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table of contents

Acknowledgements ..... **i**
Table of Contents ..... **ii**
Abbreviations ..... **iv**

1. Abstract & Outcomes **1**
1.1 Abstract 1
1.2 Outcomes 3

2. Hygienic Behaviour & Brood diseases **4**
2.1 Introduction 4
2.2 Hygienic behaviour and resistance to American foulbrood 7
2.2.1 The disease 7
2.2.2 Disease resistance 8
2.3 Hygienic behaviour and resistance to chalkbrood 10
2.3.1 The disease 10
2.3.2 Disease resistance 13

3. Genetic & Environmental Factors that affect Hygienic Behaviour **15**
3.1 Genetic factors – the Rothenbuhler studies 15
3.2 Environmental factors 18
3.3 A more complex genetic basis? 21

4. Field Studies **24**
4.1 Introduction: Breeding of hygienic bees in Australia 24
4.2 Importation 25
4.3 Experimental Cross A 26
4.4 Experimental Cross B 28
4.5 Field assays for segregating colonies 30
4.6 Results 34
4.6.1 Segregating colonies 34
4.7 Summary 38

5. Quantitative traits and Linkage Mapping **40**
5.1 Introduction 40
5.1.1 Quantitative traits 40
5.1.2 Genetic markers 41
5.1.3 Linkage Maps 42
5.2 Methodology 44
5.2.1 Generation of RAPD molecular data 44
5.2.2 Extraction of genomic DNA 44
5.2.3 Polymerase Chain Reactions 45
5.2.4 Electrophoresis of PCR products 46
5.3 Results and Analysis 47

5.4 Summary 56

6. QTL Analysis 58
6.1 Introduction 58
6.2 Methodology 60
6.2.1 Generation of phenotypic data 60
6.2.2 Generation of molecular data 61
6.3 Results and Analysis 62
6.3.1 Identification of QTLs 62
6.3.2 Testing of candidate markers 69
6.4 Summary 73

7. Subtractive Hybridisation 75
7.1 Introduction 75
7.2 Methodology 77
7.2.1 Preparation of equipment and RNase-free water 77
7.2.2 Extraction of mRNA 77
7.2.3 cDNA Synthesis 78
7.2.4 cDNA Subtraction 79
7.2.5 Cloning and sequencing of cDNA subtraction products 81
7.3 Results and Analysis 82
7.4 Summary 83

8. Conclusions & Implications 86

Bibliography 89

Appendices 99
Appendix A Phenotype data used in QTL analysis 99
Appendix B Linkage group scans for hygienic behaviour and uncapping behaviour QTL 101
Appendix C Genotype data used in mapping and QTL analysis ..... Disc

Abbreviations

°C	degrees Celsius
AFB	American foulbrood
bis	N', N' –methylene-bisacrylamide
bp	base pairs
cM	Centimorgan (unit of map distance)
CTAB	Hexadecyltrimethylammonium bromide
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	an equimolar solution of deoxynucleotide triphosphates (ie. dATP, dGTP, dCTP, dTTP)
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
IPTG	isopropylthio-b-D-galactoside
LB	Luria-Bertani (media)
MAS	Marker Assisted Selection
mRNA	messenger RNA
NaOH	Sodium Hydroxide
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RNA	Ribonucleic acid
RNase	ribonuclease
STS	Sequence Tagged Site
TBE	Tris-borate-EDTA
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactoside

1. abstract & outcomes

1.1 Abstract

Hygienic behaviour in the honeybee (*Apis mellifera*) has been shown to be an effective control mechanism against brood diseases such as chalkbrood and AFB. Chalkbrood has proven to be problematic for the Australian honey industry since it was identified here in 1993. Hygienic behaviour is a much studied trait. Rothenbuhler investigated the genetic basis of hygienic behaviour, proposing a two-gene model to explain the uncapping and removal of dead brood. His elegant experiment remains the textbook example of a behavioural genetic study. Although this model has been challenged, it is still generally agreed that a small number of unlinked genes produce a large effect on hygienic behaviour, that hygienic alleles are recessive and are inherited in a Mendelian manner.

