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CYTOLOGY, GENETIC VARIATION AND INHERITANCE STUDIES IN THE STURT DESERT PEA *Swainsona formosa*

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ABSTRACT

The Sturt Desert Pea (*Swainsona formosa*) has an attractive flower but possesses a number of characteristics that have prevented its broad scale production. An enhanced understanding of the limitations to production and application of characterised novel genetics will accelerate breeding efforts aimed at generating a series of Sturt pea varieties.

Research in this thesis encompassed three broad approaches. The first approach aimed to investigate genetic diversity within the species. Cytological investigations which comprised chromosome number determination and pairing observations revealed uniformity in chromosome number and did not detect any structural variation. AFLP analysis revealed considerable variability that was not identified in cytological studies.

A second approach involved the characterisation of a novel compact form and its inheritance. Objectives of this approach were to develop a quantitative method that differentiates compact and normal habit plants and to characterise the genetic control of the compact phenotype. It was identified that the compact form was conditioned by a single recessive gene, but was commonly inherited in a manner fitting two genes with inhibitory action.

As ornamental plants are often marketed in a series which is an important aspect of commercial success, a third approach aimed to investigate the inheritance of flower colour in Sturt pea. This approach made the assumption that the facultative outbreeding species would tolerate sufficient rounds of inbreeding to produce lines that were homozygous at flower colour loci and that the number of genes controlling flower colour would allow this approach. Furthermore, the assumption was made that genetic control could be deduced in F_2 or F_3 generations after hybridisation of homozygous parents. Homozygous lines for floral variants and a novel mutation were produced and dominance relationships were observed in F_1 and F_2 populations. Six major genes that control flower colour were identified in this study. Epistasis and pleiotropy were also recorded.

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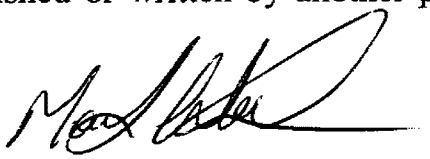
To my wife Natalie, your tolerance and encouragement has been invaluable. To my boys Nicholas and Bradley, you simply inspire me.

Certificate of Originality

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



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LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphisms
Bp	Base pairs
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
EtBr	Ethidium bromide
GLB	Gel loading buffer
HCl	Hydrochloric acid
JSC	Jaccards similarity coefficient
Kbp	Kilo base pairs
n	Haploid chromosome number
2n	Diploid chromosome number
N:P:K	Nitrogen : Phosphorus : Potassium
NSW	New South Wales
PBI	Plant Breeding Institute
PCR	Polymerase chain reaction
SA	South Australia
SDP	Sturt desert pea
TBE	(1x) = 89mM Tris borate, 1mM EDTA pH 8.0
UPGMA	Unweighted pair group method with arithmatic means
WA	Western Australia
W/T	Wild type

CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

The Sturt desert pea (Sturt pea) is an Australian native plant species that is attractive, unusual and unique. Cultural problems have prevented its widespread commercial production, however it's potential as a floriculture crop is widely recognised (Williams and Taji 1991). First described by George Don (1798-1856) Sturt pea has become well known in Australia, aided by it being the floral emblem of South Australia (Boden 1985, Ashby 1991). In foreign markets it has an identity as a unique Australian native wild flower, and the uniqueness of Australian native flora is recognised as providing considerable export potential. The potential of the Sturt pea as a cut flower or potted plant remains unfulfilled because relatively small amounts of research and breeding have been performed to overcome the factors that have prevented its production.

1.2 Classification

The Sturt pea is currently classified as *Swainsona formosa* in family Fabaceae and it is assigned to sub family Papilionoideae, and tribe Galegeae. The derivation of the name *Swainsona formosa* is *Swainsona* - after Isaac Swainson, a scientist and plant cultivator, *formosa* – from latin formosus meaning beautiful (Farrer 2000).

Tribe Galegeae comprises over 3000 species which belong to approximately 20 genera (Hutchinson 1964, Polhill 1981). *Swainsona* is one of the largest of these genera and consists of 84 species. All of the *Swainsona* species are endemic to Australia except *Swainsona nova-zealandiae* which is endemic to New Zealand (Allan 1961).

Fabaceae tribe Galegeae is part of a vast radiation into the temperate regions of both southern and northern hemispheres (Sanderson and Wojciechowski 1996). However,

much of the species diversity in Fabaceae is concentrated in the tropics and sub tropics. Genus *Clianthus* formerly included the Sturt pea when it was classified as *Clianthus formosus,* but it was re-assigned to *Swainsona* in 1990 (Thompson 1990). Alex George more recently proposed that Sturt pea is vastly different to members of genus *Swainsona* and should be reassigned to a monotypic genus and designated a new scientific name, *Willdampia formosa,* in honour of explorer William Dampier (Farrer 2000, George 1999a; George 1999b).

1.3 History of collection

The Sturt pea was first collected in 1699 on the islands of the Dampier archipelago. William Dampier described it as "a creeping vine that runs along the ground, and the blossom like bean blossom but much larger and of a deep red colour looking very beautiful" (Boden 1985). In July 1817 botanist Allan Cunningham made a second collection from a location vaguely described as the shores of Regents Lake on the river Lachlan (Cavanagh 1988). He called the plant *Kennedya speciosa* unaware of an earlier discovery (Cavanagh 1988, Williams 1996). He returned a year later on another expedition and found specimens of the plant again, this time on an island in the Dampier archipelago. When Cunningham described these plants in the transactions of the Horticultural Society of London he classified them as two different species – *Clianthus oxleyi* for the Lachlan river specimens and *Clianthus dampieri* for the Dampier archipelago specimens.

1.4 Ornamental value and interest

The Sturt pea has a spectacular flower, which is arguably one of the worlds most striking (Jusaitis 1994). Consequently this plant has great potential as a cut flower or as a container grown ornamental (Barth 1990). For some years there has been interest in developing the plant as a floricultural crop (Williams and Taji 1991; Kirby 1997). In South Australia the red and black form is considered most desirable and frequently other colours are rejected as not being authentic. The red and black colour combination has proven difficult to use in floral arrangements.

1.5 Historical cultivation

Sturt pea was imported to England by nursery growers Messrs. Veitch and sons of Exeter in 1834. A period of 16 years elapsed between the time of initial importation and the production of a flowering specimen for exhibition. The length of time between its importation and flowering was attributed to the plants unreliability and it was reported that "it dies suddenly in the most unaccountable manner" and "the treatment which produces complete failure in one case and brilliant success in another is apparently the same". It was determined that the soil media / root relationship and levels of root disturbance were often the determinants of the success in culture. Re-potting or transplanting requires care to minimise root disturbance and careful attention to watering is necessary to maintain optimum plant health.

1.6 Ornamental horticulture and requirements for large scale production

In ornamental horticulture a plant must meet certain criteria to be produced on a broad scale (Ball 1998). These criteria are often determined by demand trends and existing production protocols. The diverse plant materials grown by commercial production nurseries are often required to conform to the same or very similar production practices. Specific production considerations include ease of propagation, amenability to meeting production timeframes and scheduling, plant compactness or amenability to manipulation of habit through growth regulators, time to flower and available colour range (Ball 1998).

In recent years changes in marketing have heavily influenced crop selection, scheduling and sales. Today pre-selling large quantities plants to retail chains is common (Ball 1998). As a consequence the homogeneity of plant appearance, development and performance in production is of great importance. Production of homogeneous crops is reliant on provision of a consistent growing environment and plant varieties that are genetically uniform, at least for the loci that regulate characteristics that are relevant to plant production and plant appearance.
It is desirable to market ornamental potted plants in series that are comprised of several varieties that possess many common production characteristics and appear the

same to consumers except that they have a range of different flower colours and types (Ball 1998).

1.7 Sturt pea – cultural deficiencies

Sturt pea is a facultative outbreeding species (Thompson 1994) and plants generated from wild collected seed display variability in habit (Hamid and Williams 1997a), rates of growth and time to floral initiation (Jusaitis and Schmerl 1993), even when they are collected from a single plant. There is an enormous amount of quantitative variation in all growth characteristics (G. Kirby pers. Comm.).The use of plant growth regulators (PGR's) to control habit in the species has been investigated (Hamid 1996; Hamid and Williams 1997a ; Hamid and Williams 1997b) with results suggesting their application likely had some benefits but possibly limited commercial potential.

A range of habits have been described that extend from prostate and spreading to upright and bushy (Kirby 1996). Variability in floral characteristics and disease resistance has also been described (Jusaitis and Schmerl 1993). This effectively ensures generous scope for plant improvement exists. Collection of seed material from wild populations has in recent years diversified the gene pool available to seek solutions to long standing culture deficiencies (Kirby 1996). Whilst knowledge of important characters underpins efforts to breed commercial varieties efficiently, there are no published studies on the genetic control of any fundamental trait in this species upon which improvement programmes can be based.

No production protocol has yet been developed for this species that could be readily adapted to current commercial production. Numerous studies have been undertaken over long periods to develop commercial cut flower and potted plant varieties although to date no commercial cultivar has been reported or released.

The absence of a reliable production method, a poor understanding of the species and limited effort to develop the species by breeding has contributed to its absence from commercial production.

1.8 Seed treatments

Seed germination is one of the more difficult aspects of the growing process in commercial production (Ball 1998). Jusaitis and Schmerl (1993) undertook detailed studies into culture protocols for Sturt peas, starting with seed treatments. Like many Australian native species, the Sturt pea has a hard seed coat that acts as an inhibitor of germination. Treating seeds with boiling water is the most common pre- germination treatment to increase uniformity of and rates of germination in plants such as Senna, Acacia and all of the pea family as the seed coat is impervious to water (Wells 2000). Plants requiring this type of treatment are often native to areas where bushfires occur at regular intervals – the heat of the fire cracks the hard coat and allows moisture to reach the embryo inside. The boiling water simulates this effect (Wells 2000).

Three different types of seed treatments were assessed by Jusaitis and Schmerl (1993). Nicking the seed manually produced rapid and uniform germination. This out performed all other approaches. Seeds soaked in boiled water until cooled had a germination response dependent upon the volume of water used. This was attributed to the longer time required for a large volume of water to cool down, resulting in heat induced death of a proportion of seeds. Various sodium hypochlorite treatments failed to achieve any increase in germination above controls.

Supporting this study is work of Humphrey (1972) who found that chipping of the testa provided a germination rate of 90% as compared to untreated controls. Boden (1985) agreed that soaking seed in warm water gives variable results and boiling water should not be used as it destroys beneficial bacteria on the seed coat. Microbial associations and the role they may play were noted by Webb (1972). He commented boiling water treatments may prove more detrimental than beneficial as it would kill rhizobium bacteria on the seed surface. Kirby (1991) later established an association between rhizobia and better growth of Sturt pea but has not identified specific bacterial strains. Williams (1996) reported that there appears to be more than one rhizobia strain involved.

Sturt peas usually germinate within 2-3 days if abraded and given hot water treatment (Williams 1996). However in practice the germination of the seed is very variable partly due to variation in the quality of seed, when harvested from wild plants and plants of differing ages. This variability must be overcome in a mass production that is reliant on seed propagation.

1.9 Plant growing media

Jusaitis and Schmerl (1993) found a media consisting of 2 parts scrub sand: 1 part coarse washed river sand: 1 part sieved peat moss: 1 part vermiculite: 1.1kg / m^3 Osmocote (8-9 months N:P:K - 17:1.6:8.7) and 300 g / m^3 Micromax to be successful during seedling establishment. After plants were potted into larger containers and fertiliser treatments were undertaken, differences between potting mixes disappeared and plants grew equally well in a range of media. Huxley (1992) found an open, gritty sand based mix was required for success. Williams (1996) found a sand based media to be required but was marginally more concise adding a coarse sand is required in the garden, and media for pot culture was a 3:1 mixture of coarse washed sand : peat with perlite, vermiculite or fine composted pine bark to lighten the mix. Wells (2000) found greatest success was achieved in the U.K using a 50:50 combination of two different sands ranging in particle size from 1mm to 5mm. Kirby (1997) in pot plant trials used a commercial media of pine bark / sand and supplemented with 1 teaspoon of iron sulphate and half a cup of lime per 10L volume.

1.10 Environmental requirements

Sturt peas prefer full sun and grow most actively at temperatures of 25-30^{oC}, however, they will survive temperatures of greater than 40^{oC} providing a stable supply of water is maintained (Williams 1996). When grown under glasshouse conditions an increase in shoot growth was reported as temperatures were increased from 25^{oC}/18^{oC} day/night to 35^{oC}/28^{oC} (Jusaitis and Schmerl 1993). The increased height of plants grown at higher temperatures was attributed to an increase in vegetative growth.

Similar results were observed by Haupt (1969) in *Pisum sativum* with a delay in flowering of up to six nodes.

Williams (1996) identified that warmer night temperatures result in more compact plants while big variations in temperature between days and nights encourages longer shoots. Barth (1990a) found that if similar day and night temperatures are maintained plant height can be sufficiently controlled. Huxley (1992) recommends a minimum night time temperature of 7-10 °C.

Sturt peas can tolerate high temperatures but will often show foliar damage if placed under extreme humidity (Williams 1996). Good ventilation in and around these plants is critical (Jusaitis and Schmerl 1993; Williams 1996; Huxley 1992). They require protection from wind and rain, but these must not compromise high levels of light. Barth (1990) says a high light intensity is critical for flowering.

The Sturt pea is day length insensitive, and plants can flower all year round given adequate temperatures in a glasshouse (Williams 1996, Erwin and Warner 2002). Extending the day length with supplementary lighting did not affect floral initiation, but flowering did occur earlier, probably due to promotion of vegetative growth (Jusaitis and Schmerl 1993). After trialling a range of temperature regimes it was found decreasing temperature induced earlier flowering on a lower node resulting in a higher total number of flowers per plant (Jusaitis and Schmerl 1993). Low light intensities due to heavy shading will delay flowering (Kirby 1997).

1.11 Vegetative propagation - cuttings

Vegetative propagation of Sturt peas has proven difficult. Sturt peas exhibit vegetative dimorphism (Jusaitis 1997). Seedlings characteristically have a main stem with alternate phyllotaxy and a single pinnate leaf at each node, arranged spirally up the stem. Lateral shoots arising from axillary buds on the main stem display distichous phyllotaxy, again with a single pinnate leaf at each node. While the main stem is

generally orthotropic in growth response, lateral stems are generally plagiotropic, the degree to which depends on the clone. Cutting derived plants originating from lateral stems, or from decapitated main stems display plagiotropic growth and distichous phyllotaxy (Jusaitis 1997).

Kirby (1997) developed a cutting propagation protocol for Sturt pea, but offered the following comment. "most Sturt pea plants cannot be propagated by cuttings because the stems rot, meristems rot, leaves collapse or wilt disease kills the cutting after potting on". Jusaitis and Schmerl (1993) found it increasingly difficult to find suitable cutting wood, due to the monocarpic nature of development and the progressive production of only floral buds on adult plants. Williams (1996) identifies that the cutting type, pre-treatment, potting medium and environmental conditions were important determinants of success of vegetative propagation, but suggested that the genetic make-up and growing conditions of the stock plant were of greater importance. Williams (1996) reported good success using a propagation medium of sand : peat : vermiculite (2 : 1 : 2). In contrast to this, Jusaitis and Schmerl (1993) obtained best results using perlite with the number of roots per cutting and mean root length significantly improved. When Kirby (1997) used horticubes® for propagation he claimed that "depending on the clone being propagated, roots appear through the foam in 9-14 days". He also identified that rooting occurred more quickly when temperature was maintained at 30^{c} instead of 25^{c}. Jusaitis and Schmerl (1993) found that cuttings generally rooted in 2-4 weeks. It is unclear if both were describing timeframes of the same degree of root development. The literature of both authors states auxin not to be of great significance in increasing percentage rooting or root length above controls. For this reason Kirby (1997) says sometimes a commercial hormone is applied and sometimes no treatment is used. However, Jusaitis and Schmerl (1993) identified a significant increase in root number per cutting to occur for all tested combinations of IBA + NAA tested. Williams (1996) says a typical hormone treatment for *Clianthus* is a 5-second dip in a mixture of 1000-2000ppm each of IBA and NAA in 50% ethanol. Williams and Taji (1992) found neither gibberellic acid or benzyl amino purine increased possible cutting production.

A common theme found among the literature is the recommendation of not using mist and plagiotropic growth from axillary buds being a major problem associated with

cutting production from this species. Furthermore, rooted cuttings tend to have poor root systems (few roots, largely concentrated at the cutting base) in comparison with seedlings, leading to inferior anchorage and low root : shoot ratios (Jusaitis 1997).

1.12 Vegetative propagation - Grafted plants

Grafting is commonly used to overcome root related failure in Sturt peas. The advantage in grafting lies in providing a plant with a new root system; however it does nothing to solve above ground problems. The process is also costly and time consuming, but has been demonstrated in other species to confer pest and disease resistance, increased cold, soil type and pH tolerance (extended geographic range), greater productivity and increased lifespan (Dawson 2002; Hartmann and Kester, 1975).

Two methods have been employed to graft this species. Conventional grafting using more mature tissue with wedge or whip and tongue grafts, or wedge grafting at the cotyledon stage. Anon (1999) reports success with conventional grafting has all been with "actively growing side shoots or the terminal shoot of a young plant. When younger terminal shoots are used, the stem is firmer in the centre and actively lengthening". Anon (1999) provides a protocol for wedge grafting at the cotyledon stage.

Grafting onto *Clianthus puniceus*, the New Zealand Glory Pea (McKenzie 1981) has enabled growing of Sturt pea in the cooler and wetter parts of Australia. The main benefits of grafting using *C. puniceus* as a rootstock (McKenzie 1981) include the root system is much longer lived, better adapts to seasonal variation and has greater regrowth and rejuvenation if damaged. The grafting technique is currently being used by Bushgrafts native nursery in Victoria Australia to maintain some valuable genotypes. Kirby (1996) discusses other species for use as rootstocks to achieve greater longevity of plants in commercial cut flower systems and to reduce chances of

crop failure. *Colutea arborescens* and *Sutherlandia frutescens* have also been used as root stocks for grafting Sturt pea.

Mechanisms and symptoms of graft incompatibility in Sturt pea was investigated by Kawaguchi and Taji (2005) with key findings being a higher concentration of CO_2 being found around graft compatible unions than incompatible unions.

1.13 Vegetative propagation - Micropropagation

Development of marketable forms of Sturt pea has been severely hampered by the lack of rapid clonal propagation techniques capable of reliably preserving the genetic purity of selected lines (Jusaitis and Schmerl 1993).

Sturt pea demonstrates varying degrees of monoaxial growth depending on the propensity of the lower buds on the main stem to produce lateral stems. The leaf axils of main and lateral stems bear dormant stem buds or flowers. Once a leaf axil initiates a flower primordium, all subsequent nodes on that axis do so as well until growth is terminated (Jusaitis 1997). Floral buds proved to be unsuitable explant material as they generally failed to produce adventitious shoots (Williams and Taji 1987), and shoot proliferation from nodal sections of mature plants was very low, with 32% of cultures producing shoots with an average of 3 shoots per culture (Taji and Williams 1989).

McIntyre and Whitehorne (1974) propagated Sturt peas by tissue culture in what was probably the first attempt with a leguminous plant. Callus formation on a Knudsons C and coconut milk media occurred readily within an 8 week period, and shoots and roots produced within 3 months. It was noted that when the callus tissue just began to form on the seedlings it was white, but after a few weeks in the light it began to turn a very bright green. Callus of both colours responded quite differently. White callus on Murashige and Skoog high salt medium grew, did not become green and then some turned brown. No differentiation occurred in 4 months. Several media were tried with

no success. Green callus on the same media again did not differentiate, but did increase in size. When this callus was divided and placed on Murashige and Skoog basal medium, plus kinetin, shoots were produced in approximately 4 weeks. This study and studies of Ermayanti *et al.*, (1993), using *Swainsona galegifolia* and Nasinec and Nemcova (1990) using *Galega officinalis* and Taji and Williams (1989) on Sturt pea appear to be the only studies using species of the Galegaeae (Papilionoideae) tribe. Jusaitis (1997) and Jusaitis and Schmerl (1993) question the usefulness of work on seedlings to continued breeding by highlighting the importance of using explants from mature adult plants that have been selected on the basis of some desirable character. Important findings were made by Jusaitis (1997) for micro propagation of adult plant material.

Jusaitis and Schmerl (1993) identify explants taken from mature plants to callus readily, but be difficult to stimulate morphogenesis in, and note the few shoots that are formed often become hyperhydritic / vitrified. Williams (1996) says *Clianthus* is particularly susceptible to this disorder and it is usually associated with excess humidity and high levels of plant hormone (particularly BA) in the medium. This is supported by Taji and Williams (1989) who report a large number of cultures of Sturt pea produced from hypocotyls or cotyledon segments were vitrified. They also found the use of gelrite instead of agar increases the incidence of vitrification in this species.

The problems of plagiotropic growth in Sturt pea cuttings and distichous phyllotaxy have not been overcome using micro propagation according to Jusaitis (1997). These problems were present in primary culture plants and were present in all sub cultures. This is a commercially undesirable difficulty as it destroys the form of the plant. Gupta and Durzan (1987) showed plagiotropism in micro propagated tree shoots was determined by the position on the parent plant from which the explant was taken, while Timmis *et al.*, (1992) and Cameron and Sani (1994) demonstrated this was tied in with maturity of parent tissue. Jusaitis (1997) says this means shoots derived from more mature tissues are more susceptible to plagiotropism. Interestingly work of Taji and Williams (1989) did not report plagiotropic growth in progeny of seedling stock explants.

1.14 Pests and Diseases of Sturt pea

Root disease is thought to be the primary constraint to successful propagation and plant longevity, both of which are key requirements for commercial production (Williams 1996). During the winter water application needs to be reduced to prevent damping off to which they are particularly liable (Kirby 1997). The plant is susceptible to *Fusarium*, and *Pythium* has been isolated from affected plants. In Sturt peas *Fusarium* control is critical as infection is rapid and fatal (Angas 1996). Benlate and prochloraz-Mn have been recommended as effective controls (Angas 1996). Williams (1996) also discusses collar rot as a problem a fungicide regime can help prevent.

Williams (1996) reports high susceptibility of the plant to two spotted mites, and suggests the use of predatory mites for control in a glasshouse environment. Kirby (1997) has found two spotted mites and aphids to be serious pests of the plants before flowering. Additionally he discusses heliothis moth caterpillars being potential problems and suggests the use of carbaryl as it has minimal effect on predatory mites if used.

Angas (1996) discusses measures to be taken to control the incidence of disease in Sturt peas focusing on personal hygiene and stringent hygiene practices for the surrounding environment. Disinfection of floors and benches, cleaning tools, sterilising potting media, and the use of new / clean pots and trays rather than old has been recommended.

1.15 Floral structure of Sturt pea

In Sturt pea, the flower is up to 100mm long, flag shaped consisting of an upper standard petal (or flag) that incorporates a shiny usually black boss, and a lower keel that houses the sexual organs (Jusaitis 1994).

At the bottom of the keel are stylar brush hairs. These anterior facing hairs play an important role in pollen extrusion from the keel during bird pollination (Arroyo 1981). Stigmatic cuticle barriers to self pollination have been identified in other Papillionoid legumes by Shivanna and Owens (1989). Jusaitis (1994) has found cuticle disruption to expose the surface, or at least cuticle bruising to release stigmatic exudate was necessary for pollen germination to occur. This thick cuticle has historically reduced the probability of self pollination and has enhanced the possibility of bird pollination (Jusaitis 1994).

A close relative of the Sturt pea, *Lotus berthelotii* has been observed by Owens (1985) to contain a similar cuticular system. Anthesis has been found to occur several days prior to flower opening, usually on the same day the wings begin to separate and reflex.

Pollen of the Sturt pea was found to be desiccation tolerant (Hughes *et al.*, 1991). This should facilitate long term storage if required, as has been demonstrated in other species using the same treatment described by Towill (1985).

1.16 Floral colour in Sturt pea

The use of Sturt pea in ornamental horticulture could be greatly enhanced by the use and exploitation of the available floral colour variation combined with a suitable habit that performs well in commercial culture.

Most literary references to flower colour in the species describe the red / black floral type. This has been greatly influenced by the adoption of the flower as the floral emblem of South Australia (Boden 1985) and the subsequent publication of the associated image. The recorded range of colour is far broader, and extends from the varying shades of red, through to pinks, apricots and whites (Ashby 1991; Anon 2003; Boden 1985; G Kirby pers. Com; M Jusaitis pers. Com).

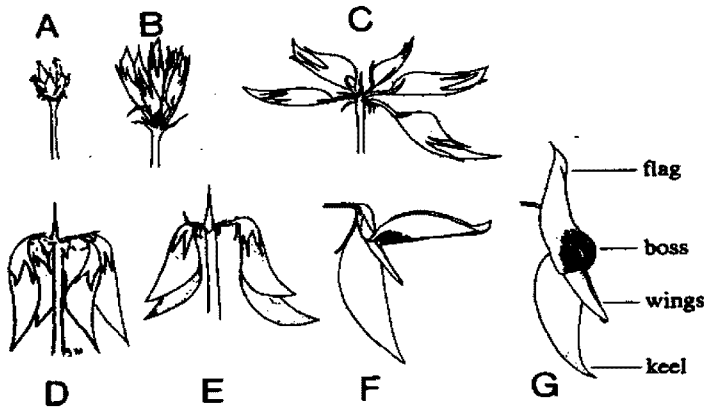
Among the pea flowers within the legumes, yellow is the most common colour, often with a red or orange centre, but colours of every shade from red through to white can be found (Wrigley and Fagg 1996). Sturt pea does not appear to contain as full an array of colours found broadly in the legumes.

The species is frequently found in populations of the single flower colour i.e red / black (or subtle variants thereof). Small populations however have been found that contained exceptionally broad diversity, ranging from reds and pinks through to white (M. Jusaitis pers. Comm). This appears a very restricted finding and seems atypical for the species.

There are no published studies that identify the number, action and interaction of genes involved in the expression of flower colour in this species.

