from contig sequences obtained. Dashes indicate that no band was observed, but that a band might be expected, if pattern is compared with results from other primer pair combinations. Observed bands that are surrounded by dashes are therefore possible artifacts.

<table>
<thead>
<tr>
<th>Unsequenced putative copy “L”</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>primers</td>
<td>CactRev1</td>
<td>CactRev2</td>
<td>zoonactRev</td>
<td>dinoactRev</td>
<td></td>
</tr>
<tr>
<td>dinoactForw</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BCactForw</td>
<td>~620</td>
<td>-</td>
<td>~700</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CactForw1</td>
<td>-520</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CactForw2</td>
<td>-520</td>
<td>-</td>
<td>-600</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CactForw3</td>
<td>~480</td>
<td>-</td>
<td>~520</td>
<td>-</td>
<td>-1250</td>
</tr>
</tbody>
</table>

| Sequenced copy 1             |     |     |     |     |     |
| dinoactForw                   | ~620 | 600 | ~680 | 680 | -  |
| BCactForw                    | ~510 | 530 | ~600 | 610 | -  |
| CactForw1                    | ~400 | 420 | -  | -  | ~900 | 890 | -  |
| CactForw2                    | ~420 | 420 | ~500 | 510 | -  | 890 | ~920 | 930 |
| CactForw3                    | ~350 | 360 | ~450 | 440 | ~850 | 840 | ~890 | 870 |

| Sequenced copy 2             |     |     |     |     |     |
| dinoactForw                   | ~550 | 530 | ~600 | 610 | -  |
| BCactForw                    | ~450 | 460 | -  | 540 | -  |
| CactForw1                    | ~330 | 350 | -  | 440 | -  | 840 | -  |
| CactForw2                    | ~350 | 350 | ~500 | 470 | ~850 | 840 | -  | 800 |
| CactForw3                    | ~300 | 290 | -  | 400 | ~730 | 720 | ~730 | 750 |

| Sequenced copy 3             |     |     |     |     |     |
| dinoactForw                   | ~500 | 500 | ~570 | 580 | -  |
| BCactForw                    | ~450 | 440 | ~500 | 520 | -  |
| CactForw1                    | ~330 | 330 | -  | -  | -  |
| CactForw2                    | ~330 | 330 | -  | 410 | ~750 | -  | -  |
| CactForw3                    | ~300 | 280 | -  | 340 | -  | -  | ~550 | -  |

| Unsequenced putative copy “S” |     |     |     |     |     |
| dinoactForw                   | -  | -  | -  | -  | -  |
| BCactForw                    | -  | -  | -  | -  | -  |
| CactForw1                    | -  | -  | -  | ~620 | -  |
| CactForw2                    | -  | -  | -  | -  | -  |
| CactForw3                    | -  | -  | -  | -400 | -  |

**Actin intron sequences and implied gene multiplicity in clade A**

The exons of clade A sequenced unambiguously by direct sequencing method, as did the second intron. However the first intron of this clade did not sequence unambiguously in a range of amplicons that had been generated with different primers and had been sequenced also with range of primers (AactForw1, ABactForw2, ABactRev1, ABactRev2 [Table 4.3]). These data imply that more than one copy of actin may be present in clade A.
Actin phylogeny

Actin sequences from cultured and in hospite Symbiodinium
All available dinoflagellate actin sequences were combined in a nucleotide alignment including the partial actin-coding sequences novel to this study. Nucleotide analyses conducted under a codon-based NY98 model implemented in Mr Bayes, resulted in grouping of all studied Symbiodinium clades A, E, B, C and F together and with Polarella (Figure 4.5). In alignments that are not shown here, sequences of two exons of actin from the symbiont of H. actiniformis (this study, data not shown), had identical DNA sequences to those of Sym-Gt (this study, genbank accession DQ174713). The Sym-Gt actin sequence was used for phylogenetic analyses because it was the longer of the two.

Investigation of phylogenetically unplaced actin sequences
Actin sequences Syact-p, Tridactpart and Tridactfull were attracted to the published Pyrocystis lumula actin sequence (Figure 4.5), and this was because of an extensive shared set of synapomorphic amino acids: approximately fourteen amino acids, shared between these four sequences, differed from the amino acids of the Suessiales of this study (data not shown). The Syact-p sequence has been reported as a Symbiodinium actin (Fukuda et al. 2002), but when long branches Pyrocystis, Tridactpart and TridactFull, were removed (Figure 4.6), Syact-p fell outside the dinoflagellate division (Figure 4.6).

Phylogenetic confirmation that host and symbiont actins segregate separately and appropriately
In order to determine the origins of individual actin sequences, an alignment of algal and metazoan actin proteins was employed. A phylogenetic analysis of this protein dataset (Figure 4.7) corroborated the nucleotide analyses (Figures 4.5 and 4.6) and together these indicated: (i) that the novel Symbiodinium and Polarella actin sequences of this study are monophyletic as expected for inclusion in Suessiales; (ii) published actins AGfact-p and AFcact-p are Symbiodinum actins; (iii) host actins group with animal actins as expected; and (iv) that none of Syact-p, Tridactpart or Tridactfull lie within the Suessiale clade, nor in the dinoflagellate clade that was supported with a posterior probability of 1.0 (Figure 4.7).
**Figure 4.5** Actin nucleotide NY98 phylogeny of *Symbiodinium* with an *Amphidinium* outgroup, and with cryptic sequences included. Sequences novel to this study are asterisked in red. Sequences of other *bona-fide* *Symbiodinium* actins sequenced by other labs, are represented by black stars. This tree is the sum of 11000 most likely Bayesian trees, after the initial 8000/19000 trees were discarded, and has a log likelihood of −7047.
Figure 4.6 Actin nucleotide NY98 phylogeny of *Symbiodinium* actins with a stramenopile outgroup, and with longest branches of Figure 4.5 excluded from the analysis. Sequences novel to this study are asterisked in red. Sequences of other *bona-fide* *Symbiodinium* actins, sequenced by other labs, are represented by black stars. This tree is the sum of 15000 most likely Bayesian trees, after the initial 15000/30000 trees were discarded, and has a log likelihood of −5965.
Figure 4.7 Actin protein phylogeny of selected protists and Metazoa. Sequences novel to this study are asterisked in red. Sequences of other *bona-fide Symbiodinium* actins, sequenced by other labs, are represented by black stars. Amino acid sequences that branched significantly differently than the corresponding nucleotides analysed in Figures 4.5 and 4.6 are boxed. This tree is the sum of 350000 most likely Bayesian trees, after the initial 50000/400000 trees were discarded, and has a log likelihood of −4258, and alpha of 0.20<0.28<0.41.
Actin utility as a within-clade marker: sequences of comparable actin intron-1 loci are variable within Symbiodinium of a single clade, from different coral hosts

The potential utility of actin introns for future study of phylogenetic species boundaries was cursorily assessed by aligning sequences sampled across strains but within a clade. This question was investigated separately for each of two clades, C and F. In the case of clade F, the investigation was undertaken on strains that had been cultured in the previous chapter (Figure 4.8), and targeting their single actin gene. Sequence variation between Symbiodinium strains was also investigated at a chosen actin intron locus in clade C strains Sym-Ha, Sym-Lp, and Sym-Gt by using Symbiodinium clade C actin primers Cact_Forw1 and Cact_Rev1. Two bands (~350bp and ~430bp) were obtained from Sym-Ha and the same two sized bands were obtained from Sym-Lp (electrophoretic data not shown). The smaller band (~300bp) was sequenced and it aligned well with copy-3 intron-1 of Sym-Gt (smallest band in Figure 4.4 lane A). The Sym-Ha/Lp sequence contained two point mutations and a 9-base indel relative to the Sym-Gt sequence (Figure 4.9). The sequences on Figures 4.8 and 4.9 represent divergence between strains, and do not represent intragenomic divergence due to multiple copies. Cultured strains of clade F used to generate data for Figure 4.8 were clonal, and the direct sequencing method indicated consistently that actin is a single copy gene in that clade. In hospite strains of clade C used to generate data for Figure 4.9 were each clonal in terms of the resolution obtainable at this locus, and the locus is orthologous across the isolates in the figure, because the locus (copy 3, intron 1) has a very different sequence than similar actin loci (other ‘copies’) present in clade C.
Figure 4.8 Actin intron-1 sequence variability within widely separated cultures of clade F Symbiodinium. Cultures Z3, Z4 and Z6 were obtained in chapter 3, and actin sequences were aligned by Clustal-X. Intron-1 sequences (boxed) are those presented in Appendix 4a, but here are carefully aligned. Asterisks indicate bases conserved across the three isolates. Variability is greater in the intron than in the neighbouring exons (not boxed).
Figure 4.9 Actin intron sequences at the copy 3 intron-1 locus compared among clade C isolates. The forward sequence read and part of the reverse sequence read of actin copy 3 from *Sym-GH2* are shown to compensate for editing of the forward sequence (pink bases) in Sequencer. Intron borders are marked by red lines. Black circles represent variable bases across strains at this locus. No variable positions occurred within the 80 bases (comprising the middle of the intron) that are not shown.
3.2 PsbA marker

Amplification of psbA coding sequence from Symbiodinium

Amplicons representing a 425 bp internal fragment of the psbA gene were obtained from Symbiodinium clades, A, B (Figure 4.10a) and C (Figure 4.10b). Those from clades A and C were sequenced. Primers 4.6FOrw and 4.3Rev, designed from the clade A sequence and oriented outward, amplified a ~2.2 kb piece of DNA from three clonal cultures of clade A, indicating the presence of a single-gene closed minicircle, containing the psbA gene (Figure 4.10c). Both ends of the large fragment from clade A culture Tm8.2 were sequenced and the data confirmed that the large fragment contained both termini of the psbA gene. These end sequences were merged with the internal psbA fragment obtained from Tm8.2, to generate the full 1035 bp ORF of Symbiodinium psbA (Genbank accession AY160084).

*PsbA phylogeny*

A Bayesian protein phylogeny of all dinoflagellate *psbA* proteins (data not shown) grouped all the *psbA* sequences obtained in this study within the monophyletic genus *Symbiodinium* with a posterior probability of 1.0. However it also showed clade A *Symbiodinium* emerging at the tip of the genus after clades BCF, rather than its expected position at the base of the genus (data not shown).

A codon based analysis of *psbA* nucleotide sequences also grouped all sequences from this study within the genus *Symbiodinium* (Figure 4.12), and branching of clade A *Symbiodinium* at the base of the genus was observed when choice of outgroup was limited to the Suessiales *P. glacialis and G. simplex*. 
Figure 4.12 *PsbA* phylogeny of Symbiodinium, under codon-based model NY98. Sequences novel to this study are asterisked. This tree is the sum of 2000 most likely Bayesian trees, after the initial 18000/20000 trees were discarded, and has a log likelihood of −3442.
The *psbA* minicircle non-coding region of clade C Symbiodinium is homogeneous within a cell, and includes dynamic double-hairpin elements

**Amplification of non-coding sequence, and subsequent alignments**

Primers 7.4Forw and 7.8Rev successfully amplified the entire non-coding side of the *psbA* minicircle from *Sym-Ha* (1202 bp), *Sym-Lp* (1133 bp), *Sym-Gt* (965-1018 bp) and *Sym-Pd* (882 bp), which included 43 bp of the C-terminus and 191 bp of the N-terminus of the *psbA* gene in each case (including primers). After initial alignment by ClustalX (Thompson et al., 1997), extensive alignment by hand was necessary due to the presence of multiple repeated sequences and large indels. All minicircle fragments that were amplified with primer pair 7.4Forw and 7.8Rev yielded unambiguous chromatograms when sequenced with the primers listed in Table 4.4. This was interpreted as indicating that only a single minicircle variant encodes the full-length *psbA* gene in each of the clade C zooxanthella strains analysed.

**Structure of the minicircle non-coding region**

An alignment of sequences from the non-coding side of the *psbA* minicircle is presented in Figure 4.13. On examination, the alignment revealed a number of distinct regions, some with apparent secondary structure. Assignment of regions as variable (V), conserved (C) and metastable (M), is based principally on the comparison of *Sym-Ha*, -*Gt* and -*Lp* sequences, as the sequence of *Sym-Pd* is very divergent (having many large deletions compared to the orthologous sequences from the other three hosts). The outlier status of *Sym-Pd* as detected in this chapter is consistent with differences already noted between *Sym-Pd* and these other OTI zooxanthellae based on rDNA sequencing (Carter et al., 2000; and chapter 1 of this thesis).
**Figure 4.13** Complete alignment of the non-coding region of *psbA* minicircles from five isolates of *Symbiodinium* clade C. Start and stop codons of the *psbA* gene are underlined. Bases conserved in all five isolates are asterisked. Each arrow represents one side of a single inverted repeat. Bold bases are intra-IR spacers not constituting part of a palindrome. Motifs within core (C), metastable (M) and variable (V) regions are named alphabetically in the order in which they occur except that analogous regions within C2, C3 and C4 are given a common suffix.
There are four main conserved or core regions, C1-4, excluding the presumed promoter region (P) of the \textit{psbA} gene, which is 48 bp in length, contiguous with the gene and conserved across all the symbionts. Two highly conserved regions, C2 and C4, are similar to each other, but not identical. Region C3 is also highly conserved but the overall sequence is not related to that of C2 and C4, except that a poly-A sequence is found in each, and a 13bp motif (ATCGCGACYTATA), designated f, is common between C2 and C3. Region C1 is only semi-conserved, with a sequence completely unrelated to those of the other C regions. Two variable regions, V1 and V2, lie between the set of cores and the \textit{psbA} gene. The sequences within any one V region cannot be unambiguously aligned across the four zooxanthellae types. The two remaining regions are those that separate C2 and C4 from C3. The sequences of these two regions are related to those of the C2 and C4 regions, but they are each either present in full or completely absent, depending on the zooxanthella strain analysed. As such they are termed metastable (M) regions.

Inverted repeats (IRs) lie in regions V1, C2, C4, M1 and M2. Five of these IRs are conserved across \textit{Sym-Ha}, -\textit{Lp} and -\textit{Gt}, and these lie in the C2 and C4 regions (Figure 4.13). In region C2 is a pair of IRs designated C2a and C2b. A similar pair in region C4 are designated C4a and C4b. IR C2a directly abuts IR C2b; similarly IR C4a directly abuts IR C4b. The term ‘twin IR’ describes this abutment. Within C2 and C4 the IR abutment is at an identical palindromic sequence: CATATG. One additional small IR, designated C4h, lies downstream of the twin IR C4a-b. The C2 and C4 regions can be considered to qualitatively mirror each other, given that a run of adenines (C2e) flanks C2 at the upstream side, and another run of adenines (C4e) flanks C4 at the downstream side. At the centre of the observed partial symmetry of the minicircle is C3e, consisting of the conserved adenine-rich sequence GAAAAAGAAAAA (positions 589 to 599 in Figure 4.13).

The arrangement of the C2 and C4 regions, relative to the central adenine-rich region C3e, can be interpreted in the sense that the sum C2+C3+C4 approximates a single large symmetrical unit, but may have intervening modular regions present as well. The modular regions, M1 and M2, contain twin IRs (M1a-b and M2a-b respectively), but are each without a flanking poly-A motif, and without an accompanying small IR like C4h. In this sense the variation present in M1 and M2 seems to be constrained. Presence/absence
switching (modularity) of M1 and M2 suggests that the twin IRs in these regions are highly evolved in that a twin pair may be gained or lost only as a unit. Likewise indicating a refined process, the IRs in M1 and M2 never contain mismatches in the inverse sequence relationship, even though the IR sequence may change between zooxanthella strains. Inverse sequence relationships in C2 and C4 IRs are also perfect. Even though sequence variation occurs between zooxanthellae with regard to these core IRs, they are always perfect inversions. Perfect inversions constituting twin IR sequences are not observed outside the partially symmetrical unit C2+M1+C3+M2+C4. There are twin IRs in the V1 region of the minicircle from Sym-Gt, -Ha and -Lp, but these IRs (V1a-V1d) are all either short, or composed of slightly imperfect inverse sequences.

In analysing the lack of inverse sequence mismatch in the core IRs, a special case must be made of IR C4b. It is an unusual core IR in that there is a conserved insertion of a single adenine (position 706, Figure 4.13) in the left hand side of the IR making it asymmetrical. After the inserted base A, the IR structure resumes for another 3 bases (positions 707 to 709 in Figure 4.13). It is assumed that these three offset bases of IR C4b are functional, because the inverse relationship at these positions is retained in Sym-Gt relative to that of Sym-Ha and -Pd, even though point mutations have occurred (i.e. CAC/GTG [Sym-Gt] versus CGC/GCG [Sym-Ha, -Lp, -Pd]).

Some core IRs are interrelated in the sequence of the 3-5bp intra-IR spacers that lie central within any given IR (Figure 4.13). There is conservation of the spacer TTC within many of the core IRs of the Symbiodinium isolates studied. All isolates have the spacer TTC within C4b and C4h. Sym-Ha, -Lp and -Pd isolates have spacer TTC within C2a. Conservation of the spacer TTC in two unrelated IR types (h and b) may indicate a function for this particular spacer motif.

Following the observation that each twin IR constitutes a unit and that this unit occurs many times in a sequence, an alignment of all the twin IRs was generated (Figure 4.14). The alignment indicates that all units but C4ab are related. When C4ab is reverse complemented, the match does not improve significantly (data not shown). Since sequence likeness occurs between all units except C4, these may be considered to have had a common mode of origination, or to have been duplicates of each other that have since diverged. A term that describes all of the IRs in the C and M regions is ATG-CAT IRs,
because ATG and CAT are invariant as the first three and last three bases respectively (the first base varies however in IRs of the V1 region).

\[ \text{Sym.} \]

\[
\begin{array}{c|c|c}
Gc_V1ab & \text{GTGGGTCACCCCATA-ATAGGGTGCCCACATGGGTA-CCTGATAGGCGTACC}
\
HALP_V1cd & \text{ATGGGTTGCC-CTTGTA-EGGGTACCCATCTG---CCCGCA-GGG---CAT}
\
HALP_C2ab & \text{ATGGGCGACCCC-TTC-GGGGTCACATGCGCCCA-CCCTTTTAGGGTCC}
\
HALP_M1ab & \text{ATGGGCGACCCC-TTC-GGGGTCACATGCGCCCA-CCCTTTTAGGGTCC}
\
HALP_M2ab & \text{ATGGGCGACCCC-TTC-GGGGTCACATGCGCCCA-CCCTTTTAGGGTCC}
\
HALPd_C4ab & \text{ATGGGCGACCCC-TTC-GGGGTCACATGCGCCCA-CCCTTTTAGGGTCC}
\end{array}
\]

** Figure 4.14 Alignment of representative twin IRs that occur in the psbA minicircles of five Symbiodinium clade C isolates. Boxed regions are common to the majority, but different in C4a-b. Bases with asterisks are common to all sequences shown. Bold bases are intra-IR spacers. Each arrow represents one side of a single IR.**

Precise abutment of C3f with M2a imitates the junction of C2f and C2a (Figures 4.13 and 4.15). This recurring juxtaposition might imply a relationship between the poly-A stretches in the e regions and twin IRs, because f regions intervene and are an exact length. Considering the recurrence of the order e-f-a-b, a high level of organisation is noted in the Symbiodinium minicircles studied here (Figure 4.15). First-order organisation is the presence of IRs. Second order is the precise abutment of two IRs to make a twin IR. Third order is the abutment of a twin unit against the f region (ATCGCGAC YTATA) and the accompanying poly-A stretch (specifically C2e-f abuts C2a-b, and C3e-f abuts M2a-b). Fourth order is the duplication of the third order complex (e-f-a-b) about an intervening unique region, C3g. A tendency for the third order complex to include a second a-b repeat is noted, but is apparently non-essential since not all isolates contain these extra repeats.

Conserved sequence elements also exist in the C1 region, including apparent relics of two to three copies of an 18 bp palindrome TAATTTTGGCCAAAATTA (relics conforming to this consensus are indicated by lower case letters on Figure 4.13). Two elements like the 18 bp palindrome also occur in other parts of the minicircles: a possible relic in V2 (lowercase, positions 816-831), and an invariantly conserved 12 bp relative TGGCCAAAATTA located at the extreme 3' end of the putative psbA promoter (lowercase, positions 953-964).
Figure 4.15 Schematic interrelationship of f regions and inverted repeats in the psbA minicircle of clade C Symbiodinium. Twin IR M1a-b is present only in Sym-Ha and -Lp isolates. Twin IR M2a-b is conserved in all isolates except Sym-GtI. Region labels are in accordance with figure 4.13.

Figure 4.16 Schematic of the proposed base pairings within one DNA strand of a twin IR, to potentially form a double-hairpin structure. This would be repeated on the complementary strand and result in four hairpins, which cannot easily be represented in two dimensions. The sequence depicted is the a-b region present in the C2 block from Sym-Gt. Boxed ATG and CAT bases are invariant in all the twin IRs present in the C and M regions of all the minicircles in this study. Each of the twin IRs in Figure 4.13 can be represented by a structure very similar to this, generally with 11 bp per putative stem, and always with no unpaired bases at the junction of the two stems.
Secondary structure prediction on the twin IRs of this study indicates they have the potential to form double hairpin structures (Figure 4.16). DNA Double Hairpin Elements (DHEs) were modelled in three dimensions (data not shown) to assess their potential to form compact structures that could pack together at close intervals such as C2ab+M1ab, or M2ab+C4ab. Two alternative folding and packing topologies were modelled, both of which allow close packing of two consecutive DHEs without steric hindrance.

The V1 and V2 sequences of the psbA minicircle are signatures specific to a symbiont strain inhabiting a particular host

Preliminary evidence was collected to assess the proposition that the variable regions V1 and V2 may constitute unique signature sequences for identifying strains specific to their respective coral host species at the location OTI. V2 regions were not always informative in the case of Sym-Ha owing to ambiguous chromatograms (detailed in next chapter), while by contrast the V1 region of this symbiont sequenced cleanly. Sym-Gt and Sym-Pd sequenced cleanly in all regions of the minicircle. An alignment of the V1 regions from several samples of these three symbionts confirmed that V1 represents a strain-specific region, for each host-specific strain (Figure 4.17). A caveat to this is that Sym-Lp OTI possesses the same V1 sequence as Sym-Ha OTI (Figure 4.13).
**Figure 4.17** Unique psbA minicircle V1 sequences are consistently specific to symbionts from particular hosts. Four independent isolates of Sym-Gt were sequenced, two of Sym-Ha, and three of Sym-Pd. The V1 region is shown in full, bounded by the psbA C-terminus and the C1 core region (large boxes). Stop codon in lower case. Asterisks indicate bases that aligned reasonably well (aligned by hand) across three sequence types shown. Bases that differed across isolates from a single host species are shown in small boxes. Sym-Pd sequences were kindly provided by K. Ferguson, University of Sydney.

**Sym-Gt contains at least 6 distinct minicircles with conserved C3 regions (Ch4)**

Based on published minicircle data from other dinoflagellate genera (Barbrook and Howe 2000; Howe et al. 2003; Zhang et al. 2002; Zhang et al. 1999) it was hypothesised that the C regions of the psbA minicircle may represent conserved cores that would also be present in Sym-Gt minicircles containing genes other than psbA. To test this hypothesis, primers were designed within the C3 region, pointing away from each other, and these were used to amplify putative whole minicircles from Sym-Gt genomic templates.

Four bands (2800 bp, 1950 bp, 1800 bp and 1550 bp) were reproducibly amplified using Expand polymerase (Figure 4.18a). Band 3 was of the correct size to be Sym-Gt psbA minicircle (1735bp, calculated by adding the size of the Sym-Tm psbA coding region to the size of the Sym-Gt psbA non-coding region). Faint bands were obtained when Taq was used. A fifth and sixth band (~3600 bp and ~920bp) were less frequently amplified (using
Taq polymerase) but were of a staining brightness sufficient to indicate potential conservation of the priming site (Figure 4.6b). A further four bands (also obtained with Taq polymerase, Figure 4.18b) were of lesser brightness and like the sixth band, were of a size barely sufficient to represent circular molecules (1300, 1150, 1000 and 800 bp).

Direct sequencing of the four bright and crisp bands that had been amplified by Expand polymerase, was attempted and yielded a short piece (~200bp) of clean sequence in one case, band 2. The band 2 sequence aligned reasonably well with the C1 and C2 core regions of the Sym-Gt psbA minicircle sequence (Figure 4.19) indicating that band 2 is of the same minicircle backbone family, and thus verifying that the primers C3F and C3R do amplify clade C Symbiodinium minicircles. Attempts to sequence the other amplified minicircles were not sufficiently thorough to determine whether sequencing problems were due to internal priming, multiple non-identical molecules per band, or other causes. Cloning was attempted but not prioritised because it was unknown whether presence of double hairpins might interfere with plasmid replication in bacteria. In total, six amplicons of sufficient brightness and size were obtained that may be Sym-Gt minicircles.
Figure 4.18 Investigation of minicircle complement of Sym-Gt. (a) Cleanly amplified minicircle bands from Sym-Gt obtained using Expand polymerase. (b) Faint bands obtained using Taq polymerase. Fourth lane deliberately overloaded to visualise dim band 5. Template DNA in all lanes was Sym-Gt OTI.

Bright bands, approximate sizes (bp): 1-2800, 2-1950, 3-1800, 4-1550
Other bands, approximate sizes (bp): 5-3600, 7-1300, 8-1150, 9-1000, 6-920, 10-800

Figure 4.19 Partial sequence of Sym-Gt minicircle band-2 compared to Sym-Gt psbA minicircle. PsbA minicircle non-coding regions are as shown in Figure 4.13. Asterisks indicate conserved positions between the psbA minicircle and the novel minicircle.

3.3 Putative mitochondrial lsu rDNA

Lsu rDNA gene amplification

Putative mitochondrial lsu rDNA sequence reads of greater than 500 nucleotides from the 3’ ends of long (~1200 bp) fragments, were obtained from cultured Symbiodinium and Amphidinium and from the zooxanthellae resident in coral tissues, using primers 11.6Forw and 11.3Rev. A shorter 3’ end sequence read (~400bp) was obtained for a single culture I.9, and was phylogenetically analysed separately from most of the longer reads.

Lsu rDNA phylogeny

The tree presented as Figure 4.20 contains the highest posterior probabilities from either of two separate Bayes runs, one with Emiliana and Thraustochytrium mt lsu rDNAs
included and one with these two taxa excluded (the latter being the topology retained for presentation). This was necessary because the 160th taxon *Emiliana* grouped spuriously within the gamma-proteobacteria, specifically attracting to the longest branch there, novel sequence Gt3. The *Thraustochytrium* sequence was stable in the mitochondrial clade but was removed from the second analysis because it was the longest remaining branch in that clade. A range of other mitochondrial lsu rDNAs generated very unstable long branches, spuriously grouping with bacterial clades: red algae - *Chondrus, Porphyra, Cyanidioschizon*, ciliate - *Paramecium* and stramenopile - *Phytophthora*. These were removed from the analysis.
Figure 4.20 Tree of bacterial 23S, and organellar 23S genes. Sequences novel to this study are marked with red asterisks. Last 300,000 of 1100,000 trees. Log likelihood -8405, alpha 0.33<0.39<0.49
Trees obtained from subsets of taxa (Appendix 5; Figures A to C) yielded very similar branching orders of taxa compared the tree obtained from the full set of taxa. This was done in order to more stringently assess the phylogenetic placement of sequences. In analyses across widely diverse organisms (here nearly all eubacteria), inclusion of a large number of taxa (Figure 4.20) is considered most accurate for discovering the clade or division in which an organism lies. However, such analyses can lack definition when establishing the position of a taxon or gene accurately within a clade of that tree. Analyses of subsets of taxa (Appendix 5; Figures A to C) did show slightly different groupings, justifying the exercise, but were overall the same as the large tree (Figure 4.20).

None of the novel sequences obtained grouped with the mitochondrial clade, rather they were all shown to be bacterial sequences (Figure 4.20 and Appendix 5; Figures A to C). One sequence obtained from coral tissues, HaSW2, grouped within the alphaproteobacterial clade close to its base (Figure 4.20 and Appendix 5; Figure B). Other bacterial sequences analysed here, representing taxa that putatively occur in association with corals or with Symbiodinium cultures, were: one member of the CFB group (Chlorobium-Flexibacter-Bacteroides), two Spirochaetes, one Planctomycete, one Alteromonadale, one Vibrionale, one member of the Firmicutes or Propionigenium, and three novel and interrelated gamma-proteobacteria with no close relatives identified by BLAST. A sequence that was putatively alphaproteobacterial was also obtained from Symbiodinium culture L9 (tree not shown, Genbank accession DQ174765). However, the sequence obtained was not of sufficient length to allow a supported tree, and did not cover the same region of the l5u nucleotide sequence as the other novel taxa analysed. Attempts to amplify mitochondrial l5u rDNA from an Amphidinium carterae culture yielded one alphaproteobacteria sequence and one CFB sequence (Figure 4.20 and Appendix5; Figures A and B).

In this study, the most common bacterial taxa identified in DNA from the dinoflagellate cultures were putative alphaprotobacteria (2/5 cultures) and CFB organisms (2/5 cultures). The most common bacteria identified in DNA from coral tissues were gamma-proteobacteria and spirochaetes. The sequences of a novel gamma-proteobacterial group (three independent novel sequences) emerged in the phyletic vicinity of Enterobacteriales, Vibrionales and Pseudomonadales with well-supported separation from the Aeromonadales and the Oceanospirillales (Figure 4.20 and Appendix 5 Figure C). No
mitochondrial sequences were obtained from cultured or in hospite Symbiodinium despite these numerous attempts being made.

4. Discussion

4.1 Development of actin as a marker in Symbiodinium

Actin nucleotide analyses and copy-number

The utility of primers (dinoactForw, dinoactRev, etc.) for amplifying zooxanthellae from hosts relied on the electrophoretic separation of host and symbiont bands, which was facilitated by the presence of variable sizes and numbers of introns in the actin genes of the symbiont. Host bands were of the size expected for a fragment with no introns, and these were sequenced and contained only a single sequence, indicating none of the detectable Symbiodinium actin loci were intron-free. Sequence variability among introns from single zooxanthella strains was observed in many cases, which allowed a preliminary assessment of the copy-number of actin in the various clades of Symbiodinium studied. These were as follows: (clade - copy number) A-2, B-1, C>=3, F-1. While copy number was determined by electrophoretic separation of copies in the case of clade C, it was determined by lack of redundant peaks in direct-sequence chromatograms in the cases of clades A, B and F.

Three sequenced copies (fragments) of actin from clade C included a candidate functional gene and a pseudogene. The definitive number of copies of the functional actin gene was not determined, as it did not affect this study. However it is assumed that the multiple loci in clade C were formed by recent duplications (after divergence of clade C from clades B and F). The existence of multiple actin copies in clade C (demonstrated in this chapter) complicated the choice of a single paralog from among the set, for use as a marker in population genetic analyses (Chapter 5). The copies of the gene that have intact reading frames (as far as determined) are presumably under selection to retain function and so may be recombining amongst each other, which could potentially confound studies. For this reason, and because of beneficial selectivity of primers positioned over an exonic 7 bp indel (positions 743-749, Appendix 4), the pseudogene locus (Sym-Go actin copy 2) was
chosen as the most suitable actin marker for use in population genetic studies of clade C in chapter 5 (using the primers GtΨactForw and GtΨactRev in Table 5.1 of that chapter).

The unsequenced L and S actin bands obtained from the F1Z of hosts bearing clade C (Table 4.6), were observed in multiple instances and so may be *Symbiodinium* bands, but they might equally well be amplification artifacts, host actin bands (with introns), or even actins from non-dominant symbionts or parasites. The absence of introns in *Heliofungia* actin (this study) and the deliberate design-bias of primers to be selective for *Symbiodinium*, argues against the possibility that the putative actin copies L and S are host actins.

As in clade C, the clade A actin is present in multiple copies, at least two. This was deduced from the fact that intron 1 of clade A did not sequence unambiguously. The second intron of clade A sequenced cleanly, and so may be a more recent intron gain than intron 1 of this clade, or otherwise the sequencing primers used may have been selective for a given copy.

Cloning of the actin copies would have been desirable, to definitively characterise each of the actin copies in each clade. However assembly of the directly sequenced bands into contigs had an outcome that was verified by electrophoretic visualisation of three actin copies in the case of *Sym-Gt* (Figure 4.4 and Table 4.6). Size consistency of amplified fragments documented in Figure 4.4 and Table 4.6, indicated that the contig assembly method allowed for the potential selectivity of primers and did not introduce artefacts.

**Symbiodinium and host actin phylogenies**

All putative *Symbiodinium* actin sequences obtained in this study grouped conclusively within the dinophyceae (Figure 4.7) and formed a well-supported Suessiale clade (Figures 4.5 and 4.6). In the actin nucleotide phylogeny (Figures 4.5 and 4.6), two previously published putative cnidarian actin genes AFcactP and AGfactP are well supported within the Suessiale clade of dinoflagellates, and in the protein phylogeny (Figure 4.7) they are well supported within the dinoflagellates, arguing that they are *Symbiodinium* actins rather than cnidarian actins as reported (Fukuda et al. 2002). The error in the previous report may
have been due to an inaccurate assumption that larvae are ‘asymbiotic’, given that larvae were the source of the DNAs used.

Remarkable selection for actin sequence orthology across all *Symbiodinium* clades has occurred at the amino acid level (Appendix 4b), while DNA sequence has varied (Figure 4.6). The actin DNA sequence variation may provide a useful marker for intra-genus phylogeny studies, given that the Bayesian tree of DNA sequences (Figure 4.6) retrieved a branching order of *Symbiodinium* clades that is partially similar to that of published nuclear and chloroplast rDNA studies (LaJeunesse 2001; Pochon et al. 2001; Santos et al. 2002b).

The actin nucleotide tree grouped clades B and F to the exclusion of clade C, as opposed to the expected grouping of clades C and F (LaJeunesse 2001; Pochon et al. 2001; Santos et al. 2002b). This conflict could be attributed to the incompleteness of actin sequences in this study, and could indicate that the Bayesian analysis of nucleotides is sensitive to these truncations even though gaps are not considered as data in Mr Bayes (Huelsenbeck and Ronquist 2001), and accordingly gap-rich sequences do not attract each other in the algorithm implemented in Mr Bayes. In PAUP distance and PAUP Parsimony analyses (not shown), a non-truncated 500bp region with no gaps, and sampled across all Suessiales and all dinoflagellates, also retrieved the B-F group with C as sister, indicating that the branching order of *Symbiodinium* clades was not sensitive to presence of some truncated DNA sequences. Sisterhood of clades B and F to the exclusion of clade C is contraindicated by analysis of intron-positions (this section, this chapter, heading: *Symbiodinium* phylogeny by actin intron presence/absence).

Potentially spurious topologies between clades B-C-F were accompanied by low posterior probabilities and this may suggest that the actin DNA sequences were of a variability only sufficient for Bayes to include them within a *Symbiodinium* monophyly, but insufficient to distinguish structure within the *Symbiodinium* genus. Alternatively, codon usage in actins of clades B and F may be convergent. A cursory analysis of codon usage in an equivalent 500bp region of taxa *Sym-A.aspera*, *Sym-L.purpurea Z6*, *Sym L.purpurea Z4* and *Sym-A.pulchella* (same ungapped region as used for analysis of the influence of gaps) did not reveal any obvious codon usage bias in the clade B-F combination as opposed to a clade
C-F combination (data not shown), indicating that the effect may be homoplastic. It is possible that sampling of actin from a larger number of taxa from each clade, especially clade B which was underrepresented here, could resolve the rDNA-derived branching order of *Symbiodinium* clades, namely that clades C and F are a group and clade B is their sister (LaJeunesse 2001; Pochon et al. 2001; Santos et al. 2002b).