Experimental backcross colonies were produced from an inbred hygienic line and an inbred non-hygienic line, both provided by Dr. Marla Spivak, University of Minnesota. These backcross colonies were assessed for hygienic behaviour using a standard assay. Statistical analyses of the field data indicated that the genetic basis of the trait was more complex than either the simple Mendelian and widely accepted two-gene or three-gene models that have been proposed previously. Molecular techniques, linkage mapping and QTL analysis then were

employed to determine how many loci directly influence hygienic behaviour and the relative level of influence and location of each locus within the genome of *A. mellifera*.

Full multipoint linkage analysis by Mapmaker v3.0 software produced a new genetic map of the honeybee comprised of 358 marker loci ordered over 25 linkage groups spanning a total distance of 3406.2 cM. The average distance between each marker was 9.5 cM. QTL analysis of the experimental data identified seven putative genetic markers associated with hygienic behaviour. QTLs located on linkage groups 2, 4, 6 and 22 were detected for both overall hygienic behaviour and uncapping behaviour only. Individually, each QTL is of relatively small effect with each explaining only 9% – 15% of the variance in hygienic levels observed. Collectively, the putative QTLs identified here explain 79.4% of the observed variance in the expression of hygienic behaviour. These results indicate that there are many genes of low to moderate effect rather than few genes of large effect involved in this complex behavioural trait. This is typical of inherited quantitative traits which do not exhibit Mendelian phenotypic ratios.

DNA extracted from the brood samples taken during testing of commercial stock, and from individual bees identified as either highly hygienic or non-hygienic in a reciprocal backcross experiment, were screened with the candidate markers associated with putative QTLs to test their diagnostic power. Unfortunately, none have produced reliably diagnostic DNA profiles. As we have now shown that hygienic behaviour is a polygenic, quantitative trait, simple

diagnostic markers for Rothenbuhler's 'uncapping' and 'removal' genes are unlikely to be achieved. Our results show that the most likely way to improve disease resistance in Australian stock is via traditional methods of recurrent selection.

The project was responsible for the importation of new genetic material into Australia from the United States. This hygienic stock has been well received by industry, has been widely disseminated, and incorporated into local breeding programs. We hope that it has lead to a general improvement in the level of disease resistance in Australian commercial bees.

1.2 Outcomes

This project attempted to elucidate the mechanisms involved in honeybee hygienic behaviour using molecular techniques. A new genetic map of the honeybee genome was constructed and used to identify quantitative trait loci (QTL) which influence hygienic behaviour. Identification of genetic markers (eg, RAPDs, STSs, microsatellites and allozymes) linked to regions that control hygienic behaviour has provided us with a better understanding of the exact number of genes influencing the behaviour, their relative level of influence, and their location in the honeybee genome.

This project further aimed to develop markers that may be used diagnostically by industry to rapidly and cheaply identify hygienic stock, and to provide a means for further study of this important behavioural trait.

2. Hygienic behaviour & Brood diseases

2.1 Introduction

Hygienic behaviour is one of the most studied and well-documented elements of the European honeybee behavioural repertoire. The term is used to describe a two-step process carried out by some workers upon detection of dead or diseased larvae and/or pupae in brood comb. This process first involves 'uncapping' or elimination of the wax cap covering the cell, followed by removal of the corpse inside (Milne 1985a; Rothenbuhler 1964a). It is distinct from undertaking or necrophoric behaviour in which dead adult bees are removed from the nest (Visscher 1983).