1.16.1 Floral development in the Sturt pea

Figure 1.1 Floral progression in Sturt pea



Source: Jusaitis 1994

Description of floral stages in Sturt pea according to Jusaitis (1994) from previous page.

A= Flowers tightly clustered in bud, petals completely enclosed by calyx.

B= Tips of petals begin to protrude through calyx, showing first signs of colour.

C= Flowers oriented horizontally during the process of downward curvature of pedicels.

D= Pedicels complete downward curvature and flowers oriented vertically.

E= The flag begins to reflex back from the keel.

F= The flag in a half reflexed position is horizontal.

G= The flag is fully reflexed and flower is open.

1.17 Floral aberrations observed in Sturt pea

Many floral mutations have been found in breeding lines of varied parentage (M.Jusaitis pers. comm.) Examples are; varying angles of floral presentation (splayed flowers), two rings of flowers on a peduncle, double flowers, extra dense or loose flower heads, droopy flags, vestigial flowers, protruding styles and split keels.

The double flowers consisted of a twin flag and boss but a single keel. None of these had their inheritance investigated. Dwarf / highly compact forms have also apparently been observed with morphology of these plants being normal and relative in size to the overall plant frame. Plants showing floral aberrations were found often to display the aberration inconsistently within and between generations (G.Kirby pers. comm.). The inheritances of most floral mutations are thought to be recessive in nature as they were exposed by inbreeding. However, this has not been statistically proven (G.Kirby pers. comm.).

1.18 Control of flowering in Sturt pea

It has been stated flowering in Sturt pea does not appear to be under photoperiodic control. Results of Jusaitis and Schmerl (1993) indicate the plant is day neutral. Interestingly their studies found flowering occurred nearly 2 weeks earlier in plants given extended lighting. The bringing forward of flowering time was attributed to the increased growth rate concurrently observed, not to a direct affect on floral induction. This can still be a commercially significant gain.

Jusaitis and Schmerl (1993) also tested the theory that by reducing / removing lateral shoot growth, the redirection of available photosynthate would encourage earlier flowering. They found removal of leaves, lead to reduced photosynthetic capacity and a delay in flowering. The minimal leaf area required to support optimal growth was found to be 5-6 pinnate leaves.

When growing from seed, Jusaitis and Schmerl (1993) found time from sowing to first bloom ranged from 73 to 170 days. Yates seed packets advise a period of 24 weeks (164 days) from seed sow to flower. Williams (1996) has observed time from seed to flowering can range from 10-16 weeks (70-112 days). Much variation has been observed in time to flower especially in prostrate forms, some flowering when still quite small (13cm runners) while others delay flowering until runners are 40cm long (G.Kirby pers. comm.)

According to Williams (1996) floral initiation takes place at a very early stage and does not appear to be affected by environmental conditions. Flowers in the lower nodes frequently abort, and will also occur at higher nodes when the plant is stressed.

1.19 Genetic diversity in Sturt pea

Of the 84 *Swainsona* species in Australia (Allan 1961), a small number have been included in three notable molecular studies, each phylogenetic in nature (Wagstaff *et al.*, 1999; Wojchieowski *et al.*, 2000; Wojchieowski 2003). Wojchieowski *et al.*, (2000) has proposed the existence of a clade that includes the genera *Carmichaelia*, *Clianthus*, *Montigena* and *Swainsona.* By examining ITS sequences and nuclear and chloroplast genes, these studies have added a new level of understanding to intergeneric relationships.

As a by-product of these studies, further light has been shed on some relationships amongst *Swainsona* species. Species examined in the study of Wagstaff *et al.*, (1999) showed two separate clusters placing *S. pterostylis*, *S. formosa*, *S. stenodonta* and *S. decurrens* together in a sub cluster removed from the 12 other *Swainsona's* sampled.

The value of these developments is unquestioned, however information provided in these studies can not be directly and immediately applied to the improvement of Sturt pea. Importantly, while the geographic distribution of the species has been documented (Ashby 1991; Boden 1985), relevant information on genetic variability across this distribution is absent and will continue to be a constraint to progress until studies dealing explicitly with the subject are undertaken.

1.20 Cytogenetic status of the species

Thompson (1994) has stated 2n=32 to be uniform within the species whilst Zulkairnain *et al.*, (2002) has reported 2n=16. It has not been established which chromosome number is typical for the species. The possibility both are correct and these findings reflect different geographic origins or an evolutionary adaptation has not been explored. These studies alone form the current knowledge on Sturt pea cytology.

Of the 23 genera in the tribe Galegae, 17 are characterised cytologically and all have a base number of x = 8 (Goldblatt 1981). Aneuploidy appears restricted to one large genus while polyploidy is more common. Lee (1948) first speculated about polyploidy in the genus *Swainsona* suggesting large floral morphology was evidence. This same character has been used as argument for re-classification into a monotypic genus (George 1999).

No studies have been undertaken to assess chromosomal number and genome structure across the broad distribution of the species on mainland Australia. This is considered essential for understanding breeding constraints and possibilities. By determining the typical chromosomal complement and any possible variations we may be more able to determine evolutionary relationships, predict most compatible candidates for exploring interspecific hybrid production and identify barriers to character introgression within the genus.

1.21 Interspecific hybridisation

Other *Swainsona* species have been identified as possible sources of useful characteristics necessary for the widespread production of the Sturt pea. However, no interspecific crosses have been performed that have resulted in true hybrids (G.Kirby pers. Comm.). Crosses with both close and far relatives in the genus *Swainsona* have been attempted. Occasionally, when the Sturt pea was the ovule parent in crosses with *Swainsona macculochiana* a few seeds would be produced, which would subsequently grow into healthy plants without any hybrid characters (G. Kirby pers. comm). *Swainsona* relatives have proved very difficult to obtain seed set with (~20spp) when artificially pollinated, but when insect pollinated will have abundant seed set. (Dr G. Kirby pers. com.)

Difficulties have been commonly reported in creating interspecific hybrids in other leguminous relatives and valuable information may be gleaned from these efforts. In *Phaseolus*, few crosses yielded seeds and results were genotype dependant (Mok *et*

al., (1978) and exhibited high seed mortality (Andrade-Aguilar *et al.*, 1988). Similar results are reported in *Vicia* (Roupakias and Tai 1986)(see Cubero 1982 for a comprehensive listing of *Vicia* interspecific crosses attempted and their outcomes). Extremely low cross-ability is reported between *Glycine* species (Ladizinski *et al.*, 1979) and in the sub genus *Glycine Willd.* (Singh and Hymowitz 1987). In grasspea (*Lathyrus sativus*) interspecific crosses were more successful if the wild parent was the maternal parent, but overall still very low (Yunus and Jackson 1991).

1.22 Intergeneric legume hybridisation

There is little evidence of successful intergeneric crosses in legumes (McComb 1975).

Knobloch (1972) listed 8 reported intergeneric leguminous crosses. All are in the sub family Papillionoideae. McComb (1975) listed another five he sourced from literature, and then methodically claimed all bar one not to be valid hybrids. Reasoning was acceptable for most, especially when a grafted scion species exhibiting some morphological changes was described as 'hybrid'. Putative pea hybrids have been heavily questioned due to the current knowledge in peas there is a high frequency of apomixis (Sobolev *et al.*, 1970).

Sobolev *et al.*, (1968) reported hybrids between *Vicia faba* and pea which had chromosome numbers between 2n = 12 and 16. Then Sobolev *et al.*, (1971) reported the non homologous chromosomes of peas and beans formed bivalents which separated to give two groups, each composed of the chromosomes of one parent.

1.23 Apomixis / Matramorphy

The Sturt pea is a facultative outbreeding species and exhibits inbreeding depression, when self pollinated. However, instant homozygosity through matromorphic seed (not apomictic) may be an alternate route. Advice from Dr G.Kirby after attempting interspecific crosses suggests this may be feasible. He reported finding putative

hybrid progeny looking like Sturt peas. Dr M.Turner (Univ of Syd) reported seed being produced after prickle pollination having a high mortality, but progeny in F_2 and F_3 generations being very similar phenotypically.

1.24 Male sterility / pollen sterility

Male sterility in Sturt pea has been identified (G.Kirby pers. comm.) in three different breeding lines. In one of these lines the sterility is temperature sensitive (warm weather facilitating the pollen sterility, cool weather aiding fertility). Temperature does not affect male fertility in the other two lines. All are usually inherited as recessive alleles of nuclear genes.

1.25 Aim of the thesis

There is a lack of understanding of the diversity and barriers to breeding in Sturt pea, and the inheritance of characters that are important to ornamental horticulture is not known. This thesis aims to generate information that will enhance the efficiency of genetic improvement of the species and expedite the generation of commercial varieties that are suitable for commercial production. The cytology of the species will be established and gross differences in genome composition will be investigated to establish if there are likely to be any breeding barriers or differences that may be exploited to generate improved types.

An investigation of genetic variation in the species will be performed using AFLP analysis on DNA isolated from accessions in different geographical regions.

A quantitative method for identifying a novel compact form was developed and this information was applied to establish the genetic control of the character. In addition, the genetic controls of the major flower colours within the species were to be characterised, as was the most novel of floral colour mutations found.

CHAPTER TWO

CYTOGENETIC CHARACTERISATION OF STURT PEA

2.1 INTRODUCTION

In recent history the Sturt pea has been assigned to, or been proposed to be assigned to, the genera *Clianthus, Swainsona, Willdampia* (Walters 1999).

In the most recent revision of genus *Swainsona* the Sturt pea was reassigned to *Swainsona* from *Clianthus* (Thompson 1990). This assignment has met some opposition and post its inclusion a proposal that the species be transferred to a new monotypic genus (*Willdampia*) was made (George 1999). Lee (1948), in the first revision of genus *Swainsona* suggested the large floral morphology characteristic of the species may be evidence of polyploidy. This was one character used in the justification by George (1999) for re-assignment. Most species in the genus have not been examined cytologically and of those that have, variation in genome structure and chromosome number is generally not well documented.

The published literature of Thompson (1994) specifically addressing the cytology of Sturt pea, identified that somatic cells had a chromosome number of 2n=32. This complement was reported to be uniform within the species but was not well supported with suitable evidence, a point acknowledged by the author (J. Thompson pers. Comm.). Support for a claim of 2n=32 being uniform in the genus is available (Yeh *et al.*, 1986 ; Ermayanti *et al.*, 1993) and this does link the right groups in the phylogenetic sense (J. Thompson pers. Comm.).

More recently a cytological study reported that twenty genotypes of Sturt pea collected as seed from South Australia had a somatic chromosome number of 2n=16 (Zulkarnain *et al.*, 2002) thus questioning previous published claims. Conflicting reports on chromosome numbers in legumes is historically common (Goldblatt 1981).

Little is understood about the variability in chromosome number and genome structure present in Sturt pea within the natural distribution on mainland Australia. The possibility both published reports are correct and is reflecting cytological diversity within the species has not been investigated.

The aim of this chapter is to determine the typical 2n chromosomal complement. An understanding of cytological variation within Sturt pea may contribute to a better understanding of evolutionary relationships between genera and will provide information that will expedite the development of improved Sturt pea varieties.

2.2 MATERIALS

2.2.1 Mitosis

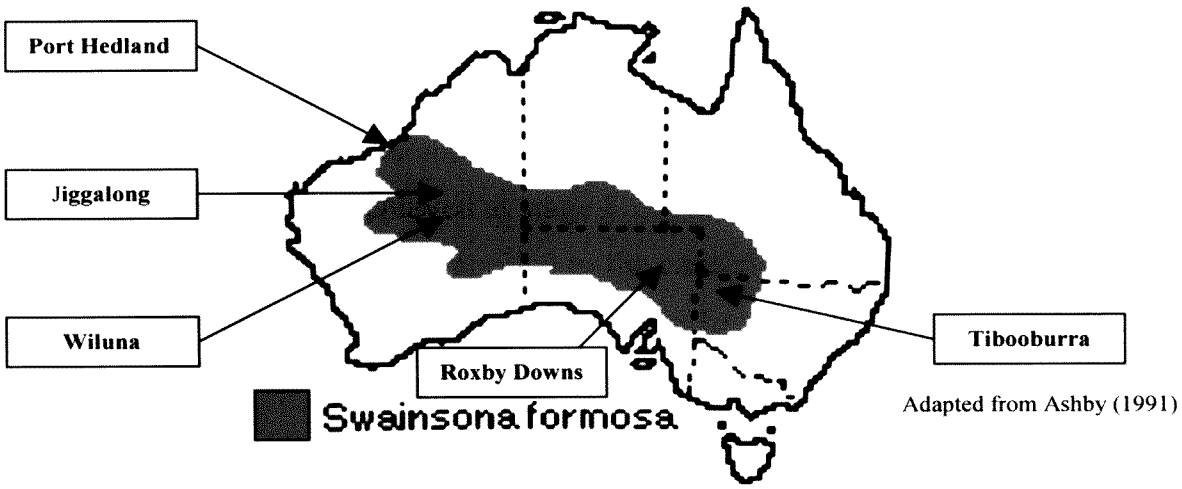
Five (5) Wild Type W/T genotypes from RoxbyDowns, South Australia (Lattitude 30.5^0S, Longitude 137^0E) and five (5) W/T genotypes from Tibooburra, New South Wales (Lattitude 29^0S, Longitude 142^0E) were analysed. All ten (10) genotypes were the standard Red/Black flowered types.

2.2.2 Meiosis

Wild type (W/T) seed of known geographic origin was sourced from five (5) localities across Australia for meiotic analysis (Figure 2.1). Ten genotypes from each region were included. In addition to those regions listed in 2.2.1, locations of accessions used were; Wiluna, Western Australia (Lattitude 26.5^0S , Longitude 120^0E), Jiggalong, Western Australia (Lattitude 23^0S, Longitude 121^0E) and Port Hedland, Western Australia (Lattitude 20^0S, Longitude 118^0E). All fifty (50) genotypes were the standard Red/Black flowered types.

Seed was sourced (with cooperation of the authors) from the same batch which provided genotypes for assessment in the study of Zulkarnain *et al.*, (2002). Ten (10) genotypes were assessed.

Figure 2.1 Distribution of Sturt pea on continental Australia and the geographic sources of accessions analysed.



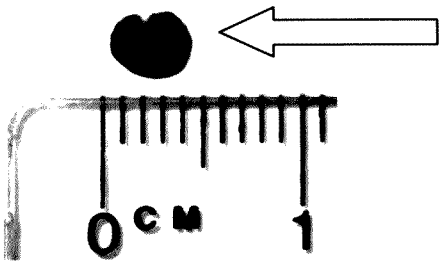
2.3 METHODS

2.3.1 Seed sampling and treatments

Seed was harvested from specified locations (Figure 2.1) and bulks of each location were made. Seed were randomly selected from these bulk samples for analysis.

The testa of all seed was manually 'nicked' with a sharp scalpel blade prior to germination. This technique broke the hard seed coat and allowed penetration of water (Jusaitis and Schmerl 1993). Seeds were nicked on the side that contained the cotyledons to ensure that the embryo was not damaged (Figure 2.2).

Figure 2.2 The morphology of Sturt pea seed and identification of the correct side to 'nick' prior to sowing.



2.3.1.1 Emasculation and pollination

Sturt peas have a floral structure that facilitates the collection and maintenance of pollen at the base of the keel petal within the confines of the flower (Fig 2.2C). This provides a neat pocket that concentrates pollen around the stigma for self pollination or is capable of being removed for use in the transfer of pollen to a selected female candidate. All candidate female flowers had their anthers removed before they began to dehisce. This is best achieved at stage E of floral progression (see Fig 1.1). During controlled crosses, pollen was applied to the candidate stigma using the pocket and was manipulated between the thumb and index finger (Fig 2.2B). This figure has the keel petal removed for the purpose of showing the methodology. To maximise success, this removal is not recommended. Figure 2.2A identifies stage G of floral progression (Jusaitis and Schmerl,1993) being the suitable stage selfing or for removal of collected pollen from a donor male parent.

Fig 2.2A

Stage G of floral progression - suitable stage for selfing or pollen collection

Image source:
Jusaitis and Schmerl 1993

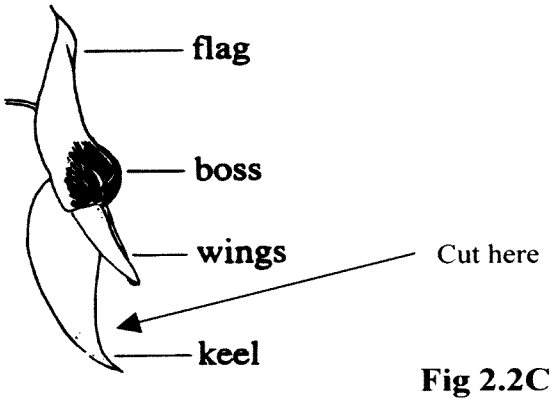


Fig 2.2B
The application of pollen to a candidate stigma

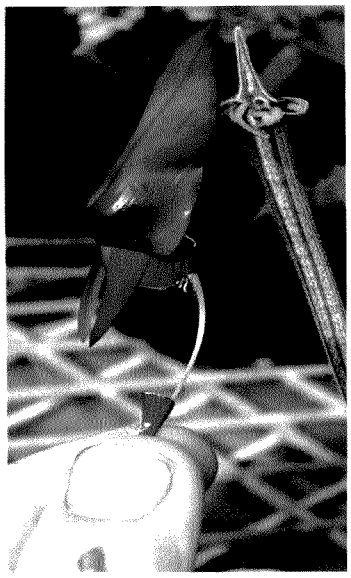
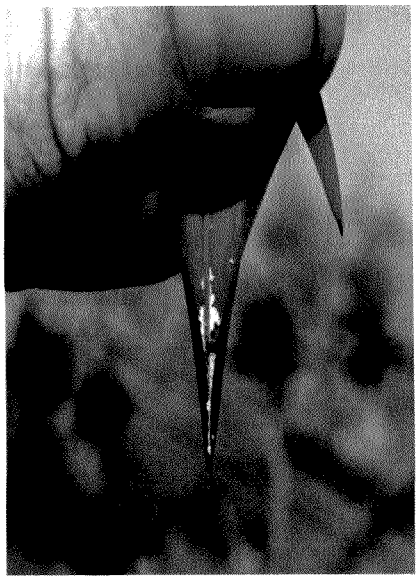


Fig 2.2C
Pollen collecting at the base of a wild type flower.



2.3.2 Growing media and environment

Plants were grown in a potting media that consisted of a mix of fine, medium and coarse composted pinebark (80%), Coir (15%), and fine sand (5%). Each cubic metre of soil media was supplemented with 0.125kg nitrogen, 1.0kg gypsum, 3.0kg lime, 0.2kg ferrous sulphate, 1kg Micromax slow release iron, 2.0kg Nutricote Red (N:P:K - 13.5 : 5.7 : 9.1), 1.0kg Nutricote Yellow (N:P:K – 14 : 6.1 : 11.6) 1.0kg , and 2.0kg Nutricote Black (N:P:K - 16 : 4.4 : 8.3). Plants were grown on raised benches in 150mm pots in a multispan shadehouse. Irrigation was by handwatering with pest and disease control subject to the commercial practice of Oasis Horticulture. Natural daylength was provided.

2.3.3 Determination of mitotic chromosome numbers

Seed was manually nicked and placed in petri dishes that were lined with filter paper (Whatman size 42). A half strength solution (manufacturers recommendation) of liquid soluble fertilizer Aquasol® was applied in sufficient quantity to dampen the filter paper. Lids were then placed on the petri dishes which were kept at 21°C under natural daylight and daylength until the root tips were excised.

Root tips were harvested when the radicle was 8-10mm long. Root sections (5-6mm) that contained the tip were removed and placed in distilled water, where they were maintained at 2^{0C} for 24hrs. The sections were then removed and placed in fixative, (3 parts ethanol alcohol: 1 part acetic acid), where they were kept at room temperature for 2hrs. The fixative containing the root sections was cooled to 4^{0C} and the root sections were maintained at this temperature for 24 hrs.

Root tips were then removed from the fixative and placed in glass vials containing pre-heated (60^{0C}) 1M HCl for 12-13 mins. The root sections were then placed in Feulgens stain (Phillips 1981) for 30 mins. Purple staining meristems (distal 0.1mm), were excised on a glass slide.

Meristems were dissected into 4-5 smaller pieces on a glass slide. One drop of Aceto-orcein (Phillips 1981) was applied to the dissected pieces which were allowed to sit

for 1min. A coverslip was then placed on top of the stained sample and tapped firmly to assist in spreading cells into a monolayer.

The prepared slide was then squashed between blotting paper and gently heated over an alcohol flame for 5 seconds. For each genotype observations were made on ten clear cells.

Cells were viewed at 100x magnification using a Zeiss Axioskop 50 130 VA TYP B microscope. Photographs were taken using a Nikon Coolpix 4500 digital camera.

2.3.4 Determination of meiotic chromosome numbers

Ten genotypes from each of 5 regions (see Figure 2.1) and 10 genotypes from a seed batch that had previously been investigated (Zulkairnain *et al.*, 2002) were examined. Four hybrid combinations (Table 2.1) were selected and five (5) genotypes of each were generated and examined.

Meiotic studies were performed as per Zulkairnain *et al.*, (2002) with minor modification. Anthers were collected from flower buds (13.7-13.9mm long), placed on a slide, cut in half and 1-2 drops of aceto-orcein applied. After 1-2 minutes a coverslip was placed on the tissue and tapped firmly around and over tissues underneath. The slide was then squashed between blotting paper and gently heated over an alcohol flame for 5 seconds. Observations were made as described in Section 2.3.3.

The chromosomes of ten (10) clear cells were counted for each genotype i.e Fifty (50) cells per hybrid combination.

Table 2.1 Hybrid combinations assessed

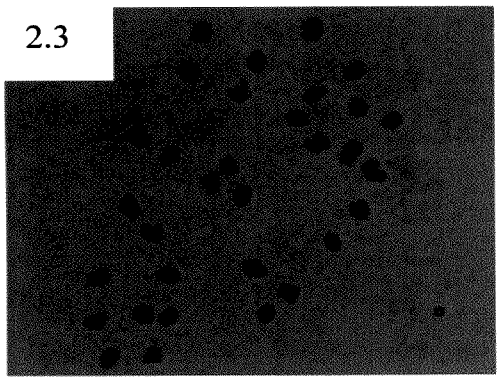
Combination	Female parent	Origin	Male parent	Origin
1.	T1	Tibooburra NSW	J1	Jiggalong WA
2.	R1	Roxby Downs SA	P1	Port Hedland WA
3.	T1	Tibooburra NSW	P1	Port Hedland WA
4.	W1	Wiluna WA	P1	Port Hedland WA

2.4 RESULTS

2.4.1 Mitotic chromosome numbers

A somatic chromosome number of 2n = 32 was identified in mitosis of all ten genotypes examined (Figure 2.3).

Figure 2.3 Genotype R4 mitosis anaphase I clearly showing 32 univalents.

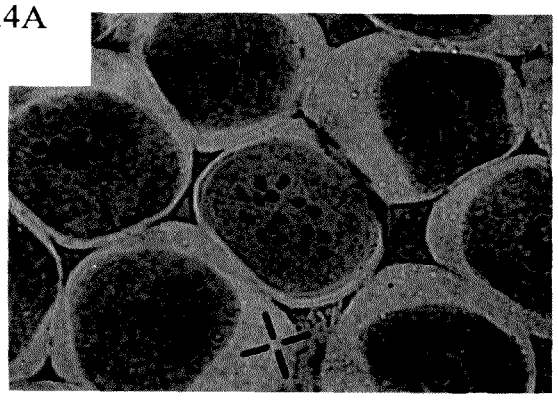


2.4.2 Meiotic chromosome numbers

All eighty (80) genotypes that were examined during meiosis possessed 16 bivalents. All cells displayed a high level of pairing affinity and no multivalent formations or other aberrations were commonly observed (Figures 2.4a – d; 2.5a – b; 2.6a-d). A summary of results and genotypes is presented in appendix 1.

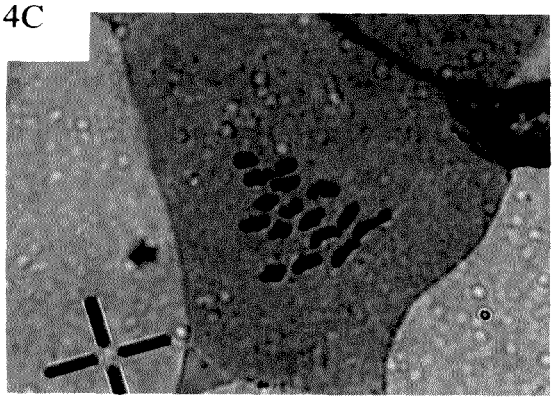
Figures 2.4A – 2.4D Observations of genotype (code R9) which display 16 bivalents at prophase 1(2.4a), 16 bivalents at metaphase 1 (2.4b), 16 bivalents at metaphase 1 (2.4c) and 32 univalents at Anaphase 1 (2.4d).

2.4A

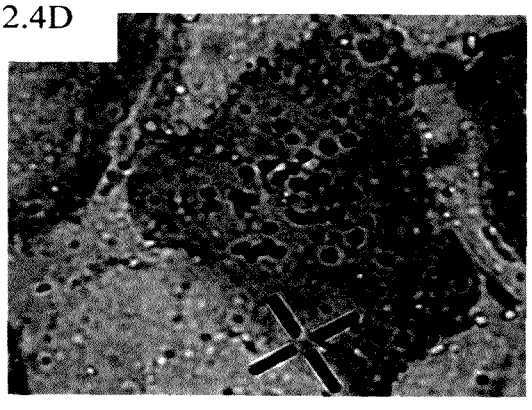


2.4B

2.4C



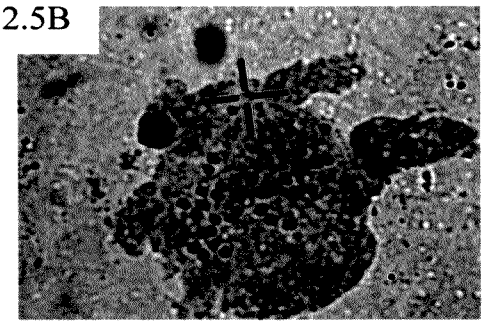
2.4D



Figures 2.5A - 2.5B Seed sourced from the University of New England had chromosome numbers of 2n = 32 for each of the 10 genotypes examined. Figures 2.5a and 2.5b shows genotype UNE02 with 16 bivalents at pro-metaphase 1 and 32 univalents at Anaphase 1.