The actin sequence putatively derived from host *Heliofungia actiniformis* (this study) grouped within the Scleractinia in the analysis of Figure 4.7. The positions of the actin sequences Tridactpart and Tridactfull, obtained from Dr D.Yellowlees (James Cook University) and of unknown origin (either zooxanthellae or their Tridacnid clam host) were investigated here as regards source, and are indicated as being host mollusc derived (Figure 4.7). However, the Ecdysozoa and Mollusca did not form the expected protostome group (Glenner et al. 2004), and so Tridactpart fell within the deuterostomes rather than the Mollusca. A broader sampling of protostome actins would be required to assess the topology in this area more accurately, and determine whether Tridactpart and Tridactfull are *bona fide* mollusc sequences.

**Revision of the phylogenetic affinities of some Myzozoan actin sequences in Genbank**

Only one actin sequence published in the study of Fukuda et al. (2002) was claimed as a putative *Symbiodinium* actin sequence, Syact-p. This sequence was found in the current study to be most likely from a basal alveolate (Figure 4.7). It is unlikely that it is a dinoflagellate sequence, given the posterior probability of 0.99 on the well-sampled dinoflagellate clade, in which it did not lie. Retrospectively, apicomplexan and dinoflagellate actins were among the top 10 BLAST hits to Syact-p. The order of BLASTp hits to the Syact-p sequence was: AFcact-p, AGfact-p followed by AAC13766 *Toxoplasma gondii* and AY436363 *Perkinsus marinus*. If the Syact-p sequence was derived from an apicomplexan, then it is implied that: (i) an apicomplexan parasitizes the clonal culture of free-living *Symbiodinium* HG39 that was isolated and used by Fukuda et al. (2002), or (ii) that the Syact-p gene is a native chimera or PCR chimera of *Symbiodinium* and apicomplexan genes, or (iii) that the Syact-p gene is a horizontal transfer from an apicomplexan to *Symbiodinium*, or (iv) that the HG39 culture itself is apicomplexan, perhaps resembling the novel Taxon-1 of this thesis (Chapter 6).
In order to keep alignment sizes manageable in the analysis program, sparse taxon sampling was undertaken for algal and other protist actins, other than dinoflagellates and apicomplexans. Nevertheless, it has been shown here (Figure 7) that the published putative *P. lunula* actin sequence (Okamoto and Hastings 2003a) may represent any of: a culture contaminant, a parasite/symbiont of *P. lunula*, or a lateral gene transfer to *P. lunula* from another protist. A retrospective BLASTp search yielded the top five hits of the *P. lunula* actin sequence as members of a phylogenetic assemblage called the Amoebozoa which are phylogenetically far removed from alveolates and are related to fungi and animals (Baldauf et al. 2000; Cavalier-Smith 1998). This is not the first *Pyrocystis* gene to show taxonomically discordant phylogeny (Fagan et al. 1998), and may indicate a history of lateral gene transfer to the dinoflagellate order Pyrocystales. The importance of gene sampling issues was not clear until the superior NY98 model (codon based) was adopted (Figures 4.5 and 4.6) which definitively removed the *Pyrocystis* actin sequence from the Myxozoaan clade. In GTR (non codon-based) analyses, *Pyrocystis*, Syact-p, Tridactpqrt and Tridactfull actins fell within the genus *Symbiodinium* (trees not shown). In general, the widening of taxonomic sampling to include other kingdoms (effectively outgroups) is justified, in that it establishes whether genes of some organisms may have been horizontally obtained (e.g. gene transfer from a protist to an unrelated protist) and thus may interfere with monophyly in analyses of the phylogenetic groups from which they were amplified. Establishment and acceptance of such instances prevents errors, such as the forcing of distant genes together, when an insufficient number of outgroups is used.

**Symbiodinium phylogeny by actin intron presence/absence**

The duplication of the actin gene is likely to have occurred independently in clades A and C because the introns are in different positions in each of these clades. So-called “intron sliding” is not able to account for the 176 bp physical distance between the clade A and clade C first introns, nor the 75 bp distance between the clade A and clade C second introns. Long range intron sliding of more than a few bases has been debunked as a valid natural phenomenon (Stoltzfus et al. 1997) owing to the improbability of creating new coding sequence simultaneously with loss of the identical (or very similar) amino acid
sequence at the other exon boundary. Furthermore, it is widely accepted that clade B *Symbiodinium* intervenes phylogenetically between clades A and C, and so intron sliding from clade A to C might have also required loss of a clade B second intron. Intron loss is thought to occur by reverse transcription from RNA, and recombination of the resulting DNA with the genome (Aravind et al. 2000; Logsdon 1998). No agreed mechanism for intron gain in eukaryotes has been proposed in the literature though this process clearly occurs (Logsdon 2004; Rogers 1989).

The lack of introns in the corresponding fragment of *Polarella* sp. actin, and *Amphidinium carterae* actin, leads to a most parsimonious model for intron gains and losses in *Symbiodinium* actins, presented in Figure 4.21. As markers for speciation events across a genus, presence/absence characters such as introns are potentially superior to variable DNA sequences since the latter are subject to multiple substitutions.
Presence/absence of intron/s at given position

- clade F *Symbiodinium*  
  1 gain

- clade C *Symbiodinium*  
  1 gain

- clade B *Symbiodinium*  
  2 gains

- clade A *Symbiodinium*  
  + - + - 

- *Polarella glacialis*  
  - - - - 

- *Amphidinium carterae*  
  - - - - 

{ No introns }

Canonical boundaries: Most recent gain?

Non-canonical boundaries: ancient gains?

**Figure 4.21** The most parsimonious evolutionary history for intron occurrences at the actin loci of *Symbiodinium* compared to other dinoflagellates. The rightmost intron (arrowed) may be the most recent gain because, of all the introns in this study, it has the most canonical sequence (GT-AG bolded). Shaded areas of the actin gene were not sequenced. The most extreme primers used for amplifications are represented by dotted lines. Therefore possible occurrence of introns in the shaded regions is unknown.
Alternative evolutionary histories to that presented in Figure 4.21, such as the hypothesis that all four introns were ancient in a proto-dinoflagellate, would require that four losses took place in *Polarella*, and four losses independently in *Amphidinium*. In a data-mining survey of dinoflagellate and apicomplexan actins (data not shown), only one taxon, an apicomplexan, possessed an intron. The *Toxoplasma gondii* actin intron (Dobrowolski et al. 1997) is at position 1155 (relative to alignment in Appendix 4a) which is 45 bp distant from the nearest *Symbiodinium* actin intron. Given that intron sliding cannot account for this distance between a *Toxoplasma* intron and a *Symbiodinium* intron (Stoltzfus et al. 1997), no evidence is available to support the idea that the near ancestor of Suessiales possessed four ancient introns at the phyletic base of the dinoflagellates.

**Gradual mutation of actin intron splice sites over evolutionary time**

The intron boundaries within *Symbiodinium* actin genes, are in many cases non-canonical. It has been suggested (Rogers 1989; Sverdlov et al. 2003) that the archetypal patterns for intron boundaries are 5'AG/GTNN (where AG is coding, and "e" represents the exon intron boundary) and NNAG/GT3' (where GT is coding and "e" represents the intron-exon boundary). The adherence or otherwise of *Symbiodinium* actin intron splice sites to this pattern is analysed in Table 4.7. Among eleven separate observed cases of an intron left boundary in this study (Appendix 4a), only three splice sites are of the canonical /GTNN type. Clade C actin copy 2 (Appendix 4a), a presumed pseudogene, has been excluded from that analysis.
Table 4.7 Sequence conservation at intron borders in *Symbiodinium* actins.

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<th>Clade, strain, intron</th>
<th>Exon</th>
<th>Intron left border</th>
<th>Intron right border</th>
<th>Exon</th>
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<tr>
<td></td>
<td>-4</td>
<td>-3</td>
<td>-2</td>
<td>-1</td>
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<tr>
<td>A (CS-158) 1</td>
<td>C</td>
<td>A</td>
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<td>G</td>
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<td>A (CS-158) 2</td>
<td>A</td>
<td>G</td>
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<td>G</td>
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<tr>
<td>B (CS-164) 1</td>
<td>C</td>
<td>A</td>
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<td>G</td>
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<tr>
<td>C (Gt) copy 1</td>
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<td>C</td>
<td>A</td>
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<tr>
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<tr>
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<td>T</td>
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<tr>
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<td>T</td>
<td>A</td>
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<td>G</td>
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<tr>
<td>F (Lp-Z6) 2</td>
<td>T</td>
<td>G</td>
<td>A</td>
<td>G</td>
</tr>
</tbody>
</table>

Key: Intron bases critical to splicing in most eukaryotes are bolded. Bases conserved across a significant majority (at least 9/11) of the *Symbiodinium* intron boundaries are underlined.

---

It has been hypothesised by Sverdlov et al. (Sverdlov et al. 2003) for a wide range of eukaryotic organisms that splicing information, initially coded in the splice-proximal bases of the exons, moves toward the intron over evolutionary time following the creation of an intron. For instance the splice site at an intron left border AG/GTN may degrade to AA/GT with concommitment fixation of a third consensus base in the intron, such as AA/GTC where the C is now required for splicing (a generalisation adapted from Sverdlov et al. 2003). This movement of splicing information from exon to intron may not be the case for *Symbiodinium* introns. Rather, at least at the left borders of these actin introns, splicing information for *Symbiodinium* introns is apparently not as complex as in other eukaryotes. The canonical left border splice sequence /GT is not present (or is degraded) in the majority of observed cases in *Symbiodinium* actin introns (Table 4.7). Non-canonical left borders (/GC, /GA,) occur in eight of ten other *Symbiodinium* intron sequences available in Genbank (high copy-number genes, accessions: AF482424, AF020781, U43532). The potential lineage-specific nature of dinoflagellate splice sequences was also recognised by Schott et al. (Schott et al. 2003) and by J. Saldarriaga and P. Keeling authors of Genbank submission AF482424 (P. Keeling, University of
British Columbia, pers. comm.). The phenomenon noted by Scott et al. (2003) was not confined to *Symbiodinium* but also occurred in other dinoflagellates: *A. carterae* (accession AJ009670) and *Cryptocodinium cohnii* (accession AF417567).

Degradation of the intron splice sequence /GT over time may correlate in at least one case with the deduced relative ages of the introns examined in this study: the second intron of clades C and F is a probable recent gain compared to the intron that they share with clade B (Figure 4.21), and accordingly this second intron contains matches to the canonical splice boundaries. If splice-site proximal sequence conservation were taken to be prescriptive for intron age (Sverdlov et al. 2003) then it could be inferred that both of the actin introns of clade A *Symbiodinium* are older than all of the other actin introns of this study, because they lack −4C and +3G at their right border, which are conserved in all the other introns of this study. However the alternative explanation is at least as good, that those −4 and +3 consenses are specific only to the clade BCD group and not to clade A.

It is premature to conclude that all splicing information is relaxed in dinoflagellates, because analysis of possible splicing ‘branchpoint’ sequences within these *Symbiodinium* actin introns has not been attempted here. Consensus branchpoints are not known in dinoflagellates, but are known for many other eukaryotes (Brown et al. 2002; Edelmann and Staben 1994; Goldstrohm et al. 2001; Spingola et al. 1999). Another reason to be skeptical that left borders are a reliable indicator of splicing information in the whole intron, is that the consensus of intron sequence at right borders is greater than at left borders (Table 4.7).

*Actin marker utility within-clade*

The verified phylogeny of *Symbiodinium* actin genes, and the unique qualities of the resident introns makes it plausible that the introns of these loci may be useful as population genetic markers. Within clade C, across-strain comparison of introns in functional actin genes is potentially subject to paralogy by lack of primer selectivity. However it was found possible in this study to discriminate between actin copies based on sequence alone. Actin copy 3, (Appendix 4a), was found to be common to *Sym-Ha*, -Lp and -Gt (Figure 4.9). This locus amplified from *Sym-Ha* and −Lp, as the smallest of two
bands (data not shown), and amplified from *Sym-Gt* as the smallest of three bands (Figure 4.4, lane A). While the choice of band excised from the gel could confound certainty of orthology if isolates were phylogenetically far apart, that was not a problem in this set of very closely related isolates.

In clades where only a single actin locus is amplified with universal primers, any intron is orthologous across strains, and is a useful population marker, as illustrated in Figure 4.8 for intron-1 of clade F. However, actin intron sequences (as opposed to actin intron positions analysis of Figure 4.21) are unlikely to be useful to compare one clade to another, as the sequences are not alignable across clades without considerable ambiguity. Within a clade, actin introns have been shown here to be promising markers at the strain level (Figures 4.8 and 4.9), and thus are also candidate markers for typing at the level of the individual (Chapter 5).

### 4.2 Development of psbA as a marker in *Symbiodinium*

**PsbA phylogeny**

Analysis of the phylogeny using *Symbiodinium psbA* protein fragments was not possible because the protein sequences were not variable enough. The near identity of *Symbiodinium psbA* protein fragments supports their origin from a single genus, and not from contaminants. In a *psbA* nucleotide analysis (Figure 4.12), all sequences obtained in this study from cultured and *in hospite Symbiodinium* grouped together monophyletically within the genus *Symbiodinium*, indicating none of the *psbA* sequences were from contaminants, such as co-symbionts or culture contaminants. This monophyletic grouping included *Symbiodinium psbA* sequences that had been published by another lab (Takishita et al. 2003a) in the course of the current study.

The branching order of *Symbiodinium* clades (Figure 4.12) using the *psbA* gene is unlike the branching order obtained using the *Symbiodinium* actin gene (Figures 4.8 and 4.9), and both are unlike the consensus order obtained using nuclear *lsu rDNA* (Pochon et al. 2001), *5.8S rDNA* (LaJeunesse 2001) and chloroplast *23S rDNA* (Santos et al. 2002b). In analyses of *ITS* (LaJeunesse 2001; Savage et al. 2002b), clades C and F also grouped
more closely together than either did to clade B, in accordance with the branching order obtained using nuclear and chloroplast rDNA markers.

During the course of this study Takabayashi et al. (2004), published a study of the *Symbiodinium* cox1 coding region across several clades, and found that the sequence of this gene evolves faster than that of nuclear rDNA, but slower than that of plastid rDNA (Takabayashi et al. 2004). The branching order obtained from that study, and those obtained from the current study are presented in Figure 4.22. In each case the data had been analysed as DNA rather than as protein. The branching orders above the basal position of clade A differ in each case. The cox1 analysis by Takabayashi et al. did not utilise a codon-triplet based model of nucleotide substitutions, and therefore it is not possible to directly compare that cox1 analysis to the analyses of actin and *psbA* in this study. Artifacts due to insufficient sampling may also be present. For instance, in the *psbA* gene analysis of the current study, the single D1 sequence is a very long branch and may have created instability, as evidenced by the very low posterior probability (0.5) of the branch that grouped clades D1, D2 and F (Figure 4.12).

![Figure 4.22](image-url) 

**Figure 4.22** Comparison of branching order of phylotypes with different *Symbiodinium* markers. Letters represent *Symbiodinium* clades, except S which represents other Suessiaceae. Branching orders were found in the following studies: 5.8S rDNA and Isu rDNA (LaJeunesse 2001; Pochon et al. 2001), cox1 (Takabayashi et al. 1998), actin (this study), *psbA* (this study).

**Symbiodinium psbA minicircles: structure and evolution**

The *psbA* minicircles from clade C *Symbiodinium* spp. share features common to other dinoflagellate minicircles but also possess unique features, making them of interest from
biological, functional and evolutionary viewpoints. One notable feature of *Symbiodinium* clade C minicircles that is shared with the minicircles of other dinoflagellates is that, like those of *Amphidinium operculatum* and *Heterocapsa triquetra*, they possess tracts of poly-A. An alignment of the poly-A section from *A. operculatum* minicircles and two regions from *Symbiodinium*: C2e-f and C3e-f, is shown in Figure 4.5. The e-f region in C3 in the *Symbiodinium* sequence has particularly high similarity to the *A. operculatum* sequence. The poly-A tract from *H. triquetra* minicircles has been proposed by Zhang et al. (1999, 2002) to be a putative origin of replication, as AT rich sequences are more easily opened to create a replication fork than are GC rich sequences. It is plausible that one or more of the *Symbiodinium* poly-A tracts could serve as origin/s of replication for the minicircle.

\[
\begin{array}{ll}
\text{Sym C2e-f} & \text{CGTATAAAAAGATCGCGACTTATA} \\
\text{Amph} & \text{AGTAGAGAAAAATCCAGG-TCATA} \\
\text{Sym C3e-f} & \text{AGAAAAGAAAAATCGCGACTTATA} \\
\end{array}
\]

**Figure 4.23** Comparison of a poly-A sequence motif present in *Amphidinium operculatum* minicircles with that found in the C2 e-f and C3e-f regions of the *Symbiodinium* clade C *psbA* minicircle. Asterisks indicate identity of top or bottom sequence compared to middle sequence. Conservative changes are noted as R (purine) or Y (pyrimidine).

The feature particularly distinguishing *Symbiodinium* clade C minicircles from those of other dinoflagellates is the presence of multiple small IRs, rather than a single very large IR, and also their arrangement, such that each IR is abutted to another very similar IR. IRs have been found in other dinoflagellate minicircles (Zhang et al. 2002; Zhang et al. 1999) but not at this density nor in twin formation. A notable feature of the IRs in the *Symbiodinium* minicircles is that they can be metastable even between isolates having otherwise identical minicircle sequences, and obtained from a single host species. The minicircles from *Sym-Gt1* and *−Gt2* had identical non-coding sequences except for an indel consisting of a twin IR, M2a-b. A possible explanation for this is that minicircle IRs can form loop regions during minicircle replication or recombination, and thus may be lost or gained as a unit. Similar modular behaviour of a twin IR is apparent in the M1 region,
where the *Sym-Ha* or *Sym-Lp* minicircles possess a twin IR in region M1 while *Sym-G1* and *-G12* do not.

DNA sequence motifs similar to the twin IRs reported here have been found in the mitochondrial genomes of a range of taxonomically diverse fungal species (Paquin et al. 1997; Paquin et al. 2000; Paquin and Lang 1996). These motifs, termed Double Hairpin Elements (DHEs) range in size from 26-79 bases and assume a secondary structure consisting of 3-5 base loops and two adjacent, helical stems, with extensive G-C pairing in at least one stem. Like the fungal DHEs, the *Symbiodinium* plastid twin IRs reported in the current study (Figures 4.13-4.16) are GC-rich in the proposed stem regions, with loops of 3-5 bases. Paquin et al. (2000) argued that DHEs are mobile elements, as multiple copies are present in all of the mitochondrial genomes where they have been detected, they are scattered in distribution and they vary in number between closely related species. The iteration of twin-IRs in the *Symbiodinium psbA* minicircle and their sporadic occurrence in closely related strains is also consistent with a mobility hypothesis. The twin IR formation appears to act as a single unit of selection, since only a single unpaired IRs of the ATG-CAT type was found in this set of sequences (IR V1d of *Sym-Pd*).

Twin IRs may be parasitic mobile elements that have accumulated in the non-coding region of this plastid minicircle. Alternatively they may have a functional role, which is suggested by their very high level of conservation compared to other regions of the *Symbiodinium* minicircle. One possible function of the twin IRs is that they could facilitate recombination between minicircles of the genomic set. In this scenario, recombination would act to maintain the nucleotide sequence and thereby maintain replicative and other essential functions of the minicircles. IRs are known to be recombinogenic in several systems, including plastid (Kawata et al. 1997) plant and fungal mitochondria (Gross et al. 1989; Lupold et al. 1999) fungal and bacterial genomes (Farah et al. 2002; Holmes et al. 2003), plasmids (Francia et al. 1997; Francia et al. 1999), and phage (Mertens et al. 1988; Smith-Mungo et al. 1994). In the current study, support for frequent recombination comes from the observation that mismatches within IR arms do not occur in the C2, C4, M1 and M2 regions, even though these IR sequences vary among strains. In contrast, IRs in the V regions contain mismatches. Strict maintenance of inverse matching in the C and M regions indicates that although these loci mutate rapidly, there is
some stabilising influence, such as gene conversion, that occurs within them but not within the V regions. Further, a role for recombination could be invoked to explain the loss of non-abutting IRs C2b and M1a in *Sym-Pd*, on the basis that these two IRs may have been loops at the time of recombination with another minicircle.

If the model of recombination among cores is correct (Barbrook and Howe 2000; Zhang et al. 2002; Zhang et al. 1999) then the hypervariable sequences of the *Symbiodinium* V regions would indicate that these do not homogenize or recombine nearly as often as the cores. It is possible that the V regions act as buffer zones lowering the probability that recombination will extend into the protein-coding regions of minicircles. Such buffers might help prevent the accumulation of truncated proteins, and of hybrid, potentially non-functional proteins at valuable sites within the plastid apparatus.

Another hypothetical function for the twin IRs is that they may be part of the essential machinery of replication origins. Zhang et al. (1999) hypothesised that AT-rich regions of *H. triqueta* minicircles may be involved in DNA unwinding, and stated that a nearby IR element (of 40 bp) might be part of the putative replication origin. In the *Symbiodinium* minicircles, poly-A sections and twin-IRs do occur together in some cases, giving some support to a functional linkage between the two sequence types.

The results of this analysis of the *Symbiodinium* psbA minicircle open up two further questions to be explored: First, do clade C minicircles possess their high level of organisation as a derived feature, or an ancestral one? It would be interesting to sequence the minicircles of a distant Suessiale such as *P. glacialis*, *G. simplex*, *G. beii* or a proto-Suessiale such as *Wolszyńska* spp., to establish whether any of the non-coding motifs observed in *Symbiodinium* minicircles are of ancient origin. Secondly, will the psbA minicircle be useful as a molecular marker for *Symbiodinium*? The data presented here indicate that the psbA non-coding region can distinguish between closely related clade C *Symbiodinium* isolates, such as *Sym-Ha* and *Sym-Lp*, that could not be differentiated using sequencing analysis of the D1/D2 region of the 16s rRNA gene (see chapter 2, General Materials and Methods). Similar to the 16s rRNA gene, the psbA minicircle may allow different levels of differentiation to be obtained depending on the region chosen: the V regions may be used to distinguish closely related strains, with the C and M regions used to assess diversity in more distant strains, clades and species.
Other minicircles in clade C Symbiodinium

Other labs (Barbrook and Howe 2000; Barbrook et al. 2001; Ellen et al. 2004; Green 2004; Howe et al. 2003; Koumandou et al. 2004; Nisbet et al. 2004; Zhang et al. 2001; Zhang et al. 2002; Zhang et al. 1999) designated conserved minicircle regions or ‘cores’, by comparing across a set of minicircles from a single strain. By contrast in this chapter, the assignment of conserved minicircle regions was made on the basis of a single minicircle, but sequenced from a population of related strains. Therefore, the conclusions that have been made above, regarding Symbiodinium psbA minicircle structure, required testing, and so partial sequences of minicircles encoding other genes were sought.

Using primers C3F and C3R, four bands were cleanly amplified, and direct sequencing of one of these yielded a sequence having C1, M1, and C2 regions that were alignable with the corresponding regions of the psbA minicircle (Figure 19). The conservation of C1, M1 and C2 across different minicircles of a single strain tentatively confirms that the assignment of C, M and V regions in Figure 4.13 was correct, and that the across-strain (rather than across-minicircle) rationale used to assign these was valid.

An exhaustive catalogue of all the minicircle band sizes was not sought. However, the set of bands that were obtained from Sym-Gt, using primers C3F and C3R, may be tentatively considered as minicircles. The sum of all observed bands in Figure 4.7 may indicate presence of at least ten minicircles in Sym-Gt. No conclusions can be drawn about the number of genes encoded, nor have all the minicircles necessarily been found, but the potential for amplification of novel minicircles using primers in the C3 region has been shown (Figure 4.7).

The smaller putative minicircle bands of 1300-800bp are unlikely to encode functional genes. ‘Empty’ (geneless) minicircles have been found in other dinoflagellates (Barbrook et al. 2001; Ellen et al. 2004; Nisbet et al. 2004; Zhang et al. 2001), and were of this size range. However, if DNA bending were facilitated by DHEs, then Symbiodinium minicircles that contain genes might tolerate smaller size than the minicircles of dinoflagellates with no DHEs, such as those of Amphidinium and Heterocapsa. The psbA minicircle of Sym-Gt is 1735 bp, significantly smaller than the 2.3 kb size in A. carterae,
and 2.2 kb, 2.15 kb, 2.3 kb and 2.3 kb sizes respectively in *H. pygmeae*, *H. triquetra*, *H. nei* and *H. rotundata* (Zhang et al. 2002). The DNA tertiary structure modelling done in this chapter (data not shown) indicated a possible conformation of DHEs such that each DHE introduces a 90° bend in DNA, while two DHEs in a row can introduce a 180° kink without steric hindrance, and could result in considerable spatial compaction of the minicircle.

Comparison of further minicircle non-coding sequences from *Sym-Gt* against the *Sym-Gt psbA* minicircle non-coding sequence, would allow confirmation of core locations, and would enable a test of the hypothesis that recombination acts to prevent inverse sequence mismatches in the core regions. In the set of minicircles from *Sym-Gt*, the expectation is to find perfect inverses in core regions. By comparison, subtly different, but equally perfect matches would be expected for cores across the set of minicircles from a closely related zooxanthella strain, e.g. *Sym-Ha*. Likewise, the sequences of other minicircles would reveal whether the buffer zone hypothesis (section 4.2 above) for the V1 and V2 regions is correct. The expectation is that in a single zooxanthella cell, identical V1 and V2 regions would not be shared between any two minicircles containing different genes.

4.3 Bacterial 23S inadvertently sequenced

*Primers for mitochondrial lsu rDNA amplified only bacterial sequences*

No *Symbiodinium* mitochondrial sequences (or other mitochondrial sequences) were obtained, though many bacterial sequences were obtained. These included some sequences from the alphaproteobacteria, a division that contains the closest extant relatives of mitochondria (Andersson et al. 2003; Andersson et al. 1998; Gray 1999; Lang et al. 1999).

Environmental sequences have been proposed to be unreliable by some commentators (Zhou et al. 2003) owing to potential PCR artifacts, that have undergone fixation by cloning. The environmental sequences obtained in this study were not cloned and thus are not subject to this artifact. Bacterial rDNA sequences from published environmental surveys were not included in the phylogenetic analysis, because they had been cloned.
No grouping was observed between prokaryote-type lsu rDNA sequences obtained in this study, and mitochondrial lsu rDNA sequences. Dinoflagellate mitochondrial rDNAs have only been found in small fragments (Norman 2000). By virtue of the negative evidence obtained here, the current study corroborates an emerging model (Norman 2000) that dinoflagellate rDNA is fragmented across the mitochondrial genome, analogous to the situation in apicomplexan mitochondrial genomes (Feagin et al. 1997; Gardner et al. 1993; Gillespie et al. 1999; Kairob et al. 1994; Ossorio et al. 1991), rather than resembling the intact rDNA genes of ciliates (Brunk et al. 2003; Burger et al. 2000).

**Significance of bacterial rDNA sequences in zooxanthella cultures and in hospite**

The lsu rDNA work shown here, demonstrates the difficulty of discriminating eukaryotic organellar bands from bacterial bands when looking for mitochondrial markers. The sources of the bacterial sequences obtained in this study may be bacteria associated with corals, or bacteria not associated with corals. The large volume of bacteria observed with the naked eye, when isolating *Symbiodinium* for culturing from *H. actiniformis, L. purpurea* and *G. tenuidens* indicates that bacteria are abundant in the tissues of all three hosts at least after transport from the GBR to Sydney, during which the bacterial population may possibly increase.

While the peridinin-type dinoflagellate nuclear genome is known to contain genes of alpha-proteobacterial origin (Morse et al. 1995; Palmer 1995; Whitney et al. 1995), the findings in the current study are probably not relevant to the evolution of those genes. Considerable variation in bacterial genotypes was seen here, and so speculation on potential bacterial endosymbionts in *Symbiodinium* is not warranted on the basis of these data, nor does lateral gene transfer need to be invoked. The bacteria sequenced are most likely simply present in the FIZ fraction or culture pellet from which DNA was extracted.

Alpha-proteobacteria, gamma-proteobacteria, CFB and firmicutes are the dominant bacterial types associated with zooxanthellate corals, as detected in three published studies (Casas et al. 2004; Rohwer et al. 2001; Rohwer et al. 2002), using ssu rDNA. The gamma-proteobacteria were the only taxa culturable by Rohwer et al. (2001), but these did not represent the bulk of bacteria detected (Rohwer et al. 2001). However, the culturable gamma proteobacterial genus *Vibrio* is implicated as a cause of coral disease (Rosenberg and Falkovitz 2004). The current study introduces one new type of bacteria, a
planctomycete, that can co-culture with *Symbiodinium*, and another new bacterial type for the *in hospite* niche, a spirochete, that was not cultured. A class detected by Rohwer et al. (2001, 2002) but not detected in this study, is beta-proteobacteria. Rohwer et al. (2002) also reported several hits to mitochondrial sequences, but since the BLAST baits they used were sequences obtained from holobions, the source cannot be attributed to *Symbiodinium*. Those authors also reported hits to plastid sequences especially of stramenopiles, and the potential relevance of these to coral ecology and symbiont evolution will be discussed in chapter 7. In the current chapter no conclusive grouping of any organellar LSU rDNA sequences with plastids or mitochondria was observed.

### 4.4 Organellar inheritance issues

Organellar genomes are uniparentally inherited in sexual organisms (Birky 1995; Birky 2001), and so it is possible that the chloroplast and mitochondrial genomes of a dinoflagellate may both be inherited from the same parent. In *Chlamydomonas*, the mitochondrial genome and chloroplast genome are each inherited from a separate parent (Armbrust 1998; Remacle and Matagne 1998), but the *Chlamydomonas* system is possibly the exception rather than the rule (Birky 2001) (and U. Goodenough pers. comm.). Plastids are maternally inherited in apicomplexans (Ferguson et al. 2005), while little is known about apicomplexan mitochondrial inheritance (van Dooren et al. 2005). Uniparental inheritance of organelles in dinoflagellates has not been studied. However, based on available protist models, the premise of the current recombination study, that the organellar genomes of *Symbiodinium* may be unlinked (i.e. may segregate independently), is questionable, and accordingly emphasis on characterising a mitochondrial locus was softened over the course of this study.

### 4.5 Homogeneity or heterogeneity of zooxanthella strain/s within each host

The coding regions of *psbA* and actin genes sequenced unambiguously in this study by direct sequencing, implying *Symbiodinium* subspecies were pure within each coral individual studied. This finding does not fully address the issue of *Symbiodinium* clonality within a host because coding regions cannot discriminate zooxanthellae at the strain level.

By contrast, the non-coding regions characterised in this chapter could reveal whether a host is unialgal or not. Finding a host that is reliably unialgal would be a prerequisite for a
sexual recombination study (at the population genetic level) using *in hospite* zooxanthellae, so as to avoid the confounding effect of multiple symbionts. Clade C zooxanthellae of four coral hosts were investigated using the *psbA* non-coding region, and three using the actin intron. Of these hosts only one, *H. actiniformis*, in a significant number of cases appeared to be polyalgal at the individual alga level (detailed in chapter 5), making it an unsuitable host for a recombination study. By contrast, three of seventy three *G. tenuidens* colonies (also in chapter 5) were shown to house two conspecific strains of *Symbiodinium* (differing only in presence/absence of the *psbA* minicircle M2 region), while the other 70 *G. tenuidens* colonies are effectively unialgal. The intron of the actin pseudogene (actin copy 2, Appendix 4) was not so effective at differentiating strains as the *psbA* non-coding region was, but is more variable than ITS. There were two point mutations and an indel in this actin pseudogene locus between Sym-Ha and Sym-Gt (Figure 4.9), versus one base change in ITS between these two strains.

4.6 Chapter summary: relevance of outcomes to the thesis and to the *Symbiodinium* research community

Available cultures for this study were of range of clades of *Symbiodinium* other than clade C (chapters 1-3). *In hospite* clade C zooxanthellae were therefore used directly. Considerable progress was made in characterising a hypervariable locus, the *psbA* minicircle non-coding region, establishing its utility as a source of variability among closely interrelated clade C strains, such that its use may contribute to a study of sexual recombination history in this clade (developed further in chapter 5). More surveying would need to be done with the *psbA* non-coding region at the population genetic level across the zooxanthellae of many more host species to establish this as a useful marker for species or subspecies. The assertion by Zhang et al. (Zhang et al. 1999) that conserved cores in minicircles are species markers, not conserved across species, presents a potential way for *Symbiodinium* community to confirm biological species boundaries that were hypothesised by the ITS recombination study of Rodriguez-Lanetty (Rodriguez-Lanetty 2003a).

Actin proved to be a multicopy locus in clade C *Symbiodinium*. This need not detract from its potential use as marker for individuals, strains, subspecies and species in a population, because primers can be designed (as demonstrated here) that discriminate between actin copies in a single cell line, and are applicable to strains other than the strain in which they
were developed. Orthologous actin introns can be targeted across strains and have been so here. Dinoflagellate mitochondrial rDNA was an elusive target in this study, but has been characterised in another dinoflagellate Cryptochodiniun cohnii (Norman 2000).

References


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Chapter 5

Application of the new markers to study of a *Symbiodinium* population

Work outlined in this chapter formed the basis of a manuscript:


Biological species boundaries are currently unresolved in *Symbiodinium*, however recombination has been shown to occur in clades A and B (Baillie et al. 2000a; Baillie et al. 1998; Santos et al. 2003c), making it likely that the biological species concept may be broadly applicable within the genus. The *Symbiodinium* phylotype most abundant in corals of the Great Barrier Reef (GBR) is clade C, followed at much lesser abundances by clades A and D (General Introduction). No published information is available on presence or extent of recombination potential in clade C.

A large volume of literature attests to the ongoing anthropogenic pressures on coral reefs (Hoegh-Guldberg 1999; Hughes et al. 2003). Chief among these pressures is global warming, which is arguably anthropogenic (Walther et al. 2002), and clearly has an adverse effect on coral reefs (Hughes et al. 2003). One way in which coral reefs may cope with the current and predicted rate of change in their environment, is that the zooxanthellae might adapt. The primary mechanisms of adaptation in eukaryotes are sexual recombination (Goddard et al. 2005; Rieseberg et al. 2003) and speciation (Swanson and Vacquier 2002; Wu 2001). Recombination involves, among other functions, the sharing of niche-specific traits among compatible members of a species (Flint et al. 1993; Kover and Caicedo 2001). In view of the dominance of clade C *Symbiodinium* in the GBR ecosystem, this chapter has the following aims:
A) To apply the markers developed in chapter 4 to a population of clade C *Symbiodinium* from Great Barrier Reef corals (One Tree Island), and to assess within-population variability at each locus;

B) To investigate whether there is evidence for a history of recombination in that population.

1. Introduction

The dominance of clade C *Symbiodinium* within Scleractinia of the GBR (General Introduction), together with the disposition of this clade to bleaching, raises the question of whether or not this ecosystem might adapt under threat from global warming. Coral bleaching linked to global warming, is associated with coral morbidity and reduced fecundity (Baird and Marshall 2002; Hoegh-Guldberg and Hoegh-Guldberg 2004; Hoegh-Guldberg 2004; Hughes et al. 2003), and is therefore often fatal (Marshall and Baird 2000). The bulk of literature on the subject asserts that the main cause of coral bleaching is photoinhibition of the symbionts, which occurs when corals encounter an extended period of concurrent high light and high water temperature (General Introduction).