Hygienic behaviour is predominantly exhibited by middle-aged workers that have not yet begun foraging (Arathi *et al.* 2000). It is usually performed by a small proportion of bees in the colony (personal observations; Arathi *et al.* 2000; Boecking and Spivak 1999), and is found at low frequency (incidence) among commercial honeybee colonies (Arathi *et al.* 2000; Oldroyd 1996a; Spivak and Gilliam 1993). It has been speculated that hygienic behaviour has evolved in honeybees as an adaptation to the repeated reuse of cells and as a mechanism

of disease resistance (Arathi *et al.* 2000; Park *et al.* 1937; Rothenbuhler 1964a; Spivak and Gilliam 1993).

The internal environment of the *A. mellifera* colony, and particularly the brood area, is highly conducive to the spread of parasites and pathogens. Thermoregulation of the nest maintains the temperature of the brood area at around 35°C at all times. Even during winter when there may be no brood present, the temperature at the center of the adult bee cluster usually remains above 20°C (Michener 1974; Seeley 1985; Simpson 1961). Brood reared outside the normal temperature range of 32°C - 36°C display developmental abnormalities or do not survive at all (Seeley 1985). Such tightly controlled thermoregulation means that the nest is consistently maintained at temperatures conducive to viral and bacterial infection.

Honeybee nests are also characterised by relatively high humidity and extensive contact between adult individuals and between adults and the developing larvae (Milne 1983). Nurse bees secrete larval food from the hypopharyngeal gland and progressively feed developing larvae resulting in regular contact between adults and brood until the cell is capped (Michener 1974). Worker to worker contact within the hive is also extensive. Nectar coming into the hive is transferred from foragers to receivers who then distribute the load to other hive bees (Michener 1974). Such extensive food transfer is probably the most obvious mechanism by which any pathogen present in the colony may be efficiently spread throughout the colony.

Honeybees are also avid recyclers. Brood cells are usually reused instead of being rebuilt each time new eggs are laid, as they are in related genera such as *Bombus* and *Melipona*. Even wax from cell caps is often saved for later use after a new adult bee emerges (Michener 1974; Seeley 1985). Beekeepers commonly maintain old brood combs in managed hives for many years, a practice that contributes to the development of brood diseases (Koenig *et al.* 1986). These factors mean that reservoirs of potentially infective agents are likely to be present in honeybee colonies at all times.

2.2 Hygienic behaviour and resistance to American foulbrood

2.2.1 The disease

Honeybee hygienic behaviour first received attention as a potential mechanism for control of American foulbrood (AFB) (Park 1936; Park 1937). AFB is caused by the Gram-positive bacterium *Paenibacillus larvae larvae* (formerly known as *Bacillus larvae*, (Heyndrickx *et al.* 1996)), which is an obligate pathogen of the honeybee and infects worker, drone and queen larvae (White 1907). Infection of larvae occurs via ingestion of brood food contaminated by bacterial spores. Spores then germinate in the larval gut. The pathogen reproduces in the larval tissues and kills the developing bee, which turns brown and liquefies due to the action of proteolytic enzymes. The bacterium undergoes sporulation in the disintegrating host. Although adult bees are not infected by the disease, nurse bees are the primary agents that transmit infective spores to brood (Bailey 1963; Bailey and Ball 1991; Bambrick 1964; White 1907). Spores may be spread to other colonies by robbing of infected honey by robber bees (Edwards 2000).

Larvae are most susceptible to infection when fed *P. l. larvae* spores when they are one day old or less (Woodrow 1942). *P. l. larvae* spores are very resilient. They exhibit considerable resistance to heat and chemical disinfectants and can remain infective for at least 35 years (Bailey and Ball 1991; Haseman 1961). Relatively few chemical agents have proven successful as treatment against AFB. The antibiotics sodium sulphathiazole and oxytetracycline (OTC) are

effective control agents against AFB, however both have problematic residues in honey. OTC is the only registered chemical for control of AFB in the United States (Bailey and Ball 1991) and in Australia is only registered for use against AFB in Tasmania. It is, however, registered for use against European Foulbrood (EFB) in all Australian states. There is evidence in the United States and Argentina of *P. l. larvae* resistance to OTC (Edwards 2000; Miyagi *et al.* 2000; Spivak and Gilliam 1998b). There is no effective chemical treatment for combs and other beekeeping equipment contaminated with *P. l. larvae* spores (Bailey and Ball 1991). Common practice in Australia to control AFB is by gamma irradiation of infected equipment or to destroy infected colonies by burning. Treatment of EFB by OTC has probably led to an increase in the incidence of AFB (Oldroyd *et al.* 1989).