2.5A

2.5B



2.4.3 Meiotic analysis of hybrids between geographically diverse genotypes

Chromosome numbers of 2n=32 were detected in all hybrids examined and no multivalent formations or other aberrations were commonly observed.

Table 2.2 Bivalent and multivalent observations in hybrids

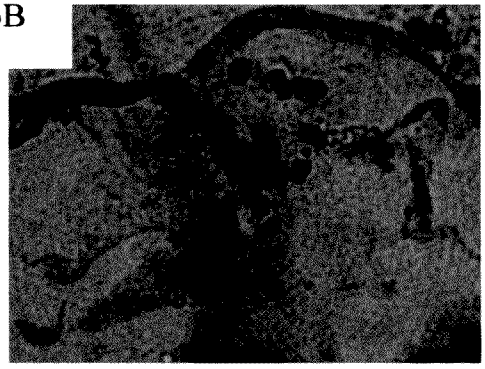
Hybrids	Genotypes observed	Cells viewed	Bivalents observed	Multivalents observed
T x J	5	50	16	0
R x P	5	50	16	0
T x P	5	50	16	0
W x P	5	50	16	0
Totals	20	200		

T = Tibooburra J= Jiggalong R= Roxby Downs W= Wiluna P= Port Hedland

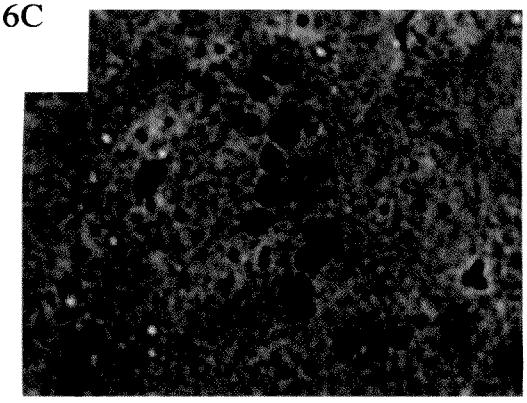
Figures 2.6A – 2.6D Meiotic examination of four different hybrids. (2.6a) Tibooburra x Jiggalong - (2.6b) Roxby Downs x Port Hedland - (2.6c) Tibooburra x Port Hedland (2.6d) Jiggalong x Wiluna

2.6A

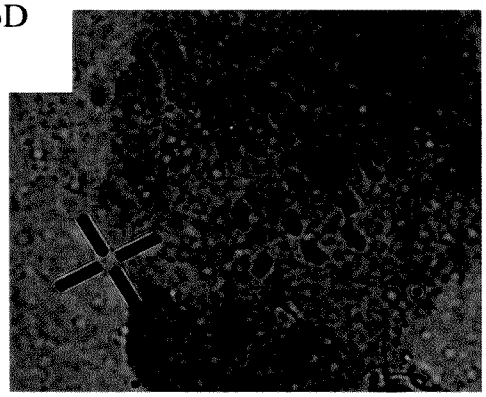
2.6B



2.6C



2.6D



2.5 Discussion

2.5.1 Chromosome number in Sturt pea

In this study a chromosome number of 2n = 32 was found in ninety (90) Sturt pea genotypes that originated from a broad spectrum of geographic origins within its known distribution. This number was found when employing both mitotic and meiotic analyses. Previous research has conflictingly identified that Sturt pea has a chromosome complement of 2n=16 (Zulkarnain *et al.*, 2002) and 2n=32 (Thompson 1994). Material was obtained from the same collections that were examined by

Zulkarnain *et al.*, (2002) and this study identified they all possessed chromosome numbers of 2n=32.

Cytological analysis of hybrids between accessions of different geographical origin did not reveal any differences in chromosome number or gross structural differences between chromosomes. This finding is consistent with the experience of others (M. Jusaitis pers. Comm.) who report no difficulty hybridising diverse lines from distinctly different provenances resulting in viable and fertile genotypes.

2.5.2 Polyploidy and Sturt pea

Legumes display variability for ploidy and base number (Paterson *et al.*, 2000) as may be expected in a large family (Goldblatt 1981). Species polyploidy is known to be overwhelmingly concentrated in legumes of temperate and cool Eurasia and it has been established the tropical Papillionoid representatives have base numbers of x = 10,11 or 12 whilst the predominantly temperate tribes (e.g Galegae) have characteristically lower base numbers of x = 6,7 or 8 (Goldblatt 1981). Most members of tribe Galegae, possess a chromosome number of n = 8. *Clianthus* and *Swainsona* are the only representatives in Galegae that are characterized by n = 16, a characteristic which is also typical of the related genera *Carmichaelia*. It appears from this evidence that Sturt pea as typically 2n=32, is a polyploid plant species. This result belatedly supports claims of Lee (1948).

2.5.3 Possible modes for evolution of Sturt pea

In this study it was identified that chromosome number and structure of Sturt pea was relatively uniform in a range of genotypes originating from geographically diverse locations. In most polyploid species there is greater opportunity for accelerated evolution of chromosome structure due to duplication of equivalent chromosomal regions that contain similar genes.

Based on the evidence of this study and other available literature it is not clear how Sturt pea evolved. However, it appears reasonable to suggest it was either a result of a hybridisation between two closely related species that have chromosome

complements of 2n=32 or that it evolved from a 2n=32 species that was an ancestor of genera *Swainsona*, *Carmichaelia* and *Montigena*.

In the first published revision of genus *Swainsona*, Lee (1948) suggested that some *Swainsona* species may be the result of interspecific hybridisation. Thompson (1994) proposed that the closest relatives of *Swainsona* may well be in the tribe Carmichaelieae which contains a number of genera typically possessing 2n=32. There is still some confusion about the relationship between members of genus *Swainsona* to members of genera *Carmichaelia* and *Clianthus*. These have both been demonstrated to be very closely related to genus *Swainsona* (Wagstaff *et al.*, 1999), and evidence is slowly being produced to broaden discussion and clarify collective phylogeny. Accordingly, results of ITS studies (Wagstaff *et al.*, 1999) may be interpreted to suggest that had Sturt pea been generated by hybridisation of two closely related parents with chromosome complements of 2n=32, candidate parents involved in such a hybridisation may include three *Swainsona* species shown to be more closely related to Sturt pea than other *Swainsona* species sampled in their study. Alternatively, closely related members of the genera *Carmichaelia* and *Clianthus* also fit the stated criteria.

To date no reports of successful interspecific hybridisation between *Swainsona* species have been substantiated and published. Hybridisations between different 2n=32 species capable of generating fertile progeny have been observed in genus *Carmichaelia*, but not *Swainsona*.

2.5.4 Chromosomal considerations when breeding with Sturt pea

In this study no cytological barriers to hybridisation were identified and geographically diverse accessions were readily hybridised. The resulting hybrids were fertile and when self pollinated generated viable progeny that possessed characteristics that were typical of Sturt pea. The implication of this finding is that because the common cytological impediments such as differences in chromosome number or ploidy were not detected in the germplasm examined, the Sturt pea may readily be improved by hybridisation of geographically diverse material without the constraints of such cytological barriers.

CHAPTER THREE

MOLECULAR VARIABILITY WITHIN STURT PEA

3.1 INTRODUCTION

The amplified fragment length polymorphism technique (AFLP) is a polymerase chain reaction (PCR) based technology (Lipp *et al.*, 2005) that is a highly reliable and reproducible means of assessing genetic variation in natural populations (Keiper and McConchie 2000). It is often used to identify genetic diversity within breeding populations or differentiate accessions that might possess similar morphological characteristics or indistinct traits (Saunders *et al.*, 2001) such as is observed in the Sturt pea.

Molecular approaches to characterise the phylogenetic relationships within Galegae, which contains genus *Swainsona*, have provided new and valuable information pertaining to the tribe (Wojciechowski *et al.*, 2000). However, significant gaps in the current understanding of relationships between and within the temperate legume tribes remain, and no molecular study specific to genus *Swainsona* has been conducted.

Based on morphological observations it has been suggested there is "generous scope" for selection and improvement in Sturt pea (Jusaitis and Schmerl 1993). No sound information about the genetic variability of the species is available to support the claim. The aims of this chapter were to establish if the AFLP technique is a suitable method to assess genetic variability in this species, and if so, to assess the levels of genetic variability within and between geographic zones of Sturt pea's distribution.

3.2 MATERIALS

Fifty genotypes (plants generated from 10 seed that were harvested from each of five locations across mainland Australia; Tibooburra NSW, Roxby Downs SA, Wiluna WA, Jigalong WA and Port Hedland WA) were analysed in the study.

3.3 METHODS

3.3.1 Cultivation of plants and harvest of leaf material

Seed were manually nicked prior to sowing as described in section 2.2.3.
Seed were sown in 150mm pots using a commercial potting mix (see section 2.2.2). Seedlings were allowed to grow until five compound leaves had developed and young leaflets were taken from the fifth compound leaf for DNA extraction. Single leaves were placed in eppendorf tubes and DNA extractions were performed immediately using fresh tissue.

3.3.2 DNA isolation

DNA was isolated using a Qiagen DNeasy mini kit® as per the manufacturers recommended protocol except that leaf material was placed in eppendorf tubes with buffer and RN*ase* and placed in a Retsch mill (MM301) for cellular interruption in place of liquid nitrogen grinding using a mortar and pestle.

3.3.3 Assessment of DNA yield per isolation

Equal volumes of genomic DNA and gel loading buffer (GLB – 19.6ml formamide, 400µl 0.5M EDTA) were mixed and electrophoresed on a 2% agarose gel (100ml) containing 2 µl Ethidium bromide (10mg/ml). DNA was quantified by comparing the intensity of genomic DNA bands with those of a standard of known concentration (Promega cat. No. D150a – conc.1ng / µl) when viewed under ultraviolet light.

3.3.4 AFLP analysis

The AFLP technique used was as described by Vos *et al.*, (1995) except that *Eco*RI was substituted with the methylation sensitive enzyme *Pst*I and adaptors were modified accordingly (Table 3.1). Pre-amplification reactions (Table 3.2) were performed in 25µl reaction mixtures containing 2.5µl each of 5µM *Pst*I + 0 and *Mse*I + 0 (Table 3.2), 2.5µl 2 mM dNTP, 2.5µl 10 x PCR buffer, 1.5µl 25mM $MgCl_2$, 2.5µl 2 mM dNTP, 1 U *Taq* polymerase (5 U / µl), 14.3µl dH_2O and 2 µl of digested / ligated *Pst* I-*Mse* I template DNA. Pre-amplification PCR reactions consisted of between 15 and 20 cycles which had the following profile; denaturation at 92° for 1 minute annealing at 56° and extension at 72°. Pre-amplification products were then diluted 1:20 with TE buffer (1mM EDTA, 10mM Tris-HCl, pH8).

Two µl of diluted *Pst-Mse* pre-amplification product was added to 18µl of selective amplification master mix bringing the total reaction volume to 20µl. The master mix comprised 2µl 10X PCR Buffer, 1.2µl 25mM $MgCl_2$, 2µl 2mM dNTP, 1µl 5uM *Pst* + 1 primer, 1µl 5uM M*se* + 3 primer (Table 3.3), 0.2µl *Taq* polymerase (5U/µl) and 10.6µl dH_2O. The number of amplification cycles required for selective amplification varied between 10 and 20. PCR reactions for selective amplification were performed with the following profile: (94° 1 min, 92° 1 min, 65-56° , 72° 1 min) x 10. (92° 30 sec, 56° 30 sec, 72° 1 min) x number of required cycles.

3.3.5 Screening of Selective primers

Eleven *Mse* +3 primers (Table 3.3) were screened using 5 genotypes that originated from Roxby Downs (genotypes 11-15 in study). One primer failed to amplify any products during preliminary testing (Fig 3.1A) Of the ten that had amplification products, four were employed for conducting the analysis (Table 3.4 and figure 3.1B).

Table 3.1 *PstI and MseI* adaptors used in AFLP analysis

Adaptor	Enzyme	Type	Sequence
*Pst*A1	*Pst* I	Adapter	CTCGTAGACTGCGTACATGCA
*Pst*A2	*Pst* I	Adapter	CATCTGACGCATGT
*Mse*A1	*Mse* I	Adapter	GACGATGAGTCCTGAG
*Mse*A2	*Mse* I	Adapter	TACTCAGGACTCAT

Table 3.2 Pre-amplification primers used in AFLP analysis

Adaptor	Enzyme	Type	Sequence
Pst-0	*Pst* I	Primer+0	GACTGCGGTACATGCA
Mse-0	*Mse* I	Primer+0	GATGAGTCCTGAGTAA

Table 3.3 Selective amplification primers evaluated for AFLP analysis

Primer	Sequence	test primer number
Mse +3	GATGAGTCCTGAGTAA + GGC	1
	GATGAGTCCTGAGTAA + GGT	2
	GATGAGTCCTGAGTAA + GTT	3
	GATGAGTCCTGAGTAA + GAA	4
	GATGAGTCCTGAGTAA + GCG	5
	GATGAGTCCTGAGTAA + GAG	6
	GATGAGTCCTGAGTAA + GGA***	7
	GATGAGTCCTGAGTAA + GAC	8
	GATGAGTCCTGAGTAA + GGG	9
	GATGAGTCCTGAGTAA + GTA	10
	GATGAGTCCTGAGTAA + GTG	11

*** primer failed to amplify any product in any of 5 genotypes used for screening

Table 3.4 Selective amplification primers used to reveal genetic variability.

Primer	Sequence	test primer number
Mse +3	GATGAGTCCTGAGTAA + GGC	1
	GATGAGTCCTGAGTAA + GGT	2
	GATGAGTCCTGAGTAA + GTT	3
	GATGAGTCCTGAGTAA + GAA	4

3.3.6 Electrophoresis and marker scoring

Three µl of gel loading buffer was added to an equal volume of selective amplification product and the resulting mixture was heated at 96° for 3 minutes, and chilled on ice (Keiper *et al.*, 2003). Selective amplification products were separated by denaturing polyacrylamide gel (5%) electrophoresis using 50 x 38cm Bio-Rad sequencing apparatus in 1x TBE buffer and were visualised by silver staining (Maniatis *et al.*, 1982). Each amplified fragment was considered to be a separate character and scored as present (1) or absent (0). Fragments that were poorly resolved, or of low intensity were not scored (Keiper *et al.*, 2003). The molecular weight of amplified bands were scored against a 25bp ladder (Invitrogen – cat. No. 10597-011).

3.3.7 Statistical analyses

Percentage polymorphic loci were calculated using a presence/absence data matrix. Jaccards similarity coefficient: GS(ij) = $a/(a+b+c)$ (Jaccard 1908) has been used to calculate genetic similarity where GS(ij) is the genetic similarity between individuals i and j, a is the number of polymorphic fragments that are shared by i and j, b is the number of fragments present in i but absent in j, and c is the number of fragments present in j but absent in i. The Jaccard similarity matrices were used to perform cluster analyses using the unweighted pair-group method using arithmetic means (UPGMA) while support for the clusters was evaluated using bootstrapping analyses with 1000 iterations (Felsenstein 1985, Keiper *et al.*, 2003). Construction of the

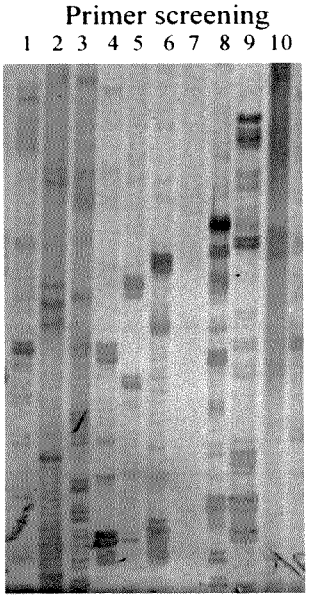
Jaccard matrices and cluster analysis were performed using the program NTSYSpc version 2.1 (Exeter software) and the bootstrapping analyses were performed using WinBoot (Yap and Nelson 1996).

3.4 RESULTS

3.4.1 Marker polymorphism

The AFLP technique generated a large number of products that ranged from 80-500kbp and revealed high levels of polymorphism in Sturt pea. A total of 221 bands were produced in reactions employing four primer combinations (Table 3.5). Of these only four were monomorphic across all genotypes. 98.2% of products were polymorphic. A mean of approximately 55 (55.25 ± 13.31 s.d) polymorphic fragments were observed per primer combination (Table 3.5). AFLP's proved to be a reliable method to assess genetic variation within Sturt pea.

Figure 3.1A Visualising banding patterns using silver staining during primer screening



Genotype used – R2

Figure 3.1B Visualising banding patterns using silver staining during AFLP analysis

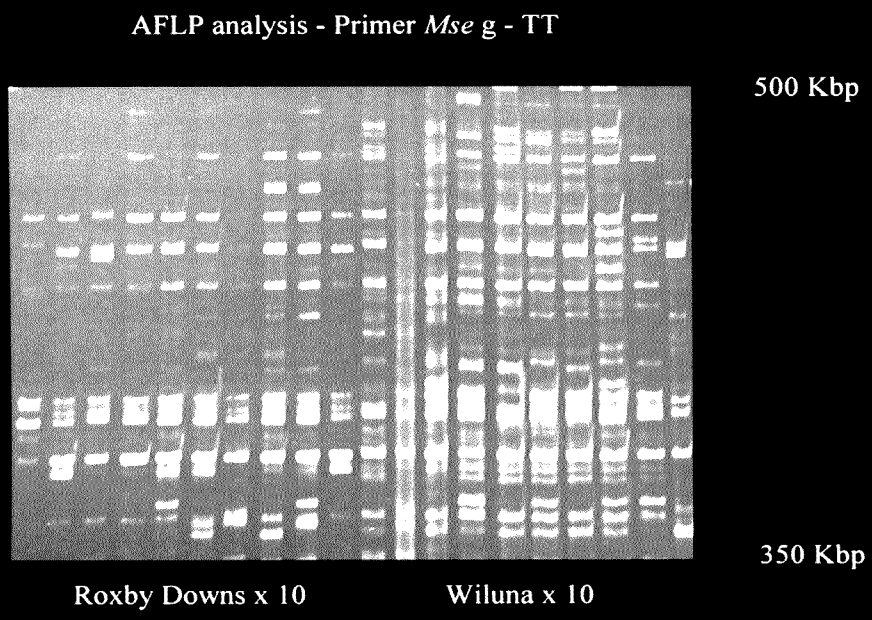


Table 3.5 Primer specific data from AFLP analysis

Mse primer	Bands produced	No. of polymorphic loci	% Polymorphic loci
ggC	46	45	97.83
gTT	77	77	100
ggT	55	52	94.55
gAA	43	43	100
Total	221	217	98.19

3.4.2 Genetic diversity

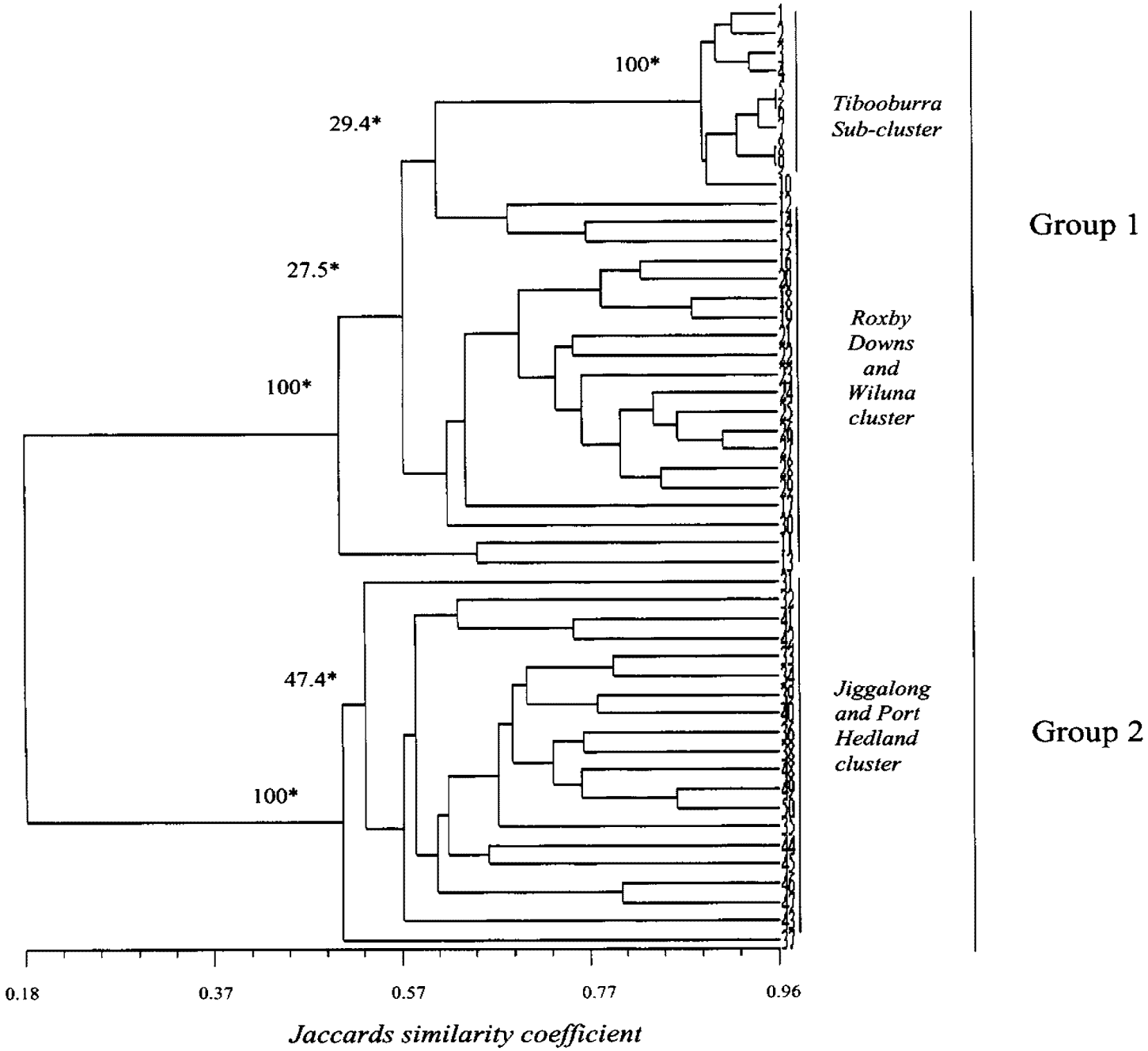
Cluster analyses of combined polymorphic markers revealed two primary clusters with the Tibooburra, Roxby Downs and Wiluna accessions (group 1) separated from the Jiggalong and Port Hedland accessions (group 2) (Figure 3.2). There was a distinct division with each cluster having a bootstrap value of 100 inferring a delineation of groups is strongly justified. This delineation had a Jaccards similarity coefficient (JSC) of approximately 0.50 supporting considerable dissimilarity between them.

The Tibooburra sub group within group 1 was distinct from other accessions in that group. Cluster analysis of the genotypes from Roxby Downs and Wiluna within group 1 revealed that there was no significant similarity (P=0.05) between any two genotypes. Mean similarity within these two classes showed a JSC of 73.6.

The Jiggalong and Port Hedland accessions of group two contained no two genotypes that were significantly similar (P=0.05). Mean similarity within this group showed a JSC of 68.75.

The Tibooburra cluster from group one had two pairs of genotypes whose similarity was significant (P=0.05). Mean similarity of these accessions was higher with a JSC of 92.1. Greatest similarity within a group was found in the Tibooburra accessions with a bootstrap value of 100 and a Jaccards similarity coefficient of 88.4 or higher within them.

Figure 3.2 Genetic similarity /dissimilarity among selected Sturt pea accessions described in a dendrogram depicting patterns of genetic similarity estimated by AFLP's with selected bootstrap values shown*



Genotypes 1-10 – Tibooburra accessions, 11-20 – Roxby Downs accessions, 21-30 – Wiluna accessions, 31-40 – Jiggalong accessions, 41-50 – Port Hedland accession

3.5 Discussion

3.5.1 AFLPs and genetic variability in Sturt pea

AFLP's have been employed to examine variation in crop legumes and their relatives. In most cases the results from AFLP analysis have been compared to those of similar studies that have employed other molecular methods and they have revealed similar results. Therefore, the results from these analyses represent appropriate comparators regarding the efficiency of AFLP's to identify genetic variation. In this research AFLP analysis revealed 221 polymorphisms (98% were polymorphic) within the accessions studied and the average number of polymorphisms per primer were 55. In studies examining *Cicer sp.* 98.6% of products were polymorphic and on average 43 polymorphisms were generated per primer (Nguyen *et al.*, 2004). Studies concerned with *Vigna* similarly identified that 86.4% of amplification products were polymorphic and that an average of 59 polymorphic bands were generated per primer (Sivaprakesh *et al.*, 2003).When compared with these results it can be concluded that AFLP's represent a useful approach to examine genetic variation in Sturt pea.

3.5.2 The importance of genetic diversity in Sturt pea

Genetic diversity helps organisms cope with environmental variability, reduces potentially deleterious effects of inbreeding (particularly applicable to Sturt pea) within populations and is the primary basis for adaptation to future environmental uncertainty. No organism is optimally adapted for all environmental conditions. Nonetheless, the current genetic composition of a species influences how well its members will adapt to future physical and biotic environments (Falk, Knapp and Guerrant 2001).

High levels of genetic variation were detected within the material examined which would infer that this species is well placed to adapt to new environments caused by natural environmental change or cultivation.

The dendrogram of relatedness that was constructed from AFLP data indicates that there are two main clusters within Sturt pea. The accessions that originated from Port

Headland (W.A) and Jiggalong (W.A) formed one cluster, whilst the accessions that originated from more Eastern and Southern locations formed a separate cluster.