1.1 Recombination potential as adaptive potential

The anthropogenic threat from global warming is widely regarded as real (General Introduction) and, as such, research on how biota may adapt is needed. The predicted fruition period of the ecosystem decline is decades only (Hoegh-Guldberg and Hoegh-Guldberg 2004; Hoegh-Guldberg 1999; Hoegh-Guldberg 2004), if current coral and symbiont genotypes continue to dominate the GBR (Baird and Marshall 2002; Hoegh-Guldberg and Hoegh-Guldberg 2004; Hoegh-Guldberg 2004; Hughes et al. 2003; LaJeunesse et al. 2004; Marshall and Baird 2000). Information on the adaptive potential, adaptive speed, and geographic recombination hotspots of the reef’s zooxanthellae would allow reef management authorities to be better informed about which reef localities require protection against further anthropogenic pressures such as overfishing (Crowder et al. 2000; Roberts 1995), sedimental or chemical pollution from estuarine runoff (Bell and Elmetri 1995; Brodie et al. 2001; Haynes and Johnson 2000; Neil et al. 2002), and diving
tourism (Rinkevich 1995; Rouphael and Inglis 1997). Estimates of adaptive speed are controversial in research on animal phyla, protist and fungal groups and in algal and plant divisions (Sniegowski et al. 2000). However, two tractable questions bearing on adaptive potential are: i) do clade C Symbiodinium have a basic sexual capacity?, and ii) can molecular markers be used to assess this?

1.2 Recombination potential in Symbiodinium

Recent studies have pointed to a history of recombination in Symbiodinium clades A and B (Baillie et al. 2000a; Baillie et al. 1998; Santos et al. 2003c). Baillie et al. used a suite of five isozymes (1998), and 163 RAPD markers (2000a) on a localised population of clade A3 Symbiodinium cultured from seven species of Tridacnid clams. The levels of isozyme and RAPD diversity across Symbiodinium isolates that had identical ITS or rDNA types (Baillie et al. 2000a) were of a level that is unprecedented if the organism is asexual, yet closely match the levels seen in a sexual population of Gymnodinium catenatum strains (Bolch et al. 1999). The authors concluded that it was likely the clade A Symbiodinium of their extended study (Baillie et al. 2000b; Baillie et al. 2000a; Baillie et al. 1998) was a single sexual species distributed throughout the Indo-Pacific.

Baillie et al. (1998), showed a Jaccard-dendrogram analysis of the allozyme data from zooxanthellae of the seven clam species examined. The groups on the dendrogram did not correlate to host-specific strain groups. Mere noise could be misconstrued as meaningful variation due to recombination, and the population might rather be clonal but very rapidly evolving. This possibility was discussed by the authors (Baillie et al. 1998). However purely clonal propagation would be expected to yield structure in a combined-locus tree in all cases: if the variable traits are under selection, or under no selection (Maynard-Smith et al. 1993). Absence of structure in this type of combined tree, that analyses variation at multiple hypervariable loci, is therefore generally accepted to indicate a sexual population (Maynard-Smith et al. 1993; Tibayrenc and Ayala 2002), provided that the strains all possess identical or near identical rDNA sequences, as did the isolates of Baillie et al. (Baillie et al. 2000b). It is clear from the data of Baillie et al. (Baillie et al. 2000b; Baillie et al. 2000a; Baillie et al. 1998) that one strain of zooxanthella is shared by seven host clam species. This is, in itself, potential evidence for sexual recombination of the
symbiont, as traits for host specificity may need to be periodically updated by sharing, if the symbiont is to retain all seven niches.

Santos et al. (2003c) employed 23 microsatellites to analyse a geographically widespread *Symbiodinium* clade B1 population from the gorgonian (octocoral) *Pseudopterogorgia elisabethae*. They found clade B1 *Symbiodinium* to undergo recombination of the 'epidemic' type rather than the 'panmictic' type. This means that rather than being frequently sexual they are maintained by alternating periods of clonal propagation (eg. in the host) with periods of intermittent recombination in an unknown niche, perhaps in the water or the sediment.

In the study by Santos et al. (2003c), noise was not an issue, as the analysis investigated linkage disequilibrium by a stringent statistical method (Haubold and Hudson 2000). They detected significant deviation from random association among alleles. Nevertheless, it is arguable that the association among alleles would have become random noise if these recombination events had not been recent. Present day sexuality of *Symbiodinium* clade B1 is therefore probable but, as noted by Santos et al. (2003c), certainty will require more support from a larger non-redundant data set, because of the epidemic/infrequent nature of the recombination.

The results of the Santos et al (2003c) and Baillie et al. (1998, 2000a) studies are valid in that recombination analyses were limited to strains of a single rDNA or ITS genotype. In view of the indications of recombination in clam symbionts of clade A and gorgonian symbionts of clade B1, a basic sexual capacity for *Symbiodinium* of other clades is predicted. The intracellular lifestyle (Trench 1993) adopted by *Symbiodinium* resident in scleractinians and octocorals, imposes limits on sexual contact but this does not seem to be a barrier for clade B *Symbiodinium* in octocorals (Santos et al. 2003c) so is unlikely to present a barrier for clade C in Scleractinia.

1.3 Clade C *Symbiodinium* variability at the field site
Symbiont phylogeny within clade C of the OTI coral population has been previously assessed (Carter et al. 2000; Loh et al. unpublished; Loi 1998). The essential outcome was that phylogenetically supported clusters within clade C correlated to vertically-transmitted zooxanthellae, in a host specific manner (Carter et al. 2000). This was interesting for two
reasons: (i) because these symbionts may lack a free-living stage or may leave and re-enter the host only rarely, and (ii) because a free-living stage is thought to be a prerequisite for sex in the genus Symbiodinium (Trench 1997). Sequences from vertically transmitted strains tended to be divergent (fast mutating) and to form a long-branch cluster with similar strains from the same host (collectively an ‘ecotype’). It was presumed that, in vertically transmitted ecotypes, the fast mutation rate might signify lack of frequent recombination between strains composing the cluster.

By contrast, three clade C Symbiodinium strains were basal on the OTI survey tree (Figure 2.3, Chapter 2); - these were the horizontally transmitted symbionts of Goniopora tenuidens (Sym-Gt), Heliofungia actiniformis (Sym-Ha) and Leptastrea purpurea (Sym-Lp). At this field site, these three horizontally transmitted strains constitute the best suited candidates for an investigation of sexual recombination, for two reasons: (i) because a free-living stage is thought to be a prerequisite for sex in the genus Symbiodinium; and (ii) because a formal population genetic analysis of sexual recombination requires that rDNA-level variability be essentially fixed in the population, a feature of the phylogenies of these strains, but not of other Symbiodinium clade C strains at OTI.

1.4 Chapter overview

This chapter focused on a localised population of a single strain, Sym-Gt for application of the new markers (actin pseudogene and psbA non-coding region) to a population. This combination of two markers was augmented by sequencing the Internal Transcribed Spacer (ITS), because the third marker that had been sought in Chapter 4, mitochondrial lsu rDNA, was never obtained. While ITS was the next most variable nuclear marker available, and was unlikely to be strongly linked to the other two markers, it was not likely to contain enough variability for assessment of allele reassortment in a population at the chosen geographic scale, and host range (=1). Rather ITS served as the baseline, an invariant marker at this scale, and its invariability was intended to contrast with (and thus emphasise) the variability expected at the other two loci. The findings of this chapter were: (i) that no variation exists either in ITS or the actin pseudogene within the Sym-Gt population at OTI, and so recombination could not be assessed on the basis of psbA variability alone; (ii) that Double Hairpin Element variation seen in the psbA non-coding locus separates a large Sym-Gt OTI population into two groups.
2. Materials and Methods

2.1 Choice of host, sampling rationale and sampling procedure

The population chosen for this study was originally a set of 80 individuals of *Heliofungia actiniformis* collected from OTI reef. However, an analysis of the hypervariable non-coding region of the *psbA* minicircle from three *Sym- H. actiniformis* isolates determined that this host was unsuitable, due to the host containing multiple strains of *Symbiodinium* in some instances (Figure 5.1). Around one *H. actiniformis* individual in every three had more than a single *psbA* non-coding sequence (this chapter), though their zooxanthellae genotypes were homogeneous under an LSU rDNA analysis (General Introduction). The abundant host *Goniopora tenuidens* was instead chosen as the most suitable for this chapter’s aims. The third alternative subject, host *Leptastrea purpurea*, was also used in marker development work of chapter 4, and occupies a basal position within clade C1 (shown in General Introduction) as required for this chapter, but is scarce at the OTI field site, and sampling of it at the required intensity might unduly harm the ecology of that host at this site.

The sampling scale and sampling pattern adopted was that geographic scale was limited to a single reef lagoon, on the assumption that sex is putatively infrequent in this genus or clade, and that distance is a barrier to recombination. These are ‘safe’ assumptions that avoid the collection of sequence data of such variability that isolates do not share any informative characters. It was planned that this scale could be increased in subsequent studies if variation within this population is shown to be insufficient.

Samples of *G. tenuidens* host tissue were collected (by O. Hoegh-Guldberg, C. Smith, M. Phillips and F. Frentiu) at OTI reef in January 2003 (see Figure 5.4). Colonies from which tissue was taken were several metres apart from each other, and were all well lit by sunlight, but no information on water flow, water temperature, water depth or turbidity were recorded. A piece of each colony was broken off and taken to shore, and an airgun used to remove tissue into a plastic bag as detailed in General Materials and Methods (Chapter 2). Tissue was preserved in 50% ethanol 50% seawater. DNA was extracted as detailed in Chapter 2, using the sarkosyl method.
2.2 PCR and sequencing

Loci targeted in this chapter were (i) the actin pseudogene intron, (ii) the C3-M2-C4-V2 region of the psbA minicircle, and (iii) the ITS region of the nuclear rDNA operon. Primers for amplification of these loci are detailed in Table 5.1. These amplification primers were also used for direct sequencing. Primers *dinoITSForw* and *ITS4Rev* lie in the extreme 5' end of the ssu rDNA gene and the extreme 3' end of the lsu rDNA gene respectively (Loh et al. unpublished; White et al. 1990). The positions of the other four primers are defined in Table 5.1. PCR amplifications, DNA sequencing, sequence editing and sequence alignments were performed as detailed in General Materials and Methods (Chapter 2).

<table>
<thead>
<tr>
<th>Primer name and direction</th>
<th>Primer sequence</th>
<th>Annealing temperature</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Actin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>GtactF</em></td>
<td>5'-ATCTTCGTTAACGTCCTCC-3'</td>
<td>55°C</td>
<td>Fig 6. This chapter</td>
</tr>
<tr>
<td><em>Gtact R</em></td>
<td>5'-TCCCTGGAAATTTGAGTGG-3'</td>
<td>55°C</td>
<td>Fig 6. This chapter</td>
</tr>
<tr>
<td><strong>psbA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.3Forw</td>
<td>5'-GAACGAACGCGAAAGGCGAGTGG-3'</td>
<td>55°C</td>
<td>*553-573 Fig 13. Ch4</td>
</tr>
<tr>
<td>10.14Rev</td>
<td>5'-GATACCAAAACCATGAGAGACG-3'</td>
<td>55°C</td>
<td>*1073-1099 Fig 13. Ch4</td>
</tr>
<tr>
<td><strong>ITS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>dinoITSForw</em></td>
<td>5'-GTTATTATTCCGGACTGACG-3'</td>
<td>55°C</td>
<td>(Loh et al. unpublished)</td>
</tr>
<tr>
<td><em>ITS4Rev</em></td>
<td>5'-CCCTCCGCTATTGATGATG-3'</td>
<td>55°C</td>
<td>(White et al. 1990)</td>
</tr>
</tbody>
</table>

* Chapter 4

2.3 Correlation analysis

Some *Sym-Gt* samples contained an M2 region in their *psbA* minicircle, and these samples tended to occur near sites of water inflow/outflow (Figure 5.4). To assess whether the position correlated with genotype, correlation tests were performed. Distance from collection site (arrow heads, Figure 5.4) to the nearest oceanic inlet/oulet (marked by Xs, Figure 5.4) was measured using the OTI map at an arbitrary fixed scale. Oceanic inlet and outlet sites were estimated by reference to published studies (Atena et al. 2002; Booth et al. 2000; Ducklow 1990; Frith 1983; Ludington 1979) and local knowledge of the author. The number A (= M2'-M2+), if positive, is the amount by which blue samples (M2') outnumber red samples (M2+), or vice versa if negative. A is positive at a location when
blues exceed reds, zero when they are equal, and negative when reds exceed blues. The quantity n is the number of samples at a given site. Quantity M was 7.8, the mean number of samples at a site (70 samples divided by 9 sites). Multiplying A by n/M, had the effect of correcting for the low sampling at areas such as G, where all samples were M2\(^+\) but there were few samples. The quantity A times n/M was the "dominance of M2\(^-\) versus M2\(^+\)". Pearson's Correlation and Spearman's Rank Correlation were performed in Minitab (Minitab, State College Pennsylvania), and linear regression and plotting was performed in Excel (Microsoft, Redmond Washington).

Table 5.2 Calculation of the quantity ‘Dominance of M2\(^-\) versus M2\(^+\)\(^+\)+

<table>
<thead>
<tr>
<th>site name</th>
<th>D Distance to inlet/outlet (metres)</th>
<th>A (B-R)</th>
<th>n</th>
<th>n/M</th>
<th>A.(n/M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>221</td>
<td>9</td>
<td>9</td>
<td>1.15</td>
<td>10.35</td>
</tr>
<tr>
<td>B</td>
<td>757</td>
<td>8</td>
<td>8</td>
<td>1.03</td>
<td>8.24</td>
</tr>
<tr>
<td>C</td>
<td>820</td>
<td>6</td>
<td>10</td>
<td>1.28</td>
<td>7.68</td>
</tr>
<tr>
<td>D</td>
<td>1170</td>
<td>4</td>
<td>10</td>
<td>1.28</td>
<td>5.12</td>
</tr>
<tr>
<td>E</td>
<td>1830</td>
<td>2</td>
<td>6</td>
<td>0.77</td>
<td>1.54</td>
</tr>
<tr>
<td>F</td>
<td>1140</td>
<td>-1</td>
<td>9</td>
<td>1.15</td>
<td>-1.15</td>
</tr>
<tr>
<td>G</td>
<td>1550</td>
<td>-3</td>
<td>7</td>
<td>0.9</td>
<td>-2.7</td>
</tr>
<tr>
<td>H</td>
<td>1170</td>
<td>-6</td>
<td>8</td>
<td>1.03</td>
<td>-6.18</td>
</tr>
<tr>
<td>I</td>
<td>1390</td>
<td>-3</td>
<td>3</td>
<td>0.38</td>
<td>-1.14</td>
</tr>
</tbody>
</table>

*\(A.(n/M)\) is defined as the dominance of M2\(^-\) versus M2\(^+\)*

3. Results

3.1 *Goniopora tenuidens* OTI contains a single symbiont clone per coral colony, while *Heliofungia actiniformis* OTI often contains many

Ninety four samples of *H. actiniformis* tissue had been collected for analysis of hypervariable sequences such as introns and minicircle non-coding regions. *Sym-Ha* had been selected as a good candidate for finescale population analyses because the host coral is a single polyp, and because the symbiont's nuclear *lsu* rDNA had sequenced cleanly under direct sequencing (Loh et al. In preparation; Loi 1998). However in the current analysis, about one in three individuals of the *H. actiniformis* population from OTI appeared to possess two or more *psbA* non-coding sequences (Figure 5.1). Given the general finding (Figure 4.13, Chapter 4) that clade C cells usually possess a single *psbA*
minicircle sequence per cell, the frequently variable sequences obtained from single H. actiniformis samples (Figure 5.1) were interpreted as indicating multiple symbiont strains per host polyp rather than multiple psbA minicircles per cell. The multiple symbiont strains in a single polyp of H. actiniformis differed only by indels, and thus appeared to collectively constitute a single symbiont ecotype.
Figure 5.1  Representative chromatograms of minicircle non-coding regions from Sym-Ha and Sym-Lp. (a) The region of chromatogram shown is within the V1 region of Figure 4.13. Isolates Sym-Ha7 and Sym-Ha9 show multiple peaks, compared to the same stretch of sequence from Sym-Lp5. (b) In part of the C3 region (Figure 4.13 chapter 4), isolate Sym-Ha7 shows multiple peaks compared to sequence of Sym-Lp5. (c) Contig assembled in the program Sequencher and showing (as boxed) the region of chromatogram from (b). The pink base G is an example of editing. Whilst the pink G was unambiguous, its upstream bases TGCATT (assigned by the ABI base calling algorithm during chromatogram creation) could be alternatively interpreted as GAACGA. Thus it appears that there are two Sym-Ha strains in this coral colony, one that has incurred a 14bp deletion represented by blue colons, and one that possesses the 14 bp intact. Such indications of polyalgal status were quite common among Ha OTI.
Choice of host for fine scale symbiont population studies was therefore altered to *G. tenuidens*, isolates of which had in every case shown unambiguous chromatograms from the *psbA* minicircle non-coding region. *Sym-Gt* isolates presented an attractive target for use of the minicircle as a unialgal marker, due to a major distinguishing feature among individuals being presence/absence of the metastable M2 region (which contains a DHE) among a population that had otherwise homogeneous *psbA* minicircle non-coding sequences (Figure 4.13, chapter 4). All sequence chromatograms from the non-coding region of the *psbA* minicircle of *Sym-Gt* had been unambiguous in chapter 4.

3.2 Sequences in the non-coding region of the *psbA* minicircle are variable within a population of *Sym-Gt*

The *psbA* minicircle M2-C4-V2 region was amplified from seventy isolates (fifty of which are shown in Figure 5.2) and directly sequenced from a selected 32 isolates (Figure 5.3) to confirm that the band size polymorphism observed was due to presence or absence of the M2 sequence and to record any sequence polymorphisms within V2. As presented in Figure 5.3, absence of M2 sequence was observed in seventeen of the isolates that correspondingly showed small band sizes in Figure 5.2, whilst presence of M2 sequence was observed in fifteen of the isolates that showed larger band sizes in Figure 5.2. In addition to the 70 *G. tenuidens* samples that showed clear M2 presence or absence, three more *G. tenuidens* samples yielded smeared PCR products possibly representing co-occurrence of both bands, and precluding their use in analyses. The double bands could be explainable by invoking that: (i) those three colonies housed cells with M2 and cells without M2; (ii) those tissue samples were cross-contaminated by other samples during airgun removal of tissues; or (iii) PCR templates were cross contaminated (in contrast, PCR negative controls -no template- showed that the master mix was not contaminated data not shown). Whichever is the case, it was not considered to be significant as the double-band samples were a very small minority of the samples.
Figure 5.2 Presence or absence of the M2 region of the *psbA* minicircle from *Sym-Gt* isolates indicates *G. tenuidens* OTI houses a single symbiont clone per coral colony. Lane numbering indicates samples *Sym-Gt*-1 to *Sym-Gt*-50. Isolates yielded amplicons of either ~520bp (lanes designated with bolded font), or ~470bp (lanes designated with plain font). Amplicons include parts of the N-terminal region of the *psbA* gene and non-coding region C3, and the entirety of each region M2 (if present), C4 and V2.

Single nucleotide polymorphisms (SNPs) were present in some isolates, the most notable being three conserved SNPs (positions 24, 353 and 373 on Figure 5.3) that together defined a subset of three *Sym-Gts* within the set of isolates that lacked M2. SNPs were not limited to the V2 region, nor especially concentrated there. Of the five SNPs observed, the SNP at position 24 (Figure 5.3) is in the otherwise conserved C3 region, while the SNP at position 408 (Figure 5.3) is in the *psbA* coding region.
Figure 5.3 Sequence alignment of *psbA* minicircle regions C3, M2, C4 and V2, from 32 isolates of *Sym-GI*. Variable single nucleotides are boxed. Bars below alignment indicate minicircle region. Numbers 1 to 413 of this figure represent numbers 574 to 1030 on Figure 4.13 Chapter 4. Colons represent alignment gaps.
3.3 Spatial distribution and ratio of Sym-Gt samples with DHE M2 versus those without

OTI reef is ponded at low and medium tides with water inflow and outflow funneled towards defined sites (Atema et al. 2002; Booth et al. 2000; Ducklow 1990). The distribution of Sym-Gt isolates across the OTI reef (Figure 5.4), indicated that those lacking an M2 sequence grow in the areas where water flow is greater while Sym-Gt not containing an M2 sequence grow mainly in areas where water putatively has longer retention times.

![Diagram of OTI reef with Sym-Gt isolates distribution](image)

**Figure 5.4** Distribution on OTI reef of Sym-Gt isolates possessing an M2 sequence in the psbA minicircle, versus those without the M2 sequence. Solid red circles = M2 present (M2⁺); solid blue circles = M2 absent (M2⁻); red ring filled with blue = bands indicate zooxanthellae with M2 present and M2 absent exist in a single Gt sample; squares = not done. Thin arrows pointing to circles indicate position of collection of subsets of Sym-Gt isolates. Thick yellow arrows show zones of high water flow (Atema et al. 2002; Booth et al. 2000; Ducklow 1990; Frith 1983; Ludington 1979). White arrows indicate largest channels. The general direction of water flow is from southeast to northwest (Frith 1983). Xs mark the points from which distances were measured for distance/flow correlation (Figure 5.5). The part of the island that is above the high tide mark is shown in green.
To assess whether distance from an inflow/outflow zone correlated with genotype, correlation tests were performed. Genotype was discerned as the dominance of blue (M2 absent, Figure 5.4) over red (M2 present, Figure 5.4), as detailed in the methods section of this chapter. Pearson’s Correlation and Spearman’s Rank Correlation were performed (Table 5.3), as well as a regression analysis in Microsoft Excel (Figure 5.5). Pearson’s and Spearman’s Rank correlation tests both showed a correlation of increasing distance with the presence of M2, and correlation was statistically significant under both methods.

**Table 5.3** Assessment of correlation between presence of M2 and distance from inflow/outflow

<table>
<thead>
<tr>
<th>Test</th>
<th>R</th>
<th>2-sided T-value</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson’s Correlation</td>
<td>-0.695</td>
<td>2.365</td>
<td>0.05</td>
</tr>
<tr>
<td>Spearman’s Rank Correlation</td>
<td>-0.661</td>
<td>2.365</td>
<td>0.05</td>
</tr>
<tr>
<td>Linear Regression</td>
<td>-0.693</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

![Graph showing the correlation between distance to inlet/outlet and dominance of M2- vs M2+](image)

**Figure 5.5** The plot of genotype versus distance from inflow/outflow has a strongly sloped trendline. Linear regression outcomes are shown.
3.4 Sequences at the actin pseudogene intron-1 locus and the ITS locus are invariable in a population of symbionts from *G. tenuidens* OTI

The ITS was directly sequenced from the same set of 32 *Sym-Gt* isolates that were analysed for the *psbA* minicircle M2 and V2 regions in Figures 5.2 and 5.3, to confirm that each isolate of the set was uninodal (sequenced cleanly), and to seek variation in the population, from a different organelle. The actin copy-2 (pseudogene) intron-1 locus was also directly sequenced from the same set of 32 isolates, for the same reason, and was amplified using primers designed to be specific for the pseudogene (Figure 5.6). For each of the 32 isolates, amplicons of the ITS and actin pseudogene intron-1 sequenced unambiguously, but they did not show any variation (actin intron deposited as Genbank accession DQ174767, ITS was identical to Genbank accession number AY237297). Homogeneity of the *Sym-Gt* population at the actin pseudogene intron-1 locus and at the ITS locus contrasted with the variability seen in this population at the *psbA* minicircle non-coding locus.
Figure 5.6 Sym-Gt sequences at both ends of actin pseudogene intron 1. The intron borders are marked by red lines and the upstream exon of actin copy 1 is shown for comparison. Black circles represent exon-bases not shared across these two actin gene copies within a Sym-Gt cell. Copy-selective primers that target the pseudogene (copy-2) are marked in boxes. The isolates shown are three that possess an M2 region in their psbA minicircle, and three that do not. Intron sequences (partially shown) were identical in all 32 isolates. Pink bases were bases edited in Sequencher.
4. Discussion

4.1 Variability is low at introns and organellar-gene flanking regions, among Sym-Gt isolates from OTI reef

An actin pseudogene intron was compared across Symbiodinium isolates from a single host - G. tenuidens. Orthology of these intron sequences was conclusive because sequences at this locus were identical across all the isolates. Similarly, no ITS variation was seen across isolates. Identity of all individuals at both these loci serves to contrast and confirm the high level of variability in the psbA non-coding locus.

The lack of variation across orthologous actin intron sequences within the Sym-Gt population was surprising given that the particular locus targeted (Figure 5.6) was a pseudogene. The actin pseudogene sequence bears a 7 bp deletion, and if translated would result in a very premature stop codon due to frameshift. Actin is a gene of known functional conservation and so a premature truncation could not conceivably yield a functional product. Indeed presence of such a truncated product could inhibit normal actin function so it is to be expected that the control regions of the pseudogene would also be mutated to prevent translation or expression. As such the actin pseudogene is presumably not currently under selection for function. The conserved sequence of the pseudogene intron is surprising but may either indicate that members of the chosen Sym-Gt OTI population are all recent clones of each other, or that there is some selection to retain this particular pseudogene sequence i.e. a selection against revertants. Perhaps a recombinational and mutational coldspot has been generated at and around the actin pseudogene, but this is very speculative, and the former explanation, that the Sym-Gt OTI populations are recent clone-mates is more preferable.

By comparison it is unlikely that the SNPs (Figure 5.3) in the V2 region of the psbA minicircle were under direct selection of any kind since they did not fall within DHEs nor within any other highly conserved part of the non-coding region. The C-A substitution in the psbA coding region is silent (synonymous) and it is possible that the gene itself may be selected semi-independently of the non-coding region but the data are insufficient to make a conclusion on this point. Given the low selection acting at both loci (neither is a protein coding region), a comparison can be drawn between mutation rates (per base) in the
plastid (psbA non-coding) versus those in the nucleus (actin pseudogene intron) of clade C Symbiodinium. In the current study this ratio is 0.001 vs 0 in comparable non-coding loci (calculated from data presented in Figure 5.3, versus no variability in actin pseudogene). This corroborates the published finding that in rDNA coding regions, the plastid mutation rate is ≤7 times higher than the nuclear mutation rate (Santos et al. 2002b), though in the current study no baseline mutation rate for the non-coding DNA was obtained due to the limited geography of the sample set, and restriction to a single host.

4.2 Assessment of future sampling practice for clade C recombination studies

To fully utilise the markers developed in this thesis, a wider geographic range of samples would need to be taken in future, if recombination in a population is to be assessed. The geography of the sampled population was deliberately kept limited in this thesis as a first step towards the goal. The next scale up will be to sample G. tenuidens outside the reef on the external walls of it, and at the neighbouring reef of Heron Island. If the markers of this thesis are to be augmented with other markers a first step may be to include the two newly developed clade C microsatellite markers of Magalon et al. (Magalon et al. 2004), which are specific to the symbiont of P. damicornis. These need to be tested on other clade C strains before assuming utility.

As long as sampling is kept within the Capricorn Bunker group of islands an area of length ~ 100 km, then it is hoped that the symbionts of G. tenuidens may be of a predictable species, equal to the species sequenced at OTI. As far as known no sequence data has yet been published to demonstrate the genotype of zooxanthellae from G. tenuidens on other sections of the Great Barrier Reef. However a DGGE genotyping survey of GBR zooxanthellae by LaJeunesse et al. (LaJeunesse et al. 2004) showed a clade C1 ITS phylotype in this host at Rib Reef 800 km northwest from OTI, the same phylotype (species) as that examined in this chapter (also presented as Sym-Gt OTI on Figure 3.2, chapter 3).

4.3 A phylogenetic approach to prediction of recombination

A way to speculate on the adaptive potential of clade C is to refer to the nearest organism proven to have a sexual capacity, and estimate the likelihood of this trait being retained across that phylogenetic distance and habitat range. Sexuality of clade A and clade B Symbiodinium has been shown (Baillie et al. 2000a; Baillie et al. 1998; Santos et al.
but not of other clades as yet. Of these two, the closest to clade C is clade B (Lesser 2004; Pawlowski et al. 2001; Wilcox 1998). Clades B and C share a common intron position in the actin gene (Chapter 4), and share a number of phenotypic traits including a preference for intracellular symbioses (Baghdasarian and Muscatine 2000; Berner et al. 1993; Carlos et al. 2000; Chen et al. 2003; Coffroth and Santos 1997; Goulet and Coffroth 2004; Jones and Yellowlees 1997; Lesser 2004; Rands et al. 1993; Rodriguez-Lanetty et al. 2004; Schwarz and Weis 2003; Trautman et al. 2002; Trench 1993; Wakefield and Kempf 2001; Wakefield et al. 1999; Weis et al. 2001), a trait not shared with most characterised strains of clade A (Baillie et al. 2000b; Carlos et al. 1999; Farmer et al. 2001; Wakefield et al. 2000; Yokouchi et al. 2003).

Within some eukaryotic genera, sexual ability of a subset of the constituent species is cryptic or in question (Burt et al. 1996; Tibayrenc 1996; Tibayrenc and Ayala 2002). There is no agreed phenotypic or genotypic guideline for the taxonomic limit of a genus, other than the requirement for monophyly. *Symbiodinium* is a large genus, with five species formally named (LaJeunesse 2001; Trench and Blank 1987; Trench and Thinh 1995), and at least five others named but not validly published (LaJeunesse 2001; LaJeunesse et al. 2005; LaJeunesse and Trench 2000; McNally et al. 1994). The number of species intervening (branching) between clades B1 and C1 *Symbiodinium* is arguably none, one, or two (Rodriguez-Lanetty 2003a), and so at this distance sexual ability is likely to have been conserved. To rephrase, the existence of sexual recombination in clade B, which are intracellular strategists, makes it likely that meiotic ability is common to other intracellular *Symbiodinium*, such as the intracellular clade C, and that adaptation to changing environments might therefore be possible in a range of *Symbiodinium* strains that inhabit scleractinians. This inference from clade B1 to clade C1 should not be mistaken with the erroneous idea that sex could occur between members of different *Symbiodinium* clades (see chapter 1, table 1.1, for proposed general limitations on recombination in the genus).

### 4.4 Possible significance of the *psb4* minicircle M2 region as a marker in *Sym-Gt*

The absence of the M2 region in some *Sym-Gt* OT1 (Figures 5.2 and 5.3) is indicated as a potential marker for thermotolerance, though only on a strain scale. This is a testable prediction. Thermal stagnation of the midwaters of OTI lagoon, as compared to the edge waters (reef flat and entrances), has been evidenced by the distribution of bleached corals
after a bleaching event in 1998 (Affendi Bin Yang Amri, University of Sydney, unpublished observations).

An alternative hypothesis for the near equivalence of counts of Sym-Gt possessing an M2 sequence and those not possessing an M2 sequence, is that the dichotomy may correlate with host gender. *G. tenuidens* is a gonochoric species, i.e. male and female individuals grow as separate colonies (S. Ward, University of Queensland, pers. comm.). Host-specificity of zooxanthellae is thought to be due to cell surface interactions (Jimbo et al. 2000; Reynolds et al. 2000; Schwarz and Weis 2003; Weis et al. 2001), but gender-specificity has not been demonstrated.

The marker is in the plastid, and so is more likely to be hitchhiking with a plastidic fitness trait than with a cell surface fitness trait. It is improbable that the M2 sequence would represent a symbiont strain marker that is gender specific to its host, and only marginally more probable that the marker is correlated with resistance to thermal photoinhibition given the lack of statistical support gained in Table 5.3 and Figure 5.5.

A method of assessing possible correlation of the M2 presence/absence with host gender would be to re-collect, this time at spawning season, and to note the gender of host by gravidity (S. Ward, University of Queensland, pers. comm.), and by isolating host nubbins in containers on the night of spawning to see which sex of gamete is produced. Sequencing of the *psbA* minicircle C3-M2-C4 region from the sexed isolates, would indicate correlation or non-correlation with host gender.

A mechanism to test whether the presence/absence of M2 is correlated with thermotolerance and or resistance to photoinhibition may be to expose nubbins of wild collected *G. tenuidens* OT1 (some possessing M2 and some not, established by PCR) to a range of thermal regimes under controlled laboratory conditions with abundant natural sunlight, and to observe relative bleaching susceptibility.

On current data (*n=70*) there is no definitive cause to reject the default assumption that the M2 deletion evolved recently in a clone of the Sym-Gt population, without any evolutionary selection for this deletion, and that this clone populated OT1 lagoon at the same time as did the clone that possesses M2. As such the two clones, being with and
without M2 are equidominant in the population, and no other causative factor for the 50:50 distribution of M2 need be invoked. However, if similar presence/absence of DHEs could be observed to be present at 50:50 ratio among clade C Symbiodinium of a single host species at another lagoon, then this unlikely ratio might be interpreted with increased meaning, and the reasons sought more intently.

Further explanations are possible for why the regulatory region of a photosynthesis gene might differ in members of the population that inhabit areas of differential water flow. These possibilities include different amounts of plankton in the different areas of the lagoon, with a subsequently lessened need for photosynthesis to satisfy the nutrition requirements of some host colonies. However, the M2 deletion would affect only photosynthesis, and a diet richer in plankton content could not replace the role of photosynthetic metabolism in enabling corals to calcify. Photosynthetic productivity is required for calcification to occur (Barnes and Chalker 1990). The G. tenuidens population sampled in this study were all well calcified, indicating photosynthesis was strong in all samples. Additionally, in well lit coral colonies such as those of this study, the contribution of planktonic nutrient to coral respiration is known to be very minor compared to the contribution by zooxanthellae carbon (Falkowski et al. 1984; Muscatine et al. 1984).

The overriding questions in assessing whether any importance should be placed on the 50:50 distribution of the psbA-M2 region across Sym-Gt of OTI lagoon, are whether there is any time dependence of water temperature, or spatial gradient of water residence time, in the lagoon. These overarching questions fully contain the component questions regarding nutrient inflow/outflow to/from the lagoon, and temperature variation across the lagoon.

An indicator of the nature of the thermal inflows and outflows was given by Atema et al. (2002) who noted that the ebb tide from the lagoon releases warmer water into the surrounding sea if the ebb tide occurs following daytime, but releases cooler water into the sea if the ebb tide occurred following a night time. So in general the temperature fluctuates much more in the lagoon than in the surrounding ocean. Bleaching events are pronounced in the lagoon even when there is no bleaching at all on the surrounding reef slopes (Affendi Bin Yang Amri, University of Sydney, unpublished observations). The
observations can be taken together to imply that the surrounding ocean is effectively a constant temperature. Atema et al. (2002) documented that during January and February of 1998, the sea outside the lagoon remained at 27°C constantly, whereas the waters of the lagoon warm to ~30°C during the day, and cooled to only 26°C at night. Plumes from the lagoon were measured as temperature spikes in the surrounding sea. These observations show that: i) at a morning flood tide, the cool water entering the lagoon is relatively close to the temperature that the lagoon water has reached during the preceding night, ii) at an afternoon flood tide the incoming cool water is a very different temperature than the warm water that has developed in the lagoon during the preceding warm day.