2.2.2 Disease resistance

Resistance to *P. l. larvae* infection was demonstrated to exist in *A. mellifera* in the 1930s (Park 1936; Park 1937). Resistance was found to be both variable between lines and heritable, making possible the establishment of lines of variable susceptibility and resistance by selective breeding (Lewis and Rothenbuhler 1961; Park *et al.* 1937; Rothenbuhler 1964a). Investigations into the mechanisms of AFB resistance revealed that the amount of diseased brood present in a colony was proportional to the time taken to remove infected material from the comb and hygienic behaviour became the popular explanation for resistance to AFB (Woodrow 1941; Woodrow and Holst 1942). The vegetative stage of the pathogen is not infectious (Tarr 1937) and resistant bees

can not only detect and remove diseased brood before spores become infectious, but can also detect infection before symptoms are even visible (Brodsgaard *et al.* 2000; Woodrow and Holst 1942). Hygienic behaviour has also been documented as a response to AFB infection in the Asian hive bee, *Apis cerana* (Chen *et al.* 2000).

Hygienic behaviour by adult bees is not the only mechanism of resistance to American foulbrood (Hansen and Brodsgaard 1999; Rothenbuhler 1967; Rothenbuhler and Thompson 1956; Woodrow and States Jr. 1943). Larvae of different lines exhibit variable levels of innate resistance and susceptibility (Bambrick and Rothenbuhler 1961, but see Woodrow, 1941 #419; Rothenbuhler and Thompson 1956). Age dependent resistance has been demonstrated (Crailsheim and Riessberger-Galle 2001). Spores are thought to be removed from infected larval food by a "honey stopper" mechanism in adult bees of some strains (Sturtevant and Revell 1953). Larvae tended by nurse bees from a resistant line have lower rates of AFB infection and higher rates of survival, possibly due to antibacterial secretions in larval food (Thompson and Rothenbuhler 1957). There is variability in the pathogenicity and spore germination of *P. l. larvae* in honeybee larvae between resistant and susceptible lines (Bambrick 1964; Bambrick 1967). Differences in larval growth rates between resistant and susceptible lines have been reported (Sutter *et al.* 1968) and variation in resistance of queen, worker and drone larvae due to differences in larval food supply have been shown (Bilikova *et al.* 2001; Rinderer and Rothenbuhler 1969).

2.3 Hygienic behaviour and resistance to chalkbrood

2.3.1 The disease

Chalkbrood is another highly infectious disease that affects honeybee brood. It is caused by ingestion of spores of the heterothallic fungus *Ascosphaera apis*, formerly known as *Pericystic apis* (Spiltoir and Olive 1955), and is thought to kill its host by competition for necessary resources such as glucose (Gochnauer and Margetts 1979). Larvae 3-4 days old are particularly affected. The spores then germinate in the larval hindgut and the white mycelium grows to cover the larvae, which become mummified (Bailey and Ball 1991; Gilliam 1978a; Gilliam 1978b). The disease is characterised by the presence of these hard, chalk-like mummies (Figure 2.1), which turn dark grey or black if fruiting bodies form through contact of spores from different strains (Gilliam *et al.* 1978; Mehr *et al.* 1976). Both larvae and prepupae are susceptible to infection, which can also occur via growth of the fungus through the cuticle. Both the mated and unmated forms of *A. apis* are infective (Gilliam 1978a; Gilliam 1990; Gilliam *et al.* 1978).