Genetic variation appeared to have a close association with the geographic location of the accessions studied. This observation is in agreement with findings that a large component of the genetic diversity in *Vicia* (faba bean) is consistent with a strong geographic effect (Kyu Huh 2001).

3.5.3 Accounting for genetic variability in Sturt pea

Physical distance between collection locations appears to account for a lot of the genetic variability that was identified in this study. Distance between plant populations has been a particular area of focus in plant population genetics studies (Klinger *et al.*, 1992) as it is recognised to play a crucial role in regulating gene flow. It is known that the distance between plant populations is inversely related to gene flow and that limited dispersal should result in genetic differences between populations being proportional to the distance of separation (Hellberg 1995).

Even though gene flow is probably a major contributor to genetic variability in Sturt pea it is difficult to separate differences in gene flow caused by distance from other environmental influences such as climate which is variable and dependent on geographic location.

In this study variation of Sturt pea accessions from different locations is also consistent with variation in climatic factors, such as temperature profiles and rainfall.

Specific results of this study indicate that factors other than distance are influencing genetic variability. For example, Wiluna (WA) and Roxby Downs (SA) clustered together yet can geographically be described as quite distant (kms) suggesting that there is an unexpectedly high gene flow or that there is selective pressure that is responsible for increasing the genetic similarity of accessions within these localities.

The level of genetic variation found within genotypes that originated from Tibooburra (NSW) was not consistent with that found at other locations. It is not clear if these

accessions displayed greater similarity because of restricted gene flow due to genetic isolation or because there is greater pressure for genetic conservation for survival in this region.

Genetic variability of the genotypes originating from Roxby Downs was greater than for other locations that were analysed. Of the regions included in this study, Roxby Downs is the closest to Maralinga, South Australia. A former nuclear test site between 1953 -1963, Maralinga could be the origin of both subtle and substantive DNA alterations. It is possible that gene transfer between the Maralinga site to genotypes from Roxby Downs occurred. The UPGMA dendrogram shows that Roxby Downs genotypes share some similarities with Tibooburra and Wiluna genotypes. Gene flow between these locations may explain the high levels of genetic variability observed within accessions from Roxby Downs.

3.5.4 Molecular and cytological variation in Sturt pea

The molecular evidence from this study has suggested that there is substantial genetic variation between Eastern and Western accessions of Sturt pea and that genetically Tibooburra genotypes are an isolated group. This is quite different to the results of the cytological study in chapter 2 which was not able to obviously differentiate accessions from Eastern and Western extremities of the distribution of Sturt pea by analysing the meiotic behaviour of hybrids.

These results highlight the benefits of employing molecular analysis to establish relationships within a species where cytological variation is not obvious. Even though cytological analyses did not reveal significant genomic differences in accessions from different localities it did establish that from a cytological perspective no barriers to hybridisation were apparent, which was supported by the efficiency of hybridisation to generate material for the study.

Therefore, it must be concluded that variation within Sturt pea is the result of subtle sequence variation rather than gross structural alteration to the genome.

CHAPTER FOUR

THE REDUCED MORPHOLOGY OF A COMPACT HABIT FORM OF STURT PEA

4.1 INTRODUCTION

A novel Sturt pea genotype that possesses a compact habit was developed by Dr Matthew Turner whilst in the employ of Oasis Horticulture Pty Ltd. The genotype has several characteristics that are desirable for ornamental horticulture such as tolerance of tissue culture, amenability to cutting propagation, and small branches and leaves.

A rapid quantitative technique that is capable of identifying compact genotypes early in their development is required to characterise the effect of the form on plant morphology, to investigate inheritance of the novel form, and to apply efficient selection in breeding.

Investigations were also to be undertaken to establish if 'normal habit' plants that when self pollinated produce populations that segregate for the novel form could be distinguished from the wild types that do not produce segregating populations.

A comparison of meiosis between compact and normal genotypes was conducted to investigate if chromosomal rearrangements may be responsible for the novel form.

4.2 MATERIALS

4.2.1 The novel form

Plantlets exhibiting a novel habit characterised by small leaflets were identified in *in-vitro* culture at the premises of Oasis Horticulture Pty Limited, Winmalee, New South Wales, Australia. After they were propagated *in-vitro*, micro cuttings were taken and resulting plantlets were multiplied by vegetative cuttings. When grown in pots after hardening (Fig 4.1a) they were found to also possess high levels of branching, thin stems, small flowers with a split keel petal, and shorter peduncles and stipules.

4.3 METHODS

4.3.1 Seed treatments

Seed treatments are as described in 2.3.1

4.3.2 Growing media and environment

Plant material that was grown at Oasis (Winmalee) was grown as described in 2.3.2. Plant material that was grown at The University of Sydney, Cobbitty was grown in a multispan polyhouse, under natural daylength in 150mm pots using a soil mix of 4:1 pine bark : coarse sand. All irrigation at Cobbitty was by hand watering.

4.3.3 Determining the difference in morphology between compact and normal genotypes

4.3.3.1 Assessment of a test population

A population of 70 F_2 seed which segregated for the mutation (Population Code -BBR 29) was used to identify differences between normal habit and compact compact plants to develop a quantitative method for classification purposes. Initially the plants were visually inspected to identify characters that were likely to differ between the two classes; keeping in mind that in breeding it is desirable to perform phenotyping as early in the plants development as possible. Measurements of the selected characters were made and data was analysed to establish if statistical differences existed between compact and normal plants.

Figure 4.1a The difference in plant morphology in early developmental stages between the standard and compact phenotype

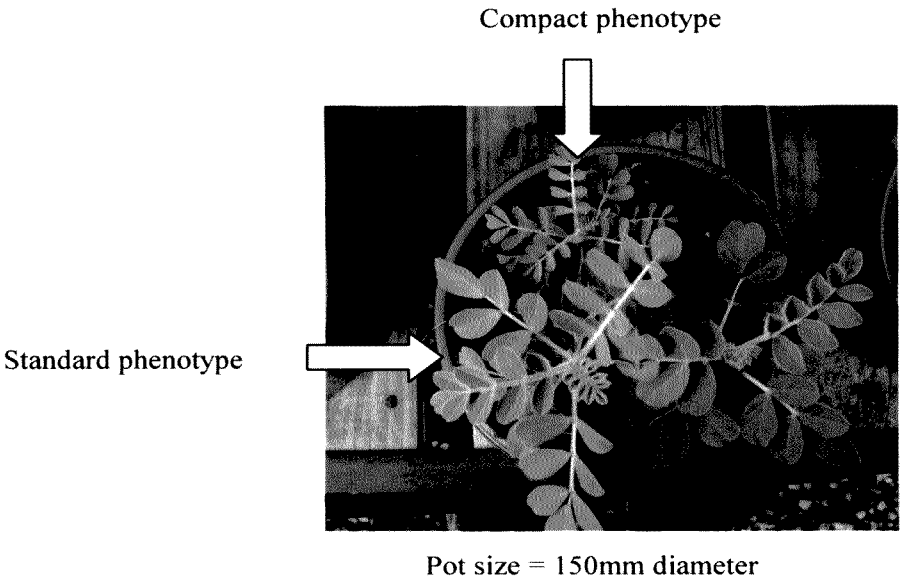
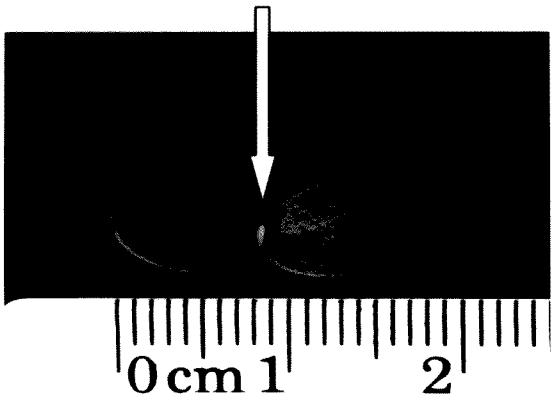


Figure 4.1b Time of cotyledon measurement as first true leaves emerge



4.3.3.2 Characters selected for measurement

In this experiment the length and width of the cotyledons (2 characters) were recorded.

The vegetative characters of length and width of terminal leaflets of fully expanded first, second and third compound leaves (6 characters) were recorded.

The vegetative characters of plant height and width was also recorded on full expansion of the third, fourth and fifth compound leaves (6 characters) of each plant. A total of 14 characters were recorded.

4.3.3.3 Cotyledon measurements

The length and width of the cotyledon of each germinating seed was measured as the first true leaves emerged (demarcated in Figure 4.1b).

Length was determined by measuring the distance from the base to the tip of the cotyledon.

Width was determined by measuring the distance across the cotyledon half way along the mid vein.

After measurements were taken, they were retrospectively collated into two groups, one containing compact genotypes and the other containing siblings that did not express the compact form.

4.3.3.4 Leaflet measurements

Length of terminal leaflets was determined by measuring from the point of attachment of the leaflet to the petiolule to the tip of the mid-vein at the leaf apex. Width of terminal leaflets was determined by measuring the distance between the leaflet margins half way along the mid vein.

4.3.3.5 Plant measurements

Plant height was determined by measuring the distance from the soil surface to the highest point of the plant. Plant width was determined by measuring the widest part of the plant on a horizontal axis.

4.3.3.6 Statistical analysis of cotyledon, leaflet and plant dimensions

Each data set was initially subjected to an F-test to examine if variances were equal or unequal. If the F value exceeded the f critical value, the data was subjected to a T-test assuming equal variances. Alternatively, the data was subjected to a T-test that assumed unequal variances.

A hypothesis was proposed that for each character either:
H_o- The means for both classes would be the same, or
H_a- The means of each class would differ
The level of significance was set at P=0.05

4.3.4 Application of developed protocol to segregating populations from different genetic backgrounds

Cotyledon measurements were taken (4.3.3.3) and statistical analyses were conducted (4.3.3.6) on data generated from 14 segregating F_2 populations. Vegetative characters were measured and analyses were conducted on eleven of these populations as described in 4.3.3.2, 4.3.3.3, 4.3.3.4, 4.3.3.5 and 4.3.3.6. Populations that were examined are listed in Table 4.5 for cotyledons and Tables 4.10 and 4.11 for

leaf and plant dimensions. Each F_2 population was derived from sibling F_1's. F_2 populations were grown as sub populations in varying number.

4.3.5 Attempts to differentiate heterozygous and homozygous normal genotypes

4.3.5.1 Graphical presentation using an XY scatter graph

To determine if there may be measurable differences between the genotypes that generated segregating populations when selfed from those that produced populations that did not segregate, every genotype was assessed for each of the 12 stated vegetative characters (4.3.3.2). A mean was determined for each of the 12 characters for all individuals and for the population in its entirety. The mean of the sum of 12 measurements per individual was calculated and this was graphed as a mean of the sum of 12 measurements for the population. The method can be suitably shown in three steps as follows:

A) Individual data

$$\sum \frac{(\text{character } 1 + 2 + \ldots\ldots\ldots 12)}{12}$$

B) Population data

$$\sum \frac{(\text{mean character } 1 + 2 + \ldots\ldots\ldots 12)}{12}$$

C) $\sum$ (A/B x 100/1) = identifier per genotype

4.3.5.2 Comparison of means by T-test

Each data set was initially subjected to an F-test and the appropriate T-test was performed to compare means. Details of the analysis are described in 4.3.3.6.

4.3.6 Meiotic characterisation of compact genotypes

Methods employed were as described 2.3.4 (Zulkairnain *et al.*, 2002) with modification. Anthers were collected from flower buds that were 8.0-10.0mm long. Slides were observed using a Zeiss Axioskop 50 130 VA TYP B microscope. Photographs were taken using a Nikon Coolpix 4500 digital camera. Individuals with the prefix BBR are recombinant F_2 compact genotypes produced as described in 5.3.3.1. The individuals described as T1 and T2 are recombinant compact genotypes produced after the hybridsation of two F_1 plants (codes BBR29 and BBR47).

Table 4.1 Compact genotypes used in meiotic analysis

	Genotype	Designation	Cells viewed
1	Compact	BBR29-23	10
2	Compact	BBR29-18	10
3	Compact	BBR29-17	10
4	Compact	BBR29-25	10
5	Compact	BBR29-34	10
6	Compact	BBR29-53	10
7	Compact	BBR29-75	10
8	Compact	BR29-80	10
9	Compact	BBR47-7	10
10	Compact	BBR47-16	10
11	Compact	BBR47-38	10
12	Compact	BBR47-49	10
13	Compact	BBR47-64	10
14	Compact	T1	10
15	Compact	T2	10

4.4 RESULTS

4.4.1 Identification of differences between compact and normal classes in a test population

4.4.1.1 Cotyledon dimensions

Means obtained for the two classes identified in the test population (compact and other) were different for both cotyledon length (7.91mm compared to 8.54mm) and width (5.17mm compared to 6.04mm). Variances were not equal (length) and equal (width). The T-tests revealed that there was a significant difference ($P = 0.01$) between means (Table 4.2).

Table 4.2 Statistical analysis of cotyledon measurements in a test population

Character	Length		Width	
	Com	Norm	Com	Norm
BBR 29-test				
Mean	7.91	8.54	5.17	6.04
Variance	0.94	0.67	1.09	0.66
P (T-test)	0.009		P < 0.001	

4.4.1.2 Leaflet and plant dimensions

Differences in length and width of leaflet dimensions of each class were recorded in all cases (Table 4.3). All characters were significantly different (P = 0.01). Results are described using abbreviated identifiers e.g L1T is the length of the first terminal leaflet upon full expansion, W1T describes the width. The compact sub class is described as 'Com' whilst the normal phenotype sub class is described as 'Norm'.

Table 4.3 Comparison of leaflet dimensions in normal and compact genotypes of population BBR29

Character	L1T		W1T		L2T		W2T		L3T		W3T	
	Com	Norm	Com	Norm	Com	Norm	Com	Norm	Com	Norm	Com	Norm
BBR 29-												
Mean	7.33	21.46	5.76	16.87	6.93	21.4	5.2	16.66	7	19.6	4.93	15.73
Variance	4.52	22.4	5.24	14.12	5.20	12.55	4.31	15.66	4.14	11.97	2.31	10.35
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	

Differences in height and width of each class were recorded in all cases (Table 4.4). Each measured character was significantly different (P = 0.01). Results are described using abbreviated identifiers e.g H3C is the height of the plant upon full expansion of the third compound leaf, W3C describes the width of the plant at the same developmental point.

Table 4.4 Comparison of plant dimensions in normal and compact genotypes of population BBR29

Character	H3C		W3C		H4C		W4C		H5C		W5C	
	Com	Norm	Com	Norm	Com	Norm	Com	Norm	Com	Norm	Com	Norm
BBR 29-												
Mean	15	39.8	25.33	79.53	19.26	50.66	29.8	94.46	22.86	60.53	35.8	112.66
Variance	25.85	32.02	54.80	198.12	23.92	149.38	81.17	495.69	41.98	178.98	117.88	823.80
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	

4.4.2 Validation of differences in other populations

4.4.2.1 Influence of the compact form on cotyledon dimensions

Differences in means were recorded in all sub populations grown and between environments used. Table 4.5 shows means and standard deviations for each sub class (compact and other).

In populations grown in the multispan shadehouse at Winmalee, a reduction in cotyledon length ranged from 9.1% to 24.3% of that achieved by the siblings. A reduction in cotyledon width ranged from 4.1% to 20.85% (Table 4.7). For the sub populations grown at Cobbitty the reduction in cotyledon length ranged from 10.8% to 12.5%. A reduction in cotyledon width ranged from 9.6% to 16.79% (Table 4.8).

The statistical analysis of cotyledon dimensions revealed significant differences between means (P = 0.05) in 20 of 28 cases (10 of 14 for length measurements and 10 of 14 for width measurements) (Table 4.6). Of the 20 cases in which a significant difference was recorded, 17 cases were significant at P = 0.01.

Environmental factors are implicated in variability as evidenced by both BBR47 sub populations and BBR14 sub populations having both significant and insignificant differences in variance across sub groups (Table 4.6).

Means of each class were compared against the population means (Tables 4.7 and 4.8) with variability in the degree of morphology reduction recorded.

Table 4.5 The length and width of cotyledons in F_2 compact plants and their normal siblings, the population means to which they each contribute and the standard deviations of each.

Oasis Grown

Population	Com L mean	s.d	Com W mean	s.d	Norm L mean	s.d	Norm W mean	s.d	Pop L mean	s.d	Pop W mean	s.d
BBR 29 sub pop 2-O	9.82	1.26	6.32	0.98	12.06	1.16	7.594	0.85	11.64	1.47	7.36	1
BBR 29 sub pop 4-O	7	1.1	4.55	0.64	8.43	1.27	5.61	0.82	8.154	1.36	5.404	0.88
BBR 29 sub pop 5-O	7.55	1.37	4.94	0.85	8.99	1.44	5.74	1	8.646	1.56	5.57	1.03
BBR 47 sub pop 1-O	11.25	1.37	6.5	0.77	12.58	1.43	7.24	0.99	12.32	1.5	7.09	0.99
BBR 47 sub pop 3-O	8.9	1.12	5.56	0.96	9.798	1.41	5.97	0.89	9.513	1.39	5.79	0.92
BBR 14 sub pop 1	9.2	0.76	5.8	0.76	11.6	0.86	7	0.69	11.14	1.24	6.75	0.82
BBR 14 sub pop 3-O	8.42	1.13	4.64	0.48	9.43	1.9	5.71	1.22	9.287	1.83	5.62	1.2
BBR 4 sub pop 3-O	9.14	1.49	5.87	1.18	10.52	1.21	6.84	0.79	10.33	1.33	5.857	0.91
BBR 18 sub pop 2-B	8.3	0.97	5.2	0.84	10.97	1.71	6.57	0.99	10.44	1.91	6.3	1.1
BBR 27 sub pop 1-O	8.96	1.3	6.03	0.88	9.89	1.89	6.29	1.17	9.74	1.83	6.24	1.12

Cobbitty Grown

Population	Com L mean	s.d	Com W mean	s.d	Norm L mean	s.d	Norm W mean	s.d	Pop L mean	s.d	Pop W mean	s.d
BBR 29 sub pop 3-C	7.87	0.94	5.625	0.76	8.824	1.01	6.32	0.68	8.568	1.08	6.135	0.76
BBR 47 sub pop 2-C	9.33	0.87	5.55	0.53	10.54	1.1	6.41	0.7	10.291	1.16	6.412	0.75
BBR 14 sub pop 2-C	8.75	0.5	5.5	0.58	10	0.84	6.61	0.82	9.71	0.94	6.353	0.9
BBR 4 sub pop 1-C	8.81	1.1	6	0.7	10.02	0.83	6.64	0.68	9.827	0.98	6.541	0.72

Table 4.6 Statistical comparison of mean cotyledon dimensions of compact and normal classes

F_2 Populations	F-test Length	P (T-test) length	F-test width	P (T-test) length
BBR-29-2-0	F<f crit	P < 0.001	F<f crit	P < 0.001
BBR-29-3-C	F>f crit	P < 0.001	F<f crit	P < 0.001
BBR-29-4-0	F<f crit	P < 0.001	F<f crit	P < 0.001
BBR-29-5-0	F<f crit	P < 0.001	F<f crit	P < 0.001
BBR47-1-0	F>f crit	0.06	F>f crit	0.07
BBR47-2-C	F>f crit	P < 0.001	F>f crit	P < 0.001
BBR47-3-0	F<f crit	0.028	F>f crit	0.16
BBR14-1-0	F>f crit	P < 0.001	F<f crit	P < 0.001
BBR14-2-C	F>f crit	P < 0.001	F>f crit	0.01**
BBR14-3-0	F<f crit	0.18	F>f crit	P < 0.001
BBRQ-1-C	F<f crit	P < 0.001	F<f crit	0.018*
BBRQ-3-0	F>f crit	0.05*	F>f crit	0.07
BBR18-2-B	F<f crit	P < 0.001	F<f crit	P < 0.001
BBR27-1-0	F<f crit	0.07	F<f crit	0.42

* Significantly different P = 0.05 ** Significantly different P = 0.01

Table 4.7 Percentage reductions in mean cotyledon lengths and widths of compacts. Values are expressed relative to the mean of the remainder of the population (Oasis grown populations)

Population	Length % reduction	Width % reduction
BBR 29 sub pop 2-O	18.57	16.78
BBR 47 sub pop 1-O	10.57	10.22
BBR 5 sub pop 2-O	13.33	14.61
BBR 1 sub pop 3-O	14.46	16.56
BBR 29 sub pop 4-O	16.96	18.89
BBR 4 sub pop 3-O	13.12	14.18
BBR 29 sub pop 5-O	16.02	13.94
BBR 47 sub pop 3-O	9.165	6.868
BBR 18 sub pop 2-O	24.34	20.85
BBR 14 sub pop 3 - O	10.71	18.74
BBR 27 sub pop 1 - O	9.403	4.134

Table 4.8 Percentage reductions in mean cotyledon lengths and widths of compacts. Values are expressed relative to the mean of the remainder of the population (PBI Cobbitty grown populations)

Population	Length % reduction	Width % reduction
BBR 29 sub pop 3-C	10.81	11
BBR 47 sub pop 2-C	11.48	13.42
BBR 4 Sub pop 1-C	12.08	9.639
BBR 18 sub pop 1-C	10.98	10.74
BBR 14 sub pop 2-C	12.5	16.79

4.4.2.2 – Influence of the compact form on leaflet and plant dimensions

A reduction in mean for the compact genotype sub group compared to their siblings was observed in all populations and environments. Variable levels of reduced morphology have been found within populations when grown in different environments as sub populations. A summary is provided describing the spectrum of morphological reduction observed within the parameters of the genetic backgrounds used. At all times a minimum buffer of 30% reduction was found. Table 4.9 summarises reductions observed for each measured character. Table 4.10 shows data specific to leaf characters and table 4.11 shows data specific to plant characters. Figure 4.2 illustrates the morphological reductions observed.

Table 4.9 Spectrum of reduction across all populations summarised.

	Range leaf length %	Range leaf width %
At first terminal leaf expansion	32-69	32-69
At second terminal leaf expansion	36-73	35-74
At third terminal leaf expansion	35-71	34-71
	Range plant height %	**Range plant width %**
At three compound leaves -	34-67	34-73
At four compound leaves -	39-70	33-69
At five compound leaves -	42-72	33-79

Table 4.10 Comparison of leaflet dimensions of compact and normal plants showed means of leaf dimensions were significantly different*** at P = 0.001 (Table 4.10).

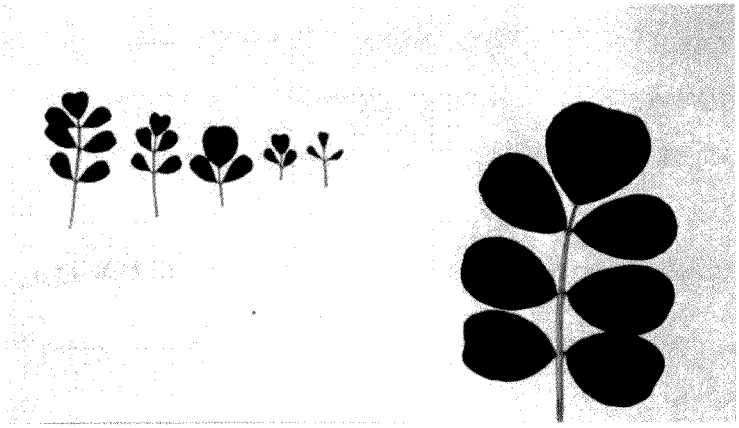
	Character											
Population	**L1T**		**W1T**		**L2T**		**W2T**		**L3T**		**W3T**	
	Com	Norm	Com	Norm	Com	Norm	Com	Norm	Com	Norm	Com	Norm
BBR 29-2												
Mean	8.1	25.4	5.9	18.5	7.6	25	6.1	19.1	7	23.6	5.4	18.4
Variance	15.9	20.6	7.29	10.45	13.38	15	6.64	7.88	6.65	14.77	4.97	8.13
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR 29-4												
Mean	8.5	18.86	5.8	13.17	8.45	19.25	6	13.5	7.6	18.75	5.05	13.02
Variance	5	10.86	2.84	7.79	5.58	10.56	4.22	6.02	4.04	10.09	2.3	8.07
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR 29-5												
Mean	7.46	20.18	5.79	18.16	7	19.98	5.17	15.08	6.79	19.33	4.72	14.25
Variance	3.89	18.86	4.15	297.3	4.64	16.67	3.93	12.47	4.45	12.51	2.62	8.25
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR47-1												
Mean	9.58	26.13	7.66	18.2	10.16	27.53	8	20.6	12.42	25.4	8.83	18.86
Variance	26.2	12.83	19.56	9.31	24.56	18.55	20.8	9.4	49.24	17.68	24.56	9.83
T-test	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR47-2												
Mean	5.16	11.5	3.77	8.15	5.33	12.95	4.16	9.35	5.83	14.55	3.88	9.9
Variance	1.8	11.21	1	5.39	4.31	6.68	2.25	3.71	2.06	8.26	2.04	5.36
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR47-3												
Mean	7.6	17.3	6.33	13.63	7.2	17.92	6	14.11	7.46	17.28	6	13.44
Variance	1.8	13.84	1.95	7.61	3.17	11.28	2.71	6.44	2.26	9.46	2	5.91
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR14-1												
Mean	8	25.8	5.9	19	7.3	27.11	5.3	20.33	7	24.44	5.4	17.61
Variance	5	8.86	0.55	6.75	7.7	4.11	3.57	2	4.12	7.77	4.42	2.61
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR14-3												
Mean	8.55	18.47	5.77	12.64	7.66	18.52	5.11	13.41	7.11	18	5	13.17
Variance	5.02	23.51	2.94	13.49	4	15.14	2.61	14	2.86	17.62	2.25	14.4
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR4-3												
Mean	10.5	19.5	9.29	15.1	9.86	20.3	8.71	16.13	9.5	20.2	8	15.43
Variance	3.58	10.92	2.99	6.74	6.48	8.93	2.15	8.83	3.42	10.22	1.91	7.38
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR18-2B												
Mean	8	19.7	5.8	14.16	8.4	19.89	5.8	14.22	9	19.33	6.2	13.55
Variance	3.5	16.41	1.2	16.73	3.3	17.4	3.2	15.24	2.5	11.41	2.2	11.79
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR27-1												
Mean	7.36	18.56	6.25	14.39	7.85	19.18	6.36	14.72	7.78	18.75	6.32	14.2
Variance	3.17	16.39	3.91	14.8	1.67	14.2	2.24	12.06	3.26	12.05	3.22	10.35
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	

Table 4.11 Comparison of plant dimensions of compact and normal plants showed means of plant dimensions were significantly different*** at $P < 0.001$ (Table 4.11).