Whether Sym-Gt in the lagoon are more challenged by incoming cool water or incoming warm water, may be answered by the general lack of reports of cool water bleaching, though these do occur (S. Ward, University of Queensland pers. comm.). Thus the observed temperature of incoming water (ranging from 1°C warmer to 3°C cooler than the lagoon) is probably not a challenge to Sym-Gt (if it were 3°C warmer there would be a challenge), rather the challenge to Sym-Gt may be from warm water that has developed within the lagoon during a warm day. The lagoons shallowness (2-10 m), and 5-6 hr ponding time in each tidal cycle (Frith 1983; Ludington 1979), both favour warming of the lagoon water by sunlight on hotter days. The ingress of ocean water 3°C cooler than lagoon water after a warm day can be expected to create a gradient of water temperature across the lagoon during the first part of the flood tide because at that time most inflow is across the reef crests neighbouring the major channels (Frith 1983; Ludington 1979) as annotated on Figure 5.4. Inflow becomes greatest after the tide height in the surrounding sea reaches the level of the reef flat, and is thereafter less spatially focussed because it occurs over a wide area of reef flat. However, the general direction of water flow is constant from southeast to northwest (Frith 1983). Flood tide contributions from the northeast and southwest directions are of lesser significance.

Additional factors that may impact the distribution of psbA minicircle M2 indel in the Sym-Gt population are water depth and sedimentation. Water flow can be considered a representative component for analysis, because depth and sedimentation in the lagoon are dependent on water flow (Frith 1983). Depth increases from south and east to northwest. Sedimentation is greatest at the south and east, where sand flats are formed by the action
of backflow along the lagoon bottom due to the year-long southeasterly surface winds (Frith 1983). Turbidity differences can potentially generate miniecologies by influencing light levels on sand flats to contrast with light levels in channels of high water flow. Light levels cannot be discounted as a contributing factor in the population variability observed, as turbidity was not measured. Similarly depth of sampling was not recorded but, like turbidity, it may influence light availability. The OTI lagoon has been suggested to function as a sediment trap and to be slowly filling (Ludington 1979). This is due to ponding but also due to the consequently long residence time. Ludington (1979) measured a water residence time of six tide cycles under neap tides and 0-15 knot wind conditions, and inferred that much greater residence times would occur when calm conditions prevailed for several days. The feature of residence time that has not been sufficiently studied is its influence on lagoon temperature during various times of the year.

4.5 Verification by minicircle sequencing, that *G. tenuidens* OTI are unialgal, corroborates that they contain multiple actin loci within a cell

Sym-Go OTI samples 4, 5, 14, 22 and 44 had been used to assess the copy number of actin for Sym-Go in chapter 4 (Figure 4.4, Table 4.6 and Appendix 3a). The clonality of each of these samples, i.e. effectively unialgal nature of host-symbiont relationship, as shown in Figure 5.3, indicates that the multiple actin copies sequenced were not derived from multiple symbionts per host colony.

4.6 Chapter summary

An actin pseudogene showed an unexpectedly high level of conservation, while ITS homogeneity was evident and was expected. Sequence homogeneity in the zooxanthella population at each of two nuclear loci, actin and ITS, contrasts with the high sequence variability neighbouring the chloroplastic locus *psbA* in this population, and this difference validates the claim made in chapter 4 that the M2 DHE of Sym-Go evolves independently and rapidly, by its insertion or deletion. Given that the Sym-Go population of OTI is highly homogeneous in genotype at two genetic loci (actin-intron, ITS), and heterogeneous at only one of the loci chosen (plastid minicircle), a wider geography of sampling (and consequent wider genetic variation) will be required in future if the actin and ITS loci are to become useful in documenting recombination history. Alternatively a
set of hypervariable markers such as microsatellites (Magalon et al. 2004; Santos and Coffroth 2003a; Santos et al. 2003c) could be developed for recombination studies of this limited-variability population from OTI. The chapter raises the questions: - how frequently do DHEs insert or delete themselves in the plastid genome?; and therefore, - are they useful markers for population studies of sexual recombination history? A question raised by the results but unrelated to the chapter aims is: - do DHEs have a regulatory role in gene expression?

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Chapter 6

Novel algal taxa obtained from corals while culturing Symbiodinium

Work described in this chapter formed the basis of the following publications:


The sexual cycle of Symbiodinium is so uncharacterised in vitro that the question must be asked whether historical de novo culturing techniques may have inadvertently selected for asexual strains, or forced adaptation of sexual strains to an asexual mode of reproduction by cumulative fixation. To allow for this possibility, during the culturing studies of Chapter 3, forms that were brown coloured but not of the expected shape for Symbiodinium were saved and isolated, and then grown further to investigate whether they might be Symbiodinium gametes or zygotes. Preliminary evidence had already been gained in Chapter 3, that selective isolation media can induce gametogenesis of Symbiodinium. The periodic motility of other Symbiodinium species can be viewed as adaptations to an intracellular lifestyle. An assumption not made in this chapter, is that periodic motility is a defining feature of all members of the genus Symbiodinium. The strict requirement for particular lifecycle traits was abandoned, and rather the light microscope features of ochre colouring and unicellularity were taken as the most basic defining features of potential zooxanthellae. In a separate rationale, some widespread and ecologically interesting symbionts other than Symbiodinium have been found in corals (Fine and Loya 2002; Lesser et al. 2004; Toller et al. 2002; Upton and Peters 1986) but have not been cultured, so any new cultures of ‘symbionts’ that might be obtained would be inherently valuable.
The aims of this chapter were therefore:

a) To culture novel algal forms from FIZ inoculated into the growth media developed in chapter 3;

b) To investigate, using molecular sequencing, whether these represent gametes, zygotes or vegetative cysts of *Symbiodinium*, or rather represent some other alga;

c) To retain all ochre-coloured unicellular symbionts (whether novel or otherwise) for future ultrastructural and pigment characterisation and submission to culture collections.

1. Introduction

1.1 Morphological features of dinoflagellate sexual stages

Sexuality of the modern Suessiales: *Symbiodinium* spp. *Gymnodinium bei*, *Gymnodinium simplex* and *Polarella* spp. is undocumented *in vitro*, though in the case of *Symbiodinium* it has been evidenced *in vivo* by population genetic studies (Baillie et al. 2000a; Baillie et al. 1998; Santos et al. 2003c). However other dinoflagellates possess enlarged zygotes that are encased within environmentally resistant walls during both the *in vitro* and environmental lifecycle. Quadriflagellate zygotes are possessed by *Woloszynska* (K. Heimann, James Cook University, pers. comm.) the next closest genus to Suessiales (Kremp et al. 2005; Saldarriaga et al. 2004).

Cells smaller than the normal vegetative forms have been observed in many dinoflagellate species, but it has not been shown in all cases that the small cells were gametes. While the gametes of dinoflagellates may often be smaller than the ‘adult’ somatic cells, and of a different shape, alternative roles for the small cells have been speculated as depauperate terminal-, or temporary interim- stages in the asexual lifecycles (Silva and Faust 1995).
1.2 Groups present in coral tissues and closely related to Dinophyceae

*Apicomplexa and their relatives*

Apicomplexans are a monophyletic group of mostly obligate parasites including *Toxoplasma*, *Cryptosporidium* and *Plasmodium*, and many others, all of which parasitise metazoans (Levine 1978; Perkins et al. 2002). The group was renamed ‘Apicomplexa’ from ‘Sporozoa’ by Levine (Levine 1970) and their relevance to this thesis is that they are phylogenetically and taxonomically sister to dinoflagellates (Cavalier-Smith 1991; Gajadhar et al. 1991; Wolters 1991), and that some apicomplexans occur in corals (Toller et al. 2002; Upton and Peters 1986). Firstly an apicomplexan was characterized microscopically in *hospite* (Upton and Peters 1986). Secondly, by genotyping, an apicomplexan has been demonstrated as resident within three separate genera of Caribbean corals (Toller et al. 2002). ‘Apicomplexa’ means ‘possessing an apical complex of organelles’ (Levine 1970; Levine 1971) which is used for parasitism of hosts.

Many apicomplexans possess relic chloroplasts called “apicoplasts” that have lost photosynthetic activity (Gardner et al. 1994; McFadden et al. 1997; Ralph et al. 2004; Wilson and Williamson 1997). Apicomplexans that possess an apicoplast biochemically rely on it, and cannot survive when it is disabled (He et al. 2001; Ralph et al. 2004). Because of the large taxonomic breadth of the division Apicomplexa, and the demonstrated monophyly of the apicoplasts among all those apicomplexan genera that possess it (Lang-Unnasch et al. 1998), it is believed that apicomplexans evolved from a single photosynthetic ancestor, perhaps resembling a dinoflagellate, the sister division to Apicomplexa (Fast et al. 2001). The most convincing early evidence for the monophyly of the peridinin dinoflagellate plastid and the apicoplast was a phylogenetic analysis of the plastid targeted GAPDH gene (Fast et al. 2001), though the suggestion has also been corroborated by a study of plastid encoded proteins (Zhang et al. 2000). The idea that Apicomplexa originated from a sister to dinoflagellates was first tentatively drawn by Levine (Levine 1978) without substantiation, and was confirmed by Gajadhar et al. (Gajadhar et al. 1991) using nuclear rDNA phylogeny, but was slightly prior to the discovery of the apicoplast (Gardner et al. 1994; McFadden et al. 1997; Wilson et al. 1991).
The division Apicomplexa formally consists of the eucarophycolans and the colpodellids (Adl et al. 2005) due to the latter possessing an apical complex of organelles, as do the Euapicomplexa. Euapicomplexans are all obligate parasites of metazoaans (Perkins et al. 2002), while colpodellids are free-living heterotrophs (Simpson and Patterson 2002) that feed on algae or on other heterotrophs. The feeding habits of colpodellids, and their basal position within the Apicomplexa, allow an alternative view opposed to the mainstream hypothesis that the plastids of peridinin-dinoflagellates, apicomplexans and stramenopiles are monophyletic and secondary (Fast et al. 2001). This alternative view is that the Apicomplexa and the peridinin-dinoflagellates may have evolved from a colpodellid-like heterotroph which endocytosed a eukaryotic alga and retained its plastid. The evidence against this alternative view is that the low number of membranes surrounding the apicoplast and the peridinin-dinoflagellate plastid, being four and three respectively, is inconsistent with a tertiary origin of the organelle (Cavalier-Smith 2000; Foth and McFadden 2003; Keeling 2004).

**Chlorophytes, bacteria and cyanobacteria**

Other algal taxa and many bacteria are also found with corals in associations that may be variously beneficial, commensal, harmful or of unknown significance (Casas et al. 2004; Knowlton and Rohwer 2003; Rohwer et al. 2001; Rohwer et al. 2002; Wegley et al. 2004). Algal taxa that have been studied in hospite are endolithic algae (chlorophyte) that reside beneath the coral skeleton, and cyanobacteria that reside within coral tissues (Bentis et al. 2000; Fine and Loya 2002; Lesser et al. 2004; Shashar et al. 1997). Neither of these types of algae are phenotypically similar to dinoflagellate somatic or sexual stages.

Lesser et al (2004), demonstrated that in a coral host there may be a minor fraction of uncharacterised symbionts (in that case cyanobacteria), that are active partners in nutritive symbiosis in multiple coral genera, alongside the majority of the symbiont population, the dinoflagellate *Symbiodinium*. Accordingly in this chapter, investigation of novel algal cultures obtained during axenisation of *Symbiodinium* from corals was a desirable end in itself, because cataloguing of the *in hospite* biodiversity of minor fractions of FIZ could identify organisms that may have a bearing on coral health. Given the aims of the chapter however, only brown coloured organisms that might potentially be dinoflagellate sexual stages were saved.
1.3 Chapter overview

It became apparent while culturing *Symbiodinium* from corals in Chapter 3, that distinctive forms not possessing a gymnodinioid zoospore stage could be reproducibly cultured from FIZ. These newly characterized algae were hypothesized to be any of: (a) sexual forms of *Symbiodinium* (zygotes or gametes); (b) immotile strains of *Symbiodinium*; or (c) Suessiales or dinoflagellates ancestral to *Symbiodinium*, and living a symbiotic lifestyle in corals. These taxa did not genotype as *Symbiodinium*, but by molecular phylogeny were found to be affiliated to the Apicomplexa, particularly to the free-living apicomplexan genus *Colpodella*. While their identities did not support any of the above hypotheses (a,b,c), these new taxa were interesting because they could represent ‘missing links’ in the evolution of apicomplexans from dinoflagellate-like ancestors, and might be ecologically important.

2. Materials and methods

2.1 Culturing

At One Tree Island reef (Gladstone, Queensland, Australia), fragments of coral skeletons were obtained from ten separate coral colonies by Karen Miller and Craig Mundy (University of Wollongong, NSW) and transported alive to Sydney. These were five samples or “nubbins” from host *Leptastrea purpurea* and five nubbins from *Pocillopora damicornis*. Three nubbins of coral host *Plesiastrea versipora* were collected from Sydney Harbour (NSW).

*The L. purpurea, and P. versipora* nubbins were placed together for three days and nights in an aquarium containing non-autoclaved Sydney Harbour seawater (SW) collected at Watson’s Bay near Sydney heads, an area rich in *P. versipora* colonies. The five *P. damicornis* nubbins were processed immediately upon arrival in Sydney, and only exposed to autoclaved seawater (ASW).

The outer mucosal surface of corals was sterilised with 1:25 or 1:100 parts of pH-neutralized household bleach in seawater – modified from York (York 1986), as detailed in Table 6.1. The detergent was allowed to precipitate overnight, and the supernatant (=
HOCI as in chapter 3) was decanted gently and added to sterilized seawater the next day. Alternatively, trichloroisocyanurate (TCIC), commonly used in swimming pools, was added to the culture medium at a final concentration of 0.01g per litre of growth medium. TCIC gains and loses Cl− ions easily (it is a buffer for Cl− ions). The base culture medium used was fEs (Chapter 3). As well as coral surface treatment by HOCI, HOCI or TCIC were also used directly in the fEs (Table 6.1). Inocula were Freshly Isolated Zooxanthellae (FIZ) obtained from corals by the methods detailed in Chapter 3.

Table 6.1 Coral preparation, symbiont nomenclature, culture medium and culture conditions

<table>
<thead>
<tr>
<th>Host species</th>
<th>P. damicornis</th>
<th>L. purpurea + P. versipora</th>
</tr>
</thead>
<tbody>
<tr>
<td>surface pretreatment I</td>
<td>1 part HOCI to 100 parts ASW, overnight</td>
<td>1 part HOCI to 25 parts SW</td>
</tr>
<tr>
<td>surface pretreatment II</td>
<td>1 part HOCI to 20 parts ASW, over 2nd night</td>
<td>-</td>
</tr>
<tr>
<td>initial culture medium</td>
<td>1 part HOCI to 250 parts fEs</td>
<td>0.1g TCIC per litre of fEs</td>
</tr>
<tr>
<td>subsequent culture medium</td>
<td>fEs</td>
<td>fEs</td>
</tr>
<tr>
<td>initial temperature</td>
<td>27°C</td>
<td>27°C</td>
</tr>
<tr>
<td>initial light</td>
<td>5-10 μEinstein, 14:10hr light:dark</td>
<td>5-10 μEinstein, 14:10hr light:dark</td>
</tr>
<tr>
<td>culture date</td>
<td>9th December 2001</td>
<td>11th December 2001</td>
</tr>
<tr>
<td>symbiont nomenclature</td>
<td>Taxon-2*</td>
<td>Taxon-1* (Lp + Pp)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Taxon-2* (Lp)</td>
</tr>
</tbody>
</table>

ASW=Autoclaved Seawater. TCIC=Trichloroisocyanurate. Lp=from L. purpurea. Pp=from P. versipora

*All cultures are in the possession of the author, and are also being maintained in parallel by collaborators, under the author’s guidance (section 3.1). The cultures are not yet public, but will be submitted to public culture collections when the formal descriptions of these taxa are accepted for publication. Underlined cultures are type cultures that were sequenced.

Coupling of fEs-HOCI or fEs-TCIC culture medium with a light:dark incubation (period of light and dark roughly equal) positively selects for photosynthetic organisms. These culturing procedures had in chapter 3 been found to create favourable conditions for Symbiodinium isolation, at the expense of grazers (such as ciliates), autotrophs (such as bacteria and fungi), and non-target free-living unicellular algae (such as diatoms and haptophytes).

In some instances, cells were sourced from the meniscus of culture wells after 1-3 months of de novo culturing. Cells of taxon 1 were in some cases purified to clonality by ‘dilution streaking’ a loopful of raw culture onto fEs agar, incubating in the light room for 1-3 months, then picking single colonies each into liquid fEs for further growth. Cells were maintained at 27°C (14:10 L:D) by the author (Table 6.1), and also maintained in parallel

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at 15°C and 18°C with an unspecified L:D cycle by David Green (Scottish Association for Marine Science, UK, data used with permission).

Other cell types were purified either by picking discreet foci of immotile (resting) cells from primary culture wells (using a 100 μl pipette tip), then placing in a fresh well, or by sourcing organisms from a primary culture well in which they were already unialgal.

2.2 Microscopy
A Zeiss Axiosvert 40 CFL inverted microscope and a Leitz Labovert inverted microscope, were used for routine observations of the proliferative state and purity of de novo cultures. A Canon Powershot G6 camera was used for routine documentation on the Zeiss Axiosvert 40 CFL microscope. The Leitz Labovert microscope had a Leitz Orthomat 35mm film camera attached. Photography of cells with phase contrast and Nomarski differential interference contrast was kindly performed by Dr K. Heimann, (James Cook University, Townsville Queensland), using an Olympus BX51 microscope with an Olympus DP70 camera. Photography of cells with a Zeiss Axioplan microscope was performed with an Axiocam digital camera by Prof. D. Patterson (Marine Biological Laboratory [MBL], Woods Hole Massachusetts), and Prof. R. Andersen (Bigelow Laboratory for Ocean Sciences, Maine).

2.3 PCR and molecular sequencing
Genomic DNAs were extracted from cultures of potentially new organisms or new algal lifestages, and used as PCR templates, according to the methods given in General Materials and Methods (Chapter 2). PCR primers listed in Table 6.2 were used to target gene fragments from four loci. Two of these loci code for chloroplast genes while the other two loci code for nuclear rDNA. All four loci were sequenced for taxon 1 whilst only three loci were sequenced for taxon 2 (Table 6.3).

All the primers used are capable of amplifying multiple eukaryotic kingdoms. Reaction conditions in all cases were: 94°C two minutes, 94°C fifteen seconds, annealing at specified temperature (Table 6.2) for thirty seconds, extension at 72°C for two minutes, repeated for 30-40 cycles, followed by a final extension of 72°C for ten minutes. Cycles were kept to thirty when amplicons were to be cloned. Bands were excised from an
agarose gel and purified using a Stratprep DNA Gel Extraction Kit (Stratagene, La Jolla California). Direct sequencing, using primers psbAf6, psbAL1, 18A1F and 1800R, was performed on the psbA amplicons of taxon-1 and taxon-2, and on 300-500 bp of the ends of the nuclear ssu rDNA amplicons of taxon-1 and taxon-2. To sequence the internal regions of each 1100 bp long nuclear ssu rDNA molecule, cloning was employed. Cloning was performed with a TOPO DNA ligation kit. Clones were sequenced with M13 forward and reverse primers, or T3 and T7 primers (Invitrogen, Carlsbad California). For the other markers: nuclear ssu rDNA taxon-1, plastid ssu rDNA taxon-1, nuclear lsu rDNA taxon-1 and taxon-2, cloning only was performed, as culture purity had already been demonstrated by the psbA and nuclear ssu rDNA sequencing.
Table 6.2. PCR primers used in this chapter.

<table>
<thead>
<tr>
<th>primer pair name</th>
<th>Primer sequence</th>
<th>Annealing temp°C</th>
<th>Target gene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 18A1F 1800R</td>
<td>5'-CCTACTCIGGTGATCCTGCCACT 5'-TAATGTACCTCCCGCAGTT</td>
<td>50</td>
<td>nuc ssu</td>
<td>(i)</td>
</tr>
<tr>
<td>B ZardF ZardR</td>
<td>5'-CCCGCIGGAATTTAAGCATATAAGTAAAGCGG 5'-GTTAGACTCTCTGTTCCCGTGTCAAGA</td>
<td>45-50</td>
<td>nuc lus</td>
<td>(ii)</td>
</tr>
<tr>
<td>C psbA16 psbAL1</td>
<td>5'-GARCCAAACAHYNTAGCAYCC 5'-CRTGCATWACTTCCATWCC</td>
<td>55</td>
<td>psbA</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>psbAOF ssuT12F</td>
<td>5'-TACAACATCGTWGCKGCTCAC 5'-CATGGCTCAMGRCCYGACTTCA</td>
<td>55</td>
<td>psbA</td>
<td>This chapter</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>nuc ssu</td>
<td>“”</td>
<td></td>
</tr>
<tr>
<td>D US14F UI492R</td>
<td>5'-GTGCCAGCGMCGCGCGG 5'-ACCTGTTACAGCTT</td>
<td>45-50</td>
<td>all ssu</td>
<td>(iii)</td>
</tr>
</tbody>
</table>

(i) (Wollscheid and Wagele 1999); (ii) (Zardoya et al. 1995); (iii) (Edgecomb et al. 2002)

Table 6.3 Strains that were sequenced, and primer pairs used.

<table>
<thead>
<tr>
<th>Taxon 1 host</th>
<th>nuclear ssu</th>
<th>primer pair</th>
<th>nuclear ssu</th>
<th>primer pair</th>
<th>plastid psbA</th>
<th>primer pair</th>
<th>plastid ssu</th>
<th>primer pair</th>
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<tr>
<td>Pp7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RM12 .......</td>
<td>C</td>
<td>RM12 .......</td>
<td>D</td>
</tr>
<tr>
<td>Lp12</td>
<td>CMS22</td>
<td>A</td>
<td>CMS22</td>
<td>B</td>
<td>CMS22 .......</td>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lp13</td>
<td>CCG15</td>
<td>A</td>
<td>CMS23*</td>
<td>B</td>
<td>CCG15 .......</td>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lp14</td>
<td></td>
<td>CMS24</td>
<td></td>
<td>B</td>
<td></td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Taxon 2 host</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>RM11 .......</th>
<th>C</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pd5</td>
<td>RM11</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lp14</td>
<td>sol2</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pd1</td>
<td>sol3</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lp15</td>
<td>sol4</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lp4</td>
<td>sol6</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Lp= Lepistrea purpurea OT1, Pp= Plesiastrea versipora Sydney Harbour, Pd= Pocillopora damicornis OTI
Independent cultures occupy each row. Clonal subcultures are italicised. Sequenced cultures are underlined.
*CMS23 is the raw parent culture of the clonal culture CCG15.

To establish whether the source of taxa may have been coral tissue, as opposed to seawater, psbA primer psbAOF was designed (Table 6.2), that possesses a 3' end dissimilar to psbA of Symbiodinium (alignment not shown) but compatible with taxon-1, taxon-2 and chlorophyte psbA sequences. Primer psbAOF was coupled with psbAL1, and used to amplify fragments of DNA from holobiont samples (coral tissue plus entire symbiont population). Tissue samples used as templates were OT1 coral extracts: Lp 14, Lp15, Pd1, and Pd4, of Chapter 3; plus another Lp tissue Lp4-OT1. These corals had all been stored for >1 week in Sydney Harbour water before culturing (see Chapter 3),
implicating Sydney harbour water as a possible source of the novel cultures. Therefore to rule out this possibility, coral extract KPd2, from *P. damicornis* OT1 was also used as a source of template DNA, because that coral sample had never come into contact with Sydney Harbour water. This DNA template was kindly donated by Katherine Ferguson, University of Sydney. To further address the question of the environmental source of taxon-1, a taxon-1 specific ssu rDNA forward primer was designed and was coupled with primer 1800R (Table 6.2) in a PCR reaction.

2.4 Phylogenetic analyses
Alignments were generated and manipulated as detailed in General Materials and Methods (chapter 2). Maximum likelihood analysis of apicoplast ssu rDNA phylogeny was performed by T. Chrudimsky and M. Obornik (University of South Bohemia, Czech Republic, tree used with permission) with the program PhyML (Guindon and Gascuel 2003; Guindon et al. 2005). Parameters used in generating the plastid ssu rDNA were the GTR model for nucleotide substitutions, and a discrete gamma distribution in 8+1 categories. All parameters (gamma shape =0.057; pinvar=0.059) were estimated from the dataset. ML bootstrap support values for branches were obtained using 50 replicates, HKY85 model of DNA base substitution (Hasegawa et al. 1985), with one category of sites.

Bayesian analyses of rDNAs were performed by T. Chrudimsky and M. Obornik (University of South Bohemia, trees used with permission), using the same conditions (GTR and gamma 8+1, burnin after maximum log likelihood reached) as commonly used by the author and detailed in General Materials and Methods. Bayesian phylogenetic analysis of *psbA* proteins was performed by the author according to General Materials and Methods.

3. Results

3.1 Culturing and microscopy

Two distinct algal cell types (designated taxon-1 and taxon-2) were isolated and were retained to investigate their identities. Each was obtained from multiple nubbins of more
than one coral species. Algal taxon-1 was obtained from corals *L. purpurea* (five of five nubbins) and *P. versipora* (two of three nubbins) as detailed in Table 6.4. Algal taxon-2 was obtained from *L. purpurea* (three of five nubbins) and *P. damicornis* (two of five nubbins). Each taxon had a stable phenotype that was different than the other, and different from the published lifestage morphologies of *Symbiodinium* cells. They were initially retained to investigate whether they were undescribed lifestages of *Symbiodinium*.

**Taxon 1**

A cell type was cultured from the FIZ of *L. purpurea* and *P. versipora* (Table 6.4) that had cell size, buoyancy and colony formation properties similar to *Symbiodinium*. It was obtained by taking material from meniscus of a tissue culture well using a sterile loop and subculturing in fresh medium, or it was obtained by picking discreet foci from the well floor. Designated taxon-1, it was retained on the assumption that it may be an immotile form of *Symbiodinium*. Since they were immotile, unlike normal *Symbiodinium*, selected cultures of taxon-1 were sent to Dr K. Heimann (North Queensland Algal Identification and culture Facility, James Cook University), Dr C. Bolch (University of Tasmania), Prof. R. Andersen (Bigelow Laboratory Maine), and Dr D. Green (Culture Collection of Algae and Protozoa, Scottish Association for Marine Science) for opinions.

Of the seven independent cultures of taxon-1 obtained (Table 6.4), six were made clonal by picking cells from agar as detailed in section 2.1. Most taxon-1 cultures reported here lacked any motility. Some taxon-1-like cultures (from *L. purpurea*, data not shown) on occasions contained rapidly moving colpodellid shaped cells of the same pigmentation of as taxon-1 vegetative cells. These occurred *en masse* in young cultures, but were rare in established pure cultures, and so were not further examined due to difficulty of obtaining sufficient number of motile cells for characterisation.

Taxon-1 has a phenotype that includes an extracellular accumulation body, which is left attached to the hemispherical cell wall at ecdysis (Figure 6.1). The accumulation body is not a bud, as the taxon 1 cells grow by equal division rather than by budding (unpublished observations). Young *de novo* taxon-1 cultures, <3 months of age since extraction from coral, showed a pronounced tendency to grow as colonies in liquid culture (data not
Colonies always appeared intact and there were no 'craters' in any colony from which motile cells may have broken free. A cratered colony phenotype is frequently seen in liquid cultures of *Symbiodinium* (unpublished observations), so the uncratered colonies of taxon-1 were distinguishable from *Symbiodinium* colonies, both in this respect and in respect of cell size which was smaller than *Symbiodinium*.

Taxon-1 changed its phenotype substantially when incubated for two weeks at 15°C and thereafter kept at 18°C (Figure 6.2b, data of D. Green, Scottish Association for Marine Science, photos used with permission), as opposed to when kept at 25-27°C without any prechilling (Figure 6.2a). The low temperature phenotype was partially depigmented by comparison to the higher temperature phenotype but the chilled cells still appeared brown to the naked eye (Figure 6.2b) and still grew in f/2 medium in the light room without added nutrients so were presumably still photosynthetic (D. Green, pers. comm.).

**Table 6.4 Raw and clonal cultures of taxon-1, and raw and unialgal cultures of taxon-2.**

<table>
<thead>
<tr>
<th>Host sp. and nubbin no.</th>
<th>Taxon-1 raw cultures*</th>
<th>Taxon-1 clonal cultures*</th>
<th>Incub. in non-sterile SH water</th>
<th>Taxon-2 Site Cultures</th>
<th>Incub. in non-sterile SH water</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. versipora</em> 7</td>
<td>Pv7</td>
<td>RM12</td>
<td>SH</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>P. versipora</em> 9</td>
<td>Pv9</td>
<td>RM2</td>
<td>SH</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>L. purpurea</em> 11</td>
<td>Lp11</td>
<td>CCG13</td>
<td>OTI</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>L. purpurea</em> 12</td>
<td>CMS22</td>
<td>RM13</td>
<td>OTI</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>L. purpurea</em> 13</td>
<td>CMS23</td>
<td>CCG15</td>
<td>OTI</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>L. purpurea</em> 14</td>
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<td>CCG16</td>
<td>OTI</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>L. purpurea</em> 15</td>
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<td>CCG14</td>
<td>OTI</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>P. damicornis</em> 1</td>
<td>RM11</td>
<td></td>
<td></td>
<td>OTI</td>
<td></td>
</tr>
<tr>
<td><em>P. damicornis</em> 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bold indicates unialgal cultures. Type cultures are underlined. SH=Sydney Harbour. OTI=One Tree Island

*All cultures are in the possession of the author, and many are also being maintained in parallel by collaborators, under the author's guidance. The cultures are not yet public, but will be submitted to public culture collections when the formal descriptions of these taxa are accepted for publication. Underlined cultures are type cultures that were sequenced.
Figure 6.1 Light microscopy of taxon-1 at 25°C. A Extracellular accumulation body. B Ecdysed cell walls with accumulation body still attached.

Figure 6.2 Light microscopy of taxon 1. A Phenotype at 25-27°C, strong pigmentation (scale bar 5μm). B Phenotype at 15-18°C contains less pigmentation, and has an accumulation body on the cell surface (Image B by D. Green, Scottish Association for Marine Science, used with permission).
Taxon 2

Cultures of a large brown cell type (15-80 μm diameter) were isolated from the FIZ of corals *C. purpurea* and *P. damicornis* (Table 6.4). These were obtained by sourcing benthic (specific gravity >1) organisms from a primary culture well in which they were already unialgal, or were obtained by picking discreet foci from the well floor. Designated taxon-2, this cell-type initially appeared to be a sexual zygocyst, and therefore was thought to be an undocumented sexual lifestage of *Symbiodinium*. Taxon-2 cells that were undergoing enlargement left behind ecdysed cell walls (Figures 6.3A,B) mirroring the ecdysis phenotype of taxon-1. The possibility that these were zygotes of *Symbiodinium* was investigated because obvious serial doubling of cell size was occurring, and this was thought to represent gradual increase in size of a dinoflagellate hypnozygote, as happens in several dinoflagellate genera (Pfiester 1984; Pfiester and Anderson 1987). The increase of cell size in dinoflagellate hypnozygotes is due to the fact that even though meiotic products are at first only four in number, they are juvenile vegetative individuals and can gain numerical advantage for subsequent survival in the environment if they duplicate many times before being released from the protective cyst.

Around two months after taxon-2 cells were placed into fresh fEs, they gradually lost their brown colour and became greenish. Brown colour did not recur. Cell morphology and division was monitored weekly and these features remained consistent during the apparent ‘change of colour’. In retrospect the colour change was only partly natural and was also partly an illusion due to unfaithful colour transmission by the microscope used (Leitz Labovert) or the equipment’s age and state of repair. Cultures of taxon-2 were sent to Dr K. Heimann (North Queensland Algal Identification and culture Facility, James Cook University), Dr S. Wright (Australian Antarctic Division), Prof. R. Andersen (Bigelow Laboratory, Maine), Dr A. Simpson (Dalhousie University, Nova Scotia), and Prof M. Melkonian (Culture Collection of Algae at the University of Cologne) for opinions.

Taxon-2 cells developed green colour by adaptation to the fEs growth medium (Figure 6.3C,D and Figure 6.4C). They ranged in size from 5 μm to 80 μm, and at the higher sizes (>20μm) were apparently multinucleate judging from their developmental fate (Table 6.5). Parent cells of taxon-2 contained up to ~20 spherical daughter cells (Figure 6.3C, numbers
of daughters approximated from photographs). Some protists (e.g. euapicomplexans) divide by schizogony (multiple fission) and it is suggested that this is what the ‘mother cells’ of taxon-2 were engaged in. The ‘daughter’ cells of taxon-2 were released passively from the parent cell through an opening designated as a ‘micropyle’ (Figure 6.3D) named after its euapicomplexan equivalent (Levine 1971). Additionally, colpodellid-like flagellates were found to be intermittently present in every isolated culture of taxon-2 (Figure 6.4A,B, - taxonomically identified by A. Simpson, Dalhousie University, via visual inspection of the high resolution LM micrographs).

Taxon-2 cultures were obtained by either of two means: (i) they appeared in a dilution-well with no contaminating algae present (cultures RM11 and RM25, Table 6.4); (ii) they took over existing healthy Symbiodinium cultures quickly and unexpectedly, after six months of propagation of the Symbiodinium culture (cultures RM24, RM 26, CCG 31 Table 6.4). Takeover events indicated that taxon-2 had been present in very low (unobservable) numbers in pre-existing Symbiodinium cultures. This was at first interpreted as a very slow adaptation of taxon-2 cultures to the fEs medium, followed by a fast outcompetition of existing Symbiodinium cultures by taxon-2. Slowness of taxon-2 to adapt to the fEs medium was similarly observed in the unialgal cultures (RM11 and RM25) as stated in section 2.1.

To be deemed ‘unialgal’ required that a culture never contained proliferating Symbiodinium. Only unialgal cultures were used for PCR and phylogenetic analyses of taxon-2. Taxon-2 cultures only proliferated in liquid medium, and failed to proliferate when they were streaked on agar and incubated for two months. RM11 is the unialgal type culture of taxon-2 used for all the DNA analyses and microscopy.

Photographic documentation of taxon-2 culture RM11 (Figures 6.4A,B and Figure 6.5A) was initially performed at a time less than 3 months since axenisation from corals, and weeks before the proliferation of spheroids by multiple fission had begun (or been observed) in this culture (Figure 6.3C), and this before greening had occurred (Figure 6.5B). A localized population of flagellates (Figure 6.4A,B) became apparent but only when the live RM11 spherical cells of 50-80 μm were squashed by finger pressure under a microscope cover slide. Figure 6.4A shows the sporangium that yielded the flagellates of
Figure 6.4B. Since these flagellates appeared to have been squashed from a sporangium, intact sporangia of this type were subsequently sought for photographic documentation. Very few cells in the taxon-2 population developed into sporangia containing flagellates. Some flagellates of taxon-2 contained pigmentation (Figure 6.6) while others did not contain as much pigmentation (Figure 6.7).