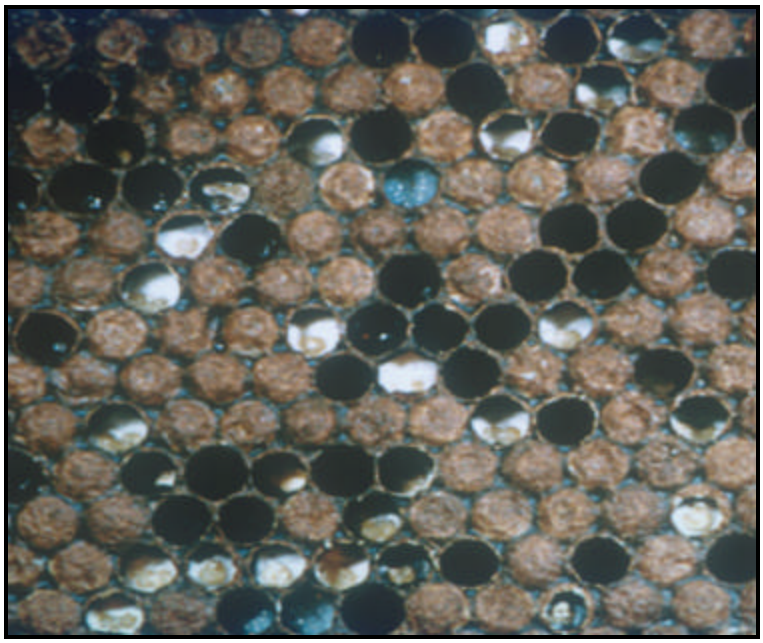


Figure 2.1: Chalkbrood mummies in brood comb. The exposed white, chalk-like dead larvae visible in this photograph are characteristic of infection by *Ascosphaera apis*.

While the disease is most apparent in summer (Heath 1982), *A. apis* spores can survive throughout the year in honey, in stored pollen, in the gut and intestine of adult bees, and are carried on the external surface of adult bees (Gilliam 1978a; Heath 1982; Koenig *et al.* 1987; Mehr *et al.* 1976; Nelson and Gochnauer 1982). These spores are highly resilient and may remain infectious for 15-38 years (Gilliam 1990). Frequent food and water-sharing among nestmates contributes to the infectious nature of the disease, as do common beekeeping practices such as reusing beekeeping equipment and brood comb, and transfer of sealed or unsealed brood, queens or workers between colonies (Gilliam 1978a; Hale and Menapace 1980; Heath 1982; Herbert *et al.* 1977; Koenig *et al.* 1986). *A. apis* is also known to infect the Asian hive bee *Apis cerana*, the alkali bee *Nomia melanderi*, the leafcutter bee *Megachile rotundata*, and the

carpenter bee *Xylocopa californica arizonensis* (Gilliam *et al.* 1994; Gilliam *et al.* 1993).

Chalkbrood seems to be stress related and certain predisposing physiological and environmental conditions seem to be required for full development of infection (Bailey 1967; Gilliam 1978b; Heath 1982). Sporulation and growth of *A. apis* are favoured at relative humidity levels above 92.5%, which may be why infection is limited to larvae that have been sealed within the brood cell (Glinski and Chmielewski 1983). The pathogen has been shown to be present in colonies that do not exhibit overt disease symptoms, with infection occurring when the colony is weak or under other stresses, for example temperature or nutritional stress (Bailey 1967; Deans 1940; Flores *et al.* 1996; Gilliam 1986; Gilliam *et al.* 1978; and reviews in Gilliam 1978b; Heath 1982).

Chalkbrood was first identified in Australia in colonies from south-eastern Queensland in 1993 (Anderson and Gibson 1998; White 1993). Within two years of its discovery, the disease had spread throughout New South Wales, South Australia and Victoria (Anderson and Gibson 1998; Oldroyd 1996a). Chalkbrood was first reported in Western Australia in 1998 and is now also widespread throughout that state (Edwards 2000). Although generally considered to be of less economic importance than American foulbrood (Gilliam and Taber III 1973), losses to honey production from chalkbrood infection have proved significant, and may be as high as 10-15% (Kleinschmidt 1996), equating to $0.5M - $0.75M p.a. in Western Australia alone (Edwards 2000).