	Character											
Population	**H3C**		**W3C**		**H4C**		**W4C**		**H5C**		**W5C**	
	Com	Norm	Com	Norm	Com	Norm	Com	Norm	Com	Norm	Com	Norm
BBR 29-2												
Mean	18.8	56.65	29.36	108.5	22.07	73.9	35.21	163.7	26.8	94.2	37.4	176.2
Variance	33.3	146.9	199.0	430.2	22.3	198.6	146.3	839.2	36.28	371.4		
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001			
BBR 29-4												
Mean	21.5	43.22	34	77.1	25.1	47.7	37.6	85.79	28.2	54.63	39.1	94.97
Variance	24.7	91.34	40.44	294.8	41.87	71.28	69.6	341.5	46.17	118.9	74.1	465.7
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR 29-5												
Mean	14.6	34.45	25.13	74.98	18.72	43	29.27	89.98	21.79	52.08	34.55	105.1
Variance	23.3	54.93	41.62	358.2	24.21	106.3	57.63	489.3	39.66	125.6	80.18	697.4
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR47-1												
Mean	28.3	52.66	40.5	113	37	64.4	49.33	128.4	40	77.13	56.83	154.9
Variance	102.	127.5	396.3	544	198.8	174.3	495.0	525.7	520	293.8	451.7	1130
T-test	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR47-2												
Mean	17.4	37.85	25.55	53.6	21.55	44.75	30	68.25	26.22	58.6	35	87
Variance	24.5	83.71	30.77	194.9	40.52	76.19	17.25	582.1	68.94	281.2	56.25	1319
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR47-3												
Mean	14.8	30.31	28.73	67.83	19.2	35.08	32.8	78.69	24.27	43.08	37.73	90.97
Variance	16.1	43.19	22.06	301.3	21.17	45.45	43.88	476.5	15.78	81.67	41.06	545
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR14-1												
Mean	20	55.89	40	128	23.2	64.45	42.2	141.1	27.2	74.22	47.4	176.8
Variance	15.5	70.86	130.5	179.3	7.7	168.3	77.7	231.6	14.7	135.7	110.8	602.4
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR14-3												
Mean	14.6	30.29	30.22	71.88	17.66	40.17	32.66	89.94	22	45.35	36	100.8
Variance	26.7	144.6	46.44	734	23.25	132.4	46	550.8	20.25	167.7	49	704.2
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR4-3												
Mean	20.3	46.98	34.29	89.13	28.57	54.53	38.29	100.7	30.71	62.35	43.29	108.9
Variance	56.8	81.82	37.9	287.7	20.28	11.03	33.9	261.3	27.57	118.1	62.24	269.7
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR18-2B												
Mean	11	32.44	27.8	71.56	15.8	40.72	30.4	88	19.4	46.33	36.2	102.3
Variance	4	58.03	61.7	353.2	19.7	84.68	47.8	579.5	43.3	136.4	33.2	824.4
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	
BBR27-1												
Mean	15.5	37.72	26	76	20.43	44.64	30.79	90.1	25.86	51.36	37.29	99.66
Variance	7.95	80.67	30.92	484.8	19.65	88.61	36.18	420.2	45.21	75.59	100.6	550.6
P (T-test)	P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001		P < 0.001	

Figure 4.2 Examples of observed leaf morphology

Mature compact genotypes Mature standard genotype



4.4.3 Comparison of classes of normal habit genotypes

4.4.3.1 Comparison using an XY scatter graph

Experiments earlier in this chapter have characterised the reduced morphology of compact genotypes compared to their normal siblings in F_2 populations. They yielded valuable information but did not differentiate genotypes that generate segregating populations when self pollinated from those that generate populations that do not segregate for compact habit. When a progeny test was conducted for inheritance studies in chapter 5 (on two sub populations derived from a heterozygous genotype designated BBR 29), the data was re-assessed to plot the relationship between these two classes based on the 12 characters analysed in the earlier part of the study (See section 4.3.3.2). This approach was performed to determine if both classes could be differentiated. This work may also determine if the compact form is codominant or recessive. From this data, the large leaf class, comprising both the heterozygotes and homozygous normals were plotted on an XY scatter graph (Figures 4.3 and 4.4). Heterozygous genotypes were evenly distributed through the range depicted in the graphs. The two genotypes could not be differentiated.

Figures 4.3 and 4.4 The genotypes that produce segregating populations when self pollinated are shown in red circles and those that generate populations that do not segregate for compact habit are shown in blue circles. The cluster of blue triangles to the lower left exclusively contains compact genotypes. The results clearly indicate that both classes of large leafed plants can not be differentiated by such an approach.

Figure 4.3

BBR29 sub pop 4

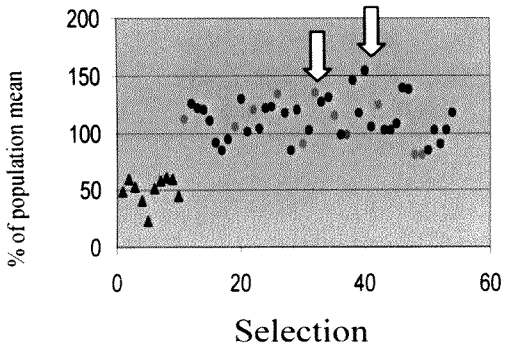
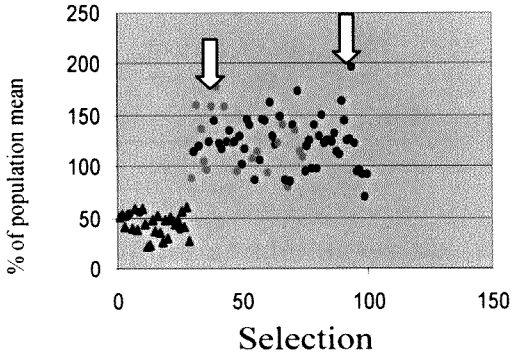


Figure 4.4

BBR29 sub pop 5



There is no evidence the genotypes that produced segregating populations when self pollinated were different to the other large leafed class.

4.4.3.2 Statistical analysis to differentiate normal genotypes

The characters listed in 4.3.3.2 (characters 1-12) were again used for this analysis. All measured characters from the population BBR29-4-0 (table 4.12) showed no significant differences (P = 0.05) between heterozygous groups (identified by Aa) and homozygous normals (Wild type) (identified by AA). In the BBR29-5-0 population 9 of the 12 measured characters were found not to be significantly different (table 4.13) whilst three were significantly different (P = 0.05). Based on results the hypothesis of their being a consistent significant difference in means is rejected. The two genotypes cannot be accurately distinguished using this method.

Table 4.12 Attempts to differentiate heterozygous and homozygous normal genotypes using variance and means - sub population BBR29 - 4

	Character											
Population	**L1T**		**W1T**		**L2T**		**W2T**		**L3T**		**W3T**	
	AA	Aa	AA	Aa	AA	Aa	AA	Aa	AA	Aa	AA	Aa
BBR 29-4												
Mean	19	18.45	13.23	13	19.06	19.82	13.42	13.9	18.67	19	13.12	12.73
Variance	11.9	8.27	8.45	6.4	9.31	15.16	5.31	8.69	8.85	15	6.98	12.22
P (T-test)	0.64		0.82		0.56		0.63		0.8		0.74	

	Character											
Population	**H3C**		**W3C**		**H4C**		**W4C**		**H5C**		**W5C**	
	AA	Aa	AA	Aa	AA	Aa	AA	Aa	AA	Aa	AA	Aa
BBR 29-4												
Mean	44.7	38.55	77.18	77	49.06	43.64	87.21	81.55	55.55	51.9	96.82	89.45
Variance	94.1	59.27	215.8	577	75.31	41.25	309.2	452.5	114.1	135.1	462.0	478.9
P (T-test)	0.06		0.98		0.06		0.44		0.37		0.35	

Table 4.13 Attempts to differentiate heterozygous and homozygous normal genotypes using variance and means - sub population BBR29 - 5

	Character											
Population	**L1T**		**W1T**		**L2T**		**W2T**		**L3T**		**W3T**	
	AA	Aa	AA	Aa	AA	Aa	AA	Aa	AA	Aa	AA	Aa
BBR 29-5												
Mean	19.6	21.88	18.74	16.43	19.66	21.13	14.94	15.63	18.91	20.75	13.87	15.56
Variance	15.9	27.58	385.4	20.53	16.19	18.78	10.47	20.78	11.67	14.33	6.34	13.73
P (T-test)	0.14		0.43		0.24		0.51		0.09		0.04*	

	Character											
Population	**H3C**		**W3C**		**H4C**		**W4C**		**H5C**		**W5C**	
	AA	Aa	AA	Aa	AA	Aa	AA	Aa	AA	Aa	AA	Aa
BBR 29-5												
Mean	33.0	38.94	73.31	80.25	41.24	48.63	87.61	98.56	50.77	56.06	101.9	117.3
Variance	51.2	44.33	385.8	266.5	79.36	169.2	464.8	537.1	90.74	239.1	565.1	1023
P (T-test)	0.004**		0.2		0.01**		0.12		0.09		0.09	

BBR 29-4

4.4.4 Cytogenetic characterisation of compact habit genotypes – meiotic analysis

It was established that 2n = 32 is typical for this species (Chapter 2). The aim of this component was to determine if a chromosomal abnormality may be associated with the compact form.

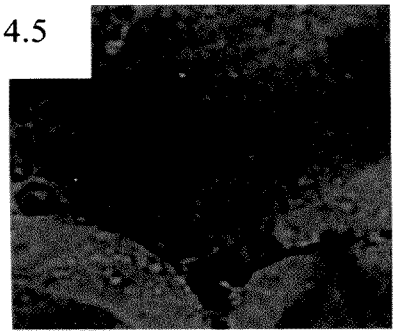
Of the 15 genotypes assessed (Table 4.14), no deviation from 2n=32 was found in individuals generated through wide hybridisation (genotypes 1-13). No multivalent observations were observed. No obvious abnormality in chromosome structure was evident (figures 4.5 – 4.10).

Table 4.14 Genotypes used in the cytology study and chromosome numbers observed in 15 compact habit genotypes generated during the study.

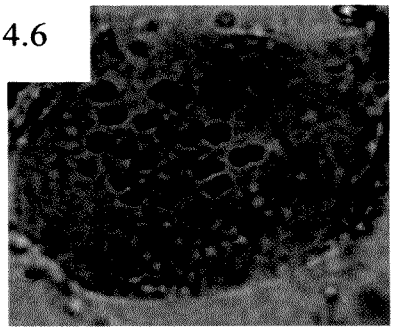
Genotypes assessed	Cells viewed	Chromosome number (2n)	Multivalents
1. BBR29-23	10	32	0
2. BBR29-18	10	32	0
3. BBR29-17	10	32	0
4. BBR29-25	10	32	0
5. BBR29-34	10	32	0
6. BBR29-53	10	32	0
7. BBR29-75	10	32	0
8. BR29-80	10	32	0
9. BBR47-7	10	32	0
10. BBR47-16	10	32	0
11. BBR47-38	10	32	0
12. BBR47-49	10	32	0
13. BBR47-64	10	32	0
14. T1	10	32	0
15. T2	10	32	0
Total	150	32	0

Figures 4.5 – 4.10 Meiotic observations in compact genotypes. Figure 4.5 shows metaphase I of genotype BBR29 –MP 23. Figure 4.6 depicts genotype BBR29- MP 53 at pro-metaphase I. Figure 4.7 is genotype BBR47- MP 38 at pro-metaphase I. Figure 4.8 shows genotype BBR47- MP38 at pro-metaphase I. Figure 4.9 shows genotype BBR47- MP16 at metaphase I and figure 4.10 shows genotype BBR47- MP 7 at metaphase I.

4.5



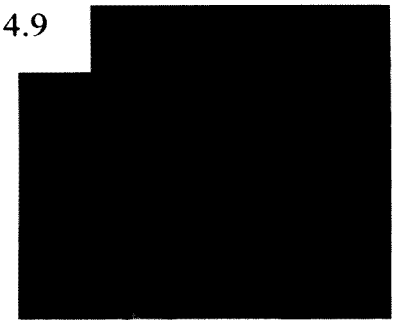
4.6



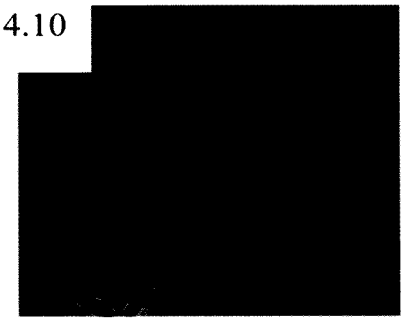
4.7

4.8

4.9



4.10



There was no evidence of any cytological abnormality being correlated with the compact trait. The small size of the chromosomes precluded specific karyotypic analysis therefore an abnormality cannot be unequivocally dismissed. It can however be stated as unlikely based on available evidence.

4.5 DISCUSSION

4.5.1 Quantitative phenotyping

This chapter set out to develop a quantitative means by which compact genotypes could be reliably differentiated from normal habit genotypes. Fourteen characters were investigated as potential means to differentiate the two classes in this study.

Cotyledon dimensions were unreliable characters for the differentiation of the compact and normal classes. However, significant differences between means of each of the classes were observed in 20 of the 28 data sets (10 for cotyledon length and 10 for cotyledon width). It appears that the form has an influence on cotyledon dimensions but in some cases that the characters are substantially influenced by genes that are not related to the form explaining why significant differences were not always detected. In all cases where significant differences were identified between the classes the compact class had lower means than the corresponding normal habit plants.

Each of the 12 vegetative characters that were examined differentiated the compact and normal habit classes in all of the populations investigated. For these characters, genetic variation conferred by loci not directly regulating the form was vastly smaller than that from loci directly involved in its expression. However none of these characters differentiated the normal habit plants that generated a population that segregated for compact and normal habits when self pollinated, from those that only generated normal habit plants upon self pollination.

The normal habit phenotype appears to be completely dominant over the compact habit and other characters that are commonly associated with it.

4.5.2 Time of effect and pleiotropy of the compact habit

It is difficult to compare the effect of genetics that influence similar characters in different species due to the large differences between them. Typically, the characters that are affected and the timing of expression of the different phenotype are

used to describe forms that are not explained by a change of function of a particular gene. In this study it is evident that the form confers differences to development as early as seed development, thus influencing cotyledon dimensions.

A number of other characters also appear to be commonly associated with the compact habit which includes small flowers with a split keel petal, reduced fertility (data not shown).

These undesirable characters represent impediments to the incorporation of the form into breeding efforts aimed at developing varieties. Some genotypes were identified in these populations which did not possess these commonly associated characters, suggesting that selected genotypes possessing the compact form may be used to develop improved commercially viable Sturt pea varieties relatively efficiently.

A compact form in the genus *Pisum* has been described which possess some similar characteristics to the compact habit Sturt pea which was the subject of this research. Plants containing the *Pisum* mutation are described as having "short, thin stems, very small leaves and profuse branching" (Symons *et al.*, 2002). The *Pisum* mutation is described as only becoming evident after 10-14 days of development (Symons *et al.*, 2002). As differences between compact habit and normal habit Sturt pea plants were expressed during seed development ie. cotyledon dimensions from compact genotypes were usually significantly less than the corresponding means of their siblings, it appears that the compact habit form examined in this study is different to the *Pisum* mutation

4.5.3 Impact of the form on breeding efficiency

Compact plants did not produce viable seed when self pollinated, however occasionally some pod development was observed. Closer examination revealed that pollen integrity and viability was lower when isolated from compact genotypes than from normal habit genotypes (see appendix 2 and 3). This attribute is not expected to impact on plant breeding strategies because it can be readily overcome by applying more pollen to stigmas when crossing (as long as some of the pollen grains are viable and capable of fertilisation) or by selection for genotypes that have greater pollen

fertility. In the course of these experiments substantial variation for pollen fertility was observed.

When the compact habit genotypes were used as female parents in crosses with normal habit plants, the rate of seed set was extremely low (data not shown). However substantial variation in the rates of seed set was observed between genotypes. The breeding efficiency of compact genotypes is extremely low, however it is genotype dependent. As compact habit female parents reduce the efficiency of hybridisation with normal habit genotypes it is recommended that any breeding strategy should include genotypes that have high rates of seed set or should preferentially use compact genotypes as the male parent in hybridisations.

4.5.4 Cytological studies and the compact habit

Meiotic metaphase cells of hybrids were examined in an attempt to identify any variation from the typical complement and behaviour that was characterised in Chapter 2. No substantial or consistent variation was observed. It appears unlikely that a chromosomal mutation is responsible for the novel phenotype. The level of congruity observed in chromosomes of compact genotypes appeared compatible with that observed in standard genotypes.

It is likely that the form is due to changes in gene sequences, or small chromosome structural changes that are not detectable using the cytological approaches employed in this study. Based on this assertion, genetic linkage or pleiotropy are the simplest and most likely scenarios that may explain the common association of compact habit and changes to many diverse characters that was observed in the populations studied.

CHAPTER FIVE

THE INHERITANCE OF A COMPACT HABIT FORM IN STURT PEA

5.1 INTRODUCTION

The influence of a compact habit form of Sturt pea on readily measured characters was investigated in Chapter 4. In this work it was identified that the compact habit was commonly associated with a number of unique characters (data not shown). Importantly, a quantitative approach to distinguish genotypes that are normal from those that possess the novel compact form was developed.

Aspects of the inheritance of the form were not explored in that study. An understanding of the genetic control of the novel compact habit will expedite breeding efforts aimed at developing improved Sturt peas for pot production.

Studies were undertaken with the objectives of establishing the inheritance of the new form in different genetic backgrounds then subjecting inheritance data to statistical analyses. Ultimately, the goal was to determine the number of genes controlling this form and establishing the pattern of transmission between generations.

5.2 MATERIALS

5.2.1 Starting plant materials

Directed hybridisation was performed to generate parents and populations for inheritance analysis. Parental material that was used in original hybridisations consisted of a vegetatively propagated compact form (plant code 18), and two normal habit genotypes (APR 1 and P/P 4.100) that were unrelated.

5.3 METHODS

5.3.1 Seed sampling and treatments

Seed sampling and treatments that were applied are described in section 2.3.1

5.3.2 Growing media and environment

Growing media and details of the growing environments utilised are described in section 2.3.2

5.3.3 Populations for inheritance studies and their designations

5.3.3.1 Generation of segregating BBR F_2 populations

An F_1 hybrid that was generated by crossing a compact habit (designation 18) ovule parent with a normal habit pollen parent (APR 1) was subsequently crossed as a pollen parent to a standard habit genotype (code P/P 4.100). Hybrids generated from this cross were self pollinated and the inheritance of the novel form was analysed in populations generated from 8 randomly selected progeny. These populations were designated BBRx,y,z.

5.3.3.2 Generation of a top cross

One of the genotypes that was generated in section 5.3.3.1 that generated a segregating population when selfed was used as a pollen parent (genotype BBR47) in a hybridisation with an unrelated normal habit genotype (code B13). This plant was part of the B series plants generated for floral studies discussed in chapter 6. Twenty plants that were generated from this cross were flowered and self pollinated to produce F_2 seed. A sample of between 12 and 16 F_2 seed was sown from each of the 20 plants to classify the generated populations as segregating or not segregating for the compact habit.

5.3.3.3 Generation of a segregating BBR29 F_3 population

50 F_2 plants from population BBR29 that possessed normal habits were self pollinated to generate F_3 plants. Segregation within each family was evaluated to classify parent F_2 plants into 2 classes (segregating or non-segregating).

5.3.3.4 Generation of supplementary F_2 populations derived from diverse parents

Amongst a range of attempted crosses using F_2 recombinant compact genotypes as ovule parents, two crosses involving the same compact genotype yielded viable hybrid seed. These crosses utilised a compact genotype with the code BBR47mp38 derived from crosses outlined in 5.3.3.1. This genotype was hybridised as an ovule parent with a normal habit wild type plant from Wiluna, Western Australia (designation W14) and a wild type from Roxby Downs, South Australia (designation RD12). Both normal habit plants were grown from wild collected seed.

5.3.3.5 Generation of an F_2 population using a compact habit genotype as a male parent

The same compact genotype (BBR47mp38) that was described in section 5.3.3.4 was used as a pollen parent in a cross with a normal habit plant (B30). This plant was part of the B series plants generated for floral studies discussed in chapter 6. One of the hybrids was self pollinated and produced seed for evaluation of segregation in F_2 plants to determine if a cytoplasmic influence is present.

5.3.4 Phenotyping

Plants were classified as either possessing a compact or normal habit after they had at least attained full expansion of the 5th compound leaf. At this stage the classes were readily differentiated (Chapter 4).

5.3.5 Statistical analysis

5.3.5.1 Chi^2 tests

Chi^2 tests were conducted to test the agreement between a stated hypothesis and observations. This test assesses the likelihood that a hypothesis applies to a data set by comparing predicted and actual frequency of classes within a population. A goodness of fit test was conducted by applying the following formula (Bailey 1995) where O and E are the observed and expected numbers in any group respectively and Σ indicates the sum of all groups. The ratios of 13:3 and 3:1 were tested.

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

5.3.5.2 Tests of homogeneity / heterogeneity across plant families

To establish if the patterns of statistical variation that groups within a population display are homogeneous or heterogeneous, where appropriate, plant families were subjected to heterogeneity tests (Bailey 1995). When these analyses were conducted, heterogeneous groups were removed and further analyses (chi tests) were undertaken.

5.3.5.3 Construction of an inheritance model using data from the BBR29 family in the F_2 and F_3

Fifty of the normal habit plants from the BBR29 F_2 population were classified into two classes based on the presence or absence of segregation for habit in F_3 populations which were generated by self pollinating each of the F_2 plants (See section 5.3.3.3). An observed inheritance model for the F_2 was reconstructed by adding the compact habit fraction, which was derived from the proportion of compact habit plants of the BBR29 F_2 population, to the observed numbers of normal habit F_2 plants that generated segregating or non segregating F_3 populations which was determined by progeny testing.

The mathematical approach to construct this three class model relies on other results as set out below:

A) In the F_2 generation of BBR 29 the compact fraction was quantified as 20.6% of the total (95 / 460) x 100/1. Thus the remaining fraction to be characterised = 79.4% of the total population.

B) The progeny test comprised 50 F_2 genotypes therefore these 50 genotypes = 79.4% of the reconstructed population. (Of these 50, 37 were characterised as heterozygous and 13 homozygous normal).

C) It follows that 1% of the population is determined by 50/79.4 = 0.6297

D) Therefore 20.6% of this reconstructed population is 20.6 x 0.6297 = 12.97

The observed frequencies of each class in the reconstructed population were subjected to chi^2 analysis as described 5.3.5.1 for goodness of fit to one and two genes.

5.3.6 Cytological assessment of heterozygous hybrid genotypes

All cytological assessments were conducted as described in section 2.3.4. The sixteen (16) genotypes assessed are described in Table 5.1. Genotypes that contributed to the inheritance study are denoted with an asterisk.

Table 5.1 Hybrid genotypes assessed cytologically

Ovule parent	Genotype	Pollen parent	Genotype	Genotypes assessed
Combination A				
BBR47mp38	Compact	Wiluna W/T	Normal	F_1-10*
*	*	*	*	F_1-12*
*	*	*	*	F_1-15
*	*	*	*	F_1-16
*	*	*	*	F_1-17*
*	*	*	*	F_1-31
*	*	*	*	F_1-33
*	*	*	*	F_1-40*
Combination B				
BBR47mp38	Compact	Roxby Downs W/T	Normal	F_1-1*
*	*	*	*	F_1-4
*	*	*	*	F_1-7*
*	*	*	*	F_1-9*
*	*	*	*	F_1-10*
*	*	*	*	F_1-11
*	*	*	*	F_1-20*
Combination C				
B30	Normal	BBR47mp38	Compact	F_1-1*

5.4 Results

5.4.1 Inheritance of compact habit in BBR F_2 populations – 2 class analysis

Chi^2 analysis revealed that a two gene control model (13 normal habit : 3 compact) was acceptable for all of the populations examined (Table 5.2). A one gene model (3 normal habit: 1 compact) could also be accepted for 5 of the smaller populations. However, seven of the eight populations showed a better fit to 13:3.

Table 5.2 Inheritance of the compact habit mutation in BBR F_2 populations

Genotypes	Expected proportion 3 : 1	Expected proportion 13 : 3	Observed frequency	Expected frequency 3 : 1	Expected frequency 13 : 3	χ^2 3 : 1	χ^2 13 : 3
BBR29							
Compact	1	1	95	115	86.3	3.47	0.87
Normal	3	4.33	365	345	373.69	1.15	0.2
Totals	4	5.33	460	460	460	4.63	1.07
P value						P = 0.03	P = 0.30
BBR47							
Compact	1	1	37	49	36.77	2.93	0.001
Normal	3	4.33	159	147	159.22	0.97	0.0003
Totals	4	5.33	196	196	196	3.91	0.001
P value						P =0.05	P = 0.97
BBR4							
Compact	1	1	34	52.75	39.58	6.66	0.78
Normal	3	4.33	177	158.25	171.41	2.22	0.18
Totals	4	5.33	211	211	211	8.88	0.97
P value						P = 0.003	P = 0.32
BBR14							
Compact	1	1	17	23	17.26	1.56	0.003
Normal	3	4.33	75	69	74.73	0.52	0.0009
Totals	4	5.33	92	92	92	2.08	0.004
P value						P = 0.15	P = 0.95
BBR18							
Compact	1	1	6	10	7.5	1.6	0.3
Normal	3	4.33	34	30	32.49	0.53	0.06
Totals	4	5.33	40	40	40	2.13	0.37
P value						P = 0.14	P = 0.54
BBR 1							
Compact	1	1	16	16.75	12.57	0.03	0.93
Normal	3	4.33	51	50.25	54.42	0.01	0.21
Totals	4	5.33	67	67	67	0.04	1.15
P value						P = 0.84	P 0.28
BBR27							
Compact	1	1	15	20.75	15.57	1.59	0.021
Normal	3	4.33	68	62.25	67.42	0.53	0.004
Totals	4	5.33	83	83	83	2.12	0.025
P value						P = 0.14	P = 0.87

All of the $BBRF_2$ populations were pooled and chi^2 analysis was conducted. This analysis revealed that a 2 gene inheritance pattern ie 13:3 was the most appropriate fit ($\chi^2 = 0.11$, $P = 0.74$) to the observed inheritance pattern.