To address whether the flagellates present in the RM11 culture were conspecific with the green alga in that culture, cells were placed into sterile seawater in a petri dish for single-cell picking via a mouth micro-pipettor. After two weeks the number of flagellates present had increased very greatly. While picking cells it was observed that the flagellate population was sporadic. That they appeared sporadically (presumably each wave from a separate sporangium) was not surprising, but the lack of buildup of the flagellate population drew attention to the short duration of the flagellate phase. It was secondarily observed that spherical cells which were adhered to the petri dish were no longer in clumps as they had been when inoculated, rather there was an evenly dispersed layer of small algal cells each 5 μm across the whole dish. Based on a model that the flagellates had morphed, and become 5 μm algal cells, this morphing was subsequently observed on many occasions (see table 6.5 for details). Isolated flagellates are currently being monitored for regeneration of the algal mother cells.
Figure 6.3 Light microscopy of taxon 2. A. Initial observation of the taxon as it appeared in cultures from *L. purpurea*. The imaging system used may have made cells look browner than they were (photo by the author). Obvious serial doubling of cell size evidenced by the ecdysed cell walls was thought to represent gradual increase in size of a dinoflagellate hypnozygote, thus putative evidence of a sexual stage for *Symbiodinium*. B. Ecdysed cell wall of a new daughter cell. C. ‘Meront’ containing ‘daughter’ cells produced by schizogony (scale bar 5 μm) (Photo by the author). D. Three views of micropyle (arrowed in first image), with young cell emerging passively (arrowed in second image), scale bar 15 μm. Sources: Image A and B by the author. Images C and D by K. Heimann (James Cook University), used with permission.
Figure 6.4 Release of flagellates from taxon-2 cells by finger pressure. A The material put under pressure was the putative cysts of Figure 6.3 A. Only a single field was investigated in obtaining the taxon 2 images in Figure 6.5 A and B, and that field is shown here in full. The field contains mother cells (Table 6.5), and a single sporangium (Table 6.5). Inset is a magnification of the burst sporangium which produced the stream of flagellates shown by the curved arrow, after finger pressure had been applied to the coverslip. B Magnifications of flagellates from frame A. B Nomarski differential interference microscopy of flagellates released by finger pressure from a sporangium (Figure 6.4) that were present in the same culture as Figure 6.5A (for increased detail of flagellates see Figures 6.6 and 6.7). Photos A and B were produced by Prof. D. Patterson (MBL), in conjunction with the author. Prof. Patterson was at University of Sydney when photographs of Figures 6.4A,B, and Figure 6.5A were taken.
Figure 6.5 Taxon 2 colouration. A Taxon 2 culture RM11 was initially brown at its first *de novo* culturing in a tissue culture well, because of its nutrient deprived state (shown here), then green pigment increased after first subculture with fresh medium. Green pigment was present in the brown form, but appeared diffusible and patchy. B Taxon 2 in a subsequent well-fed state (weeks-months later) was distinctly green and the pigment had become pervasive. Sources: Photo A by D. Patterson (MBL, Massachusetts), in conjunction with the author. Photo B by D. Patterson (MBL, Massachusetts) and R. Andersen (CCMP, Bigelow Laboratory) used with permission.
Figure 6.6 Light microscopy of taxon-2 ‘sporangium’ with moderately pigmented contents.  
A Sporangium half burst after finger pressure had been applied to the microscope coverslip.  
B Contents of sporangium of A, were colpodellids containing pigmented material. Next to each  
brown-orange organelle in each pro-flagellate is a light green organelle implicated to be the  
chloroplast.  
C A sporangium (right) next to an algal mothercell (left). The latter contained multiple  
‘daughters’ or ‘autospores’. Photos by D. Patterson (MBL, Massachusetts) and R. Andersen  
(CCMP, Bigelow Laboratory) used with permission.
Figure 6.7 Light microscopy of apparently lightly pigmented pro-flagellates of taxon-2 in a sporangium next to taxon-2 vegetative algae. Next to each brown-orange organelle in each pro-flagellate is a light green organelle implicated to be the chloroplast. This image is simply of an overall lighter tone, and lower contrast than that of Figures 6.6A,B,C Photo by D. Patterson (MBL, Massachusetts) and R. Andersen (CCMP, Bigelow Laboratory) used with permission.
<table>
<thead>
<tr>
<th>Cell size</th>
<th>differentiation state</th>
<th>phenotypic character</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Taxon 1</strong></td>
<td></td>
</tr>
</tbody>
</table>
| 5-8 μm   | somatic immotile      | - in both warm (27°C) and cool (18°C) temperatures an accumulation body often occurs on the surface of the cell  
- ecdysed cell wall often bears an accumulation body  
- in cool temperatures cells partially lose pigmentation  
- in cool temperatures or under nutrient limitation, radial symmetry increases: ring of organelles are putative carbon-storage globules |
| 5-8 μm   | somatic flagellate     | - flagellate with straight translational motion and constant roll, not unlike the translational motion of many dinoflagellates  
- colpodellid-like morphology. More bullet-like than banana-like  
- the flagellate is pigmented as for the spherical form  
- the flagellate is induced by low nutrient concentration, such as a one fourth dilution of 0/2 in sterile seawater (= 1/8) |
|          | **Taxon 2**            |                                                                                                                                                      |
- flagellate roll is very fast. Rear of cell is furthest from the longitudinal axis of roll. Front of cell is on the longitudinal axis of roll  
- flagellate does not have straight translation, there is constant yaw. As a result the flagellate swims erratically often in circles of >100 μm.  
- seems to investigate sessile self-alga as potential prey but does not actually prey on its own sessile algal stage.  
- flagellate ontogeny takes place in 50 μm - 80 μm sporangium (below)  
- the flagellate is pigmented as for the spherical form  
- the flagellate is readily induced by low nutrient concentration, such as sterile seawater, and is abundant under these conditions  
- the flagellate preys on other (non-self) alga, but in the absence of prey it attaches to the substrate via a flagellum, and while gyrating about that fixed point its form changes gradually to spherical, while remaining attached to the flagellum which has become a stalk. The stalk is resorbed slowly, until the algal cell is attached by its wall to the substrate. Thus it becomes a somatic sessile alga. |
| 5-15 μm  | somatic sessile alga   | - growing cell ecdysed its old cell wall  
- under nutrient stress radial symmetry is apparent, as a ring of organelles |
|          | (i.e. immotile form)   |                                                                                                                                                      |
| 15-50 μm | somatic sessile alga   | - beginning of multinucleate phase, cell walls ecdysed as cell volume increases  
- multinucleate phase preceeds immotile autospore (daughter cell) formation  
- thickening of cell wall accompanies production of immotile daughter algal cells inside mother cell  
- algal daughter cells passively exit through a large micropyle |
| 50-80 μm | somatic algal mother cell |                                                                                                                                                      |
| 30 μm    | somatic sporangium     | - 'sporangium' which contains developing flagellates  
- flagellates swim out through a micropyle when mature  
- flagellates are colpodellid-like and are pigmented in the same degree as the 5 μm sessile alga |

| 276 |
Figure 6.8 Comparative structure of taxon-1 and taxon-2 algae. A Documentation of planar radial symmetry in 6 μm taxon-1 cells (upper) for comparison to that of 10 μm and 5 μm cells taxon 2 (lower). Organelles of taxon-1 often lie in planar rings of 5-6 (circled) surrounding a central dark pigmented region. This putatively synapomorphic phenotype of radial symmetry (in taxon-1 and taxon-2) occurs in taxon-1 only at certain lifestages and under certain temperature conditions (see Figure 6.2B and Table 6.5). B Contents ‘spilled’ from an ~80 μm taxon-2 cell after finger pressure had been applied to the coverslip. Putatively, each planar module of ~10 μm circled in B (note scale bar 10 μm) is packaged into a daughter cell (Figure 6.8A and Figure 6.3B,C,D). Other clear organelles present in large numbers are putative carbon-storage globules, while tiny pigmented organelles are of unknown identity. Sources: Images of taxon-1 in A are by D. Green (Scottish Association for Marine Science), used with permission. Images of taxon-2 in A are by the author. Photo in B is by D. Patterson (MBL, Massachusetts) used with permission.
3.2 Comparatitive morphology of taxon-1 and taxon-2

The low temperature phenotype of taxon-1 brought attention to a diagnostic synapomorphy that links taxon-1 and some aging cultures of taxon-2. Under these stress conditions a planar radial symmetry was seen in the cytoplasm of each taxon, consisting of a circle of ~6 organelles lying consecutively adjacent to each other and forming a planar ring around another organelle which is at the centre of the cell (Figure 6.8A,B). This radial symmetry is referred to as a ‘stellate’ phenotype. In bright field microscopy of taxon-1, the ‘nuclear’ hexagon had a dark grey tone while the spokes of the radial pattern had a lighter tone (Figure 6.8A). A similar stellate phenotype was visible in stressed young cells of taxon-2 (Figure 6.8A). Similarly, in a cell squash of an 80 μm mother cell of taxon-2, stellate objects of ~10 μm were visible (Figure 6.8B), and are presumptive developmental precursors of the algal daughter cells of taxon-2. Both taxa display ecdysis of transparent cell walls (Figures 6.1, 6.3A,B). Colpodellid-like flagellates are more banana-shaped in taxon-2 (Figure 6.5B), and more bullet-shaped in taxon-1 (not photographed). The flagellates’ translational motion is erratic in taxon-2, but linear (straight-travelling) in taxon-1, and this is predicted to reflect flagellar structure and flagellar orientation.

3.3 Molecular detection

The sequence data initially collected in order to genotype the raw algal cultures were plastid psbA sequences (~400bp). A BLAST search using the psbA fragment of taxon 1 culture CMS22 (Table 6.6) retrieved top hits to the plastid of Spatoglossum, a stramenopile, Compsopogon a rhodophyte, and Halopteris a stramenopile. Identical taxon-1 psbA sequences were gained from two independent taxon-1 cultures: CMS22 (host L. purpurea 12), CCG15 (host L. purpurea 13) [see Table 6.3].

A BLAST search using the psbA fragment of taxon-2 culture RM11 (Table 6.7) retrieved top hits to Padina, Odontella and Skeletonema which are stramenopiles (e values of e^−77 and e^−74 respectively). Identical taxon-2 psbA sequences were gained from four independent taxon-2 cultures: RM11 (host P. damicornis 5), sol2 (host L. purpurea 14), sol3 (host P. damicornis 1) and sol4 (host L. purpurea 15) [see Table 6.3]. A non-identical sequence but very similar (99% identical over 407 bp) was obtained from culture sol6
(host L. purpurea 4), and obtained similar BLAST results to the RM11 psbA sequence (Table 6.8).

### Table 6.6 BLAST results for non-Symbiodinium psbA amplicon from taxon-1 culture CMS22

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<th>species name</th>
<th>stramenopile class</th>
<th>score</th>
<th>expect value</th>
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Key: R-Rhodophyta, S-Sireptophyta
BLAST query using novel sequence X, fragment obtained using PCR primers: forward psbA universal (psbA6 Table 6.2), reverse psbA universal (psbAL1 Table 6.2)

### Table 6.7 BLAST results for non-Symbiodinium psbA amplicon from taxon-2 culture RM11

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*Tertiary plastid is Coscinodiscophyceae-derived (Chesnack et al. 1997; Chesnack et al. 1996; Inagaki et al. 2000)
BLAST query using novel sequence X, fragment obtained using PCR primers: forward psbA universal, reverse psbA universal
Table 6.8 BLAST results for non-*Symbiodinium* psbA amplicon from taxon-2 culture sol6.

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*Tertiary plastid is Coscinodiscophyceae-derived (Chesnick et al. 1997; Chesnick et al. 1996; Inagaki et al. 2000)*

BLAST query using novel sequence X, fragment obtained using PCR primers: forward psbA universal (psbAf6 Table 6.2), reverse psbA universal (psbAL1 Table 6.2)

The psbA BLAST data therefore showed that taxon-1 and taxon-2 were not *Symbiodinium*. These molecular data were obtained concurrently with the photographic data. However, even though they were then clearly not *Symbiodinium*, cultures of taxon-1 and taxon-2 had each been obtained from two hosts species each, and from several coral nubbins of each species, so the taxa were considered to be common in corals and of potential ecological or evolutionary significance, so were investigated further.

A psbA primer, psbAOF (Table 6.2), was designed that excludes *Symbiodinium* (primer 3’ end specificity), but amplifies taxon-1, taxon-2, and chlorophyte psbA. Primer psbAOF was used to amplify fragments of DNA from FIZ genomic DNA and holobiont genomic DNA of hosts *L. purpurea* and *P. damicornis*. A psbA gene sequence identical to that of new taxon-2 was detected in uncultured algal samples and holobiont samples from both hosts consistent with this taxon being a tissue-dwelling symbiont (Appendix 6). Primer psbAOF did not amplify taxon-1 from *L. purpurea* tissues obtained from OTH, nor from *P. versipora* tissues obtained from Sydney Harbour, even though these tissues were the source of FIZ used to generate the taxon-1 cultures. A taxon-1 and-2 specific ssu rDNA primer ssuT12F (Table 6.2) also did not amplify taxon-1 from *L. purpurea* and *P. versipora* holobiont samples.
It was not initially clear how the psbA BLAST results or the light microscopy results could be taxonomically interpreted, therefore small subunit rDNA fragment of ~1600bp, were sequenced from each new taxon (Table 6.2). Additionally, the plastid small subunit rDNA gene of ~700 bp, and nuclear large subunit rDNA fragment of ~600bp were amplified from taxon-1.

The nuclear ssu rDNA of both taxa were sequenced directly from an amplicons excised from agarose electrophoresis gels. A single unambiguous sequence was present in each chromatogram obtained by direct sequencing, indicating that the type-cultures RM12 and CCG15 (taxon-1) and RM11 (taxon-2) may each be pure, containing only a single species each (‘monospecific’).

Taxon-1 sequences obtained using cultures derived from _L. purpurea_-OTI were identical to those obtained using cultures derived from _P. versipora_—Sydney Harbour. This was surprising as the host corals were obtained from sites ~2000 km apart. If they are symbionts then the strain is pandemic. Otherwise they are not symbionts in both taxa, and may have entered _L. purpurea_-OTI from Sydney Harbour water (in which the corals were stored) that may be rich in taxon-1 cells if _P. versipora_ is their natural host.

3.4 Nuclear phylogeny of taxon-1 and taxon-2

**Taxon 1**

Nuclear-ssu phylogeny indicated with a posterior probability of 0.93 that taxon-1 is a close sister to the apicomplexan clade (Figure 6.9). Specifically taxon-1 grouped as sister to the colpodellids with a posterior probability of 0.90. The same analysis also predicted with a 0.99 posterior probability that taxon-1 is not within the dinoflagellate-perkinsid clade.
Figure 6.9 Bayesian analysis using nuclear ssu rDNA resolves the phylogeny of taxon-1. Phylogenetic analysis and tree by T. Chrudimsky and M. Oborník (University of South Bohemia, used with permission), based on taxon-1 sequence supplied by the author.
Figure 6.10 Bayesian analysis of the phylogeny of taxon-1, using nuclear LSU rDNA. Phylogenetic analysis and tree by T. Chrudimsky and M. Obornik (University of South Bohemia, used with permission), based on taxon-1 sequence supplied by the author.

Bayesian analysis of LSU rDNA sequences (Figure 6.10) grouped taxon-1 with apicomplexans with a posterior probability of 0.86. This analysis did not resolve well between taxon-1 and the Perkinsids, as it was relatively unsupported with a posterior probability of 0.60 (Figure 6.10). LSU rDNA sequences were not available for colpodellids, perhaps limiting the support that can be gained for grouping of taxon-1 with apicomplexans using this molecule.

Taxon-2
Analysis of ssu rDNA grouped taxon-2 as sister to the colpodellids with a moderate posterior probability of 0.82 (Figure 6.11), but firmly within the alveolate clade with full Bayesian support.

**Figure 6.11** Bayesian analysis using nuclear ssu rDNA indicates the phylogeny of taxon 2. Phylogenetic analysis and tree by T. Chudimsky and M. Oborník (University of South Bohemia, used with permission), based on taxon-2 sequence supplied by the author.
Figure 6.12 Bayesian analysis using the nuclear ssu rDNA resolves the phylogeny of taxon-1 and taxon-2 together. Phylogenetic analysis and tree by T. Chrudimsky and M. Obornik (University of South Bohemia, used with permission), based on taxon-1 and taxon-2 sequences supplied by the author.

When ssu rDNAs of taxon-1 and taxon-2 were subjected to a Bayesian analysis together, the position of taxon-2 as sister to the colpodellids strengthened to a posterior probability of 0.91 (Figure 6.12), the same level of support that taxon-1 had when used alone.
3.5 Plastid phylogeny of taxon-1 and taxon-2

Analysis of plastid psbA sequences via protein phylogeny tentatively supported a grouping of both new taxa with stramenopile plastids (Figure 6.13) but only when used together in one analysis. The support was low, at a posterior probability of 0.84, that the taxa were within the stramenopile clade rather than sister to it. However, when taxon-1 was used without taxon-2 in a psbA analysis the plastid did group between peridinin-dinoflagellates, and fucoxanthin-dinoflagellates (analysis not shown). Conversely, when taxon-2 was used without taxon-1 in a psbA analysis the plastid grouped with stramenopiles. Most interestingly, the psbA sequences of the two new taxa grouped together with a perfect Bayes score (Figure 6.13), and thus are each other’s closest sisters. When taken with the nuclear ssu rDNA analysis (Figure 6.12), this suggests i) that the plastid and nuclear lineages of both taxa are are each others closest sisters, ii) that the plastids of the two taxa may therefore be related by direct descent, and iii) that the range of plastid diversity represented by the two new taxa may be very large, and that the two may have branched off the colpodellid line separately, and with a considerable evolutionary time period between them.
Figure 6.13 Plastid phylogeny of Taxon-1 and Taxon-2 by analysis of a partial psbA fragment.

Three UGA-tryptophan codons occurred in the presumptive psbA protein fragment of taxon-1. This may be a presence/absence character linking protein-coding genes of the taxon-1 plastid and those of the *Toxoplasma* and *Eimeria* apicoplasts (Cai et al. 2003; Kissinger Online; Kissinger et al. Unpublished genome sequence NC_001799) because UGA-tryptophan codon usage has not been demonstrated in any other fully or partially sequenced algal genome (Figure 6.14). However, the abundant instances of UGA-tryptophan codon usage in *Toxoplasma* and *Eimeria* apicoplast genomes do not include a
*psbA* gene, because these organisms are not photosynthetic, and so the codon usage as a taxonomic character in this particular gene is predictive only. Rather the *Toxoplasma* and *Eimeria* UGA codons occur in other genes, such as genes for RNA polymerase subunits. Taxon-2 does not show UGA-tryptophan usage at any of the three tryptophan positions in the fragment of *psbA* that was sequenced.

| *Kareния b* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Gymnodinium m* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Thoracosphaera b* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Scaphiella c* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Protoceratium r* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Alexandrium t* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Gonyaulax p* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Heterocapsa p* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Heterocapsa f* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Symbiodinium sp* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Prorocentrum m* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Taxon-1* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Taxon-2* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Heterosigma c* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Fylaicella l* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Heterosigma l* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Hamillieropsis f* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Vaucheria l* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Ectocarpus s* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Prymnesium p* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Pleurochrysis c* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Isochrysis sp* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Phaeocystis a* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Pavlova l* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Pavlova g* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Emiliania h* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Rhodomonas a* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Pyrnesiolum s* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Chlamydomonas p* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Dinophysis n* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Stylorella r* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Rhodesmus m* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Rhodella v* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Porphyridium a* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Dinoungelia l* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Campeogonum c* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Palmaria p* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |
| *Bangia f* | NSRLHFTLNGPGYIGGWLTSNGVSTMAMNLNLHGFQQUINGDICPSTWADIL |

**Figure 6.14** Presumptive UGA-Trp codon usage in the taxon-1 *psbA* gene (from DNA sequence) suggests the plastid is closer to the apicoplasts than to any algal division. The only plastids known to possess UGA-Trp codon usage are the apicoplasts of *Eimeria* and *Toxoplasma* (Cai et al. 2003; Kissinger Online; Kissinger et al. Unpublished genome sequence NC_001799 ). Amino acid 'W' represents UGG-Trp codon. Asterisk represents UGA-Trp codon. Colour key: blue = peridinin dinoflagellate, red = stramenopile, green = haptophyte, purple = cryptophyte, brown = rhodophyte.
Support for sisterhood of the taxon-1 plastid to apicoplasts was obtained using plastid ssu rDNA sequences in a maximum likelihood (ML) analysis (Figure 6.15). The analysis subordinately grouped euglenophyte plastids with those of apicoplasts and taxon-1, but the euglenophytes were not monophyletic with the other green algae, indicating that the branching of the euglenophyte plastids as sisters to apicoplasts was artifactual. ML bootstrap support for the grouping of Euglenophytes with apicoplasts decreased from 92 to 53 when the new taxa were added (Figure 6.15, compare to Figure 1 of Obornik et al. 2002), which corroborates the notion that the euglenophyte/apicoplast grouping is an artifact. Peridinin-containing dinoflagellates could not be included in the plastid ssu rDNA analysis because they are represented by only a single available sequence, that of *Heterocapsa circularisima*, which is a highly divergent sequence compared to all other Myzozoan sequences available for this locus.
4. Discussion

4.1 Microscopic and molecular purity of cultures

**Taxon-2 purity and morphology**

Establishment of culture purity is encumbent on anyone trying to establish the molecular phylogeny of protistan -heterotrophs, -parasites, or -symbionts. This is particularly the case for heterotrophic protists which are known to feed on other protists. In the case of colpodellids the food is protistan algae (Simpson and Patterson 1996).
All taxon-2 cultures contain colpodellids, and could be hypothesised to be co-cultures in which a heterotrophic colpodellid enters a large algal cell, and feeds on its contents before eventually occupying the entire space inside the algal cyst wall. D. Patterson (Marine Biological Laboratory, Woods Hole, Massachusetts) and R. Andersen (Bigelow Laboratory, Maine) undertook a preliminary test of this hypothesis by isolating a colpodellid from a taxon-2 culture and offering it live cells of the haptophyte *Isochrysis* as food, which it duly ate (data not shown). That experiment tentatively supported the hypothesis that the colpodellid-packed cysts of Figures 6.6 and 6.7 may not be stably photosynthetic organisms, because the experimental result is consistent with what is known of colpodellid feeding on unicellular algae (Simpson and Patterson 1996). However characterised colpodellids feed on unicellular uninucleate algae (Simpson and Patterson 1996), whereas the alga co-culturing with colpodellids in taxon-2 cultures is often multinucleate and divides by schizogony (multiple fission). Furthermore, other colpodellids do not reside inside the cell wall of their prey, rather they siphon the cytoplasmic contents of their prey, a process called myzocytosis (Cavalier-Smith and Chao 2004; Simpson and Patterson 1996).

Taxon-2 cultures were self purifying, yet neither green-hued alga nor the co-culturing colpodellid ever completely dissapeared, and conversely no taxon-2 algal culture was obtained that was free of the colpodellid. By contrast, in taxon-2 cultures where coexistence with *Symbiodinum* had previously occurred, *Symbiodinum* cultures were completely overtaken by taxon-2 (section 2.1). A genus-specific competitive interaction between taxon-2 and *Symbiodinum* cannot be ruled out, perhaps involving a simple growth-rate differential and/or generation of symbiocidal chemicals by taxon-2, so called allelopathy as occurs widely between various algae (Fistarol et al. 2004; Kubanek et al. 2005; Legrand et al. 2003; Rengefors and Legrand 2001; Sole et al. 2005; Sukenik et al. 2002). However, some taxon-2 cultures (RM24 and RM 26) did retain a minute number of *Symbiodinum* cells. The most parsimonious explanation for the fate of *Symbiodinum* cells in taxon-2 cultures is that the *Symbiodinum* may have been predated by the colpodellids.
Molecular data obtained in this chapter, by direct sequencing, indicates that the taxon-2 culture RM11 is monospecific, - the RM11 flagellates and RM11 alga are one species. Selectivity of the nuclear ssu rDNA primers 18A1F and 1800R for colpodellids over stramenopiles is tentatively dismissed because the primers are known to amplify mollusc DNAs (Wollscheid and Wagele 1999) and Symbiodinium DNAs (W. Loh, University of Queensland, pers. comm.) therefore they are reasonably universal. The possibility of primer selectivity cannot be ruled out, but on current evidence, monospecificity of taxon-2 is the most supported interpretation.

Interest has been shown by several labs (M. Obornik, University of South Bohemia; K Heimann, James Cook University; B. Melkonian, Culture Collection of Algae at the University of Cologne; personal communications) in further purifying taxon 2 in the near future, by picking and growing single cells. This avenue is also being pursued by the author. If single flagellate cells regenerate the spherical alga, then monospecificity of the flagellate and the spherical alga will have been shown. Conversely if spherical algae of RM11 can be cultured in the absence of the colpodellid flagellate then the conspecificity of these two cell types will have be refuted. Other ways to further investigate the conspecificity of the cells in culture RM11 may include: fluorescence in situ hybridisation of the spherical alga and the flagellate using a colpodellid-specific variable region of the ssu rDNA molecule; or PCR gene amplification and sequencing from hand-picked flagellate cells.

**Taxon-1 purity and morphology**

Purity was not an issue for cells of taxon-1, which were clonal, having been derived from single algal colonies grown on agar after dilution streaking (section 2.1). Taxon-1 differed microscopically from Symbiodinium in two obvious ways: i) its zoospore (flagellate) did not gyrate about a fixed anchor to the substrate and was not of gymnodiniod morphology, ii) its flagellates did not appear diurnally but rather periodically, and then only in certain strains (data not shown). This study focused on cultures of taxon-1 that did not contain frequent flagellates in fEs medium, and therefore appeared pure. In numerous cases these immotile cultures of taxon 1 had been obtained by picking colonies from agar, which may have selected against motile capacity, even though the parental strains had a high capacity.
for motility. Regarding the source of taxon-1, it was unclear whether having placed live corals *L. purpurea* and *P. versipora* together in aquaria with unautoclaved Sydney Harbour water, may have affected the outcomes of the culturing experiments. However, regarding the source of taxon-2, nubbins of live *P. damicornis* were not exposed to Sydney Harbour seawater in aquaria at any stage.

At least four additional microscopic features distinguished taxon-1 from *Symbiodinium*: (i) no pyrenoid was visible; (ii) an apparent extracellular accumulation body was present; (iii) the cell wall was ecdysed with the accumulation body attached; (iv) cell division was asymmetric (personal observations). All these features differ from *Symbiodinium* morphology (Farmer et al. 2001; Freudenthal 1959; Freudenthal 1962; Kevin et al. 1969; Trench and Blank 1987; Trench and Thinh 1995; Wakefield et al. 2000).

Since collection of the data presented here, transmission electron microscopy has been performed on taxon-1 by Thomas Chrudimsky, Marie Vancova, and Miroslav Obornik, University of South Bohemia, Czech Republic (M. Obornik, pers. comm., reported here with permission). The plastid of taxon-1 was surrounded by four membranes. Subsurface alveoli were present at the periphery of the cell resembling those of *Oxyrrhis* (Dodge and Crawford 1970), and ciliates (K. Heimann, James Cook University, pers. comm.). The mitochondrion and cytoplasm contained acidocalcisomes resembling those of the Trypanosomatid *Phytomonas franci* (Miranda et al. 2004). The acidocalcisome is an organelle known to be involved in pH homeostasis, calcium regulation, and osmoregulation, and is commonly abundant in parasitic protozoa (Docampo et al. 2005; Docampo and Moreno 2001). The accumulation body (positioned as in Figures 6.1 and 6.2B) was likewise shown by TEM to have the same structure as an acidocalcisome.

### 4.2 Phylogenetic placement of the novel taxa

Direct sequencing indicated that there is only one nuclear lineage per type-culture of each of these novel taxa (specifically RM13, RM12, CCG15, and RM11 nuclear markers were directly sequenced), and therefore that consortia were not present in any of these three cultures. Phenomena such as kleptopasty, parasitism, or heritable endosymbiosis between
two protists therefore cannot explain the data. All of the results obtained for several loci were consistent.

Taxon-1 was found to be a close sister of the apicomplexans (Figure 6.9) and to contain a putatively photosynthetic plastid (Figure 6.13). Capacity for photosynthesis was confirmed by S. Wright (Australian Antarctic Division, pers. comm.), and N. Davies (University of Tasmania) by means of Pulse Amplitude Modulation (PAM) fluorometer data (reported here with permission). PAM measurements indicated active photosynthesis in taxon-1 (manuscript in preparation). The plastid of taxon-1 is monophyletic with the apicoplasts (Figures 6.14 and 6.15), and contains UGA-Trp codons as coccidia do, so it may represent the only original (photosynthetic) apicoplast yet characterized. However, it is difficult to formally call the taxon-1 plastid an ‘apicoplast’ because no indication is available as to the presence/absence of an apical complex of organelles in taxon-1 (TEM has not yet been done on the flagellae). However, colpodellids have recently been formally inducted into the Apicomplexa (Adl et al. 2005) because they possess an apical complex of organelles homologous to those of euapicomplexans (Leander and Keeling 2003).

Taxon-2 is a colpodellid (Figure 6.11 and 6.12) with a plastid that is indicated to have originated from the same lineage as the stramenopile (Figure 6.13) and peridinin dinoflagellate plastids (Note in proof; plastid data more informative than the partial psbA fragment reported here has subsequently been gathered and analysed by T Chrudimsky, J. Janouskovec, M. Obornik and R. Moore, and indicates that the plastid of taxon-1 has a phylogenetic position midway between that of stramenopile plastids and peridinin dinoflagellate plastids, manuscript in preparation). Accordingly, Figure 6.13 may be interpreted to indicate that taxon-2 represents a photosynthetic colpodellid form that is ancestral to taxon-1. The existence of taxon-1 and taxon-2 supports the hypothesis that alveolate and stramenopile plastids share a common linear ancestry (Cavalier-Smith 1999).

Using ssu rDNA the lower Bayesian support for taxon-2 alone as sister to the colpodellids (Figure 6.11), than for taxon-1 alone as sister to the colpodellids (Figure 6.9), supports a deeper branching of taxon 2 within the alveolates than of taxon-1 within the alveolates.
More sampling of additional (as yet uncultured) taxa in this region of the tree may be required to resolve these relationships. Bayesian analysis of the ssu rDNA of both taxa showed no tendency for the pair to split the colpodellid clade, and rather indicated that the pair are both basal to colpodellids (Figure 6.12). A recent review of the biology, phylogeny and taxonomy of colpodellids was undertaken by Cavalier-Smith (Cavalier-Smith and Chao 2004). No other colpodellid has the large sporangium containing tens of flagellates (that taxon-2 has), rather other colpodellids ‘round up’ in cyst form as a tetrad after consuming an alga (Simpson and Patterson 1996).

4.3 The novel taxa are parsimonious within the evolutionary tree of algal protists.

To place the new taxa of this chapter in context it is necessary to review current theories of plastid endosymbiosis, as well as the evolutionary position and biodiversity of alveolates. Endosymbiotic partnerships involving alveolate hosts have led to transfer of genes from each acquired plastid to its new host over evolutionary time, a feature also common to endosymbioses in other lineages (Bachvaroff et al. 2004; Cavalier-Smith 2003; Doolittle et al. 2003; Green 2004; Hackett et al. 2004a; Huang et al. 2004a; Huang et al. 2004b; Martin et al. 2002; Palmer 2003; Zhang et al. 1999).

The dinoflagellates are prominent examples of frequent loss and gain of plastids (Saldarriaga et al. 2001). Distinct lineages of dinoflagellates contain unrelated plastids with varying numbers of membranes surrounding the organelle (Cavalier-Smith 2003; McEwan and Keeling 2004; Palmer 2003; Tamura et al. 2005). These have been obtained by a mechanism known as ‘plastid replacement’ that has occurred in many dinoflagellate genera (Hackett et al. 2004b) due to a common habit of feeding by siphoning the contents from prey alga, a process known as ‘myzocytosis’ (Cavalier-Smith 2004).

The division dinoflagellata, at its monophyletic root, possessed a plastid that it inherited from its stramenopile-like ancestors (Figure 6.16) (Fast et al. 2001; Hackett et al. 2004b). Ancestral possession of a stramenopile-type plastid by dinoflagellates is known from inspection of the number of membranes surrounding the organelle in those genera of dinoflagellates that still possess the original plastid. The original plastid of dinoflagellates is exemplified by the plastid in Symbiodinium, and is known as the peridinin-type plastid.
owing to presence of the unique pigment peridinin, not found outside the dinophyceae (Chang and Trench 1982; Jeffrey et al. 1975; Loeblich 1976; Schnepf and Elbrachter 1999). The peridinin plastid is surrounded by three membranes in total (van den Hoek et al. 1995).

In Figure 6.16, the term stramenopile (Patterson 1999) encompasses chrysophytes (e.g. diatoms), oomycetes (e.g. Phytophthora), and others. A prominent theory (Cavalier-Smith 1999; Harper and Keeling 2003) is that stramenopiles and alveolates were ancestrally photosynthetic and that the plastid lineage was monophyletic with that of algal divisions Haptophyceae and Cryptophyceae, i.e. that all these plastids represent a single ancient endosymbiotic event (Figure 6.15). Cavalier-Smith had previously already instated the group ‘Chromista’ (=stramenopiles, haptophytes, and cryptophytes). His larger and more current theory is therefore known as the ‘chromalveolate hypothesis’: that chromists and alveolates were all descended from a single ancestral photosynthetic. The chromalveolate theory was partly based on parsimony principle, and partly on the evolution of protein and lipid targeting to organelles (Cavalier-Smith 1999). It has since been has been supported by molecular data (Harper and Keeling 2003; Yoon et al. 2002b), and the existence of taxon-1 and taxon-2 now adds weight to that support, at least in regard to the sisterhood of alveolates and stramenopiles.
Figure 6.16 Evolutionary history of the Myozoa. Myzozoaans ancestrally fed by siphoning the contents out of prey. At least three individual instances of plastid replacement have occurred in dinoflagellates - each endosymbiotic event was from an engulfed chromalveolate. Additionally, a fourth event serial secondary endosymbiosis of a green alga in a dinoflagellate is shown in chapter 1 (Figure 1.16). The parasitic perkinsids are an example of a plastid being lost from a pre-dinoflagellate and not regained. Plastid losses have been frequent and so are not shown here. The heterotrophic colpoddellids feed on protistan algae and bodonids (see chapter 7, Figure 7.6), while many euapicomplexans have a relic plastid. The term Apicomplexa covers colpodellids plus euapicomplexans. Consensus favours that the apicoplast was a secondary plastid, derived linearly from the stramenopiles and not replaced. Key: Primary endosymbioses (1°) are exemplified by Archaeplastida, secondary endosymbioses (2°) by chromalveolates and perdinin dinoflagellates, and tertiary endosymbioses (3°) by euapicomplexans and by specific dinoflagellates: e.g. the fucoxanthin dinoflagellate lineage (haptohyte plastid), Dinophysis (cryptophyte plastid) and Kryptoperidinium (stramenopile plastid). Publications on these topics include: (Adl et al. 2005; Cavalier-Smith 1999; Douglas and Turner 1991; Fast et al. 2001; Funes et al. 2004; Hackett et al. 2004b; Harper and Keeling 2003; Morden et al. 1992; Saldarriaga et al. 2001; Saldarriaga et al. 2004; Yoon et al. 2002b). Figure by the author.
4.4 Co-evolution of Myzozoa with Metazoa?

In this chapter, culturing of basal Myzozoans from corals (corals represent basal metazoans) raises the question: Was the common ancestor of dinoflagellates and apicomplexans a photosynthetic symbiont that was resident in early metazoans? Data presented here allows this hypothesis to be raised, but not answered. It is unrealistic to expect to determine conclusively whether the common ancestor of dinoflagellates and apicomplexans lived inside early metazoans. However, further culturing of intermediate forms like taxon-1 and taxon-2, and characterisation of them, may provide more data on which to build stronger models.