Chalkbrood cannot be effectively treated by the chemotherapeutic or comb sterilisation techniques often employed to treat other brood diseases and encouraging results for potential chemical treatments have not been repeatable under different conditions (Gilliam 1978b; Gilliam 1990; Heath 1982). Even if these methods were developed, problems with residues in honey might preclude the use of most chemical treatments in the commercial sector. Mostly, recommendations for control of the disease have centered on management practices designed to minimize physiological and environmental stresses in the colony, and selective breeding of strains which exhibit resistance to the pathogen (Gilliam 1990; Heath 1982; Warhurst 1998).

2.3.2 Disease resistance

Some stocks of honeybees are far less affected by *A. apis* than others and larvae show variable levels of resistance to chalkbrood infection (Gilliam 1986; Gilliam *et al.* 1978; De Jong 1977, cited by Heath 1982). As with American foulbrood, resistance to chalkbrood is correlated with hygienic behaviour, particularly with the removal step (Gilliam *et al.* 1988; Gilliam *et al.* 1983; Milne 1983; Palacio *et al.* 2000; Spivak and Gilliam 1991; Spivak and Reuter 1998b). Evidence suggests that hygienic colonies have less substrate contamination and lower rates of *A. apis* survival than non-hygienic colonies (Gilliam *et al.* 1983). It has also been shown that levels of infection caused by contaminated honey and pollen are also influenced by hygienic behaviour (Gilliam *et al.* 1988).

Resistance to chalkbrood disease is not purely behavioural and some colonies exhibiting poor hygienic behaviour have been found to be resistant to the *A. apis* pathogen (Gilliam *et al.* 1988). In these colonies, various physiological mechanisms of resistance are also involved (Harbo 1995; Milne 1983; Spivak and Gilliam 1991; Spivak and Gilliam 1998a), and it has been suggested that few colonies are both highly hygienic and physiologically resistant to chalkbrood (Spivak and Gilliam 1993). Identified secondary mechanisms of resistance include filtering of *A.apis* spores by adult bees (Rath, 1985, cited in Spivak and Gilliam 1998a); larval resistance to spore infection (Harbo 1995; Spivak and Gilliam 1998a); addition of inhibitory moulds and bacteria by adult bees to pollen stores (Gilliam 1990; Gilliam *et al.* 1988); and up-regulation of brood-comb temperature - essentially a preventative fever response to infection indicating that workers can detect infection before symptoms are visible (Borges 2000; Starks *et al.* 2000).

Although other mechanisms have been demonstrated, hygienic behaviour remains the primary mechanism of resistance to American foulbrood, chalkbrood and other brood diseases (Gilliam *et al.* 1988; Spivak and Gilliam 1993; Spivak and Gilliam 1998a; Woodrow and Holst 1942). Hygienic behaviour is also a main mechanism of defense against the parasitic mite *Varroa jacobsoni* for its original host *A. cerana*, and hygienic *A. mellifera* colonies have also shown some resistance to infestation (see review in Boecking and Spivak 1999).

3. genetic & environmental factors that affect Hygienic behaviour

3.1 Genetic factors – the Rothenbuhler studies

The expression of hygienic behaviour is variable between honeybee colonies and the expression of the character can be increased by selection, indicating that some genetic mechanism must underlie the trait. In a series of elegant studies conducted during the 1960s, Walter Rothenbuhler and his colleagues investigated the genetic and environmental bases of hygienic behaviour (see reviews in Rothenbuhler *et al.* 1968; Spivak and Gilliam 1998b).