The single gene pattern ie 3:1 was rejected $\chi^2 = 20.99$, ($P \leq 0.001$).

5.4.1.1 Inheritance of compact habit in a subset of the BBR29 F_2 populations – 3 class analysis

50 normal habit genotypes from the BBR 29 F_2 population were classified by self pollinating them and determining if the F_3 population generated from each segregated or did not segregate for compact habit (Table 5.3).

Of the 50 normal habit genotypes that were self pollinated, 37 generated F_3 populations that segregated for habit and 13 generated populations that did not segregate.

The heterozygous class exceeded 50% of the population (37 of 50). The frequency of compact genotypes in individual F_3 families varied (Table 5.3) as can be expected with small sample sizes.

Three class chi^2 analysis (2 degrees of freedom) was performed on a subset of the BBR29 F_2 population (see methodology 5.3.5.3). This analysis revealed that a single gene control (3:1) appears to be the most appropriate inheritance model for the data obtained. The 13:3 inheritance mode was rejected based on this analysis (Tables 5.4 and 5.5).

Table 5.3 Classification of F_2 plants from the BBR29 population based on the presence of segregation in F_3 populations.

Test no.	Genotype	Sown	Germ	Germ %	Compact	Normal	Segregation
	BBR29-						
1	1	16	16	100	3	13	Yes
2	2	16	16	100	4	12	Yes
3	5	15	15	100	3	12	Yes
4	6	17	15	88.23	0	15	No
5	7	14	14	100	5	9	Yes
6	8	16	16	100	6	10	Yes
7	9	16	16	100	3	13	Yes
8	10	16	16	100	3	13	Yes
9	11	13	7	53.84	2	5	Yes
10	12	16	16	100	2	14	Yes
11	13	16	16	100	0	16	No
12	14	16	16	100	4	12	Yes
13	14a	16	16	100	3	13	Yes
14	15	16	16	100	2	14	Yes
15	16	16	16	100	5	11	Yes
16	17	16	16	100	0	16	No
17	19	16	16	100	2	14	Yes
18	20	16	16	100	1	15	Yes
19	22	14	11	78.57	4	7	Yes
20	23	17	16	94.11	4	12	Yes
21	26	16	15	93.75	4	11	Yes
22	28	16	16	100	3	13	Yes
23	29	16	16	100	0	16	No
24	30	16	13	81.25	2	11	Yes
25	31	16	16	100	4	12	Yes
26	32	15	15	100	4	11	Yes
27	36	16	15	93.75	2	13	Yes
28	39	16	16	100	5	11	Yes
29	42	15	14	93.33	0	14	No
30	44	8	6	75	0	6	No
31	44a	15	14	93.33	0	14	No
32	46	15	15	100	5	10	Yes
33	48	16	15	93.75	2	13	Yes
34	52	16	14	87.5	0	14	No
35	53	15	15	100	2	13	Yes
36	54	6	5	83.33	0	5	No
37	54a	16	16	100	0	16	No
38	55	16	16	100	1	15	Yes
39	56	8	8	100	3	5	Yes
40	57	16	16	100	4	12	Yes
41	57a	16	16	100	0	16	No
42	63	8	7	87.5	0	7	No
43	64	4	3	75	1	2	Yes
44	67	5	5	100	2	3	Yes
45	69	16	16	100	6	10	Yes
46	72	14	14	100	5	9	Yes
47	74	16	13	81.25	3	10	Yes
48	77	16	16	100	2	14	Yes
49	79	15	15	100	3	12	Yes
50	84	11	11	100	0	11	No

Table 5.4 Testing a hypothesis of a single gene

Single gene model

Genotypes	Expected proportion	Observed frequency	Expected frequency	χ^2
Compact	1	12.97	15.74	0.489
Segregating	2	37	31.49	0.967
Normal	1	13	15.74	0.478
Total	4	62.97	62.97	1.93

χ^2 1:2:1, 1.93, P = 0.38

Table 5.5 Testing a hypothesis of two genes

Two gene model

Genotypes	Expected proportion	Observed frequency	Expected frequency	χ^2
Compact	3	12.97	11.80	0.12
Segregating	4	37	15.74	28.63
Normal	9	13	35.42	14.23
Total	16	62.97	62.97	42.97

χ^2 9:4:3, 42.97, P $\leq$ 0.001

5.4.2 Inheritance of compact habit in F_1 hybrids produced by hybridising genotypes that when selfed produce segregating (Parent A) and non segregating (Parent B) populations

In an attempt to validate the result of the 3 class chi^2 analysis of the BBR29 F_2 population inheritance analysis was performed on F_1 plants that were generated by crossing a hybrid normal habit plant (BBR47 pollen parent) that when self pollinated generated a F_2 population that segregated for habit with a normal habit plant that when self pollinated did not segregate for habit. It was determined that the F_1 progeny of this cross consisted of 10 plants that produced a segregating population when self pollinated and 10 plants that did not. Chi^2 analysis (Table 5.6) revealed that this pattern was consistent with the model that would be expected if the character was controlled by a single gene and one parent was a heterozygote and the other parent was a homozygote at the controlling gene. In this analysis the 13:3 inheritance pattern was rejected based on the results of chi^2 analysis χ^2 2 gene, 6.66, P = 0.098.

Table 5.6 Chi^2 analysis of 20 F_1 genotypes from the cross B13 x BBR47

Phenotypes	Expected proportion 1 gene	Expected proportion 2 gene	Observed frequency	Expected frequency 1 gene	Expected frequency 2 gene	χ^2 1 gene	χ^2 2 gene
Segregating	1	1	10	10	5	0	5
Homozygous	1	3	10	10	15	0	1.66
Totals χ^2 1:1, 0, P = 1	2	4	20	20	20	0	6.66

In an attempt to establish that this finding was relevant to the BBR F_2 populations that were previously analysed the inheritance of the F_2 populations derived from the plants that produced segregating populations was investigated. To enable this, F_2 data from the segregating component (10 genotypes) of the cross B13 x BBR47 was pooled (Table 5.10). The proportion of normal to compact plants was 111:26 (χ^2 13:3, 0.004, P = 0.95) which is statistically not different to that observed in the BBR47 self F_2 population (χ^2 13:3, 0.001, P = 0.97). Both 13:3 and 3:1 models were acceptable

explanations of the observed pattern according to chi^2 analysis (P = 0.05). However it has again been demonstrated 13:3 is a better fit (Table 5.7).

Table 5.7 Pooled F_2 data B13 x BBR47 chi test for inheritance model

Phenotypes	Expected proportion 3 : 1	Expected proportion 13 : 3	Observed frequency	Expected frequency 3 : 1	Expected frequency 13 : 3	χ^2 3 : 1	χ^2 13 : 3
Compact	1	1	26	34.25	25.70	1.98	0.003
Normal	3	4.33	111	102.75	111.29	0.6	0.0007
Totals	4	5.33	137	137	137	2.64	0.004

χ^2 13:3, 0.004 P = 0.95

5.4.3 Inheritance of compact genotypes in F_2 populations produced in crosses (5.3.3.4) BBR47mp38 x Roxby Downs W/T parent (RD12)

If another factor is influencing transmission of a single gene in meiosis, it was considered that examination of populations that were generated from a diverse set of parents may reveal a typical single gene inheritance pattern for some of them.

To examine this hypothesis five (5) F_2 populations derived from F_1 plants that were generated by crossing a compact habit plant (BBR47mp38) with Roxby Downs W/T (W14) were examined.

Chi^2 analysis (Table 5.8) revealed that four of the five populations examined had a better fit to a 13:3 model. The largest of these populations did not fit a 13:3 inheritance pattern.

Table 5.8 Inheritance of compact genotypes in F_2 populations - BBR47mp38 x Roxby Downs W/T (RD12)

Phenotypes	Expected proportion 3 : 1	Expected proportion 13 : 3	Observed frequency	Expected frequency 3 : 1	Expected frequency 13 : 3	χ^2 3 : 1	χ^2 13 : 3
F_1-9							
Compact	1	1	84	86.25	64.72	0.05	5.73
Normal	3	4.33	261	258.75	280.27	0.01	1.32
Totals	4	5.33	345	345	345	0.07	7.06
P Value						P = 0.79	P = 0.08
F_1-7							
Compact	1	1	35	48	36.02	3.52	0.02
Normal	3	4.33	157	144	155.97	1.17	0.006
Totals	4	5.33	192	192	192	4.69	0.03
P Value						P = 0.03	P = 0.86
F_1-10							
Compact	1	1	24	30.75	23.07	1.48	0.03
Normal	3	4.33	99	92.25	99.92	0.49	0.008
Totals	4	5.33	123	123	123	1.97	0.04
P Value						P = 0.16	P = 0.84
F_1-20							
Compact	1	1	28	40	30.01	3.6	0.13
Normal	3	4.33	132	120	129.98	1.2	0.03
Totals	4	5.33	160	160	160	4.8	0.16
P Value						P = 0.03	P = 0.69
F_1-1							
Compact	1	1	22	30.75	23.07	2.48	0.05
Normal	3	4.33	101	92.25	99.92	0.82	0.01
Totals	4	5.33	123	123	123	3.31	0.06
P Value						P = 0.07	P = 0.81

It appears that compact habit is inherited differently in population F_1-9 to the other populations examined in this experiment that were generated from sibling F_1's. The data from population F_1-9 is consistent with a single gene. The remaining four groups appear homogeneous. Data from this sub-group was pooled for further assessment in Table 5.9.

Table 5.9 Pooled data BBR47mp38 x Roxby Downs W/T (RD14) F_1- 1, 7, 10, 20

Phenotypes	Expected proportion 3 : 1	Expected proportion 13 : 3	Observed frequency	Expected frequency 3 : 1	Expected frequency 13 : 3	χ^2 3 : 1	χ^2 13 : 3
Compact	1	1	109	149.5	112.19	10.97	0.09
Normal	3	4.33	489	448.5	485.80	3.65	0.02
Totals	4	5.33	598	598	598	14.62	0.11

χ^2 13:3, 0.11, P = 0.74

Pooled data from the four sub groups with homogeneous inheritance were consistent with a 13:3 inheritance ratio ($\chi^2 = 0.11$, P = 0.74)

5.4.4 Inheritance of compact genotypes in F_2 populations produced in crosses (5.3.3.4) BBR47mp38 x Wiluna W/T parent (W14)

Of the four F_2 populations assessed (Table 5.10) that were produced by the hybridisation of BBR47mp38 x Wiluna W/T parent (W14), two clearly showed a better fit for 13:3 inheritance (F_1-10 and F_1-12). Data from a third population (F_1-17) showed a better fit to a single gene model (3:1). An inheritance model of 13:3 or 3:1 could be accepted for the fourth population (F_1-40) with equal confidence.

Table 5.10 Inheritance of compact genotypes in the F_2 populations produced after the hybridisation of compact genotype BBR47mp38 x Wiluna W/T parent W14

Phenotypes	Expected proportion 3 : 1	Expected proportion 13 : 3	Observed frequency	Expected frequency 3 : 1	Expected frequency 13 : 3	χ^2 3 : 1	χ^2 13 : 3
F_1-17							
Compact	1	1	20	20.25	15.19	0.003	1.51
Normal	3	4.33	61	60.75	65.8	0.001	0.35
Totals	4	5.33	81	81	81	0.004	1.86
						P = 0.95	P = 0.17
F_1-10							
Compact	1	1	30	43	32.27	3.93	0.15
Normal	3	4.33	142	129	139.72	1.31	0.03
Totals	4	5.33	172	172	172	5.24	0.19
						P = 0.02	P = 0.66
F_1-12							
Compact	1	1	42	59.5	44.65	5.14	0.15
Normal	3	4.33	196	178.5	193.34	1.71	0.03
Totals	4	5.33	238	238	238	6.86	0.19
						P = 0.009	P = 0.66
F_1-40							
Compact	1	1	25	28.75	21.57	0.48	0.54
Normal	3	4.33	90	86.25	93.42	0.16	0.12
Totals	4	5.33	115	115	115	0.65	0.66
						P = 0.42	P = 0.42

5.4.5 Investigation of cytoplasmic influence on inheritance in an F_2 population

An F_2 population that was generated from a hybrid which had a compact habit pollen parent (B30 x BBR47mp38) was examined to assess if cytoplasm influenced the inheritance of the compact habit. Results show a compact plant: normal habit plant ratio better fit 13:3 (χ^2 0.02, P = 0.89) in this population (Table 5.11). The single gene model was discarded after chi^2 analysis (χ^2 6.29, P = 0.01). The 13:3 ratio was

consistent with the previously observed pattern of inheritance. The result is consistent with that of other F_2 populations examined and suggests there is no cytoplasmic influence.

Table 5.11 Chi test of the F_2 population x B30 x BBR47mp38 F_1-1

Phenotypes	Expected proportion 3 : 1	Expected proportion 13 : 3	Observed frequency	Expected frequency 3 : 1	Expected frequency 13 : 3	χ^2 3 : 1	χ^2 13 : 3
Compact	1	1	49	66.75	50.09	4.72	0.02
Normal	3	4.33	218	200.25	216.90	1.57	0.005
Totals	4	5.33	267	267	267	6.29	0.02

χ^2 13:3, 0.02, P = 0.94

5.4.6 Cytology of heterozygous hybrid genotypes produced in 5.3.3.4 -5

No chromosome structural abberations or pairing irregularities were detected in meiotic cells of hybrids between unrelated genotypes where the compact genotype was a female parent or male parent (refer to table 5.1). Sixteen bivalents were consistently observed (figures 5.1-5.3).

Observations were made at both diad and tetrad formation stages to assess consistency of cell division through meiosis I and meiosis II. No obvious abnormality was observed in any individual studied (figures 5.4 – 5.5).

Figure 5.1 16 bivalents formed during meiosis I in genotype BBR47mp38 x RD12 F_1-9

5.1

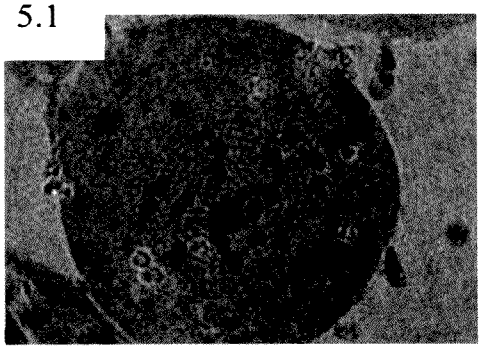


Figure 5.2 16 bivalents formed during meiosis I in genotype BBR47mp38 x RD 12 F_1-20

5.2

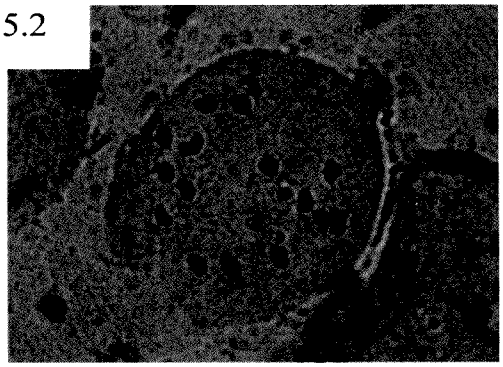
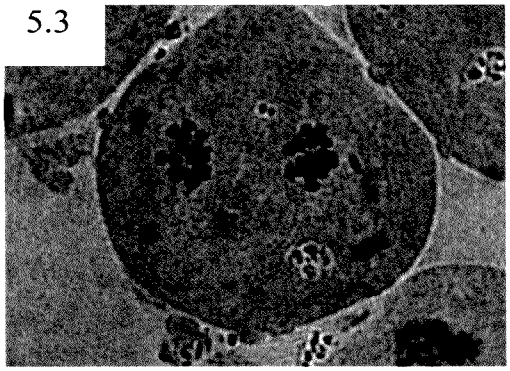


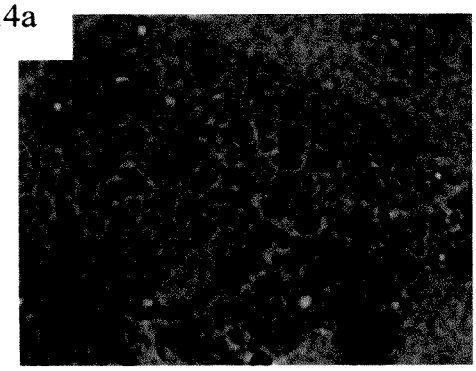
Figure 5.3 16 univalents observed after moving to opposing poles in Anaphase I in genotype BBR47mp38 x RD 12 F_1-9

5.3

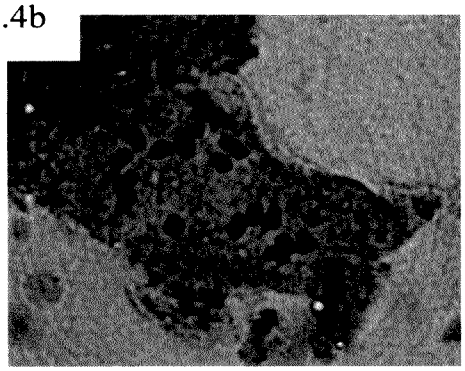


Figures 5.4a - b 16 bivalents observed pre-metaphase I in the hybrid produced using a compact form genotype as a male (B30 x BBR47mp38 F_1-1)

5.4a

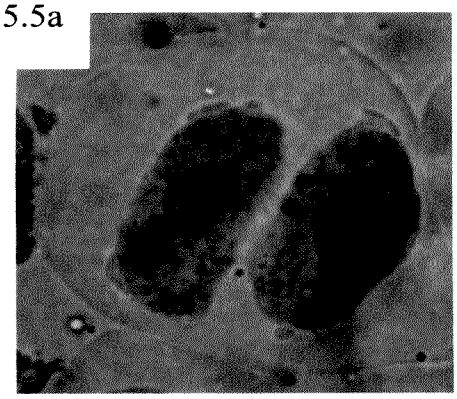


5.4b

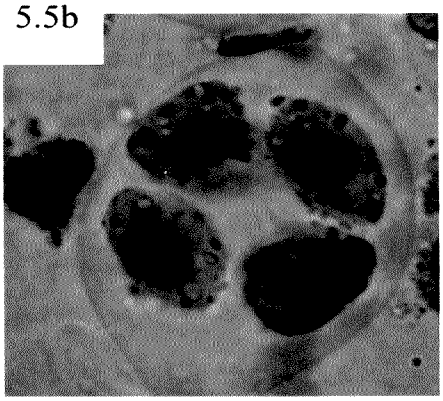


Figures 5.5a - b Diad and tetrad formations in the hybrid produced using a compact form genotype as a male (B30 x BBR47mp38 F_1-1)

5.5a



5.5b



5.5 Discussion

5.5.1 Aspect of the inheritance of compact habit

This chapter set out to investigate the inheritance of the compact habit. Two types of inheritance were identified depending on the population types that were examined and the number of classes that could be determined, which affect the degrees of freedom of a chi^2 analysis.

5.5.2 Inheritance in F_2 populations

When the first set of F_2 populations were classified using two classes viz. normal habit and compact habit a 13:3 ratio fit all of them. A three to one model also fitted

most of them (5 of 8 if confidence limit is 0.05); however this model was rejected after pooled analysis. Three class analysis, which separately classified normal habit plants that produced populations when self pollinated from those that did not, was conducted on a subset of the BBR29F_2 population. It revealed that the observed frequencies in this population inheritance were consistent with a pattern of single gene inheritance viz. 3 normal habit: 1 compact habit. Heterozygote excess was identified in this sub population which implies that there is an irregularity. However, allele frequencies were even if a single gene control is assumed. F_1 data, generated by crossing a heterozygote with a homozygote, fit a 1:1 ratio, which implies that compact habit is under single gene control. Only one F_2 population was identified that did not fit a 13:3 inheritance pattern ((Roxby DownsW/T (RD12) x BBR47mp38 F_1-9, χ^2 7.06, P = 0.08)) when two class chi^2 analysis was conducted. When data was compared between F_2 and F_3 generations from the same family (BBR29) differences were identified between them. It can be concluded that single gene control is most likely and that it is probable an uncharacterised factor is affecting the rate of transmission of the gene conferring the compact habit when heterozygotes are self pollinated. The observation of an F_2 population that is consistent with a single gene inheritance and inconsistent with a two gene inheritance pattern suggests the factor modifying transmission in most of the genotypes may be separated from the compact habit gene. These results are consistent with those of Lambrides *et al.*, (2004) who investigated inheritance of testa colour in the mungbean, which demonstrates that such a phenomenon has previously been observed in other legumes.

5.5.3 Heterozygote excess and meiotic drive

The process leading to heterozygote excess is not understood well, however it is not uncommon in plant species (Weeden and Marx 1987 ; Kiss *et al.*, 1993 ; Liu *et al.*, 1994 ; Paredes and Gepts 1995 ; Kearsey *et al.*, 1996 ; Faris *et al.*, 1998 ; Kalo *et al.*, 2000).

Any alteration to the normal meiotic process that results in the excess transmission of one genetic variant over the other was coined "meiotic drive" by Sandler and Novitski (1957). This phenomenon is thought by some to be common (Subudhi and Huang,

1996), particularly in wide or interspecific crosses (Onus 2000). Whilst Mendel's rules require that meiosis is fair – that is, each copy of each allele for each gene has equal probability of being passed on to offspring, results from this study infer unfair transmission. Observations in progeny testing found an over-representation of heterozygous individuals in a segregating F_2 population. This demonstrates a preference or bias for the maintenance of such genotypes. Taylor and Ingvarsson (2003) discuss the possibility that 'non costly' alterations of meiosis are common and spread rapidly to fixation. Tao *et al.*, (2001) concurs suggesting that fixation may result despite possible deleterious pleiotropic effects on fertility. The system in *Sturt pea* seems to differ in that the preference for heterozygosity precludes fixation. This preference shares some similarity with the observation of Bouton and Sledge (2001) on molecular markers in alfalfa. They identified a high degree of segregation distortion favouring heterozygotes, in some cases upwards of 50%. Taylor and Ingvarsson (2003) point out that although there may be many ways to achieve distorted segregation, we have a tendency to detect those traits in which the phenotypic manifestation is easily discerned.

5.5.4 The application of a new model gives better goodness of fit

Whilst the model is unusual, in the BBR 29 F_2 population statistically a better fit can be found if a hypothesis of ¾ of the heterozygous genotypes segregating in a 13:3 manner and ¼ segregating 3:1 is considered ($\chi^2 = 1.60$). This betters the single gene chi value ($\chi^2 = 1.71$), and both are vastly better than 13:3 ($\chi^2 = 4.93$). Such a model can explain pooled F_3 data not fitting 3:1 but gravitating towards it, whilst not ignoring a very good fit to 13:3 in the previous (F_2) generation. This hypothesis requires considerably more work to validate but is currently statistically plausible.

5.5.5 Possibility of a second gene being implicated in inheritance

If compact habit is controlled by a single gene it would be expected that F_2 populations and the segregating fraction of the F_3 would share a common inheritance pattern. Furthermore, if this was the case then 2 class chi^2 analysis of the F_2 would also indicate that single gene control is appropriate. Because of some discrepancies in

the results of these tests it is likely that a factor which is probably genetic is responsible for these deviations from simple single gene inheritance. However statistically it is very difficult to show.

The ability to separate the putative drive factor from the gene conferring the mutation immediately implicates a second gene / factor and confirms the compact trait as being governed by a single recessive gene. The results show the factor does not affect the expression of the compact phenotype. Rather it affects the frequency of the compact genotype in segregating populations.

5.5.6 Conclusion on heritability

A possible point of conjecture in this study is there were many populations in which both hypotheses proposed could be accepted at 95% confidence limits, a limit accepted in biological science. Given it is recognised the large amount of variation in biological material necessitates the use of statistical methods (Bailey 1995), it is important to accept their value and argue only what can be better substantiated, not necessarily that which is more convenient. The statistically more likely has been accepted in each case. As such, it is concluded the compact phenotype is controlled by a single recessive gene but can be inherited as a monogenic recessive trait or in a manner resembling two gene inhibitory behaviour. This second (uncharacterised) gene affects only its numeric representation in segregating populations. Future efforts to characterise this putative second gene would be of great value.

CHAPTER SIX

INHERITANCE OF FLOWER COLOUR IN STURT PEA

6.1 INTRODUCTION

In the potted plant industry it is typical for a set of varieties with similar attributes to be marketed as a series which offers a range of flower colours. The biosynthetic pathways which determine flower colour have been investigated in many plant species. For example, in *Lathyrus* at least 12 genes are implicated in the determination of flower colour (Levko 2001), in *Vigna* two floral colours have been characterised as controlled by a single gene with white flower the recessive phenotype (Sangwan and Lodhi 1998), in Soybean (*Glycine*) expression of several flower colours ranging from white to magenta, pink and purple have been characterised as determined by at least five genes acting epistatically (Fasoula *et al.*, 2004) and flower colour in *Pisum* was examined by Gregor Mendel with simple inheritance identified for major floral forms.

Studies that have attempted to characterise the inheritance of flower colour and the biosynthetic pathways involved have revealed numerous differences exist among related genera (Rauscher *et al.*, 1999, Liu and Rausher 2003).