To pose the coevolution hypothesis, it needs to be first shown whether or not the novel taxa of this study were truly symbiotic. The alternative explanation for a potential natural ecology of the two cultured taxa is that they may be free-living autotrophs that have entered the coral tissues via the Sydney Harbour water used to store the nubbins prior to culture. To partly address this issue taxon-2 psbA sequences were amplified directly from coral tissues including a sample that had never been exposed to Sydney water, and the results indicated that plastids similar (but not identical) to that of taxon-2 are detectable in hospite.

Coevolution of the whole euapicomplexan phylum with Metazoa, was hypothesized by Levine (Levine 1978; Levine 1988), maintained by Perkins et al. (2002), and also held by Escalante and Ayala (Escalante and Ayala 1995) among others. The hypothesis has not been corroborated using molecular techniques but is a reasonable assumption because the taxonomic branching order of apicomplexans mirrors the developmentally-based branching order of their host metazoans (Levine 1987; Perkins et al. 2000). The additional suggestion raised by the current study is that the proto-apicomplexan may have been a photosynthetic symbiont associated with an early diploblast.

All hypotheses that Myzozoans co-evolved with metazoans are subordinate to assessments of the timing of the Myzozoan heyday. The divergence of the dinoflagellate/perkinsid clade from the apicomplexan/colpodellid clade is required to have occurred at the same time as the diploblast to triploblast transition. An analysis by Escalante and Ayala (1995)
gives the timing of the divergence of the Myzooza at ~1000 mya, the same timing as the
radiation of multicellular metazoans at ~930-1070 mya, obtained by Nikoh et al. (Nikoh et
al. 1997) and corroborated by Feng et al. (Feng et al. 1997), and reviewed by Blair-Hedges
(Blair-Hedges 2002). Douzery et al. (Douzery et al. 2004) used an increased rate of
change in timeline calculations in order to better marry molecular clock analyses to the
fossil record, and whilst they concluded different timings than Escalante and Ayala
(1995), and Nikoh et al. (1997), nevertheless the overlap of the Myzoozan and metazoan
heydays in their analysis is again striking at 600-800 mya and 700-850 mya respectively.

4.5 Apicoplast origin

Corroboration that the plastids of taxon-1 and taxon-2 may be related to each other by
direct descent was gained by Simon Wright and Noel Davies (Australian Antarctic
Division, and University of Tasmania respectively, personal communications.). They
characterised the pigments from both taxa, and found that both taxa lack chlorophyll \(b\) and
chlorophyll \(c\), and possessed only chlorophyll \(a\) plus accessory pigments (unpublished
data, S. Wright pers. comm.). The novel taxa did not contain peridinin that might indicate
affiliation with peridinin-dinoflagellates, nor fucoxanthin, that might indicate affiliation
with fucoxanthin dinoflagellates. The data could be interpreted as an evolutionary origin
of apicoplasts from an alga that possessed chlorophyll \(a\) only. However, from current
evidence in the literature (Fast et al. 2001; Zhang et al. 2000), it is more parsimonious to
assume that chlorophyll \(c\) has been lost in the apicoplast lineage, after apicomplexans split
from peridinin dinoflagellates (Figure 6.15). The only modern day stramenopiles that
possess chlorophyll \(a\) without chlorophyll \(c\) are the eustigmatophytes, and \textit{Ochromonas}
(B. Green, University of British Columbia, pers. comm.). Other extant eukaryotic algae
that possesses only chlorophyll \(a\) are the rhodophytes, as do the prokaryotic cyanobacteria
(Wright et al. 1991).

Since the discovery in the early 1990’s that apicomplexans contain a relict chloroplast
genome, a debate has arisen about apicoplast origins. This is the the red-versus-green
debate, one side claiming that a green (e.g. chlorophyte or euglenozoan) plastid was the
original apicoplast (Funes et al. 2002; Kohler et al. 1997) while the other side claims a red
(e.g. chromist or rhodophyte) plastid was the original apicoplast (Fast et al. 2001; Zhang et
al. 2000). Assuming that the phylogeny of taxon-1 and taxon-2 are correct and that these taxa contain the photosynthetic predecessor of the apicoplasts, a resolution of the red versus green debate is apparent. The origin of the apicoplast was apparently by direct inheritance from the common ancestor of apicomplexans and peridinin dinoflagellates. This resolution does not rule out the possibility that a green plastid-containing organism may have donated nuclear genes to apicomplexans at some time during the evolution of the apicoplast (Bhattacharya et al. 2004; Cai et al. 2003; Funes et al. 2004). However, it does require that the monophyletic apicoplasts are secondary and derived from the red plastid lineage.

The origin of the plastids in taxon-1 and taxon-2 can currently only be interpreted via the molecular phylogenies (Figures 6.13, 6.15, and unpublished data of M. Obornik and R. Moore). Pigment data for these taxa (S. Wright unpublished) leaves open questions about the history of the apicoplast, since both taxa contained only chlorophyll a. If the new taxa had contained chlorophyll b, that would have been prescriptive for a green origin, whilst presence of chlorophyll c would have been prescriptive of a red (e.g. chromist) origin (Wright et al. 1991). Similarly, while a suite of accessory pigments was seen in both taxa (S. Wright, unpublished data) the major ones are novel and will require further work for identification, so give no information about affiliation with known plastids.

4.6 Biochemical significance of the new cultures

Much attention has been focussed on the potential for targeted drug application that the apicoplast provides. Some of the apicomplexans that possess the apicoplast depend on it and cannot survive without it (He et al. 2001; Ralph et al. 2004). The biochemical interplay between the cytoplasmic and apicoplast compartments is well understood, but mainly in the model organisms Plasmodium and Toxoplasma (Coppin et al. 2005; Dzierszinski et al. 1999; Ferguson et al. 2005; Foth et al. 2003; He et al. 2001; Ralph et al. 2004; Striepen et al. 2000; van Dooren et al. 2005). The existence of taxon-1 and taxon-2 should allow more general conclusions to be drawn about the last common biochemical pathways in the ancestral apicoplast of all apicomplexans, and by this means new avenues of attack for model apicomplexans and other apicomplexans of medical or veterinary importance such as Sarcocystis, Eimeria and Babesia could be revealed. The lack of an
apparent apicoplast in *Cryptosporidium* (Zhu et al. 2000) makes the analogy to the new taxa of less relevance for that particular organism, though there is direct evidence that a plastid was present in the ancestors of that genus (Huang et al. 2004a). The ability of the new taxa to serve as phase-1 drug-screening dopplegangers for apicomplexans other than *Cryptosporidium* is apparent. These algae are easy to grow, inexpensive, and their use as models obviates the need for metazoan cell culture or metazoan infection procedures that are required for culture of most other apicomplexans. In a similar vein Brett Baillie (Australian National University, pers. comm.) has tested antimalarial drugs against *Symbiodinium*, with preliminary results that were promising.

Taxon 1 should serve as a better model for apicoplast metabolism than taxon 2, because the UGA-tryptophan usage in some apicoplasts (Cai et al. 2003; Kissinger Online; Kissinger et al. Unpublished genome sequence NC_001799 ) may mean that taxon-1 contains a closer relative of apicoplasts than does taxon-2.

While the new taxa may have potential to facilitate drug discovery in vitro, a more subtle yet equally compelling avenue of research will be to establish the mechanism of resistance to high concentrations of HOCl, evident in the culturing procedure that selected for the growth of these cultures. A simple overarching explanation for resistance of symbionts to oxidants is that the host cell may mitigate against some stages of the lifecycle of the symbiont, by attacking it with oxidants. Evidence for this explanation is lacking in the symbiosis field, but apicomplexan parasites have been shown to possess extraordinary antioxidant activities (Kwok et al. 2004) that are presumably defensive. The acidocalcisomes of apicomplexans and other protistan parasites may also play a role in counteracting attack from host chemicals (Docampo and Moreno 2001).

Finally, study of alveolate carbon-storage metabolism may be aided by availability of the new cultures (Figure 6.7b), and may begin to open up the specific roles and mechanisms of carbon storage in the lifecycles of euapicomplexans. Carbon storage reserves are a consistent feature of both of the new taxa and are also common in many euapicomplexans (Coppin et al. 2003; Harris et al. 2004; Levine 1978; Perkins et al. 2002). The demonstrated anti-*Cryptosporidium* activity of beta-cyclodextrin (Castro-Hermida et al. 2000) might indicate inhibition of polysaccharide formation, and therefore potential
therapeutic utility of beta-cyclodextrin. In the new taxa, the origin of the carbon storage globules is presumably via photosynthesis, whereas in euapicomplexans the source, need and mechanism for collecting vast quantities of carbon into a dedicated storage compartment is less clear, but is arguably to fuel survival of sexual-cyst phases during dormancy as shown by Fayer et al. (Fayer et al. 1998). The ability of euapicomplexans to store carbon, could be related to the possession of the apicoplast, or of apicoplast derived genes that have been transferred to the nucleus (Coppin et al. 2005).

4.7 Lifecycles

A summary of the putative lifecycle of taxon-2 is shown in Figure 6.17. This is more complicated than the lifecycle of taxon-1, the simpler one is therefore not shown. A direct indication that the flagellates and green-hued algae in taxon-2 culture RM11 are one species was that the PCR of the pair yielding only a single unambiguous chromatogram trace by direct sequencing of culture RM11 nuclear ssu rDNA. Another corroborative feature that indicated the colpodeellid and the alga in the taxon-2 culture RM11 are conspecific was the observation that flagellates adhere to substrate, then round up and become of identical morphology to taxon 1, though of a different colour. It is noted that the two taxa also share shared chlorophyll a as their only chlorophyll, and are closest sisters in psbA phylogeny (Figure 6.13). These factors together are arguably sufficient to describe the complex lifecycle of taxon-2 as reducable to that of taxon-1: a photosynthetic apicomplexan displaying inheritance of the chloroplast.

The study of euapicomplexan lifecycles is complicated by the need to maintain the relevant metazoan host in the laboratory, and by the many instances of heteroxenous (Barta 1989) (multi-host) lifecycles (Barta 1989; Barta et al. 2001). Cultures of taxon-1 and taxon-2 demonstrated not only a stable heritable plastid, but also lack of complication by sexual stages, as none have been observed.
6. Flagellates - contain tiny plastids
5. 'Sporangium' - is packed with colpodellid-like flagellates
2. 'Mothercell' - contains 'daughters cells' of 10-15 μm
Pre-daughter, including radially symmetric array of organelles

3. Growth to by successive shedding 'ecdysis' of the cell wall

7. Attachment to substrate, and growth by successive shedding 'ecdysis' of the cell wall

1. Mothercell (multinucleate?)
- has ceased doubling volume
- begins schizogony
- predaughters are not yet enclosed within a membrane

4. Juvenile
- formed from daughter
- periodically doubles volume.
- multinucleate?

Figure 6.17 The twofold vegetative lifecycle of taxon-2. Both cycles occur in a single culture, to varying degrees. In the cycle on the left hand side, flagellates develop inside a sporangium and are released when mature. They travel to a new location, and while doing so they hunt for algal prey such as Symbiodinium or Isochrysis. If no prey are found the flagellate attaches to the substrate and rounds up, becoming a 5 μm algal spheroid. It is unknown what is the morphological fate of flagellates that do find prey. In the cycle on the right hand side, daughters are immotile, highly pigmented and are about twice the diameter of the small alga that flagellates become. A cue for transition from the immotile cycle to the motile cycle is nutrient limitation. Vice versa the cue to adopt the immotile cycle is a nutrient replete environment. If a mothercell has passed 30 μm in diameter, then it is no longer able to develop into a sporangium.

4.8 Potential ecological significance of the new taxa

Coral health was the context in which the culturing work of this thesis was performed, and while the evolutionary implications of the novel taxa of this chapter are interesting, there is a need to assess whether they may be an integrated part of coral health or disease. The potential role of non-photosynthetic apicomplexans in coral disease has been previously raised (Toller et al. 2002).
Potential allelopathy of photosynthetic apicomplexans against *Symbiodinium* or consumption of *Symbiodinium* by colpodellids, was evident by the fast takeover of *Symbiodinium* cultures by taxon-2. If such taxa were important agents of bleaching they should have been detected in studies of bleached tissues, but have presumably not been seen or reported. Data presented in Appendix 6 supports the presence of taxon-2-like algae in coral tissues at levels detectable by PCR of holobiont genomic DNA. The primary relevance of these taxa to the thesis is the demonstration that chlorine-rich isolation media can lead to culture of a range of photosynthetic symbionts. The secondary relevance is that they may be present in ecologically meaningful numbers inside corals.

4.9 Chapter review and summary

No novel sexual forms of *Symbiodinium* were obtained or documented in this chapter. However, this chapter documents the culturing of two new genera, at least one of which (taxon-1) might be a missing link in the evolution of apicomplexans from phototrophic ancestors. Taxon-1 has been extensively studied by collaborators since the work presented in the results section of this chapter was performed, and is proposed to be formally named as a novel genus and species in the near future. Taxon-2 has a myxotrophic life strategy: to parasitise other alga (as other colpodellids do) but also carry its own heritable chloroplast: photographs of green material inside the taxon-2 flagellates indicate that they bear heritable chloroplasts. Similarly, taxon-2 flagellates were observed morphing into spherical form, consistent with the flagellates being the vegetative motile phase of the alga. Taxon-2 may represent an earlier evolving form of the same endosymbiosis that is evident in taxon-1.

The circumstance in which both taxa were cultured, as symbionts from corals, provides tentative support for the established model that apicomplexans co-evolved with metazoans (Levine 1978). Taxon-1 and taxon-2 could become useful as first screening tools for development and testing of anti-apicomplexan drugs that are aimed at the apicoplast. The ease and safety of the maintenance of taxon-1 and taxon-2 in culture surpasses that of maintaining lineages of parasitic apicomplexans in a laboratory situation.
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Chapter 7. General Discussion

Molecular ecology and phylogeny of protistan algal symbionts from corals

The goals of this general discussion are to examine microevolution and macroevolution of Symbiodinium, in reference to the findings of chapters 3-6. The specific aims are to integrate those findings with existing literature, as regards:

i) Symbiodinium lifecycle and population genetics
ii) Symbiodinium ecology
iii) Symbiodinium species boundaries

Additionally, the similarities between protist lifecycles allow investigation of the broader question “are protists generally sexual?” ‘Species’ boundaries in protists are related to the breadth of the ecological niche that each protist inhabits. In sexual species of protist, the genetic variability within the species is a measure of the breadth of its existing ecological adaptation. Therefore species boundaries can be estimated using a single locus across a range of isolates, and this method is becoming common. It is also useful here to address the larger questions “what are dinoflagellates?” and “how did they evolve” because data obtained in chapters 3-6 are relevant to those questions. The fourth and fifth goals for this chapter are therefore to discuss:

iv) The biological species concept in protists in general
v) Dinoflagellate evolution and myzoozoan evolution

1. Central contributions of the thesis to Symbiodinium biology and ecology

Four aspects of this thesis work have influenced the field of Symbiodinium biology and ecology and are thus concrete contributions to the field:

1.1 The utility of the psbA minicircle as a genetic marker
1.2 Comparison of the *Symbiodinium* plastid genome to those of other peridinin dinoflagellates

1.3 Benefits of selective and minimal culture media

1.4 Sexual stages of *Symbiodinium*: relevance to maintenance of host-zooxanthella pairings

### 1.1 The utility of the *psbA* minicircle as a genetic marker

**Host-specificity of *Symbiodinium* strains: the minicircle as an ecotype marker**

‘Ecotype’ was defined in Table 1.1 of chapter 1, as ‘host-specific strain’. The non-coding sequences of plastid minicircles have considerable potential to serve as ecotype markers for *Symbiodinium* and to thus divide subspecies (table 1.1, chapter 1) into smaller ecologically meaningful units. In chapter 4 Figure 4.13, it was shown with the *psbA* non-coding region marker, that *Sym-Ha* and *Sym-Lp* possess sequences differing by a long indel (bases 892-953) and a short indel (bases 381-388). Because of these sequence differences, the question arises whether the strains *Sym-Ha* and *Sym-Lp* may be distinct from each other, and whether each strain is dedicated specifically to its respective host, i.e. whether each strain is actually a distinct ecotype. By contrast, using ITS the most sensitive marker previously available, it was not possible to discriminate between these particular strains (Bui et al. 2003), and the combined ecotype would have been regarded as a host-generalist. LaJeunesse classified zooxanthellae into three broad groups: ‘host-generalists’, ‘host-specialists’ and ‘opportunist’ (LaJeunesse 2005a; LaJeunesse et al. 2004b; LaJeunesse et al. 2003; LaJeunesse et al. 2004a). Opportunist essentially means similar to weeds, having no well developed niche. LaJeunesse (2003-2005) demonstrated that host-generalists are subspecies of zooxanthellae that are very successful, in the sense that they occupy many coral hosts. His classification defined levels of host-specificity, but these definitions were so far based on only one genetic locus, the rDNA operon including ITS. Analysis of further loci such as the *psbA* non-coding region or actin introns might uncover specific host-zooxanthella affiliations that have not been possible to detect with the rDNA operon alone. Some cryptically adapted host-generalists may then be reclassified as recently evolved host-specialists. Importantly, their ability to recombine with the actual host-generalist population (their closest relatives) can be tested, by means of an allele reassortment study, using the markers developed in this thesis.
The minicircle as a marker at the level of the individual

SNPs observed in chapter 5 Figure 5.3 indicated that the psbA minicircle can be used to discriminate between individual zooxanthellae clones occupying a single coral colony. Precedents for this level of sensitivity have not previously been obtained in clade C though similar discrimination of individuals was shown possible in subclade B1 using microsatellite sequences (Santos et al. 2003c). The utility of a hypervariable marker to discriminate individuals across a whole clade has yet to be shown. While the psbA minicircle holds promise in this respect, it should not be expected to yield alignable sequence for SNP detection across a whole clade (e.g. Sym-Gt versus Sym-Ha Figure 4.13 chapter 4), rather only within a subspecies (e.g. Sym-Ha versus Sym-Pd, Figure 4.13 chapter 4).

Minicircle cores as Symbiodinium species markers

Zhang et al (2002) showed that minicircles core sequences (analogous to the C 1-4 regions of the psbA minicircles of Symbiodinium, Figure 4.13 chapter 4) differ across species within the dinoflagellate genus Heterocapsa (Zhang et al. 2002). The species H. triquetra, H. pygmeae, H. niei and H. rotundata each contained different conserved cores. While the cores were conserved across the set of minicircles from any single culture, they were unalignable across the species in the genus. Zhang et al (2002) accordingly proposed that the conserved sequences of minicircle cores are “species markers”.

As introduced in chapter 4 section 4.4, plastids are often uniparentally inherited in eukaryotes (Birky 1995; Birky 2001). For instance, in the green alga Chlamydomonas reinhardtii which has two mating types called + and - (Harris 2001), there is preferential segregation of the plastid from a single parent (mating type +) into all the offspring, and exclusion of the plastid that was inherited from the other parent (mating type -) is accomplished by means of a restriction system that degrades the plastid genome (Nishimura et al. 2002; Nishimura et al. 1999; Nishiyama et al. 2002; Nishiyama et al. 2004; Sager et al. 1981; Sano et al. 1981).

The number of mating types in any species of Symbiodinium is unknown. Additionally, how plastid inheritance rules might be modified in protists that have multiple mating types has not been studied. Assuming there is some system for retaining the plastid of a
preferred parental mating type, over a non-preferred one, the plastid genotype in a given biological species will tend to be a constant as long as sex is occurring frequently. This constancy would be due to homogenisation of the plastid in the population, and would explain the observation of Zhang et al. (2002) that minicircle cores are "species markers" (Zhang et al. 2002). Nevertheless, modification of binary mating systems to multiple mating types or homothallism, might enhance the degree of plastid diversity possible in a biological species. While stability of a plastid genotype within a species complex might be affected by non-binary mating systems, it might also be affected by the phenomenon of biparental inheritance of chloroplasts, which has recently been demonstrated conclusively in photosynthetic stramenopiles (Kato et al. 2006; Peters et al. 2004).

By providing tools (primers and PCR conditions) to amplify minicircle non-coding regions from several clades of *Symbiodinium* (chapter 4), this thesis has laid the groundwork for use of minicircle cores as species markers within the entire genus *Symbiodinium*, despite the fact that mating types and strain intercompatibilities are as yet unknown. Such an exercise could considerably shorten the process of defining species groups in this genus.

1.2 Comparison of the *Symbiodinium* plastid genome to those of other peridinin dinoflagellates

Molecular characterisation (chapter 4) indicated that *Symbiodinium* probably contains a typical peridinin plastid genome. The work in this thesis has helped re-establish *Symbiodinium* as a model peridinin dinoflagellate. It has supported the affiliation of *Symbiodinium* with mainstream peridinin dinoflagellates on a molecular basis (by rDNA and actin phylogeny and by minicircle detection), an affiliation that had previously been established on the grounds of pigments, proteins, ultrastructure, other genetic loci and physiological data (Blank and Trench 1988; Chang and Trench 1982; Freudenthal 1959; Jenks and Gibbs 2000; Leggat et al. 2004; Montresor et al. 1999; Norris and Miller 1994; Reichman et al. 2003; Rowan et al. 1996; Smith et al. 2005; Takishita et al. 2003b; Takishita et al. 2003a; Wakefield et al. 2000; Whitney et al. 1995; Whitney and Yellowlees 1995; Wilcox 1998; Zhang et al. 2005). Subsequent to this study, several other research groups that do comparative studies within the division Dinoflagellata have since adopted *Symbiodinium* as a typical peridinin dinoflagellate in molecular evolutionary
studies (Barbrook et al. 2005; Saldarriaga et al. 2003; Saldarriaga et al. 2004; Zhang et al. 2005; Zhang and Lin 2005). Potential has also been shown (in chapter 5, Figure 5.4 and Table 5.3) for the *Symbiodinium psbA* non-coding region to be involved in regulation of photosynthetic function across an ecological gradient, a study which may synergize with studies by others of photosynthesis regulation in this organism (Fitt et al. 2001; Fitt and Warner 1995; Hoegh-Guldberg 1999; Leggat et al. 2004; Warner et al. 2002; Warner et al. 1996; Warner et al. 1999).

DHEs had formerly only been found in mitochondrial genomes (section 4.2 chapter 4) (Bullerwell et al. 2003; Paquin et al. 1997; Paquin et al. 2000; Paquin and Lang 1996). The existence of DHEs in a plastid genome was first shown here (chapter 4), and has influenced the design of a subsequent study by Nelson and Green (2005) who found DHEs in another peridinin dinoflagellate (Nelson and Green 2005). They raised recombination-dependent-replication (RDR) as the possible function of the DHEs. The phenomenon requires every replication to include recombination, and vice versa (Bendich 2004; Kowalczykowski 2000; Norman and Gray 2001; Oldenburg and Bendich 2001; Preiser et al. 1996; Robison and Wolyn 2002). If the RDR hypothesis of DHE function is combined with the finding (in chapter 5) that DHE presence/absence correlates to an ecological gradient (i.e. gene regulation), it can be inferred that DHEs may be involved in the regulation of minicircle copy number, similar to the way that bacteria use plasmids as a way to control gene copy number and therefore transcript number and protein turnover.

1.3 Benefits of selective and minimal culture media

Culturing media used in this work covered a range of conditions, the two extremes being the most successful: a selective medium (chlorinating), and a very unselective medium (seawater only). Both novel approaches showed promise. The chlorinating medium appears to stimulate symbionts into their environmental lifecycle. Utility of the seawater-only medium was not investigated rigorously, but several of the observations in chapters 3 and 6 regarding seawater medium combine to form a consistent story.

A comparison is made between the potential response of *Symbiodinium* to seawater, and the observed response of photosynthetic colpoddellids to seawater. The response in each case was transition to motility. The eventual culturing of clade C *Symbiodinium* in this
study (albeit too late to be useful in marker development) was achieved by using plain seawater as the culture medium, and waiting for *Symbiodinium* cells to proliferate and adsorb at the meniscus of the culture tube. Similarly, it was noted in chapter 3 section 4.9 that tank water in which an *H. actiniformis* coral was housed, contained abundant motile *Symbiodinium*. The implication for the environmental lifecycle of symbiotic algae in corals and clams, is that seawater is a natural stimulus for cell development (motility).

The successful culture of a clade C zooxanthella showed that ecologically dominant strains can be cultured using a low-nutrient strategy. Culture in seawater (or very lightly supplemented seawater) may be a helpful device to observe the lifecycle of any marine microalgal symbiont. This work has therefore indicated that the widespread use of f/2 and similarly nutritious medium Asp-8A (Santos et al. 2001) may impede observation of the full spectrum of lifestages in this genus.

**How might *Symbiodinium* resist HOCl?**

The chlorinating agent HOCl was used to prepare the nubbin that yielded the successful seawater culture, and thus may be helpful for *de novo* culturing of *Symbiodinium*, including clade C. Use of HOCl in the growth medium increased the *de novo* culture rate of non-dominant *Symbiodinium* strains. Hypochlorite of similar levels to that used in this study are routinely used to sterilise agricultural explants and agricultural seeds (Hsiao 1979; Lang et al. 2000), nematode eggs (Barstead 1999), apicomplexan oocysts (Fayer 1995; Jenkins et al. 1997; Upton et al. 1994) and dinoflagellate sexual cysts (Okolodkov 1999). However none of those, other than seed sterilisation, employ hypochlorite overnight, and none use hypochlorite as an additive in a growth medium. The ability of *Symbiodinium* to survive HOCl treatment in this study raises the question of how this is achieved.

HOCl-resistance has been evidenced for many algae. It appears to be a general trait of photosynthetic eukaryotes to be able to resist higher chlorine levels than metazoans. In particular, the macroalgae *Sargassum* and *Caulerpa* have been shown to resist the action of HOCl, or NaOCl which is readily interconvertible with HOCl (Boudouresque et al. 1996; Ribera and Boudouresque 1995). An antioxidant possessed in unusually high concentrations by dinoflagellates is melatonin, which has specific anti-HOCl activity (Antolin et al. 1997; Balzer and Hardeland 1996; Hardeland et al. 1995; Hardeland et al. 2003; Zavodnik et al. 2004). Another candidate for antioxidant protection is tocopherol.
which it has been argued is synthesised by photoautotrophs specifically to capture free radical ions (Trebst 2003; Trebst et al. 2002) such as produced by mistimed or errant photosynthesis. Thus, HOCI-resistance might be a general property of photosynthetic organisms, or Symbiodinium may actively produce antioxidant compounds to survive inside host digestive systems, or to survive short periods of photoinhibition.

The chemical action of HOCI in media at neutral pH (such as used in this study) is unclear: does it function as an oxidant or a reductant at this pH? However, the finding by several groups that melatonin counteracts the chemical action of HOCI in vivo in several organisms lessens these concerns. Presumably HOCI equilibrates to a physiological pH in the cytoplasms of dinoflagellates, and from inspection of the literature the in vivo interaction between HOCI-melatonin is chemically conserved across eukaryotic groups (Antolin et al. 1997; Balzer and Hardeland 1996; Hardeland et al. 1995; Hardeland et al. 2003; Zavodnik et al. 2004).

1.4 Sexual stages of Symbiodinium: relevance to maintenance of host-zooxanthella pairings

The observation of gametes fusing, made on several occasions in this study indicates that some weedy strains of Symbiodinium are probably homothallic, isogametic, and may be stimulated to gametogenesis only in young cultures. Parallel work (cited in chapter 3) done by K. Heimann, though not on stable cultures, has led to a new collaboration in documenting these stages for publication. Several of the fusion-competent strains were donated to a public culture collection, to facilitate such collaborations.

Environmental location of gametogenesis and recombination

It was hypothesised by Baillie et al. (Baillie et al. 1998) that Symbiodinium recombination might occur within the zooxanthellar tube system of clams, which is a modified stomach. This idea may be compatible with the HOCI resistance of coral symbionts (chapter 3). The connection is the importance of a digestive environment or digestive chemicals to cue gametogenesis. Assuming that each clade of Symbiodinium may have similar set of cues for gametogenesis, it follows from the clam case that the digestive systems of corals-grazers (fish, echinoderms) and the digestive apparatus of corals themselves may be
important in the sexual lifecycles of the intracellular symbionts of corals, at least in the sense of inducing gametogenesis, if not perhaps being the site of mating itself. The flaws in this model are: i) that recombination within the zooxanthellar tube system of clams has not been examined separately from recombination in the water, so it is not known which is the real site, and ii) chlorine chemistry has not been directly studied either in the zooxanthellar tubes, or in the HOCl medium.

In clams, including the Tridacnid clams, the zooxanthellae are not in the hemolymph fluid (circulatory system), but rather are in a confined space called a 'zooxanthellar tertiary tubule' separated from the hemolymph (Norton et al. 1992) by a continuous layer of cells (Farmer et al. 2001). The tubules are an outgrowth of the clam stomach (Morton 2000; Norton et al. 1992). As the tertiary tubules are microscopic in size, the chemistry of this space remains unknown. The way that zooxanthellae obtain access to the zooxanthellar tertiary tubules is directly via the gut (Morton 2000), where they are likely exposed to digestive fluids. Tridacnids in particular retain a fully functioning digestive system, while other zooxanthellate clams often reduce this function in favour of reliance on the zooxanthellae (Schneider 1998). Tridacnids are the most abundant zooxanthellate clams, and it can be speculated that their retention of digestive fluids is beneficial to the sexual and environmental lifecycle of zooxanthellae. By maintaining possession of digestive fluids tridacnids may have retained a competitive advantage through facilitating genetic outbreeding of zooxanthellae. Other zooxanthellate clams may have potentially boxed themselves into an evolutionary dead-end by not facilitating this.

Recombination cues for zooxanthellae that reside in corals can be modelled with reference to the clam system, noting the similarities. Even maternally transmitted zooxanthellae of corals might be induced into gametogenesis, chemically, by the guts of the hosts or the guts of grazers. It was established by Steele (1977) that zooxanthellae are induced to become motile by that digestive environment (Steele 1977). Motility is likely to be a prerequisite for sex, by comparison to other dinoflagellate lifecycles. Zooxanthellae freed by grazers are available to infect new hosts.

If the phenomenon exists, co-infection of maternally-transmitting zooxanthellate hosts by homologous free-living zooxanthellae would be very selective, and therefore difficult to detect over maternally transmitted background. However, the experiment could be easily
achievable with isotopic labelling of live cells. If it occurs, the process could allow the coral to benefit from homologous zooxanthellae that had sexually recombined in the environment. The contribution of chapter 3 of this thesis, and studies by Augustine and Muller-Parker (1998) and Steele (1997) on potential cues for the environmental lifecycle, allow framing of a new suggestion: that ‘interrupted maternal-transmittance’ could be a major evolutionary benefit to hosts by allowing adaptive recombination of zooxanthellae alongside concurrent maintenance of host-fidelity. On this basis, selection may exist for corals i) to be capable of active uptake of zooxanthellae, and ii) to maintain active digestion systems for stimulation of the motile and sexual lifecycles of zooxanthellae, as stated above for clams. Faculties (i) and (ii) are interrelated, because active digestion of plankton by corals is the route by which free-living zooxanthellae enter juvenile and adult corals (section 1.1 chapter 1, and Figures 1.1 and 1.3a).

2. Future directions for studies of Symbiodinium adaptive capacity and evolutionary history

To withstand global warming, symbiotic dinoflagellates may need to adapt. They have adapted to the niche they currently hold, over hundreds of millions of years of selection. The potential impact that this thesis may have on studies of dinoflagellate evolution, additional to the contributions already discussed, is that several clear avenues of future work are suggested. Discussion of these research avenues below is in the context of several taxonomic ranks within alveolates: *Symbiodinium*; Suessiales, Dinoflagellates; Myzozoa; Alveolates.

2.1 Species boundaries in *Symbiodinium*

Population sampling for recombination studies

The technique of comparing isozymes from all *Symbiodinium* clades together, was used by LaJeunesse (2001) to ask whether their reassortment (phylogeny) was random and thus whether the markers were potentially being sexually shared. Though not intentionally, the analysis of the genus as a set assumed that all *Symbiodinium* species are comparable, and effectively asked the question: Is the genus a single species swarm? By analysing all constituent species of a genus together, artifactual randomness of association may be seen
among markers, and randomness can be mistaken for recombination. The analysis may thus lead some readers to conclude, wrongly, that recombination occurs between clades. The method of comparing isozymes (or any other marker) across a broad set of isolates can yield artifactual randomness if the following conditions are present: i) the sampled group is more diverse than a species (e.g. if the data is from a whole genus), ii) the markers used are of insufficient sensitivity and so occur in more than one species (homoplasy). An alternative and equally misleading outcome, can be that a genus-wide population would appear artifactually clonal, if the markers are not homoplastic but also not shared between species. The isozyme study of LaJeunesse (2001) appears to have had the former of these outcomes, - artifactual randomness of association among homoplasious markers.

In a population genetic recombination study, sampling should start off conservatively non-diverse until the boundaries of a species are found. The isozyme association analysis of LaJeunesse (2001) was intended (T. LaJeunesse pers. comm.) to show that strains within any particular Symbiodinium clade are not detectably distinct from each other using biochemical markers, but the analysis may have been confounded or adversely affected by presence of too many across-clade comparisons, too few within-clade comparisons, and general homoplasy of the markers. The central conclusion drawn was that phylogenetic similarity is not related to isozyme similarity. The set of isolates being compared did not consist of a species group, and so it can be argued that the randomness of isozyme similarity was a strong indication that the data was homoplastic.

It was discussed in that paper (LaJeunesse 2001) that the isozyme study of Baillie et al. (1998) on subclade A3 along with a consequent DNA hypervariability (RAPD) study (Baillie et al. 2000a) would not stand up to scrutiny except that they were followed by and a molecular phylogenetic study using ITS (Baillie et al. 2000b), and that the latter study showed the isolates were closely related types differing by no more than one or two bases. The results of the molecular phylogeny paper by Baillie et al. (2000b) in combination with their isozyme paper (Baillie et al. 1998) therefore effectively ruled out homoplasy as the explanation for the randomness of isozyme patterns. The same cannot be said of the genus-wide isozyme analysis (LaJeunesse 2001), which would benefit by being repeated without multiple clades and subclades being present in the same analytical matrix. If so reanalysed, the extensive data used may allow preliminary assessment of sexuality within
the coherent ITS group A2 (7 strains). This ITS subclade could be analysed as an individual set, for association of isozyme profiles among the constituent strains. For other ITS groups- A1 C1 and C2,- isozyme data is not extant for a sufficient number of strains in each to allow analysis. Similarly, this method may not produce meaningful analyses within a set of only 3 strains of ITS group B1, or a set of only 5 strains of ITS group A3, though it would be interesting to confirm the recombinogenicity of these groups (Baillie et al. 2000b; Baillie et al. 2000a; Baillie et al. 1998; Santos et al. 2003c) if the numbers were greater.

The genus *Symbiodinium* is known to consist of differing morphospecies (Trench and Blank 1987; Trench and Thinh 1995) and multiple samples of a single morphospecies should be used as the set of target samples for a population genetic recombination study. The ITS is unalignable pairwise between clades A and B, B and C, A and C (LaJeunesse 2001) corroborating that the morphospecies present in those clades are genetically isolated from each other. The only ITS clade sequences partially alignable to each other, are those of clades C, F, and H, but the number of variable positions in even that alignment far exceeds the level usually diagnostic of a protistan species swarm (Coleman 2005), indicating C, H and F are unlikely to represent a single swarm. Clade C (*S. goreauti*) and clade F (*S. kawagutii*) are also distinct morphospecies and thus genetically isolated from each other.