Rothenbuhler demonstrated that the level of expression of hygienic behaviour was variable between colonies from different lines and is correlated with resistance to AFB (Rothenbuhler 1964a). Hygienic bees removed large numbers of dead pupae just as quickly as smaller numbers (Jones and Rothenbuhler 1964), and hygienic behaviour assorted independently from stinging behaviour (Rothenbuhler 1964b). It was also shown that the behaviour was innate to hygienic bees and not learned (Trump *et al.* 1967). The behavioural trait was not a specific colony response to AFB-killed brood as colonies from lines selectively bred for the hygienic phenotype also performed the behaviour when challenged with cyanide-killed brood (Jones and Rothenbuhler 1964; Momot and Rothenbuhler 1971). Later studies have

demonstrated that hygienic behaviour is also expressed in colonies challenged with freeze-killed brood (Newton *et al.* 1975; Taber III 1982), pin-killed brood (Newton and Ostasiewski 1986), brood injected with dead drone pupal extract (Titera and Kokkoris 1994), brood treated with macerated chalkbrood mummies (Taber III and Gilliam 1987), and brood freeze-killed by exposure to liquid nitrogen (Spivak and Reuter 1998a). All these elements are suggestive of a crucial role of genotype in the expression of hygienic behaviour.

Of central interest to these investigations into behavioural genetics were the mode of inheritance of hygienic behaviour and the number of genes that influenced the trait. Based on selective breeding experiments and using instrumental insemination to ensure reliability of matings, Rothenbuhler created crosses between AFB resistant and susceptible lines to produce F1 hybrid colonies. Larvae were inoculated with *P. l. larvae* spores. Colonies that removed all (or most) of the inoculated brood 2-3 days before eclosion were said to be hygienic. Those that did not remove all of the inoculated brood were termed non-hygienic. Rothenbuhler found that his test colonies showed very little hygienic behaviour, similar to that of the susceptible line and thus concluded that the trait was inherited in a recessive manner (Rothenbuhler 1964b). This conclusion is somewhat naïve in that even a cursory examination of his data might suggest a more quantitative mode of inheritance.

Backcrosses of 29 single drone progeny of an F1 queen to 29 queens of the hygienic line were investigated for distribution of the uncapping and removal phenotypes. Results indicated that the colonies could be broadly categorised

as exhibiting complete hygienic behaviour, as capable of uncapping cells only, capable of removal only and non-hygienic in the ratio 6:9:6:8 respectively (Rothenbuhler 1964b).

Based on these investigations, Rothenbuhler proposed that hygienic behaviour is controlled by two unlinked recessive loci – one governing the uncapping behaviour (*u*) and the other removal (*r*). In order to express the hygienic phenotype, an individual must be homozygous recessive at both the 'uncapping' and 'removal' loci (Rothenbuhler 1964b). Rothenbuhlers' two-gene model is considered the classic example of Mendelian inheritance of a complex behavioural polymorphism and was the first study to address the inheritance of a social behaviour (Figure 3.1).