Many different flower colours have been identified in Sturt pea (Ashby 1991; Anon 2003; Boden 1985; G Kirby pers. Com; M Jusaitis pers. Com). However, the genetic control of flower colour in Sturt pea is not well understood. At present, there are no published works describing the number, function or interaction of flower colour genes in Sturt pea. A better understanding of the inheritance of flower colour will expedite breeding efforts aimed at generating commercial varieties.

This work aims to investigate and characterise the genetic control of flower colour in Sturt pea by generating a range of fixed flower colour lines by inbreeding, and examining the segregation of flower colour in F_2 populations that are derived from hybrids between them.

6.2 MATERIALS

Seed from the collection of Oasis Horticulture Pty Ltd. was used to develop fixed flower colour lines. Seed that was generated from crosses of the same flower colour or that had been generated by self pollination were used as starting material because it was considered that fixed flower colour lines would be developed more efficiently than from random selections. This selected material originated from a wide range of crosses and generations.

6.3 METHODS

6.3.1 Seed collection and treatment

Seed was collected from hybrids and self pollinated flowers when pod colour changed from green to grey / brown and hardening of the pod caused longitudinal splitting and drying. Prior to germination seeds were nicked as described in section 2.3.1.

6.3.2 Growing media and environment

During screening for suitable parental genotypes plants were grown in 150mm pots in a polyhouse at The University of Sydney, Plant Breeding Institute, Cobbitty NSW using potting media that consisted of composted pine bark and coarse sand mixed in a ratio of 4:1. Plants were grown under natural daylight conditions.

All F_1 and F_2 populations that were used to determine the genetics of flower colour were grown as described in section 2.3.2

6.3.3 Determination of flower colour

Flower colours were determined on fully reflexed flowers (stage G as described in Jusaitis and Schmerl 1993) and assigned RHS colour chart codes. To ensure that assignment of colours was accurate three determinations of the flower colour of the standard petal were made in natural daylight over a period of 10-14 days.

The use of keel petals was unsatisfactory for determination in some genotypes because the colour of these petals was not always uniform thus making determinations unreliable. This is particularly evident in genotypes that have a novel tri-colour variant in their parentage which often results in a streaked dilution of colour in the keel petal (see figure 6.1b). Therefore, all designations were based on standard petal colour only.

6.3.4 Generation of fixed flower colour lines

Flower colour lines were generated by germinating, growing and subjecting selected plants to rounds of self pollination. At least 20 seed were grown per self pollination to examine uniformity of flower colour. Five different fixed flower colour lines were developed, which represented three colours and a novel tri-coloured flower mutation (Table 6.1). Each parental series used in this study was designated an alphabetical letter as a prefix for identification. Lines that were used in crossing were selected when all plants generated from self pollination grew true to parental phenotype.

Table 6.1 Generation of fixed flower colour lines

Group	Parental series	Genesis plant	Progeny Gen 1	Progeny Gen 2
1	I series white	White 6.12 self	F_2 Segregated	F_3- lines fixed*
2	W series white	White 4.7 self	F_3 lines fixed	F_4 lines fixed*
3.	B series RHS 41	7.4 self	F_3 lines fixed*	
4	P series RHS 34	7.86 self	F_3 lines fixed	F_4 lines fixed*
5.	Tricolour series	78.2 self	F_3 lines fixed*	

The code of the genesis plant that was selected correlates with pedigree data from Oasis Horticulture Pty Ltd. An asterisk is used to signify the round in which fixed flower colour parents were selected for hybridisation.

6.3.5 Statistial analysis - Chi^2 tests

Chi^2 tests were conducted to test the agreement between a stated hypothesis and observations. This test assesses the likelihood that a hypothesis applies to a data set by comparing predicted and actual frequency of classes within a population. A goodness of fit test was conducted by applying the following formula (Bailey 1995) where O and E are the observed and expected numbers in any group respectively and Σ indicates the sum of all groups.

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

6.4 RESULTS

6.4.1 Observations in F_1 plants

6.4.1.1 Dominance relationships in the F_1

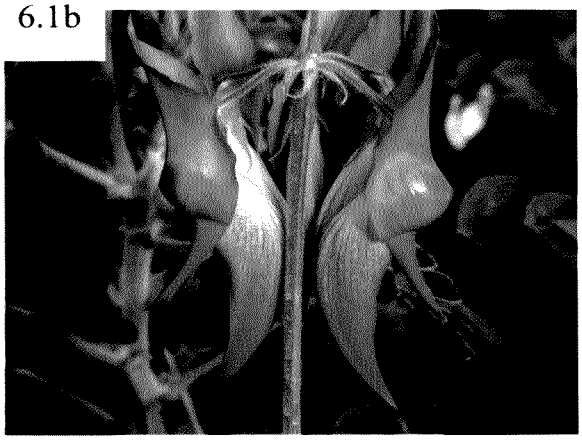
In all F_1 hybrids that were examined dominance of flower colour was observed. Red petal (RHS34a) colour was dominant over pink + (RHS41a) and pink – (RHS39a) and white.

In all crosses that involved parents with white and coloured flowers, white flower colour was recessive. The tri-colour mutation which has a coloured standard petal and a predominantly white keel petal was recessive when coloured and tri-coloured plants were hybridised (See Table 6.2). With regards to boss colour, maroon was dominant over black.

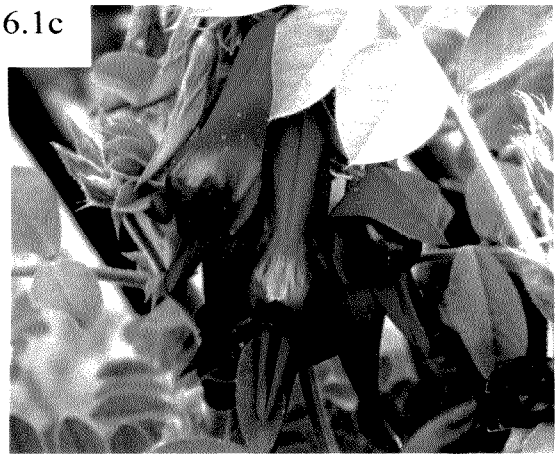
Figures 6.1a – 6.1d (6.1a)- Changing pigment intensity in consecutive peduncles of an F_1 plant (I7.11 x P4.3). (6.1b)- F_2 genotype from cross W10.15 x Tri – diluted petal colour along rib of keel petal. (6.1c)- Red 34a 53a/b (from B35 x P4.4) and (6.1d)- F_2 Tri-colour genotype with all white streaked boss often indicative of a white parent.

6.1a

6.1b



6.1c



6.1d

Table 6.2 Dominance relationships revealed in the F_1 generation

	Ovule parent details			Pollen parent details						Mode of Inheritance	
	Code	Petal Colour	Boss Colour	Code	Petal Colour	Boss Colour	F_1 PETAL	F_1 BOSS	F1's grown	PETAL	BOSS
1	B35	41a	53c/d	P4.4	34a	Black	34a	53a	40	*Complete dominance*	*Incomplete dominance*
2	B29	41a	53c/d	P4.9	34a	Black	34a	53a	34	*Complete dominance*	*Incomplete dominance*
3	B17	41a	53c/d	I7.19	White	White	41a	53c/d	32	*Complete dominance*	*Complete dominance*
4	I7.3	White	White	B17	41a	53c/d	41a	53c/d	25	*Complete dominance*	*Complete dominance*
5	B17	41a	53c/d	Tri	41a/tri	53c/d	41a	53c/d	28	*Complete dominance*	*Complete dominance*
6	W10.15	White	White	Tri	41a/tri	53c/d	41a	53c/d	22	*Complete dominance*	*Complete dominance*
7	P4.7	34a	Black	I7.26	White	White	34a	53a	40	*Complete dominance*	*Incomplete dominance*

6.4.2 Data from F_2 segregating populations

6.4.2.1 Cross designated B35 x P4.4

The petal colour of the ovule parent B35 in this cross was RHS 41a (pink+) and the petal colour of the pollen parent P4.4 was RHS 34a (Red). The petal colour of all F_1 plants was RHS 34a. Table 6.3 shows the frequency of flower colours that was observed in the F_2 population, and results of χ^2 analysis. The data best fitted a four gene model, where RHS 34a was dominant over RHS41a which was dominant over RHS39a. In this model, when petal colour was RHS 34a maroon boss was dominant over black boss.

Table 6.3 Observed and expected frequencies of F_2 genotypes from cross B35 x P4.4

Phenotype	Observed frequency	Expected frequency	Observed proportion	Expected proportion	χ^2
RHS 34 - Maroon	170	172.33	172.33	171	0.03
RHS 34 Black	59	57.44	57.44	57	0.04
RHS 41	22	21.16	21.16	21	0.03
RHS 39	7	7.05	7.05	7	0
Total	258	258	256	256	0.11

$\chi^2 = 0.11$ P=0.99.

6.4.2.2 Cross designated B29 x P4.9

The petal colour of the ovule parent B29 in this cross was RHS 41a (pink +) and the petal colour of the pollen parent P4.9 was RHS 34a (Red). The petal colour of all F_1 plants was RHS 34a. Table 6.4 shows the frequency of flower colours that was observed in the F_2 population, and results of χ^2 analysis. The data best fitted a three gene model, where RHS 34a was dominant over RHS41a which was dominant over RHS39a. In this model, when petal colour was RHS 34a maroon boss was again dominant over black boss.

Table 6.4 Observed and expected frequencies of F_2 genotypes from cross B29 x P4.9

Phenotype	Observed frequency	Expected frequency	Observed proportion	Expected proportion	χ^2
RHS 34 - Maroon	152	156	39	39	0.1
RHS 34 Black	55	52	13	13	0.17
RHS 41	40	36	9	9	0.44
RHS 39	9	12	3	3	0.75
Total	256	256	64	64	1.47

$\chi^2 = 1.47$, P= 0.69

6.4.2.3 Cross designated B17 x I7.19

The petal colour of the ovule parent B17 in this cross was RHS 41a (pink +) and the petal colour of the pollen parent I7.19 was white. The petal colour of all F_1 plants was RHS 41a. Table 6.5 shows the frequency of flower colours that was observed in the F_2 population, and results of χ^2 analysis. The data best fitted a two gene model, where RHS 41a was dominant over RHS39a, and coloured petal was dominant over white.

Table 6.5 Observed and expected frequencies of F_2 genotypes from cross B17 x I7.19

Phenotype	Observed frequency	Expected frequency	Observed proportion	Expected proportion	χ^2
RHS 41	175	177.75	8.86	9	0.04
RHS 39	55	59.25	2.78	3	0.3
White	86	79	4.35	4	0.62
Total	316	316	16	16	0.97

$\chi^2 = 0.97$, P= 0.62

6.4.2.4 Cross designated I7.3 x B17

The petal colour of the ovule parent I7.3 in this cross was white and the petal colour of the pollen parent B17 was RHS 41a (pink +). The petal colour of all F_1 plants was RHS 41a. Table 6.6 shows the frequency of flower colours that was observed in the F_2 population, and results of χ^2 analysis. The data best fitted a three gene model, where RHS 41a was dominant over RHS39a and coloured petal was dominant over white.

Table 6.6 Observed and expected frequencies of F_2 genotypes from cross I7.3 x B17

Phenotype	Observed frequency	Expected frequency	Observed proportion	Expected proportion	χ^2
RHS 41	386	380.39	45.66	45	0.08
RHS 39	27	25.35	3.19	3	0.11
White	128	135.25	15.14	16	0.39
Total	541	541	64	64	0.58

$\chi^2 = 0.58$, P= 0.75

6.4.2.5 Cross designated B17 x Tri-colour

The petal colour of the ovule parent B17 in this cross was RHS 41a (pink +) and the petal colour of the pollen parent tri-colour was RHS 41a with the tri-colour mutation. The petal colour of all F_1 plants was RHS 41a. Table 6.7 shows the frequency of flower colours that was observed in the F_2 population, and results of χ^2 analysis. The data best fitted a three gene model, where RHS 41a was dominant over RHS 39a and normal keel petal was dominant over keel petal with tri-colour mutation.

Table 6.7 Observed and expected frequencies of F_2 genotypes from cross B17 x Tri-colour

Phenotype	Observed frequency	Expected frequency	Observed proportion	Expected proportion	χ^2
RHS 41	184	186.46	54	54	0.03
RHS 39	11	10.35	3	3	0.04
RHS 41 Tri	22	20.71	6	6	0.08
RHS 39 Tri	4	3.45	1	1	0.09
Total	221	221	64	64	0.24

$\chi^2 = 0.24$, P= 0.97

6.4.2.6 Cross designated W10.15 x Tri-colour

The petal colour of the ovule parent W10.15 in this cross was white and the petal colour of the pollen parent tri-colour was RHS 41a (pink +) with tri-colour mutation. The petal colour of all F_1 plants was RHS 41a lacking tri-colour mutation. Table 6.8 shows the frequency of flower colours that was observed in the F_2 population, and results of χ^2 analysis. The data best fitted a two gene model, where RHS 41a with normal keel petal was dominant over RHS41a containing the tri-colour mutation and coloured petal was dominant over white.

Table 6.8 Observed and expected frequencies of F_2 genotypes from cross W10.15 x Tri-colour

Phenotype	Observed frequency	Expected frequency	Observed proportion	Expected proportion	χ^2
RHS 41	215	211.5	9.14	9	0.06
RHS 41 Tri	71	70.5	3.02	3	0
White	90	94	3.82	4	0.17
Total	376	376	16	16	0.23

$\chi^2 = 0.23$, P= 0.89

6.4.2.7 Cross designated P4.7 x I7.26

The petal colour of the ovule parent P4.7 in this cross was RHS 34a (Red) and the petal colour of the pollen parent I7.26 was white. The petal colour of all F_1 plants was RHS 34a. Table 6.9 shows the frequency of flower colours that was observed in the F_2 population, and results of χ^2 analysis. The data best fitted a four gene model, where RHS 34a was dominant over RHS41a which in turn was dominant over RHS39a. Coloured petal was dominant over white. In this model, when petal colour was RHS 34a maroon boss was dominant over black boss.

Table 6.9 Observed and expected frequencies of F_2 genotypes from cross P4.7 x I7.26

Phenotype	Observed frequency	Expected frequency	Observed proportion	Expected proportion	χ^2
RHS 34 - Maroon	109	104.62	112.51	108	0.19
RHS 34 - Black	37	34.87	38.19	36	0.13
RHS 41	36	43.59	37.16	45	1.37
RHS 39	3	2.90	3.09	3	0
White	63	62	65.03	64	0.02
Total	248	248	256	256	1.71

$\chi^2 = 1.71$, P= 0.89

6.4.3 White flowered genotypes contain no pigmentation

Pigmentation was absent in all plant parts of white flowered genotypes that were examined during screening for parents (Table 6.10 – no asterisk), examination of hybrids between genotypes thought to be fixed for white flowers (Table 6.11) and those white genotypes produced in populations used in F_2 evaluation of gene segregation (Table 6.11 – denoted by asterisks). In total 455 white flowered genotypes were grown with this relationship unchanged.

Table 6.10 Populations establishing white flowered genotypes

Cross or Self	Generation	No. of genotypes Obs.
7.48 self	unknown	8
6.12 self	unknown	3
R/B x Wht orig	F_1	3
6.12 self	unknown	7
5.37 x sp2	F_1	7
7.48 self	unknown	8
4.100 self	unknown	5
7.76 / I7	F_3	38
7.76 / I8	F_3	18
I7.18 self	F_4	17
I7.12 self	F_4	6
I7.34 self	F_4	6
I7.35 x I7.29	F_1	6
I7.35 x I7.18	F_1	8
W10.13 self	F_4 or F_5	7
I7.10 x W10.11	F_1	8
B17 x I7.19*	F_2	55
I7.3 x B17*	F_2	128
W10.15 x Tri*	F_2	54
P4.7 x I7.26*	F_2	63
TOTAL		455

* Crosses with an asterisk are those incorporated into inheritance studies

6.4.4 Allelic relationship tests of two white flowered lines

Two lines of true breeding white flowered genotypes were produced from unrelated parents. A cross was undertaken to assess whether the recessive alleles that conferred white petal colour were alleles of a single locus or were alleles of different loci. All hybrid progeny were true to parental phenotypes indicating they were the same gene (Table 6.11). All hybrid progeny also exhibited an absence of pigmentation.

Table 6.11 Allelism checks after hybridising two white flowered genotypes (I7.15 x W10.15)

I7.15 x W10.15

Prog. Phenotype	Observed frequency	Observed proportion	Expected proportion	F_2 Genotype / s	χ^2
White	43	100	100	----------ee--	0
Total	43	100	100		0

6.5 DISCUSSION

6.5.1 Flower colour in Sturt pea

The results of this study indicate that at least six major genes are involved in the control of flower colour in Sturt pea. It appears that depending on the combination of alleles involved seven different phenotypes may be determined by these loci (Table 6.12).When compared to *Ipomoea indica* (the morning glory), which has been extensively used as a model, the genetic control of flower colour in Sturt pea appears to be relatively simple because only two functional alleles at each of the controlling loci were identified and complete dominance was common. At least 21 floral phenotypes are known in *Ipomoea* which are determined by five genetic loci (Brown and Clegg 1984; Schoen *et al.*, 1984; Clegg and Durbin 2000).

The expression of pigment in plant tissues other than flower petals appeared pleiotropicaly controlled by the gene conferring white flower. No pigmentation was observed in leaves, stems, petioles or any other part of the plant in any white flowered type generated in the study (Figure 6.3). The gene conferring white flower (ee) appeared capable of masking the expression of all other flower colour genes when in a completely recessive state. The tri-colour mutation (ff) that is expressed in the keel petal also appeared to be masked by the presence of homozygous dominant alleles at genes conferring the pink forms (CCDD).

Table 6.12 A genotype and flower colour model

RHS Colour	Colour class	Genotype(s)
34a	Red	**ABCDEF, AbCDEF, aBCDEF**
41a	Pink +	**abCDEF**
39a	Pink -	**abcdEF**
	White	**----ee--**
	Tricolour	**----E-ff** *(Except with CCDD)*

* Pink – may be arbitrarily described as an apricot by previous investigators

In this study it appears that dominant alleles of two of the genes that were identified (designated A and B in Table 6.13), facilitate the same floral colour (Red RHS34a). A slightly darker shade of red (RHS 34/44a) sometimes occurs in the species and it is possible that this phenotype is the result of the AABB genotype which confers a colour of greater intensity. Variability in petal colour intensity was noted when Red/Black phenotypes were self pollinated in consecutive generations. It is likely that wild plants that have dark red flower colour are heterozygotes at some of the genes that confer the intense red colour and that when self pollinated combinations of the alleles of genes which confer the intensity of petal colour may be modified. However, this would imply that colours such as RHS 41a should appear 1 in every 16 times from the double heterozygote (AaBb). Detailed experiments to investigate this hypothesis were not conducted in this study.

6.5.2 The white flowered phenotype

In the populations examined the white flowered phenotype was recessive. It is plausible that the recessive form of the gene (designated e) is a lesion in the biosynthetic pathway or a functional gene which converts substrates for the colour reaction to colourless products that are not able to be converted to coloured compounds by the pathway. The gene that confers white or colour flowers, depending on the allele, appears to pleiotropically control pigmentation in other plant parts.

White flowers are sometimes caused by mutations that are only expressed in the flower Rogers (2004), however, white flowered genotypes in *Medicago* (alfalfa), were identified which lacked anthocyanin in all plant parts including seeds (Barnes 1974).

The expression of the white flower phenotype in Sturt pea is almost identical to that in *Medicago*, except that the seed of white flower genotypes can not be distinguished from others in Sturt pea but can be in *Medicago*.

White flower colour is uncommon in wild Sturt pea populations (M. Jusaitis pers. comm). This is likely due to the combined effect of recessive genetic control in a facultative out breeding species and the under visitation of pollinators to those that display the phenotype. In *Ipomoea*, such under visitation by the bumblebee pollinator is correlated with an increased frequency of self fertilisation by white maternal parents (Brown and Clegg 1984; Epperson and Clegg 1987). Such observations infer that white loci can act as mating system modifier loci and consequently bias their transmission to subsequent generations. The pigments are therefore important to reproductive success and hence to gene transmission, as it is known they act as visual signals for pollinators and in some species seed dispersal (Saito and Yamazaki 2002). In the short term this could result in an increase of white flower genotypes in a localised environment, but longer term would be countered by the deleterious effects of inbreeding depression in this species, which ensure that fixation within populations (Fisher 1941; Holsinger 1996; Karlin and McGregor 1974) can not occur.

6.5.3 Maroon boss is dominant over black boss

In this study it was identified that maroon boss colour dominant over the common black boss. This result suggests that if there was no selection pressure by pollinators that it is likely that plants with flowers that have a maroon boss would dominate in natural populations. Anecdotally, shades of maroon are reasonably prevalent but not overwhelmingly so and much variability in boss colour is found (M. Jusaitis pers. Comm.). This would suggest that for the black boss to dominate in any area that the pollinators must travel from flowers with a black boss to other flowers with a black boss. Alternatively, the level of self pollination may well be much higher than is thought. The deleterious effect of inbreeding in this species would suggest the former is more plausible and a significant selective pressure by pollinators is a major factor in determining boss colour.

6.5.4 A gene that confers pink petal colour pleiotropically affects boss colour

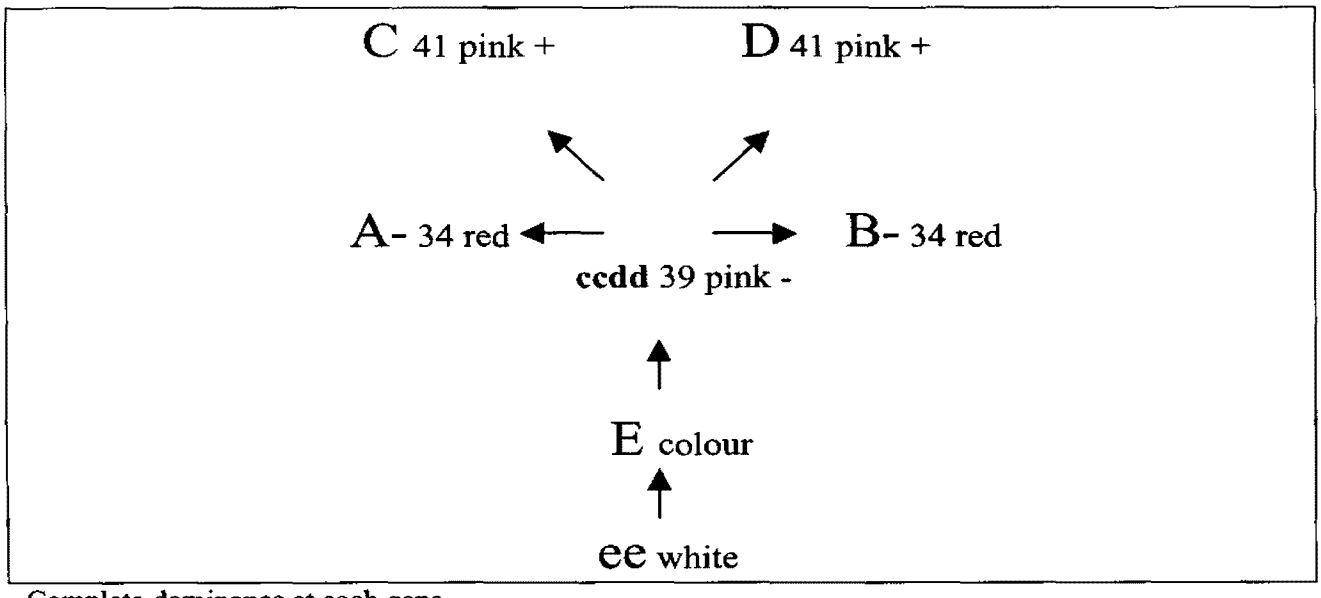
In the F_2 population derived from a hybrid between P4.7 and I7.26 it was identified that the model of best fit involved 4 genes. According to this model one of these genes acted pleiotropically (gene C or D) to control boss colour (maroon / black) in the red petal phenotypes. These genes confer pink petal classes. Similar results in the population derived from a hybrid between B35 and P4.4 appears to confirm the theory of pleiotropy. This cross has a model in which 4 segregating genes are required. The maroon / black boss again was segregating 3:1 respectively and all four genes have a petal colour attributed to its presence. Only one of the four candidate genes could be responsible based on modelling and again it was one of the pink genes.

6.5.5 Evidence the pathway for gene flow in flower colour is not linear in Sturt pea

Cross P4.7 x I7.26 in this study suggests functional / dominant alleles are not needed at genes C and D to achieve red petal as coded by A and B. This implies that the biochemical pathway is not linear and that multiple branch points may exist. All other crosses used in this study have been selected because they do not segregate at the A and B genes. This allows us to observe genes specific for colours and ignore those genes not actually involved in a given cross. By doing so a continuity of progression was observed from white to pink - to pink + and a linear development seemed most plausible.

It became evident a plant could have E enabling colour, but could be ccdd and still display a red phenotype due to a dominant allele at A or B. Based on these observations it appear likely that many enzymes conferring flower colour in Sturt pea may act on a common substrate and that the colour pathway in Sturt pea is not linear. A possible flower colour pathway for Sturt pea is illustrated in Figure 6.2.