Sampling (restricted to *Sym-Gt* OTI) of a population in the current study was perhaps too conservative, whilst that of LaJeunesse (2001) may be considered to have sampled too liberally to assess recombination. Sampling of multiple *Symbiodinium* isolates, at an appropriate taxonomic scale for sexual recombination studies, was achieved by Baillie et al. (1998, 2000a, 2000b) and Santos et al. (2003c). Each study used a haplotype population, comprised of a single ITS type, and only within those limits was reassortment of hypervariable markers investigated. The central future goal of work that will proceed from this thesis is to identify a suitable target population of clade C *Symbiodinium* for an allele reassortment study using the *psbA* and actin markers developed here. In Figure 7.1 a strategy is presented for choosing such a population. Full length ITS1 of 223 bp (containing no 5.8S rDNA and no ssu rDNA) from *Sym-Gt* OTI (Bui et al. 2003) was used as a query in an NCBI BLAST search, in order to retrieve a ranked list of the closest
relatives of *Sym-Gt* OTI. The resulting list comprises the target population for extension studies of sexual recombination that will follow on from this thesis.

Five observations notable from the BLAST results of Figure 7.1 are: i) no identical ecotype of *Sym-Gt* from any other host is present among genbank records, implying *Sym-Gt* OTI may be a host-specialist; ii) all *Sym-Gt* hits are from OTI. The *Sym-Gt* sampled at Rib Reef GBR (LaJeunesse et al. 2004b) which was found, by Denaturing Gradient Gel Electrophoresis analysis, to have the same ITS type as *Sym-Gt* OTI (chapter 5, section 4.2) has not been sequenced; iii) a wide range of hosts from across the Pacific Ocean house the same ITS subspecies as *Sym-Ha* OTI (in Figure 7.1, scores of 434 have identical base sequences), indicating this ITS subspecies is putatively a host generalist; iv) among symbionts showing the same BLAST score as *Sym-Ha* OTI are symbiont types from the same lagoon at OTI; v) among the top hits are many species from the dominant genus of coral on the GBR, *Acropora*.

Features (iii-v) indicate that the list of Figure 7.1 is a relevant sample set in which to investigate recombination, because that set will also facilitate investigation of biodiversity, zooxanthella dispersal, and host-zooxanthella ecology of coral reefs. The genetic coherence of the set of clade C *Symbiodinium* of BLAST score 434 will need to be assessed empirically and objectively, because it is not a foregone conclusion. Differing ecotypes (referred to as ‘cryptic species’ in parasitology see glossary in Appendix 8) can show identical ITS sequences. This is the case for the fungal pathogen *Cryptococcus gattii*. The morphospecies contains four genetically isolated groups, but all have the same ITS sequence (Campbell et al. 2005).
Using ITS1 of Sym-Gt OTI as BLAST search query, choose symbionts that comprise the top ~20 highest scores. Assume there is host specificity (each host counted once).

<table>
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<tr>
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Collect 20 samples of each host, and then use direct sequencing of the symbiont’s psbA minicircle non-coding region as the determinant of whether a host is unialgal.

If the host is unialgal then also apply the actin-intron copy-3 marker to the symbiont.

Define actin and psbA “alleles” as stretches of conserved synapomorphic base sequence.

Assess for allele reassortment

Figure 7.1. Methodology for further assessing recombination in clade C Symbiodinium. Using Sym-Gt OTI (Bui et al. 2003) as a search sequence, six Sym-Gt OTI strains were the top hits. All hosts are Scleractinian corals, except oct=octocorals, biv=bivalve mollusc, zoa=zoanthid. Where no. of bases divergence (from Sym-Gt)=1, this is the same base position in all hits. Where no. of bases divergence=2, this consists of that same base position shared the hits above it, plus one more base position. Five more hits to octocoral host species, of score 434, were omitted for space, and for lack of relevance of octocorals to reef structure, so only some of the octocorals were retained as representatives. Single bivalve and zoanthid hits were obtained. Symbiodinium of score 438 are probably identical to those of score 434, because the 438 population contained a base denoted R (purine) where the 434 population had a guanine base.
Ploidy as a species marker

Copy number of the actin gene could be used to indicate ploidy, history of polyploidy, and species boundaries. In chapter 4 it was shown that two functional copies of actin gene potentially exist in clade C zooxanthellae (indicated by long ORFs). Do two functional gene copies indicate diploidy? Two explanations are possible for the existence of two (putatively) functional copies: i) gene multiplication of those particular genes only; or ii) genome duplication/ polyploidy. Selective gene multiplication implies some special selection on actin multiplication, which should not be raised without identification of such a selection. This leaves (ii) as the most easily investigated hypothesis.

Santos et al. (2003c) demonstrated that a set of subclade B1 Symbiodinium were haploid. Clade B yielded only a single actin amplicon in chapter 4, tentatively suggesting that members of that clade may have only one copy of the gene. Genome-size measurements by LaJeunesse et al. (2005b) can be interpreted with respect to ploidy. A doubling of ploidy may have occurred in subclade C1 (4.8 ng of DNA per cell) relative to subclade F1 (3.0 ng) and subclade B1 (2.0 ng). Doubling may also have occurred independently in subclade A2 relative to subclade A1 (3.4 ng versus 2.2 ng). While an approximate doubling of genome-size is not necessarily an indicator of a doubling of ploidy, neither does doubling of ploidy require an exactly doubled genome-size to be maintained over the long term (see below, this section). Rather, DNA content may shrink gradually after ploidy doubling.

In the hemiascomycete ancestor of the model genetic organism Saccharomyces cerevisiae, a genome duplication event that occurred <100 mya years ago was followed by rapid decay of much of the duplicated information (Cliften et al. 2006; Wolfe and Shields 1997; Wong et al. 2002). Such transitional ploidy events are among the potential causes of speciation in organisms. In the case of Saccharomyces ('sensu stricto'), the event preceeded formation of the genus. A similar event could provide a partial explanation for the multiple actin copies in Sym-Gt OTI. Regardless of their origin, actin copy numbers may be useful as synapomorphies distinguishing Symbiodinium species, as may actin-intron lengths and actin-intron positions.
2.2 The biological species concept in free-living protists

Measurable constraint of variation in rDNA (or ITS) sequences can be used as a measure of the potential species boundaries in the dinoflagellate *Symbiodinium*. This principle was the basis of the pioneering study by Rodriguez-Lanetty (Rodriguez-Lanetty 2003a) in which ITS phylotypes were identified, which are presumptive biological species. It is appropriate to review here whether that principle was well based, i.e. has ITS constraint been used as a species indicator in any other alveolates? A caveat to this question is the assumption that zooxanthellae have a free-living stage, and so can be treated as equivalent to free-living alveolates. This assumption may apply well to *Symbiodinium* that are host generalists and opportunists. It has also been argued above (section 1.4, this chapter) that it potentially applies to maternally-transmitted host specialists as well. A simplifying assumption here is therefore that free-living is universal for all *Symbiodinium*.

ITS was used by Katz et al. (2005) to determine variation within many ciliate morphospecies in the genera *Halteria* and *Strombidium* (Katz et al. 2005). Each morphospecies was called a ‘clade’, analogous to the situation in *Symbiodinium*. In all the *Halteria* and *Strombidium* analyses reported by Katz et al. (2005), ITS variation within each clade that is thought to be sexually isolated was less than 0.05 %, whereas variation among clades was always ≥ 2 % and ranged up to 16 %. ITS was also used by Coleman (2005) to analyse the genetic relatedness of sibling groups within the breeding swarm of ciliate *Paramecium aurelia* (Coleman 2005), and intra-swarm variation was again found to be ≤ 0.05 %.

Lsu rDNA has been used earlier than ITS, to determine the genetic constraint within alveolate species. Studies done with the dinoflagellate *Cryptecodinium cohnii*, showed that it undergoes Mendelian inheritance (Beam and Himes 1974; Beam and Himes 1980; Beam and Himes 1982; Beam and Himes 1984; Himes and Beam 1975), and a subsequent molecular study investigated the amount of variation in the D2 section of Lsu rDNA within the interbreeding complex (Beam et al. 1993; Nanney et al. 1998). The combined data directly related the degree of rDNA sequence variation in a dinoflagellate swarm, to sexual recombination in that swarm. The *Cryptecodinium* analysis (Nanney et al. 1998) was accompanied by similar studies of species complexes in the ciliate genera *Paramecium* and *Tetrahymena*, and accordingly each of these are shown in Figure 7.2,
and compared to the level of D1/D2 lsa rDNA variation seen in clade C *Symbiodinium* (LaJeunesse et al. 2003). The variation documented in *Symbiodinium goreau* is very similar to that occurring in *Paramecium aurelia* (Coleman 2005) and *Cryptocodonion cohnii* swarms. Since the D2 region of lsa rDNA contains most of the variability in the D1/D2 region, the comparison to the ciliate swarms indicates that *S. goreau* (all of clade C) represents the limits of swarm, as also indicated by Rodriguez-Lanetty (2003a).

Classical studies (Coleman 2005; Nanney et al. 1998) of the limits of genetic variation in alveolate species (including a dinoflagellate *C. cohnii*), tend to contradict the possibility that the entire genus *Symbiodinium* may constitute a single interbreeding swarm, since ITS sequences differ between *Symbiodinium* clades by >10-50%. While it may be theoretically considered that some maternally transmitted *Symbiodinium* may evolve more quickly in sequence than free-living alveolates, that factor need only be considered in analyses of host specialists, not of host generalists. Furthermore, empirically *Symbiodinium* host generalists and host specialists demonstrate similar scales of genetic variation anyway.
Figure 7.2 Variation in D1/D2 region of *Symbiodinium* LSU rDNA compared to that in the D2 region of *Cryptocodonim cohnii*, *Tetrahymena thermophila*, and *Paramecium aurelia* species swarms. The D2 region contains the vast majority of the variation in the D1/D2 region. Orange circles are known species swarms (A, B, D) or inferred species swarm (C). The *S. goreau* phylogram is comparable to the cladograms of the other species, because the numbers of bases differences (numbers on branches, emphasized by green triangles) are under investigation here. Branches without numbers represent one base change. Squares are artificially constructed ancestral sequences. Binary versus cladal branching patterns are not the subject of the comparison. A, B, D reprinted from: (Nanney et al. 1998). C reprinted from: (LaJeunesse et al. 2003), bracketed numbers show bootstrap support.
Secondary structure determination benefits analysis of ITS as a species marker

Data for the ciliate species complex *Paramecium aurelia* provides a very convenient example of the degree of ITS variation that can be expected to exist between sibling strains of an alveolate species with a global niche. In addition it is the classic example over which the concept of biological species in protists was fought (Schloegel 1999; Sonneborn 1957) (see next heading). Coleman has reviewed the mating reactions, isozymes, and mitochondrial introgression data for *Paramecium aurelia* as well as performing an analysis of ITS clades within the species complex (Coleman 2005). Coleman’s findings indicated: i) that the sexual intercompatibility levels of pairs of sibling species were partially supported by the ITS groupings, and ii) that the ITS variation among compatible strains was constrained to 2 - 6 bases (1.2 - 3.6 %) in ITS2.

Coleman’s analysis (2005) of variation within an alveolate biological species swarm was based on a secondary structure alignment of ITS sequences across strains, so that each informative base was detected, and so that compensatory base changes could be identified in the double stranded regions of stem-loop structures. Secondary structure analysis was not attempted in the study of ITS phylotype boundaries in *Symbiodinium* (Rodriguez-Lanetty 2003a). It may be desirable to establish the secondary structure of ITS for *Symbiodinium* clades of interest to facilitate correct ITS alignment and discrimination of potential species boundaries. This mirrors the task of establishing the structure of the dinoflagellate lsu rDNA D2 region (proposed in section 2.4, this chapter) to address evolutionary questions, but which could also assist in establishment of species boundaries.

Recent developments in the debate over the biological species concept for protists

In 1942 Ernst Mayr proposed the ‘biological species concept’: that “species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups”, a definition which, at face value, focuses on eukaryotes (Mayr 1942). Species of aquatic marine protist in many cases occupy a global niche. Selection may occur for these species to develop and maintain a wide breadth of genetic variation, in order to continue to occupy that global niche against competition from other species (McCormick and Cairns 1991). Many protists, including *Symbiodinium* spp. are in this category. Each protistan morphospecies may be maintained by sexual recombination among the genetically (but not morphologically) diverse range of subspecies that comprise it. This description of a ‘cosmopolitan’ protist species does not apply to obligately
parasitic protists or obligately symbiotic protists. As far as known *Symbiodinium* are not obligately symbiotic, they may all have autonomy, and biogeographic data shows that many subclades are global or hemiglobal in distribution (Baillie et al. 2000b; Karako-Lampert et al. 2005; LaJeunesse 2001; LaJeunesse 2005a; LaJeunesse et al. 2003), presumably due to inherent motility (Fitt 1985; Fitt et al. 1981; Fitt and Trench 1983; Freudenthal 1959; Kawaguti 1944) coupled with the action of water currents, and carriage by larval coral hosts (Baker 2003; LaJeunesse et al. 2004b; Loh et al. 2001), foraminifers (Gast and Caron 1996; Langer and Lipps 1995; Lee et al. 1995; Pochon et al. 2004; Pochon et al. 2001), crustaceans (Fitt 1984), and grazers (Augustine and Muller-Parker 1998).

The question can also be asked, are certain protist supergroups ('kingdoms') more capable of sexual recombination than others, and if so then where do dinoflagellates fit in? Sexual reproduction has been shown to occur readily in many dinoflagellates (Anderson et al. 1984; Beam and Himes 1974; Beam and Himes 1984; Blackburn et al. 2001; Blackburn et al. 1989; Chesnick and Cox 1987; Coats 2002; Destombe and Cembella 1990; Figueroa and Bravo 2005; Figueroa et al. 2006; Fritz et al. 1989; Giacobbe and Yang 1999; Kelley and Pfiester 1990; Kim et al. 2004; Kremp and Heiskanen 1999; Ouchi et al. 1994; Parrow and Burkholder 2003b; Pfiester 1984; Pfiester 1989; Pfiester and Anderson 1987; Probert et al. 2002; Sako et al. 1984; Sako et al. 1987; von Stosch 1973; Walker 1981; Walker and Steidinger 1979; Xiaoping et al. 1989). It also occurs widely in other alveolates (Coleman 2005; Dini and Nyberg 1992; Doerder et al. 1995; Meyer and Garnier 2002; Perkins et al. 2002; Preparata et al. 1983; Smith et al. 2002; Sonneborn 1937; Sonneborn 1957) and in stramenopiles, the sister division to alveolates (Chepurnov et al. 2004; Erwin and Ribeiro 1996; Hansen et al. 1986; Kato et al. 2006; Peters et al. 2004; Sansome and Brasier 1973). Even so, the ubiquity of meiosis is always under question. For instance the question forms the basis of the current study.

Protist meiosis has been a subject of controversy in the biological research community ever since Mayr proposed his definition of biological species (Mayr 1942). The basic problem is that in unicellular organisms, it is not a simple matter to observe mating types. Protist cells of differing mating types are often of identical cellular appearance, even though they vary at the genetic level. The lack of visible differences between sexes could imply that sex is a less well-developed character/faculty in protists than it is in
multicellular eukaryotes (Hillebrand et al. 2001; Raikov 1995; Sonneborn 1957). Such a view is conditioned on an absence of evidence, which should not be mistaken for evidence.

One notable case of objection to Mayr’s definition of species (1942) was the protozoologist Sonneborn, whose extensive knowledge of the sexual cycle of a ciliate Paramecium aurelia gave him an insight into the non-binary nature of mating types in some protists (Sonneborn 1937; Sonneborn 1957; Sonneborn 1974). Paramecium aurelia has 14 mating types each of which is compatible with subsets of the other mating types to varying degrees (Coleman 2005; Sonneborn 1974). This multisex strategy befits an organism that has a global range and must inhabit a large number of niches and microhabitats. The drawback of multisex is that it could lead to speciation events if mating types become too incompatible, but this drawback is avoided in global species of protists (in particular marine protists) because contact between all the subsets is frequent enough to prevent runaway speciation.

Frequency of contact is proportional to the ubiquity of a given species, and it has been proposed that many marine microbes of size less than 1 mm are globally dispersed by physical processes (Fenchel and Finlay 2003; Fenchel and Finlay 2004; Finlay et al. 1996; Finlay and Fenchel 2004), as well as by sheer ability to replicate quickly with consequent spread (Fenchel and Finlay 2003; Fenchel and Finlay 2004). The concept of globally cosmopolitan microbe species has been opposed in the case of freshwater and terrestrial microbes (Coleman 2002) due to restricted range, even though these species complexes closely parallel the marine microbes in terms of degree of genetic variation and intercompatibility (Coleman 2000; Coleman 2002; Coleman et al. 1994; Proschold et al. 2005).

A now-extinct ancestral unicellular eukaryote was an interim stage in the evolution of higher eukaryotes (Ramesh et al. 2005). Therefore the question naturally arises: do protists need sex, or are they more like bacteria? In 1965 Dodge proposed on the basis of chromosome condensation phenotype unique among protists, that dinoflagellates were ‘mesokaryotes’, midway between prokaryotes and eukaryotes (Dodge 1965; Hamkalo and Rattner 1977). This idea was promptly shown to be mistaken (Hackett et al. 2004b; Hinnebusch et al. 1981) but is still around in current literature (Wong and Kwok 2005).
The mesokaryote idea appeared in an era when the eukaryotic tree of life was not clear. Initial versions of the eukaryotic tree of life based solely on rDNA and with limited sampling of taxa (Gressel et al. 1975; Hori 1975; Woese and Fox 1977; Woese et al. 1975) have now given way to very much better resolved trees with greater sampling (Gajadhar et al. 1991; Olsen and Woese 1993; Sogin 1991; Sogin and Gunderson 1987; Van de Peer et al. 2000; Woese et al. 1990) which indicate that dinoflagellates are by no means a transitionary state between bacteria and higher eukaryotes. Ribosomal RNA trees are being supplanted by trees that use protein data, and these are refining the branching orders of kingdoms, and their constituent phyla (Adl et al. 2005; Baldauf et al. 2000). The gap between eukaryotes and prokaryotes remains as large as it has ever seemed, with a huge increase in the number of genes having apparently taken place at the time of the origin of eukaryotes from prokaryotes, including genes for meiosis (Ramesh et al. 2005).

A hypothesis that the first eukaryotes may have been sexual, has been supported by Ramesh et al. (2005), who utilised the genome sequence databases of several protists including protistan parasites in which sex has not been microscopically observed. They retrieved full-length genes that are normally meiosis-specific, such as the recombination genes Spo11 and DMC1 (Ramesh et al. 2005). The finding was that meiosis is a conserved process, which evolved only once during eukaryotic history. Thus the genes that define meiosis can be sought by PCR in a range of organisms including dinoflagellates, and the presence of these genes may be an indicator of active sexual recombination in a species. Application of this method to Symbiodinium will be exciting, and may discriminate whether some are obligately symbiotic and some not, or whether meiosis, and therefore by implication free-living, is ubiquitous in the genus. The method would complement the method of using conserved minicircle cores, ITS, or the D2 region of lSU rDNA as species markers.

2.3 Thermal evolution of Suessiales

As discussed in chapter 1, the thermal properties of Symbiodinium species are a major factor in their ecological vulnerability. There has been interest in establishing the genetic and cell biological basis for this (Douglas 2003; Leggat et al. 1999; Leggat et al. 2004; Ralph et al. 2001; Smith et al. 2005; Tchernov et al. 2004; Warner et al. 1996; Warner et al. 1999). Work done in this thesis will contribute to the understanding of Symbiodinium
evolution by facilitating studies of the branching order of the various *Symbiodinium* clades (Figure 4.21 chapter 4), as well as by corroborating that *Symbiodinium* are Suessiales (Figure 7.3 this chapter, and Figure 4.6 chapter 4). Additionally, a cool origin of the genus *Symbiodinium* is indicated. The basal *Symbiodinium* of subclade AT characterized by Savage et al. (Savage et al. 2002b) occurs in high latitudes, where conditions are cold, and temperate East Australian waters also contain many clade A *Symbiodinium* (W. Loh, unpublished observations). Similarly the basal clade E, (e.g. *Symbiodinium varians* CCMP421, and ‘*S. californium*’) has members that occur in arctic (Okołodków 1998) and temperate waters (LaJeunesse 2001; LaJeunesse and Trench 2000) as well as some that occur in tropical waters (Baker 1999; Savage et al. 2002b; Toller et al. 2001a). The actin sequence from clade E *S. varians* used in appendix 4a was obtained by Saldarriaga et al. (2003) from genomic DNA, not from cDNA (Saldarriaga et al. 2003). Thus the actin introns noted in clades A, B, C and F are absent from clade E. Assuming that clade E never had an intron, that clade is tentatively indicated as more basal within the genus *Symbiodinium* than any of the clades A, B, C or F.

A review of the collection sites for cultured Suessiales and closely allied taxa, indicates a predominantly cool niche for these organisms (Table 7.1). In view of the ancestral link between *Symbiodinium* and Suessiales, it may be interpreted that *Symbiodinium* is likely to have had a cool origin, and that Suessiales have demonstrated an ability to adapt to thermal change over evolutionary time (or alternatively that sampling and culture of free-living Suessiales has been relatively neglected in tropical and equatorial regions). Successive clades in the genus *Symbiodinium* have adapted to warmer temperatures, though this rule is not strict, merely a pattern. *Symbiodinium* of clade B can occupy temperate, tropical and equatorial niches (Baker et al. 1997; Goulet and Coffroth 2003b; LaJeunesse 2001; LaJeunesse et al. 2003; Rodriguez-Lanetty et al. 2001; Savage et al. 2002b; Van Oppen et al. 2005), while clades F, C, H, D, and G are warm water specialists (Baillie et al. 2000b; Fabricius et al. 2004; LaJeunesse 2005a; LaJeunesse et al. 2003; Pawlowski et al. 2001; Pochon et al. 2004; Pochon et al. 2006; Ulstrup and Van Oppen 2003; Van Oppen et al. 2005; van Oppen et al. 2001). Within clade A, a development in tolerance from cool to warm (AT→other As) also tracks the branching order of the subclades (Figure 3.1 chapter 3) (Savage et al. 2002b). It will be interesting to see whether any strict cold-water clade (‘*X*’) basal to clades E, D and G may be obtained, and
therefore whether a thermostolerance progression X→E→DG may mirror the pattern of thermostolerance progressions: B→FHC, and AT→other As.

The generalities of cladewise thermal tolerance may be partially misleading, given that hyperevolvability of thermal tolerance within-clades has been shown biochemically and phylogenetically by Tchernov et al. (Tchernov et al. 2004). Echoing the assertion of Tchernov et al. (2004), a novel tropical species of *Symbiodinium*, Z1 was obtained in the present study, and is phylogenetically nearest to the temperate subclade AT (Figure 3.1 chapter 3). Similarly, the thermo-geographic distribution of clade E *Symbiodinium* is unclear, perhaps because of oversampling of tropical hosts relative to temperate hosts. A resolution to the apparent contradiction of observations and approaches is that present-day clades may derive from strains that were ecologically dominant in a given thermal regime during the clade's genesis, and that a few non-dominant strains within any given clade have since independently overcome that inherited limitation or tendency. Non-dominant here means not ecologically widespread in the present day.

A challenging task will be to examine the phylogenetic position of Scleractinia at high latitudes, and at very cool depths to test the idea that Scleractinia and *Symbiodinium* may have co-evolved. The prediction is that some (though not necessarily all) of the scleractinians in cool niches should be basal scleractinians. Further it is predicted that the zooxanthellate subset of these (they are not all zooxanthellate) should house basal *Symbiodinium* or organisms phylogenetically resembling *Gymnodinium simplex*, *Gymnodinium bei*, *Wolszynskia* sp. or *Scrippsiella* sp. (see Figure 7.3 this chapter). This prediction is being tested, with regard to both latitude and depth, by Dr Kevin Strychar (Texas A&M University) in collaboration with the author.

Table 7.1 Thermal niches of marine dinoflagellate genera closely related to *Symbiodinium*

<table>
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<th>Organism</th>
<th>Latitude</th>
<th>Reference</th>
</tr>
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<td><em>Gymnodinium varians</em></td>
<td>Arctic</td>
<td>(Okolodkov 1998)</td>
</tr>
<tr>
<td><em>Gymnodinium simplex</em></td>
<td>Temperate</td>
<td>(Hada 1967)</td>
</tr>
<tr>
<td><em>Gymnodinium bei</em></td>
<td>Temperate</td>
<td>(Spero 1987)</td>
</tr>
<tr>
<td><em>Polarella glacialis</em></td>
<td>Polar</td>
<td>(Montresor et al. 1999)</td>
</tr>
<tr>
<td><em>Wolszynskia halophila</em></td>
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<td>(Kremp et al. 2005)</td>
</tr>
<tr>
<td><em>Wolszynskia micra</em></td>
<td>Temperate</td>
<td>(Leadbeater and Dodge 1966)</td>
</tr>
<tr>
<td><em>Aureodinium</em> sp.</td>
<td>Temperate</td>
<td>(Dodge 1967)</td>
</tr>
</tbody>
</table>
The relationship of *Symbiodinium* to cultured Suessiales and *Woloszynskia* can be exploited not only for studies of thermal tolerance but also for studies of lifecycle. In view of the phylogenetic grouping of Suessiales and *Woloszynskia* in recent publications (Kremp et al. 2005; Lindberg et al. 2005; Saldarriaga et al. 2004), and the finding that *Woloszynskia* are among the closest relatives known to have an observable *in vitro* sexual lifecycle (Lindberg et al. 2005; von Stosch 1973), the sexual cycles of these two groups could be compared in detail, as regards the cues for gametogenesis, the duration of zygotes, and cues for germination.

### 2.4 Relatives of Suessiales: rDNA phylogeny and the evolution of peridinin dinoflagellates

To make inroads into the lifecycles of Suessiales, it is useful to know what their closest relatives are among dinoflagellates. One of the problems in the study of dinoflagellate evolution, is that while the organisms contain many unique features among eukaryotes, such as a different mechanism of DNA compaction than that of other eukaryotes (Chudnovsky et al. 2002; Geraud et al. 1991; Hackett et al. 2005; Michel et al. 1996; Rizzo and Burghardt 1982), and they are therefore very interesting, a branching order of evolution among dinoflagellate orders has been elusive (Daughjerg et al. 2000; Fensome et al. 1999; Taylor 2004). Discussions in this chapter help to distill an emerging model that peridinin dinoflagellates in general, and Suessiales in particular, may resemble the prototype dinoflagellate, from which most other modern dinoflagellate orders developed (Saldarriaga et al. 2004).

The alignment of nuclear *lsu* rDNA sequences that was used to produce Figure 3.1 (chapter 3) contained many other dinoflagellates in addition to *Symbiodinium*, Suessiales and *Woloszynskia*. The set of additional dinoflagellate sequences in the alignment (Gymnodiniales Prorocentrales and Peridinales) was obtained by BLAST searching with *Symbiodinium* search sequences, and retaining only the top hits (arbitrary cut-off). The retrieved sequences thus constituted the full set of closest relatives of Suessiales available in Genbank at that time. That alignment was analysed by Bayesian phylogeny (as described in General Materials and Methods) and yielded a result that has not been previously reported: the closest sister group to Suessiales/*Woloszynskia* in the analysis was Peridinales (Figure 7.3). This finding is compatible with the D1-D3 *lsu* rDNA phylogeny
of dinoflagellates presented by Daugbjerg et al. (Daugbjerg et al. 2000), in which the relationship is present but in which Peridiniales are polyphyletic, unlike in Figure 7.3.

In Figure 7.3 the grouping of Suessiales/Woloszynskia with Peridiniales was supported by a perfect Bayesian posterior probability, and relied on a synapomorphy of a large indel of ~120 base pairs in the D2 region of the nuclear lsu rDNA. Dinoflagellate taxa that would not align with Symbiodinium in the D2 region were excluded from the initial alignment, that which produced Figure 7.3. However after examination of Figure 7.3, and in order to re-test whether the D2 indel was putatively specific to the group Peridiniales, Suessiales and Woloszynskia, further taxa were added and the region was aligned carefully by hand. The more widely sampled alignment (Appendix 7) confirmed that the shared indel is highly conserved in length (± 2 bases) among the group Suessiales, Peridiniales and some species of Woloszynskia and Gymnodinium. Further work will be required to determine whether the group underwent a deletion at this locus, or whether the other dinoflagellates underwent an insertion there. Examination of the D2 regions of closely related non-dinoflagellates (Oxyrrhis, Perkinsus, colpodellids, apicomplexans, ciliates and stramenopiles) may clarify this question, by showing whether or not the ~120 bp region pre-existed.

Peridiniales includes the symbiotic species Scrippsiella vellela (associated with Scyphozoa) and Scrippsiella spp. (associated with Radiolaria). lsu rDNA has not been sequenced from those particular Scrippsiella spp., and so were not available for the alignment. The phylogeny of those organisms had been analysed using ssu rDNA (Gast and Caron 1996; Gast and Caron 2001; Gast et al. 2000). In view of the tree of Figure 7.3, and the alignment in Appendix 7, the evolutionary history of symbiotic dinoflagellates from non-symbiotic dinoflagellates may become amenable to investigation. The position of Suessiales with respect to Peridiniales and the polyphyletic Gymnodiniales will be investigated, and a tree of major dinoflagellate orders may be obtained by pinpointing the phyletic origin of the lsu rDNA D2 indel. The synapomorphy of the D2 indel will be tested with greater stringency by generating an RNA secondary structure of this region similar to the phylogenetic analysis of several protistan algal genera by Ben Ali et al. (Ben Ali et al. 2001), which included no dinoflagellates. The study of Daugbjerg et al.
(Daugbjerg et al. 2000), which did use dinoflagellates and also considered secondary structure, omitted any regions that contained indels.

**Figure 7.3** Bayesian phylogeny of the dinoflagellate Gymnodiniale-Peridiniale-Prorocentrale (GPP) complex using D1 and D2 regions of nuclear LSU rDNA (Appendix 7). Only taxa that are alignable in the D2 region of LSU rDNA were included in the analysis, and interestingly this consisted of members of the GPP complex. In the subsequently expanded alignment (Appendix 7) there is a clear synapomorphy between Peridiniales and Suessiales that is not shared by Prorocentrales or *Kareania*. The tree is the sum of the final 300, 000 trees from a run of 500,000 trees that were generated, and has an alpha of 0.66 < 0.91 < 1.22, and a log likelihood of -10499.
Peridinin dinoflagellate plastid genomes: *Symbiodinium* as a functional model

This has been the first study to show that Suessiales possess minicircles, which will facilitate further study of the plastid biology of the emerging model organism *Symbiodinium*. The body form and pigment content of the genus *Symbiodinium* typifies the simplest of dinoflagellates, and its plastid and photosynthetic functions are among the best studied of any dinoflagellate. Laboratory manipulation would go further to establishing the functions of minicircle non-coding regions in peridinin dinoflagellates. Minicircles are potentially among the many sites at which transgene integrations occurred in the study by TenLohuis and Miller (ten Lohuis and Miller 1998), in which an artificial construct of bacterial DNA plus a plant reporter gene (beta-glucuronidase ‘GUS’) was transformed into *Symbiodinium* and was integrated stably. Recombinogenicity of the minicircle non-coding regions, (inferred in chapter 4), could have played a role if integration of the GUS gene occurred in the *Symbiodinium* plastid genome. A hypothesis of Nelson and Green that constitutive replication of minicircles may be obligately linked to recombination (Nelson and Green 2005), suggests that recombination activity may be a constant process in the *Symbiodinium* plastid, and if so this activity could facilitate high efficiency of foreign gene integration. Of all the dinoflagellates in Figure 7.3, *Symbiodinium* is well placed as a model system amenable to laboratory manipulation, owing to transformability (ten Lohuis and Miller 1998) and a small genome (LaJeunesse et al. 2005b), though so far it has only been asexually propagated which currently prevents heredity studies and ploidy manipulation.

2.5 Carbon fixation in peridinin dinoflagellates is unusual

An unusual feature (Morse et al. 1995; Rowan et al. 1996; Whitney and Yellowlees 1995) of the metabolism of *Symbiodinium* and other peridinin dino-flagellates, is the possession of an alpha-proteobacterial form ('form II') of Ribulose bis-phosphate carboxylase (Rubisco). Rubisco is a key enzyme in the pathway for fixation of reduced carbon. All other protistan algal lineages contain the eukaryotic form of Rubisco ('form I'), which was originally derived from a cyanobacterium (Badger et al. 1998; Delwiche 1999; Palmer 1995). The events surrounding acquisition of the peridinin dinoflagellate Rubisco have been in question, though there is consensus that the gene was transferred from an alpha-proteobacterium to the dinoflagellates (Badger et al. 1998; Delwiche 1999; Morse et al.
1995; Palmer 1995; Whitney et al. 1995). The mode of origination of the peridinin dinoflagellate plastid genome, and Rubisco is pursued further in section 2.6.

It has been previously suggested that the peridinin dinoflagellate lineage and particularly Suessiales are among the most basal dinoflagellates (Saldarriaga et al. 2004; Taylor 2004; van den Hock et al. 1995) though in the case of Suessiales this position was not supported conclusively (Zhang et al. 2005). It is of dubious meaning to ascribe ancestral status to a modern lineage. Suessiales may have originated only ~200-300 mya (Montresor et al. 1999; Morbey 1975; Palliani and Riding 2003), while the origin of the division Dinoflagellata is likely much older than that (Fensome et al. 1999). Nevertheless, could all dinoflagellates have originated from a symbiotic pre-dinoflagellate in early metazoans? If so, then acquisition and maintenance of form II Rubisco may have been an adaptation to survival inside host metazoan cells. O₂ concentrations are very high inside modern-day host coral cells (Leggat et al. 1999). All forms of Rubisco are inefficient and vulnerable: CO₂ fixation occurs at the active site of Rubisco, but this same site preferentially binds O₂, so outcompeting CO₂ especially if O₂ levels are high (Badger et al. 1998). Dinoflagellates have solved the vulnerability of Rubisco to O₂ by localising Rubisco to the pyrenoid and co-locating there a carbonic anhydrase enzyme for HCO₃⁻ (inorganic carbon) concentration activity (Leggat et al. 1999; Leggat et al. 2002). This particular isoform of carbonic anhydrase is therefore a key to the success of the peridinin dinoflagellate lineage and one question is whether it was proteobacterial in origin, like form II Rubisco.

An NCBI BLASTp search with *Heterocapsa triquetra* plastid carbonic anhydrase as a query (accession AAW79301) yielded only proteobacterial hits (this study), indicating that horizontal transfer from bacteria was the source of this enzyme. Patron et al. (Patron et al. 2005) noted that the *H. triquetra* enzyme is plastid located, by means of its plastid targeting peptide. Since the pyrenoid is part of the plastid in peridinin dinoflagellates, the *H. triquetra* enzyme may be targeted there and so is potentially the true homologue of the *Symbiodinium* pyrenoid carbonic anhydrase. Confirmation of this homology awaits publication of *Symbiodinium* EST sets (which may contain the gene), an accurate phylogeny of the gene and its family, and further in situ probing for this specific carbonic anhydrase protein sequence. If the carbonic anhydrase found in the pyrenoid is the key to a carbon concentration mechanism in peridinin dinoflagellates, as suggested by Leggat et al. (1999; 2002), then the specific form of the Rubisco that is used may be of less
importance than previously thought, and the ability of this lineage to sequester both enzymes in the pyrenoid may be one factor in the success of the lineage. If the two genes have a common time of evolutionary origin, this may have facilitated the sequestration of both at one organelle, the pyrenoid.