Figure 6.2 Genetic flow for flower colour as suggested by results obtained in inheritance studies



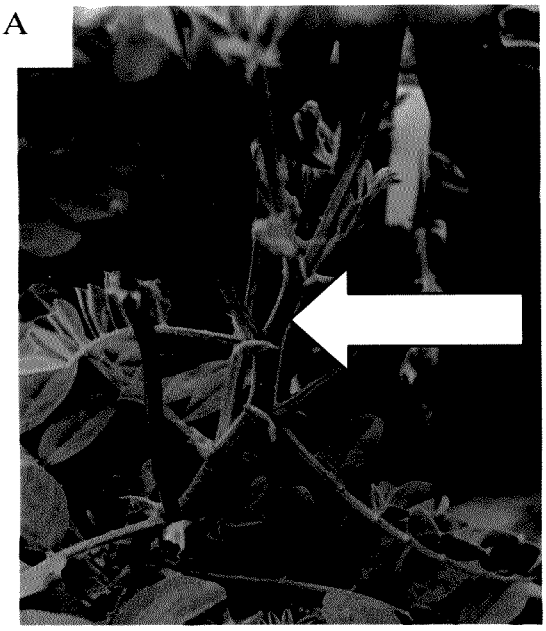
Complete dominance at each gene
RHS 39 results when * **aabb** No red * **(ccdd)** Double recessive for pink - * **E** Enabling colour

6.5.6 Additional recessive gene causing increased colour (Red)

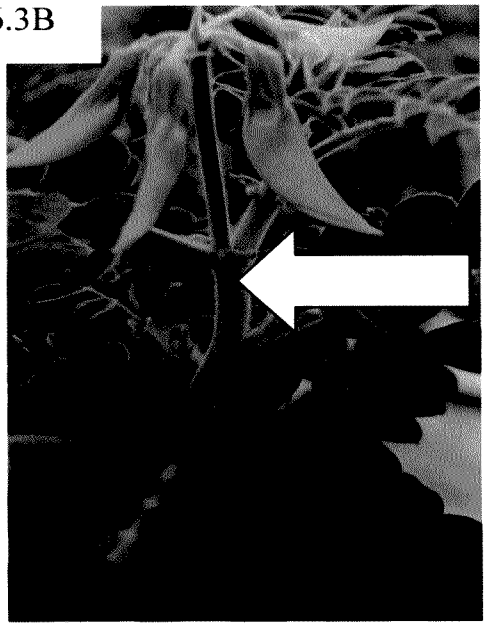
In *Ipomoea*, a modifier locus has been characterised that is an intensifier locus which doubles the anthocyanin pigmentation in the recessive genotype (Schoen *et al.*, 1984). There is some evidence to suggest that a single recessive gene exists that can produce a red phenotype in the absence of the two major red phenotype genes A and B which were identified in other crosses. It is possible that the identified recessive allele of this gene is not coding for red, but is an intensifier of colour in the petals. However, the action of this gene was not thoroughly investigated in this study.

Figure 6.3a-b Two genotypes depicting the difference in stem colour possible due to the pleiotropic action of the ee gene. A) shows genotype F_1 - I7.11 x P4.12 (Flower colour Red 34a) with a pigment throughout the stem. B) shows a white genotype F_3 - I7.34 self with a complete absence of pigment in all plant parts.

6.3A



6.3B



6.5.7 The tri colour mutation and epistasis

The tri colour mutation is a novel mutation representing a significant change in flower phenotype in Sturt pea which may have an application in ornamental horticulture. In crosses involving this form the models that best fit the populations suggested a single recessive gene conferred the phenotype. It may be expressed in a range of genetic backgrounds and appeared to have very subtle variations such as the boss streaking illustrated in Figure 6.1d depending on parentage.

When developing inheritance models for segregation data from crosses involving the tri-colour mutation, it became evident the gene conferring the novel form was subject to an interaction determined by the allelic constitution of the C and D genes (which confer pink + and pink -). Whenever there was at least one recessive allele at either C or D the tri-colour mutation altering pigment production in the keel petal would be expressed. When both genes carried only dominant alleles (CCDD) the homozygous

recessive state of the compact gene (ff) that would normally facilitate expression of the mutation was masked.

6.5.8 Phenotyping in different environments

Early in this study whilst developing phenotyping protocols, it was recognised light intensity impacted upon both the intensity of colour in petals and the eyes interpretation of these colours. Due to this phenotyping was only ever undertaken in bright natural light. Dong *et al.*, (1997) demonstrated both dark treatment and UV blocking prior to flower bud break in apple (*Malus domestica* Borkh.) reduced expression of 6 genes and inhibited anthocyanin biosynthesis, in doing so highlighting the link between levels of light intensity and resultant intensity of colour in petal tissues. Light was also demonstrated to be a significant factor influencing the intensity of flower colour in *Medicago* (alfalfa) (Barnes 1972).

6.5.9 Conclusion and future breeding efforts

The understanding of the floral genetics in Sturt pea provided by this study will facilitate accelerated efforts when combined with germplasm containing suitable habits for pot culture. Based on the information provided, it is possible to predict F_1 flower colour of numerous combinations if hybrid seed was ever pursued. Numerical representation in F_2 populations of all colours can be predicted if parental genotypes are known. A model of genetic control that accounts for most of the flower colours in the species was proposed. The study demonstrated that it is feasible to produce genotypes that are fixed for flower colour loci, despite reports of the detrimental effects of inbreeding depression.

CHAPTER SEVEN

GENERAL DISCUSSION

7.1 AIM OF THE THESIS

The aim of the thesis was to provide fundamental information about Sturt pea that will contribute to the formulation of new strategies for its development. Three broad approaches were employed in this research. The first approach employed cytogenetics and molecular genetics to assess species diversity and to investigate barriers to breeding such as differences in chromosome number and structure. The second approach aimed to develop a quantitative method to identify genotypes possessing a compact habit in segregating populations and to establish the genetic control of the novel form. The third approach aimed to characterise the genetic control of flower colour.

7.2 Genetic diversity in Sturt pea

The genetic diversity within Sturt pea is uncharacterised. Reports on the chromosome number of Sturt pea are conflicting and molecular investigations have focused on relationships within the family and tribes that contain Sturt pea, however they have not investigated variation within Sturt pea.

7.2.1 Estimating genetic diversity through cytogenetic studies

This study aimed to resolve conflicting reports of the typical chromosome number of the species. The Sturt pea accessions and hybrids that were examined in this study possessed a chromosome complement of 2n=32. It appears that this complement is uniform in the species.

This study has generated new information that may be helpful in resolving classification of Sturt pea. It is well documented that Sturt pea is an outlier of its genus based on taxonomic characters (George 1999a), and that genus *Swainsona* appears to be an outlier of tribe Galegae. Joy Thompson in her review of the genus

(Thompson 1994) made brief comment on current classification and based on taxonomic characters suggested that genus *Swainsona* may find its closest relatives in tribe Carmichaeleae. Members of tribe Carmichaeleae possess a chromosome number of 2n = 32. *Swainsona* is the only genus in tribe Galegae that possesses this chromosome number (apart from *Clianthus*) and the rest of the members of the tribe have complements of 2n=16 supporting Thompson's hypothesis (Thompson 1994). Previous molecular investigations have revealed variation in genus *Swainsona* (Wagstaffe *et al.*, 1999); however, they have not helped to clarify if it should be assigned to Tribe Galegae or Carmichaeleae.

Although no satisfactory conclusion can be drawn yet, this study when combined with available published studies was enough to allow the suggestion of two different hypotheses concerning the evolution of the species, for others to consider, and if possible, explore.

The hybrids between parents that originated from diverse geographic origins appeared to possess structurally uniform chromosomes, displayed normal pairing patterns, and were fertile. No barriers to breeding were detected in this material.

The finding of 2n=32 in Sturt pea, and the knowledge x = 8 is basic for many relatives (Goldblatt, 1981), both have important and interesting implications. Zulkarnain *et al.*, (2002) commented that the production of large amounts of pollen grains and the subsequent shedding of these grains in transport has been a major impediment to the commercialisation of the species as a cut flower. The net effect of this problem being reduced vase life as a consequence of floral degeneration after self pollination.

To overcome this problem, Zulkarnain *et al.*, (2002) suggested the production of triploid genotypes. This however, was proposed after what appears to be an erroneous premise of the species being characterised by 2n = 16. The fact that this species has been shown to be characterised by 2n = 32 and is therefore very likely tetraploid means further chromosomal doubling would generate octoploid genomes. Large genomes, be they artificially induced or naturally occurring commonly have unpredictable behaviours and capabilities (Lewis 1980; Levin 1983; Leitch and Bennet 1997; Ramsey and Schemke 2002).

Although the target of this thesis was potted plant development, the results have applications and implications for other areas of investigation in the species, and this particular example highlights the importance of correctly establishing the fundamentals of a species such as its chromosome number before embarking upon more complicated projects.

7.2.2 Estimating genetic diversity through molecular genetics

The study of Wagstaffe *et al.*, (1999) was useful when looking very broadly at inter-relatedness and diversity within tribes. However, it did not contribute information about diversity within Sturt pea. Prior to this study, it was not known how genotypes originating from Tibooburra (the most Eastern occurrence of the species) may differ from genotypes from Port Hedland (the most Western occurrence on continental Australia).

Three main groups were revealed in the study. New South Wales genotypes were different to South Australian and Southern West Australian genotypes which were quite different again to Northern West Australian genotypes. It is likely that the differences between the New South Wales genotypes and those of other regions were contributed to by genetic isolation. A change in genetic constitution was detected within West Australian genotypes the closer they got to the coast and as latitude increased.

The levels of genetic variability that were revealed by AFLP analysis in this study were much greater than expected after cytological characterisation did not detect variation between accessions.

The rate of polymorphisms and the number of polymorphisms identified per primer were similar to what was reported in investigations involving other legumes (Nguyen *et al.*, 2004; Sivaprakesh *et al.*, 2003).

7.2.3 Speculation on the causes of diversity patterns found

The clusters that were observed in the study were aligned with climatic zones of Sturt pea's distribution. This implies that climate has contributed greatly to genetic

variability of Sturt pea and probably played an important role in the evolution of the species. It is possible that both changes in latitude and colonisation of coastal areas may have been working in tandem to further expand total diversity within the species, particularly in the accessions originating from the North Western areas of Sturt pea's distribution, manifesting itself in the genetic division between groups 1 and 2 identified in the study. With Jiggalong sitting just above the tropic of Capricorn and Port Hedland well above, their climate is considered sub tropical, whilst Tibooburra, Roxby Downs and Wiluna are well below the tropic and hence have a climate described as temperate. These latitudinal differences would contribute to climatic variability, with each region requiring different genetic combinations to adapt to the prevailing seasonal conditions enabling colonisation and prosperity.

A high level of genetic diversity within the Roxby Downs accessions may allow the inference that this could be an area of species origin. This theory would infer a significant change in genetic composition had to occur during colonisation of the coastal environment as the distribution moved towards Port Hedland, and would entail a shift from adaptation for drought to adaptation for saline tolerance. This however would oppose a hypothesis from Burbridge (1960) that the arid zone flora radiated from ancestors with cosmopolitan coastal distributions, and accordingly this adaptation to salinity would pre-adapt them to an arid climate (Crisp *et al.*, 2004). Burbridge's theory does not fit well with the current distribution of Sturt pea on continental Australia as one would expect a greater presence in coastal areas north of Port Hedland if the Sturt pea ancestors were adapted to coastal climates.

Climate although heavily implicated, could not be considered to be the sole determinant of diversity. Other factors such as the physical distance of separation and continuity of distribution between the sampled sites may largely influence the rate of genetic transfer between locations. The past radiological activities in regions nearby Roxby Downs (Maralinga) may have also played a role in contributing to the molecular differences observed today. No sound scientific evidence from robust studies can be found to support such a hypothesis however the possibility should not be discounted.

If there were any validity in a theory of 'assisted' diversity, it would be extremely difficult to quantify, but published literature is available that should be noted when forming a hypothesis for future investigations. Grosch and Hopwood (1979) found lethal radiation exposure was inversely related to the amount of DNA per chromosome i.e acute affects are greater the larger the chromosome and embryos are more sensitive than the differentiated plant. Sturt pea has comparatively small chromosomes and could therefore theoretically survive exposure rates lethal to another species with molecular alterations passed to following generations. Additionally, it is known biological effects such as genomic DNA damage occur in organisms at dose rates well below those required to obviously impair reproduction (Whicker and Hinton 2003) and the results of radiation are often not seen in the exposed generation but those that follow (Whicker *et al.*, 1999).

It has been established that radioactive contamination does produce large dose variations in space and time (Whicker and Hinton 2003) making establishment of cause and effect near impossible. Although this is a fascinating area, and from a breeding point of view should be further explored as a possible source of novel traits, caution has been suggested in the use of molecular end points to make inferences about radiation effects (Hinton *et al.*, 2004). Primarily this is due to the inability to accurately quantify effect and arbitrary response interpretations (Whicker and Hinton 2003).

7.3 Characterisation of the compact form of Sturt pea

This study developed a quantitative means to reliably differentiate normal habit and compact plants, and quantified the reductions in plant parts and dimension in compact plants.

A mutation exhibiting similar characteristics to the compact form of Sturt pea is documented in *Pisum* (Symons *et* al., 2002). The study in *Pisum* found compact genotypes were not different during days 1-14, well into vegetative growth. The study presented in chapter four demonstrated a statistical difference between genotypes that was significant at the cotyledon stage in 75% of populations studied, thus inferring the mutation is 'effective' earlier in development than the *Pisum* mutation. The

continuation of the study unequivocally showed that compact plants were different to their heterozygous and homozygous counterparts throughout early vegetative growth stages.

No differences were detected between normal habit plants that were heterozygous and homozygous. Therefore, the only accurate way of genotyping these individuals is to conduct progeny testing.

7.4 Inheritance of the compact form

Efficient breeding is reliant upon an understanding of how a character may be passed from generation to generation. In this study a number of conflicting results were obtained which indicated that 1 gene (3:1) or two genes (13:3) were regulating the character. Inheritance ratios of 13:3 and 3:1 are so similar, it would be difficult to determine which of these is likely unless large populations were examined and the three classes could be separated for analysis. 13:3 ratios are describing a type of inhibitory gene action that is not often reported and is often not accepted.
The genesis of the form is not well understood as no direct means was employed to 'induce' such a change. Furthermore, the material that it appeared in was not characterised prior to identification of the novel compact habit. As such it is currently difficult to pinpoint an underlying factor. The study did however demonstrate that the compact habit can be expressed in a population fitting a 3:1 model which implies a single recessive gene governs the traits expression and a second (uncharacterised) factor can modify its frequency in segregating populations.

This modification remains a great curiosity. The description of 'meiotic drive' as described by Sandler and Novitski (1957) fits observations in this study as excess transmission of a particular allele is suspected. However, the results do not allow the timing of this event to be determined (pre, during or post meiosis). An important distinction between gametic selection (the superiority of gametes carrying the favoured allele) and meiotic drive has been made (Birchler *et al.*, 2003) showing that segregation distortion can take many forms. There are numerous reports of meiotic drive systems, in which the principle of both homologues from a heterozygous pair having equal chance of transmission are violated (Ganetzky 1977; Lyttle 1991;

Kusano *et al.*, 2002; Lambrides *et al.*, 2004), but this appears to be the first observation in this species.

7.5 Investigations into the control of flower colour in Sturt pea

The research in chapter six identified the genetic control of four petal colours, two boss colours and a novel mutation. It was unknown how efficient production of homozygous parental material would be as the number of cycles of self pollination required and the number of genes controlling flower colour were unclear. Although not vigorous, the inbred parents were produced and successfully hybridised.

The study identified two genes that both confer a red petal phenotype (designated A and B). One of the two genes conditioning pink petal (C or D) also influenced boss colour in some crosses. This was first noted in one population (B35 x P4.4) and validated in another (P4.7 x I7.26). It was established that the tri-colour mutation (designated ff) was subject to an epistatic interaction in which its expression was suppressed in genotypes possessing only dominant alleles at the C and D genes. The recessive allele of the locus that conditioned the white flower phenotype (ee) also affected pigmentation in other plant parts.

7.6 Summary

Efforts to improve the cultural characteristics of this species to generate commercial varieties have traditionally yielded modest results. To a great extent this has been caused by a lack of knowledge in areas that underpin traditional plant breeding and the resultant inability to align individual objectives into a single breeding strategy.

The level of genetic diversity within this species has probably been under-estimated in the past based on the diversity discerned through observing phenotypic variability. It is genetic diversity a breeder will try to evaluate when assessing opportunity for gains and this species may have fooled some into thinking little physical variability was the manifestation of genetic homogeneity. Whilst the cytology of the Sturt pea revealed in chapter two would alone seem not to dispel such a notion, this same finding is actually a key to future developments if employing traditional techniques.

Chromosomal congruity enhances opportunity for viable and fertile recombinant genotypes to be produced that exceed current performance standards and enable continued generational development. For efficient selection and adaptation, chromosomal congruity need be combined with a level of molecular variability sufficient to facilitate the generation of genuinely different genotypic combinations. Results produced in chapter two showed hybridisation to be barrier free and results of chapter three suggested exploitation of available genetic resources is still a worthwhile endeavour. Precisely how worthwhile such efforts may be can not be quantified at this time as another critical question remains unanswered. Do the differences observed in genetic constitution of accessions originating from diverse geographic origins actually reflect the available level of variability in genes specific to traits of cultural and agronomic importance? It is this answer that ultimately will determine the level of potential gains.

Chapter four provided an insight into what could be possible from a cultural and agronomic viewpoint. A compact habit form of the species was characterised that addresses one key deficiency in the species, that being, the ability to be grown in, and remain within, a 150mm pot. The thin stems, small leaves and overall condensed dimensions inherent in this form make it the greatest stepping stone yet towards a production capability that can conform to standard nursery practice.

If one considers understanding genetic capability (cytological and molecular) as the raw ingredients in a breeding prospect and the production of a vastly different and culturally amenable form the next significant obstacle addressed (even if only in a preliminary fashion), then knowing the information is now available to accurately produce and predict floral colour variants underlines how significant a gain this collective body of work can potentially be if momentum is passed onto further investigations.

7.7 Suggested future experiments

7.7.1 Further molecular diversity investigations

The results of these studies have exposed a number of opportunities for new research. The results obtained precipitate questions such as where the centre of diversity may be for this species. In essence this is looking at evolution and centres of origin. Genetic variability discovered in West Australian genotypes as the distribution progresses toward the coast is of interest as these genotypes may show a better resistance to root disease after adapting to a wetter climate. Studies that again employ AFLP's should be considered with sampling of genotypes from more locations across continental Australia. Genotypes from islands off the West coast of Australia should also be included.

The central South Australian areas surrounding Roxby Downs and Maralinga should be a focus, possibly as a separate sub experiment. It would be of great interest to resolve if this area represents a genuine centre of diversity or contains an unusual level of unique DNA content as sample sites get closer to Maralinga.

For the general experiment it would be easier to identify changes in diversity within and between regions if more sample sites were included and the distances between each site shortened. In the current experiment five sites were selected. Further investigations should incorporate twelve or more sites. This would also allow a better pinpointing on whether changes are dramatic over a short distance and can be explained by topographical factors or conform to a more progressive and gradual genetic shift inferring climatic forces. The same may apply for New South Wales genotypes in terms of explaining an apparent genetic isolation.

7.7.2 Closer inspection of a suspected segregation distorting factor

Work to characterise the mode of inheritance affecting numerical representation of compact genotypes concluded the presence of a distorting factor was very likely. Evidence could not be furnished on the mechanism/s behind this distortion. More inheritance studies using increased numbers of diverse backgrounds are suggested. In tandem with this further cytological investigations should be conducted paying more

attention to cell division, particularly just post metaphase I. It is possible the small chromosomal size in this species may still preclude finding a cytological aberration. However, the study conducted in this thesis did not set out to examine cell division exclusively; the goal was to observe the chromosome number of Sturt pea, which was determined at and before metaphase I. A study exclusively aimed at observing anomalies in cell division may yield new information about the nature of the segregation distortion that was observed in inheritance studies.

7.7.3 Identification of a molecular marker for compact habit

Bulk segregant analysis would be the logical and most desirable first step towards marker identification. Identification of markers that are linked would enable them to be cloned and used to for mapping in other legume genomes. This work may lead to the identification of regions that contain the gene that is responsible for the changes in habit. Efforts aimed at isolation of the gene/s in question would then be possible. Even though extensive effort would be required to conduct this work it could contribute enormously to our understanding of a range of biological pathways as the pleiotropic effects of the gene/s in question were shown to be so many and varied.

Appendix 1 – Codes and sources of genotypes used for cytology and molecular studies

Appendix table 1.1 Genotypes that were characterised cytologically as 2n = 32

Code	Bivalents	2n=	Multivalents	Geography	Source
P01	16	32	0	Port Headland	W.A
P02	16	32	0	Port Headland	W.A
P03	16	32	0	Port Headland	W.A
P04	16	32	0	Port Headland	W.A
P05	16	32	0	Port Headland	W.A
P06	16	32	0	Port Headland	W.A
P07	16	32	0	Port Headland	W.A
P08	16	32	0	Port Headland	W.A
P09	16	32	0	Port Headland	W.A
P10	16	32	0	Port Headland	W.A
J01	16	32	0	Jiggalong	W.A
J02	16	32	0	Jiggalong	W.A
J03	16	32	0	Jiggalong	W.A
J04	16	32	0	Jiggalong	W.A
J05	16	32	0	Jiggalong	W.A
J06	16	32	0	Jiggalong	W.A
J07	16	32	0	Jiggalong	W.A
J08	16	32	0	Jiggalong	W.A
J09	16	32	0	Jiggalong	W.A
J10	16	32	0	Jiggalong	W.A
W01	16	32	0	Wiluna	W.A
W02	16	32	0	Wiluna	W.A
W03	16	32	0	Wiluna	W.A
W04	16	32	0	Wiluna	W.A
W05	16	32	0	Wiluna	W.A
W06	16	32	0	Wiluna	W.A
W07	16	32	0	Wiluna	W.A
W08	16	32	0	Wiluna	W.A
W09	16	32	0	Wiluna	W.A
W10	16	32	0	Wiluna	W.A
R01	16	32	0	Roxby Downs	S.A
R02	16	32	0	Roxby Downs	S.A
R03	16	32	0	Roxby Downs	S.A
R04	16	32	0	Roxby Downs	S.A
R05	16	32	0	Roxby Downs	S.A
R06	16	32	0	Roxby Downs	S.A
R07	16	32	0	Roxby Downs	S.A
R08	16	32	0	Roxby Downs	S.A
R09	16	32	0	Roxby Downs	S.A
R10	16	32	0	Roxby Downs	S.A
T01	16	32	0	Tibooburra	NSW
T02	16	32	0	Tibooburra	NSW
T03	16	32	0	Tibooburra	NSW
T04	16	32	0	Tibooburra	NSW
T05	16	32	0	Tibooburra	NSW
T06	16	32	0	Tibooburra	NSW
T07	16	32	0	Tibooburra	NSW
T08	16	32	0	Tibooburra	NSW
T09	16	32	0	Tibooburra	NSW
T10	16	32	0	Tibooburra	NSW

Appendix 2 – In-vitro pollen germination study

Appendix table 2.1 In-Vitro pollen germination percentages achieved by three standard, heterozygous and compact genotypes over 24 hours. The table describes from left to right the genotype, the genotypes designation, and then the percent germination at 3,6,12 and 24 hour observations. The performance of compact genotypes was poor as compared to heterozygous and homozygous W/T genotypes.

Genotype	Designation	3hrs %	6hrs %	12hrs %	24hrs %
Standard W/T	A	20.8	69.9	81.2	91.6
Standard W/T	B	0	27.5	37.1	75.7
Standard W/T	C	0	34.9	91.9	92.6
Standard W/T	D	43	84.2	94.2	95.2
Standard W/T	E	6.6	29.1	35.3	51.2
Standard W/T	F	2.2	46.7	52.6	65
Standard W/T	G	0	19	92	99.2
Heterozygous	BBR 27	17.3	25.1	74.3	75.6
Heterozygous	BBR 29	19.4	89.6	90.6	98.9
Heterozygous*	F1-1	10.7	17.3	56.1	58.3
Heterozygous*	F1-2	2.2	9.1	19.3	29.7
Heterozygous*	F1-12	2.5	31.8	63.8	65
Heterozygous*	F1-10	0	8.05	16.2	16.3
Heterozygous*	F1-14	0.27	8.09	10.98	16.6
Heterozygous	47-38 x 9	23	46	63	71
Heterozygous	47-38 x 10	39	57	89	96
Heterozygous	47-38 x 7	18	43	77	96
Heterozygous	47-38 x 4	0	0	2.1	19
Heterozygous	47-38 x 2	22	44	65	70
Compact	29-74	0.84	1.2	3.5	12.5
Compact	29-25	1.2	2.8	5.62	11.2
Compact	29-27	0	0	0.92	11
Compact	47-7	0	0	0	0
Compact	14-23	0	0	0	1.4
Compact	14-9	0	0	0.87	1.97
Compact	29-16	2.65	17.9	21	36.6
Compact	29-13	0	0	0	0

BBR 27 and BBR 29 were F_1 heterozygous genotypes.
Heterozygous genotypes denoted by an asterisk were demonstrated to be this genotype through progeny testing and were produced through the intermation of related F_1 parents known to be heterozygous.

Appendix 3 - Reciprocal cross study

Appendix table 3.1 – Genotype and identifiers of individuals used in reciprocal cross study

Genotype	Code	Genotype	Code
Wild type - 1	K57+4+	Compact	14a
Wild type - 2	SDP APR1	Compact	14d
Wild type - 3	E42+5+	Compact	15a
Wild type - 4	K56+4+	Compact	16d
Wild type - 5	A42+2	Compact	18
Wild type - 6	E94+1+		

The genotype codes are proprietary information -property of Oasis horticulture Pty Ltd

Appendix table 3.2 - Pollination results using original compact clones as male parents in the reciprocal cross study.

		1	2	3	4	5	6
Compact types	♂ 14a	X	X	X	X	X	X
	♂ 14d	X	X	X	X	X	X
	♂ 15a	X	X	X	X	X	X
	♂ 16d	X	X	X	X	X	X
	♂ 18	X	X	X	X	X	X

Appendix table 3.3 - Pollination results using original compact clones as female parents in the reciprocal cross study.

		14a	14d	15a	16d	18
Standard types	♂1	X	X	X	X	X
	♂2	X	X	X	X	√
	♂3	X	X	X	X	X
	♂4	X	X	X	X	X
	♂5	X	X	X	X	X
	♂6	X	X	X	X	X

√ = 1 pollination resulting in 2 seed

Appendix table 3.4 - Data produced using recombinant F_2 compact genotypes as female parents in crosses with W/T male parents

Genetic Combinations	Pollinations undertaken	Pollinations with pod set	% Successful pollinations
67	310	3	0.97

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