The origin of the peridinin-dinoflagellate pyrenoid is likely to become an important subject of research in dinoflagellate evolution and coral bleaching theory (Jones et al. 1998; Leggat et al. 2004). As a first approximation, the evolution of the specialised pyrenoid/Rubisco function in this lineage may have been linked with the evolution of the peridinin plastid genome. The possible mode of origination of the plastid genome, and Rubisco is examined in the following section.

2.6 Myzozoa: an endosymbiotic melting pot for the generation of unique organelles

The taxonomic term Myzozoa has been defined in the General Introduction, and in Appendix 8. Myzocytosis, as a feeding mechanism, has not been directly observed in Symbiodinium or Suessiales, but a peduncle is present (an organelle putatively used in myzocytosis) in S. kawagutii, S. goreaui, S. pilosum, and S. microadriaticum (Trench and Blank 1987). The free-living Suessiale documented by Loeblich and Sherley also possesses a peduncle (Loeblich and Sherley 1979) though because of lack of molecular typing it is unclear whether this strain has affinity either with Gymnodinium simplex or Symbiodinium spp., most likely the former (Lee et al. 2003). A particular form of the retracted peduncle is known as a microtubular basket (Kubai and Ris 1969; Schnepf and Deichgraber 1984; Schnepf et al. 1985; Schnepf and Winter 1990; Spero 1982; Ucko et al. 1997). The peduncle and microtubular basket appear absent from Gymnodinium bei and Polarella (Montresor et al. 1999; Spero 1987; Trench and Blank 1987).

Because the peduncle and the microtubular basket occur in many dinoflagellates [(Hansen and Calado 1999; Jacobson 1999; Jacobson and Anderson 1996; Larsen 1988; Parrow and Burkholder 2003a) in addition to the above], these organelles are considered an ancestral character of Dinoflagellata (Cavalier-Smith and Chao 2004; Leander and Keeling 2003). The presence of only a single Symbiodinium species (S. limucheae) with a well-developed microtubular basket may be taken to mean that reliance on the myzocytotic system has
degenerated in the Suessia lineage. The largest extended peduncle that has been documented in the genus is that of *S. kawaguti* (Trench and Blank 1987), an opportunist (LaJeunesse et al. 2004a)(similar to isolates Z3, Z4 and Z6 of chapters 3 and 4), and by implication a free-living specialist rather than a specialised symbiont. The relative autotrophy of *Symbiodinium* that are host specialists may have stabilized their organelles and nuclei by preventing ingress of genes from eukaryotic and prokaryotic prey. Strict autotrophs are perhaps the kind of symbiont best suited to survival inside a host cell.

The myzocytosis character has been raised in this general discussion because the thesis has revealed a phototrophic myzocytotic colpodellid, taxon-2, (it was observed feeding on prey) which broadly supports the concept that the basal Myzozoan may have been photosynthetic (Fast et al. 2001). The existence of taxon-2 unites the previously vulnerable theories of Chromalveolata and Myzozoaa (Cavalier-Smith 1999; Cavalier-Smith and Chao 2004), by using the case of the mixotrophic colpodellids as a missing link between photosynthetic stramenopiles and alveolates.

Another more central feature of the thesis that leads to discussion of colpodellid lifestyles is the source of recombinogenic DHEs in peridinin dinoflagellate evolution. DHEs have been previously found only in mitochondria (Bullerwell et al. 2003; Paquin et al. 1997; Paquin et al. 2000; Paquin and Lang 1996). Two clues to the potential origin of DHEs in *Symbiodinium* (this study) and another peridinin dinoflagellate *Adenoides cludens* (Nelson and Green 2005) are; i) that *Symbiodinium* contains a euglenozoan glyceraldehyde-phosphate dehydrogenase (GAPDH) gene (Takishita et al. 2003b); and ii) that an American Type Culture Collection culture of *Colpodella* feeds on a bodonid (J. Cole ATCC pers. comm.), and bodonids (Euglenozoa) contain an unusual mitochondrial genome composed of minicircles the ‘kinetoplast’. Euglenozoa includes two orders that contain kinetoplasts, the Bodonidae and the Kinetoplastidae. Figures 7.4 -7.6 depict a model that can explain the origin of minicircle recombinogenicity (chapter 4) in dinoflagellates and thus explain reduction of the dinoflagellate plastid genome. The hypothesis posed in Figure 7.4, is that euglenozoan may have been the source of recombinogenic proteins that bind IRs or DHEs in dinoflagellates. Euglenozoans and dinoflagellates are unrelated (Cavalier-Smith 1999) but share numerous striking similarities as backed up with additional observations from literature, shown in Figure 7.6.
In the kinetoplast minicircles of *Trypanosoma brucei*, conserved sequence blocks (CSBs) are present that bind replication proteins only when single stranded (Abu-Elneel et al. 1999; Abu-Elneel et al. 2001; Avrahami et al. 1995; Onn et al. 2004; Tzfatì et al. 1995). 'Conserved' sequence here means short sequences of 9 to 12 bases conserved across at least three genera: *Trypanosoma*, *Leishmania* and *Crithidia*. Each single strand of DNA in the CSB1 and CSB2 regions plus flanking sequences, potentially folds into a stem loop structure (Figure 7.5) resembling a double hairpin element (this study). This was tested both for Group I minicircles and Group II minicircles as defined by Hong and Simpson (Hong and Simpson 2003). Kinetoplast minicircles were proposed to be recombinogenic (Chen and Donelson 1980) and may putatively recombine in pairs (this study), each pair consisting of a Group I and Group II minicircle. Recombination among minicircles could occur outside the kinetoplast disc where minicircle replication happens (Liu et al. 2005). The structures depicted in Figure 7.5 show the location of the critical CSB1 origin in a putative loop region. The formation of the loop may be achieved in a dynamic series of stem loop structures, with the lowest energy state being followed by the higher energy states, low facilitating transition to high. Could the CSB sequences in kinetoplast minicircles be maintained by frequent recombination? If so, this would be reminiscent of the system of maintenance proposed for dinoflagellate plastid minicircles [chapter 4, and (Nelson and Green 2005; Zhang et al. 2002)] and would be a character uniquely shared between kinetoplasts and peridinin dinoflagellate chloroplasts.

In Figure 7.4 and 7.5, the set of observations together can be interpreted to suggest that a euglenozoan may have donated recombinogenic proteins to a pre-dinoflagellate in a horizontal gene transfer (HGT) event by endosymbiosis. The model indicates that possession of minicircle IRs may be an ancestral state of all peridinin dinoflagellates, and also makes clear predictions about the order of endosymbiotic events that putatively influenced the evolution of peridinin dinoflagellates (Figure 7.6). Laatsch et al. (Laatsch et al. 2004) have shown that minicircles of peridinin dinoflagellates may be present in both the nucleus and the chloroplast, while Howe et al. (Howe et al. 2003) proposed that the minicircle system is an evolutionary shuttle for DNA between cellular compartments. As such, the mobility and implied recombinogenicity of minicircles is generally agreed.
**Figure 7.4** Model for evolutionary source of *Symbiodinium* minicircle recombinogenicity. Rightmost column indicates development of DHEs. Potentially bifurcating stem-loop structures are found in kinetoplast mitochondrial minicircle replication origins (Figure 7.5) that may resemble DHEs in function. The model invokes horizontal gene transfer of minicircle recombination proteins. It does not imply that mitochondrial genes need to have been transferred, only the DNA recombination system for these, which it is proposed is nuclear-encoded in those euglenozoans that have kinetoplasts. The term ‘genome’ here refers to the set of peridinin chloroplast-encoded genes, as well as to the peridinin dinoflagellate mitochondrion.

**Supporting data for an evolutionary model regarding the origin of the unusual organellar genome architectures of peridinin dinoflagellates**

While the peridinin dinoflagellate nucleus and plastid (chapter 1, Figure 1.13) have both been inherited from the chromalveolate lineage (Cavalier-Smith 1999; Fast et al. 2001; Zhang et al. 1999), the evidence is here reviewed that the peridinin dinoflagellate nucleus may have taken on genetic material from a euglenozoan as well (Takahita et al. 2003b). Although it is unknown whether gene transfer from a Euglenozoan occurred by endosymbiosis or viral transfer, the myzocytotic lifestyle of ancestral dinoflagellates would favour the former. To examine the broader influences of such an endosymbiotic transfer, requires a small amount of additional background on the organellar genomes of dinoflagellates and other protozoa.
Figure 7.5 A dynamic secondary-structure prediction shows potential double-hairpin structures in the DNA replication origins of *Trypanosoma brucei* (kinetoplast) minicircles. The folded region includes Conserved Sequence Block 1, (CSB1, a replication origin), CSB2 and their flanking regions. The putative folding series for a Group I minicircle origin is shown connected by blue arrows. The putative folding series for a Group II minicircle origin is shown connected by an orange arrow. Initial (longest) hairpins are the lowest energy states. Subsequent (bifurcated) hairpins are higher energy states. Initial low energy states could facilitate transition to the subsequent high energy states. The mechanism shown here may melt the dsDNA for replication proteins to bind to single stranded CSB1. Conserved sequences are: CSB1 - GGGCGTKC; CSB2 - CCCCITNC. The sequences analysed were accessions L16542 and L16540 (Corell et al. 1993; Hong and Simpson 2003). Folding was analysed using the original Mfold server http://www.bioinfo.rpi.edu/applications/mfold/old/dna/ (Zuker 2003) on the default settings for DNA. Mfold ranked the energy states from low to high, and all the Mfold results are shown here.

The mitochondrial (Gray 2003; Lin et al. 2002; Zhang and Lin 2005) and chloroplast (Wang and Morse 2006a; Zauner et al. 2004) gene transcripts of some peridinin dinoflagellates that have been examined undergo RNA editing, as do the mitochondrial transcripts of kinetoplastid protozoa (Alfonzo et al. 1999; Benne et al. 1986; Gray 2001; Gray 2003; Lukes et al. 2005). Dinoflagellate plastid genes may undergo only ‘substitutional’ editing, and not ‘insertional’ editing (Wang and Morse 2006a). Both mechanisms occur in kinetoplastid mitochondria and are mediated by guide RNAs, which have not yet been identified in the chloroplasts or mitochondria of dinoflagellates. The
presence of extensive RNA editing in dinoflagellates and kinetoplasts has been suggested as coincidental (Gray 2001), given that RNA editing also occurs to a lesser extent in various genomes of plants, fungi, animals and Amoebozoa (Gray 2001; Gray 2003). The question of whether RNA editing is a convergent, or a divergent character, awaits evolutionary/biochemical analyses of the mechanisms involved in the process in these various lineages. However, it is notable that polyuridylation of the 3' UTRs of plastid mRNAs in a peridinin dinoflagellate *Lingulodinium* (Wang and Morse 2006a), mimics the 3' polyuridylation of guide RNAs in kinetoplastids (Adler et al. 1991) and of mitochondrial mRNAs in Amoebozoa (Horton and Landweber 2000), and has been found only in these three lineages. Finally, as mentioned in chapter 4, the mitochondrial genome of dinoflagellates is fragmented (Norman 2000), though it is not known whether this happened independent of the fragmentation of the plastid genome into minicircles.

With this background, the full hypothesis can be stated: that a proto-dinoflagellate bearing a chromalveolate plastid may have endocytosed a non-photosynthetic euglenozoan (such as *Rhynchomonas, Bodo,* or a kinetoplastid) and that the conflict in managing many organellar genomes, may have selected for the fragmentation of the organellar genomes of peridinin dinoflagellates, by co-option of some features of the minicircle system and RNA editing from the euglenozoan (kinetoplast) to the dinoflagellate (chloroplast and mitochondrion). Such a scenario is supported by five observations: i) that dinoflagellates uniquely share with Euglenozoa a paraflagellar rod structure forming the basis of a ribbon flagellum (Cachon et al. 1988; Gadelha et al. 2004; Godart et al. 1992a; Maga and LeBowitz 1999) even though the two phyla are clearly unrelated phylogenetically (Baldauf et al. 2000; Cavalier-Smith 1999); ii) that the dinoflagellate 70 kDa paraflagellar rod protein is cross reactive with an antibody to the 70 kDa *Euglena* paraflagellar rod protein (Godart et al. 1992a). iii) that RNA editing occurs in dinoflagellate chloroplasts and mitochondria Gray 2003; Lin et al. 2002; Zhang and Lin 2005)(Wang and Morse 2006a; Zauner et al. 2004) and euglenozoan mitochondria (Gray 2001; Gray 2003), but probably did not occur in the predecessor of dinoflagellates because it does not occur in the modern ciliate or stramenopile mitochondria as far as known; iv) that RNA editing and transcript uridylation occur in dinoflagellate plastids (Wang and Morse 2006a; Zauner et al. 2004) uniquely among all other plastids, indicating possible mitochondrial origin of the apparatus; v) that the replication origins of kinetoplast minicircles contain potential bifurcated hairpin structures resembling the DHEs of peridinin dinoflagellate plastid
minicircles (this study). In the model (Figure 7.6), the recombinogenicity thus acquired by the plastid genome of peridinin dinoflagellates, may have aided the mass transfer of plastid genes to the peridinin dinoflagellate nucleus.

The merit of the model is that kinetoplast function and kinetoplast division, both intimately require the participation of the euglenozoan flagellar basal bodies (Kohl et al. 2003; Matthews 2005). Thus a possible selection is apparent for co-transfer (hitchhiking) of kinetoplastid flagellar components along with the genomic regulation and recombinogenicity functions. The hypothetical transfer of genes for (some but not all) flagellar proteins from Euglenozoa to a pre-dinoflagellate is testable by comparative genomics. The genes of the unique kinetoplastid flagellar proteome (Broadhead et al. 2006) are suitable probes for PCR or RNA-interference analysis of peridinin dinoflagellates, or for searching of their ESTs.

In this model, initial reduction of the peridinin dinoflagellate plastid genome may have been facilitated through acquisition of euglenozoan DNA-control machinery. Subsequently, this acquired and fixed recombinogenicity may have aided transfer of photosynthesis genes from nucleus to nucleus during tertiary endosymbiosis events (plastid replacements) that subsequently occurred in several dinoflagellate lineages (chapter 1, Figure 1.13).

The hypothesis of extensive gene transfer from a euglenozoan to a proto-dinoflagellate is raised here as a result of the discovery of recombinogenic DHEs and minicircles in the Symbiodinium plastid, and unites several additional lines of existing evidence: taxonomic distribution of the paraflagellar rod, presence of euglenozoan GAPDH in Symbiodinium, and presence of RNA editing in Symbiodinium. An alternate hypothesis, - that RNA editing arose independently in each lineage that possesses it -, has been discussed by Gray (2001).
**Figure 7.6** Model for origin of peridinin dinoflagellates and apicomplexans. *Symbiodinium* belongs in the group peridinin-dinoflagellates. Gains are shown as brown lines. In this model, the euglenozoan (kinetoplastid) 'symbiont k' was not photosynthetic. The emergence of Euapicomplexans is tentatively shown as polyphyletic on account of normal UGG-Trp codon usage in the taxon-2 chloroplast.
3. Thesis summary

The central goal of this thesis was to assess presence or absence of recombination in a population of clade C *Symbiodinium*. Sampling of a population for recombination analysis was deliberately conservative (to prevent artefacts of comparison among non-conspecific isolates), and so leaves open the final phase for future work. The hypervariable loci and the culturing methods developed led to novel findings regarding the lifecycle, evolutionary genetics, and biodiversity of the algal symbionts of corals.

Specifically, the structure and potential ecological significance of a *Symbiodinium* minicircle was assessed in detail, and the first systematic evolutionary analysis of dinoflagellate introns was undertaken; both are likely to be useful species markers for *Symbiodinium*. The first photographic documentation of *Symbiodinium* sexual cell-cell fusion *in vitro* was obtained, supported by the unpublished observations of Dr K. Heimann, and the cultures in which this occurred were donated to public collections. The ecological significance of the recombination question is arguably greater for *Symbiodinium* clade C, than for *Symbiodinium* clades A and B for which the recombination question has been solved. Assessment of the presence or absence of sexuality in a model strain of clade C, *Sym-Gt* OT1, has been advanced by development of markers. The markers can be applied again in future to study the recombination question in clade C *Symbiodinium* and also (separately) any other clade of *Symbiodinium* as all clades possesses the actin and *psbA* loci. The priority now is to apply the markers developed in this thesis to a wider sample of clade C *Symbiodinium*.

Additionally, new taxa of photosynthetic symbionts (at the taxonomic level of Class) were cultured by capitalising on the success of a novel culture medium that apparently selects for growth of symbionts that may be ecologically spread via vertebrate and invertebrate digestive systems. By combining the phylogenetic analysis of the new taxa and the finding of double hairpin elements and recombinogenicity in *Symbiodinium* plastids, a hypothesis on the evolution of peridinin dinoflagellate and apicomplexan plastids and their genomes was constructed.

The utility of these contributions to the field of *Symbiodinium* research is that they have helped maintain the status of the genus *Symbiodinium* (in their niche, coral reefs) as a
prominent model dinoflagellate for the study of: photosynthesis, plastid gene regulation, nuclear and organelar genomics, sexuality, recombination, and evolution.

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Appendix 1

Phylogeny of *P. damicornis* symbiont

Unrooted nucleotide distance Neighbour-Joining (NJ) tree of LSU rDNA sequences from clade C symbionts of selected corals of OT1 atoll. Sequence origin is from Loh and Loi (Loh et al. In preparation; Loi 1998), and the comparator sequence is from Baker (Baker 1999). Host species nomenclature is as in Figure 1.3, and the additional host represented here is *Pocillopora damicornis* (Pd). The asterisked branch obtained 56 percent bootstrap NJ support in a similar analysis that was rooted with clades A and B (not shown). The low bootstrap value is due a single base change between *Sym-Gt* and *Sym-Ha*. The analysis was done using PHYLIP (Felsenstein 1989).

References

## Appendix 2

Genbank accessions for nucleotide sequences in this thesis

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Chapter 5

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Ch6

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| RM11    | P. damicornis, OTI | Colpodella              | nuc ssu rDNA, partial | DQ174732 |
| CMS22   | L. purpurea, OTI | Apicomplexa              | nuc ssu rDNA, partial | DQ174733 |
| CMS22univ| L. purpurea, OTI | Apicomplexa              | nuc ssu rDNA, partial | DQ174734 |
| RM11univ| P. damicornis, OTI | Unculturable plastid    | cp psbA, partial     | DQ174735 |
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| soil10tisschrom | L. purpurea, OTI | Unculturable plastid    | cp psbA, partial     | DQ174737 |
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*nuc=nucleus, cp=chloroplast, bac=bacterial
Appendix 3

Synapomorphic bases in the D1/D2 region of 16S rDNA within _Symbiodinium_ clade A phylogenies by comparison across these. Base positions were numbered according to the alignment (not shown). Accession numbers of the sequences that were aligned in order to tabulate these synapomorphies were those listed in Table 3.5 chapter 3. Underlined = true synapomorphy. Italic = synapomorphy within the A2/AT group.

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## Appendix 4

### Actin nucleotide and protein sequence alignments

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373
(A) Suessiæae actin genes, with exon-intron and intron-exon boundaries annotated.

Boxed sequences are unique to this study, and were obtained by direct sequencing of genomic DNA. Assignment of exon-intron and intron-exon boundaries was done via reference to the sequences above the boxes, which were available Symbiodinium cDNAs (Fukuda et al. 2002; Leggat et al. 2005; Saldarriaga et al. 2003) and genomic actin lacking introns (Leggat et al. 2005; Saldarriaga et al. 2003). The fourth line is from a cDNA encoding the full length actin ORF of the symbiont of Acropora sp. (Leggat et al. 2005). Dashes denote alignment gaps within a sequence. Many sequences are partial. Genbank accessions are given in Appendix 2. Sequences: G. varians CCMP 421 and CladeC_Sym-CMS became available retrospectively and were not used in primer design.
(B) Suessia actin proteins.

Boxed sequences are translations of DNA sequences unique to this study [see (A)]. Reference sequences (Fukuda et al. 2002; Leggat et al. 2005; Saldarriaga et al. 2003) were translated in BioEdit (Hall 1999). Dashes denote alignment gaps within sequence. Many sequences are partial.
References


Appendix 5

Phylogeny of bacterial large subunit rDNA sequences

(A) Non-proteobacterial section of 23S tree, reanalysed. Sequences novel to this study are marked by red asterisks. 400,000 of 1000,000 trees. Log likelihood -4337, alpha 0.32<0.41<0.58.
(B) Alpha-proteobacterial section of 23S tree, reanalysed. Sequences novel to this study are marked by red asterisks. 700,000 of 1100,000 trees. Log likelihood -3513, alpha 0.42<0.53<0.76.
(C) Gamma-proteobacterial section of 23S tree, reanalysed. Sequences novel to this study are marked by red asterisks. 500,000 of 1000,000 trees. Log likelihood -1861, alpha 0.14<0.17<0.24.
Appendix 6

Evidence supporting presence of taxon-2-like organisms in coral tissues

(A) BLAST results for non-Symbiodinium psbA amplicon from *L. purpurea* tissue 13

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Key: Ch-Chlorophyta, S-Streptophyta, H-Haptophyta,

*Tertiary plastid is Coscinodiscophyceae-derived (Chenick et al. 1997; Chenick et al. 1996; Inagaki et al. 2000)
#Phylogeny of the uncultured algal RED122-4-8 is demonstrated in (Zeidner et al. 2003)
BLAST query using novel sequence DQ174737, fragment obtained using PCR primers: forward psbA non-Symbiodinium (psbAOF Table 6.2), reverse psbA universal (psbAL1 Table 6.2)

(B) BLAST results for non-Symbiodinium psbA amplicon from *L. purpurea* tissue 15

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Key: Ch-Chlorophyta, S-Streptophyta, Cr-Cryptophyta,

*Tertiary plastid is Coscinodiscophyceae-derived
BLAST query using novel sequence DQ174738, fragment obtained using PCR primers: forward psbA non-Symbiodinium (psbAOF Table 6.2), reverse psbA universal (psbAL1 Table 6.2)
(C) BLAST results for non-\textit{Symbiodinium} psbA amplicon from \textit{P. damicornis} tissue OT1

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Key: Ch-Chlorophyta, S-Streptophyta, C-Cyanobacteria,
*Tertiary plastid is Coscinodiscophyceae-derived

BLAST query using novel sequence DQ174739, fragment obtained using PCR primers: forward \textit{psbA} non-\textit{Symbiodinium} (psbAOF Table 6.2), reverse \textit{psbA} universal (psbAL1 Table 6.2)

References:


Appendix 8

List of abbreviations

O/N – overnight
SW – (Sydney) seawater
ASW – Autoclaved (sterile) seawater
fEs – (fE selective) growth medium ‘f’ with EDTA as iron chelator, and lacking silicate
CSIRO – Commonwealth Scientific and Industrial Research Organisation, Australia
LM – Light microscopy
TEM – Transmission Electron Microscopy
CCMP – Culture Collection of Marine Phytoplankton, Maine USA
NQAF – North Queensland Algal Identification and culture Facility

OTI – One Tree Island
Gt – Goniopora tenuidens
Ha – Heliofungia actiniformis
Lp – Leptastrea purpurea
Pd – Pocillopora damicornis
Pv – Plesiastrea versipora
Tm – Tridacna maxima
Sym-Gt – symbiont of Gt
Sym-Ha – symbiont of Ha
Sym-Lp – symbiont of Lp
Sym-Pd – symbiont of Pd
Sym-Pv – symbiont of Gt
Sym-Tm – symbiont of Tm

ORF – Open reading frame (complete gene)
DNA – deoxyribonucleic acid
cDNA – cloned ‘copy’ DNA, reverse transcribed from messenger RNA
RNA – ribonucleic acid
rDNA – gene encoding rRNA
rRNA – ribosomal RNA

lsu – large subunit
ssu – small subunit
PCR – (DNA) Polymerase Chain Reaction
psbA – gene coding for protein D1 of photosystem II
23S – bacterial large subunit rDNA
IR – (DNA sequence) Inverted Repeat
DHE – (DNA) Double Hairpin Element
bp – base pairs
kb – kilobase pairs
rpm – revolutions per minute
GTR – General time reversible model of DNA base substitutions
γ 8+1 – gamma correction of variable sites, in eight variable categories and one invariable category
WAG – Whelan and Goldman model of amino acid substitution weighting 2001
NY98 – Nielsen and Yang model of (amino acid) codon conservation 1998
RAPD – Randomly Amplified Polymorphic DNA: A fragment of DNA is amplified, then ‘restriction digested’ with DNA-cutting enzymes that recognize specific four-base or six-base sequences.
kDa – kiloDalton, 1000 Daltons: one Dalton (Da), is the mass of one hydrogen atom, or more precisely 1/12 of the mass of one atom of Carbon-12.
EST – Expressed Sequence Tag, a single cDNA. The process of obtaining ESTs usually involves the sequencing of many thousands of cDNAs randomly

PMA – Phorbol 12-Myristate 13-Acetate
TCIC – Trichloroisocyanurate
EB – (DNA) Extraction Buffer
HOCI – Hypochlorite
HCl – Hydrochloric acid
NaOCl – Sodium Hypochlorite
NaOH – Sodium Hydroxide
NaCl – Sodium Chloride
KCl – Potassium Chloride
**Tris** - Tris(hydroxymethyl)methylamine

**EDTA** - Ethylene-diamine-tetraacetic-acid

**mQ** - ‘milliQ’ (low cation) water

**Scleractinia** - a monophyletic family of hard or stony corals. Calcification of the hard skeleton is usually facilitated by presence of microalgae.

**Dinoflagellate** - A monophyletic protist group defined by: i) possessing two flagella of which one is a ribbon flagellum. A proteinaceous paraflagellar rod gives the transverse flagellum its ribbon shape and function; ii) having condensed chromosomes in interphase, unlike other alveolates.

**Protist** - a unicellular eukaryote. The term is in dispute, but is most widely used under this meaning. Polyphyly (convergence) of multicellularity means the term protist is not of taxonomic significance.

**Alveolate** - A monophyletic protist group possessing alveoli beneath the plasma membrane. An alveolus here means a membrane delinated sac. These usually abut each other, and are found around the full circumference of the cell. Organisms within the group Alveolata include dinoflagellates, *Oxyrrhis*, perkinsids, apicomplexans (sporozoans), and colpodellids

**Myzocytosis** - A process of siphoning the contents from a prey organism.

**Myzozoa** - The monophyletic group of protists that contains all alveolates except the Ciliata. The major groups of Myzozoa are the dinoflagellates, *Oxyrrhis*, perkinsids, apicomplexans and colpodellids

**HGT** - Horizontal Gene Transfer (also known as lateral gene transfer)

**Stramenopile** (Heterokont) - The closest sister group to Alveolates. A stramenopile is defined by the fine hairs along one of its flagella.

**Chromalveolate** - A monophyletic supergroup or ‘kingdom’ of protists and their derivatives. It includes four groups that were hypothesized by Cavalier-Smith to have been ancestrally photosynthetic, bearing the same plastid, and thus are implied to be single nuclear lineage as well. It consists of the Chromists and the Alveolates (thus ‘Chrom-alveolate’). Chromista contains 3 divisions Cryptophyta, Haptophyta, and Stramenopila.

**Euglenozoa** - A monophyletic group of protists that are unrelated to alveolates, but that share the feature of the paraflagellar rod with dinoflagellates. The group includes the Kinetoplastida, the Bodonida, and others.

**Kinetoplast** - A monophyletic group of protists that has a particular kind of mitochondrion - the kinetoplast - in close association with the flagellum.

**Kinetoplastid** - A monophyletic group of protists that has a particular kind of mitochondrion - the kinetoplast - found in close association with the flagellum of many Euglenozoa. The kinetoplast contains maxicircle of DNA coding for mitochondrial genes, and also contains minicircles of DNA that code for guide RNAs used to edit the information-content of genes on the maxicircle.
Allelopathy — natural antagonism between microbes, via production of species-specific antimicrobial chemicals by one of the microbes

Biological species — a group of actually or potentially interbreeding natural populations, which is (fully) reproductively isolated from (all) other such groups (Mayr 1942). The term does not apply well to prokaryotes, which are promiscuous and never link sexuality directly to reproduction as eukaryotes do.

Morphospecies — if enough morphological data are available for enough species in a genus, then authorities try to agree on the set of characters that define each species within the genus (von Linne 1735/1964). In this thesis it is held that the most likely means by which the set of characters are maintained (that define a morphospecies) is sexual recombination.

Subspecies — within a biological species or a morphospecies, certain individuals may possess diagnosable traits that segregate mainly in a subpopulation of the species, and less frequently in the full population. This subpopulation is a subspecies.

Sibling species — two or more subspecies of a morphospecies

Species swarm — the full set of known subspecies making up a species. A species swarm is thus roughly equivalent to a biological species, but the term only applies to species which have many diagnosable subspecies. Variation among subspecies of a swarm is infrequently shared with the other subspecies, thus a high level of variation is maintained in the swarm, which may aid adaptation and exaptation especially over a global scale in which habitat of the species may vary greatly.

Syngen — a particular subspecies

Phylogenetic species — an irreducible cluster of reproductively cohesive organisms that is diagnosably distinct from other such clusters. Unlike a biological species, which can include two or more evolutionary units, a phylogenetic species is a discrete entity in space and time (Cracraft 1987). By Cracraft’s definition, a phylogenetic species is like a subspecies, but compatibility with other subspecies is not assumed (nor is it ruled out).

Cryptic species — A phylogenetic species that is reproductively isolated from another phylogenetic species, but sexual recombination since the separation event is doubted. It is a particularly useful concept in the study of parasites, which are often clone-mates in the host individual, and cannot survive outside a host. A cryptic species can only be distinguished using molecular genetic methods e.g. DNA sequencing, because the cryptic species contains no detectable morphological variation and no significant habitat variation.

Ecological species/ Ecospecies/ Ecotype — a lineage (or a closely related set of lineages) which occupies an adaptive zone minimally different from that of any other lineage in its range and which evolves separately from all lineages outside its range (Van Valen 1976).
**Adaptation** – the fixation of genotypic and phenotypic characters under the influence of selection.

**Exaptation** – the generation and accumulation (without selection) of genotypic and phenotypic characters that can subsequently serve as a basis for selection (Gould and Lloyd 1999). Effectively means ‘neutral selection’ and ‘neutrally selected characters’.

**Selection** – an interaction between environment and species, or an interaction between species and species, that causes proliferation or maintenance of a species, or causes speciation.

Alternatively and not contradictory - an interaction between environment and individual, or an interaction between individual and individual, that causes proliferation or maintenance of an individual.

**Species** – biological species (the two are synonymous in this thesis). This is not the Linnean use (morphospecies) but tends to overlap considerably with morphospecies in terms of limits of variability, and it additionally ascribes causes, so is the preferred modern use.

**Speciation** – The generation of a new biological species, presumably through selection. It is generally accepted that this happens through the separation (reproductive isolation) of a subspecies from the parent species.

**Phenotype** – A trait. Usually a particular character. The degree to which a phenotype is reducible to a single character varies for each phenotype. Phenotype is similar to the Linnean notion of a particular measurable and definable aspect of a morphospecies. May sometimes be broader than genotype but is reliant on genotype.

**Phenome** – The full set of phenotypes that define an individual

**Genotype** – a heritable element (or set of heritable elements) of DNA that leads to expression of a trait (see phenotype). Usually specifies a particular character. The degree to which a genotype is reducible to a single gene varies for each genotype. Genotype, by definition, determines phenotype. However, the genotype is often not fully characterised, then only the known or relevant part (that effectively determines the phenotype) is reported

**Genome** –
Organism level: The set of genes and genetic control elements that fully define an organism’s phenome

Organelle level: the set of genes and genetic control elements that are encoded in an organelle.

(Note: an organellar genome does not include [genes for] the extra set of proteins imported to the organelle, which are rather encoded in the nuclear genome)

**Homoplasy** – unrelated convergence of a genotype or phenotype, as opposed to divergence from a common ancestor

**Sister taxon or sister group** – an organism or group that is the closest
known relative of another organism or group

**Character** – A measurable entity possessed by an individual or a set of individuals. It may be as small as a single base change in a gene, or as large as the neck of a brachiopod dinosaur, i.e. it may be phenotypic, genotypic, or subgenotypic.

**Strain** – A cell line begun from a single cell and propagated clonally. Clone means:

**Group** -  
*Symiodinium* group: Defined in table 1.1

Phylogenetic group: A set of organisms that potentially derived from a single common ancestor.

**Monophyly** – Using the fullest set of taxa available in a given test lineage, an evolutionary tree of those taxa is derived based on some objective criteria. ‘Outgroups’ (other lineages) are also included as part of the analysis. If the test lineage makes a group containing no members of the outgroup/s, then the test lineage is declared as monophyletic. In the absence of a fuller test set or better criteria, monophyly can be interpreted as evidence for descent from a single common ancestor.

**Polyphyly** – Opposite of monophyly. Test lineage shown to be not monophyletic. The group may formerly have been believed to be monophyletic and thus may have been given a group name.

**Phylogeny** – The science of using genes to determine the branching order of ‘phyla’ (groups). Genes may be analysed by their sequences, by indels, or by presence/absence of the gene.

**Molecular ecology** – The science of investigating an organism’s ecology, population genetics, lifecycle, biogeography or evolutionary ecology usually in organisms living in the modern era.

**Indel** (insertion/deletion) – a section of DNA sequence that is present in one cell and absent in another, even though the sequences flanking the indel seem closely related.

**Clone** 
DNA clone; A piece of DNA that has been artificially ligated (joined) to a carrier DNA to facilitate propagation and purification.

Organism clone (see ‘strain’). Clone means: asexually related set, asexually derived set, or asexually propagated set.

**Cell line** – strain, or organism clone

**Clade** - 
*Symiodinium* clade:

**Subclade** - 
*Symiodinium* subclade:

**Phylotype** = Cohesion species = Biological species. Many would argue that phylotype = phylogenetic species = subspecies, but the argument in this thesis is that *Symiodinium* phylotypes as determined by Rodriguez-Lanetty match roughly to morphospecies so in this case are more likely to be biological species than phylogenetic species.
Independently evolving lineage – = Phylotype. Again the term “independently evolving” closely resembles the accepted notion of phylogenetic species, but see the explanation that accompanies the entry for phylotype.

Endosymbiont/endosymbiosis – for the purposes of this thesis: an endosymbiont is a chloroplast or mitochondrion. A chloroplast may have come into a eukaryotic cell by uptake of either a cyanobacterium, or a eukaryotic alga. In every case the uptake (endosymbiosis) is likely to have been before 1 billion years ago.

Plastid – = chloroplast. May be used to describe green or non-green chloroplasts. May also be used to describe photosynthetic or non-photosynthetic organelles. For relic chloroplasts the term ‘plastid’ is better than ‘chloroplast’ because the organelle may lack photosynthetic capability but retain a genome.

Homothallic – Gametes derived from a single clone can fuse to each other and create viable offspring.

Heterothallic (opposite of homothally) – Gametes require an ‘opposite’ mating type (in a binary system) or a ‘compatible’ mating type (in a multisex sytem) in order for two gametes to fuse together and create viable offspring.

Isogamous – Two fusing gametes are of the same size and shape.

Anisogamous – Two fusing gametes are of different sizes and/or shapes.

Hologamous - gametes are of the same size and appearance as the somatic cells

Homoplasy – The convergent acquisition of a character or a suite of characters, in a polyphyletic way.