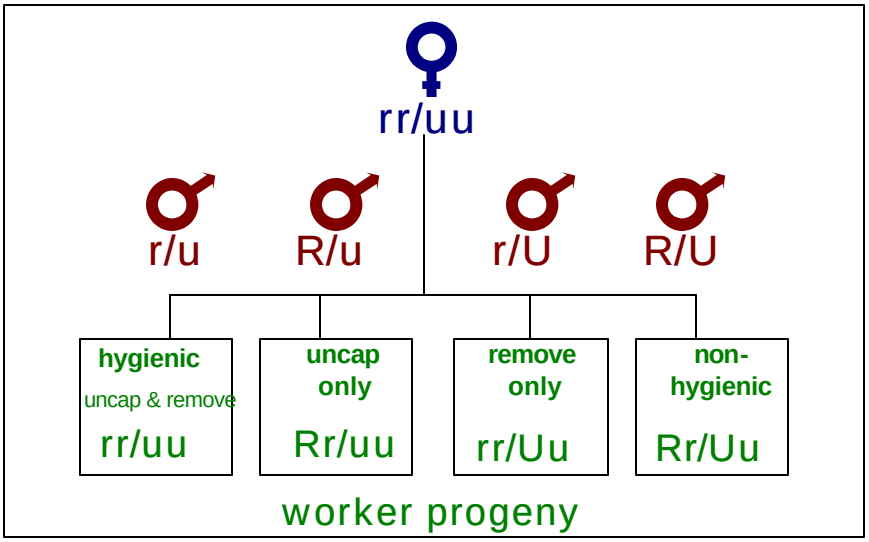


Figure 3.1: Rothenbuhlers' two-gene model for hygienic behaviour. Expected phenotypic classes as predicted by Rothenbuhlers' two-gene model for hygienic behaviour, based on an experimental backcross. Capital letters designate the dominant allele and lower case letters the recessive allele (r or R for removal and u or U for uncapping). The segregating drones from the heterozygous F1 queen would be one of four possible genotypes (**shown in red**). When backcrossed to a hygienic queen (**blue**), the resulting worker progeny (**green**) would all fall into four distinct genotypes. Only those that are homozygous recessive at both the removal and uncapping loci would express the hygienic phenotype.

3.2 Environmental factors

Hygienic behaviour is a social trait and as such its expression must be considered in a group or colony context. Studies have found that non-additive worker interactions and environmental factors also have a strong effect on colony level expression of hygienic behaviour.

Age polyethism is the major mechanism behind division of labor among workers, a central feature of the social organization of the honeybee colony. From the time of emergence, worker bees progress through a range of distinct tasks from nest cleaning to brood rearing to food storage to foraging (Seeley 1985). This ontogenetic division of labor among honeybee workers involves both behaviour and physiology (Michener 1974), and the rate at which individuals progress through this ontogeny is genetically variable (Calderone and Page 1991; Robinson and Page 1988). Hygienic behaviour is performed primarily by middle-aged workers up to 28 days old (Arathi *et al.* 2000; Thompson 1964). Thus, genotype-age interactions are important in the expression of hygienic behaviour. Despite this, older bees can be induced to express the behaviour during a nectar flow (Momot and Rothenbuhler 1971; Thompson 1964), perhaps due to changing cell space requirements within the colony, indicating that genotype-age-environment interactions are also involved in the expression of the trait.

Wide variability in the expression of hygienic behaviour is also illustrated by studies in which colony strength and worker composition were manipulated. Mixed colonies composed of both hygienic and non-hygienic workers still expressed the behavioural phenotype as long as the proportion of bees from the hygienic line was within the range 13-50% (Trump *et al.* 1967). Spivak and Gilliam also found that adding non-hygienic adult bees to colonies from the hygienic line suppressed the behaviour to some degree (Spivak and Gilliam 1991; Spivak and Gilliam 1993). Expression of hygienic behaviour is also dependent on colony strength. When hygienic colonies become weak or less populous the hygienic response is significantly reduced (Spivak and Gilliam 1993).

More recently, it has been shown that the colony environment and, more specifically, the genotypic composition of the colony affects the persistence of performance of hygienic behaviour, the partitioning of hygienic behaviour, the age at which bees perform the behaviour, and the efficiency of task performance (Arathi and Spivak 2001). Essentially, colonies comprised of a low proportion of workers of the hygienic genotype (i.e. 25%) require these bees to work longer and harder at all the tasks involved in order to express the hygienic phenotype. At the other end of the scale, colonies comprised of 100% hygienic bees exhibit the same phenotype with fewer individuals performing the task, which can be partitioned into subtasks such as uncapping and removal activities. Such studies illustrate the plasticity of the hygienic phenotype, the expression of which is adjusted according to environmental cues and influences.

If hygienic behaviour is beneficial to the colony, why isn't it fixed in populations? Spivak and Gilliam (1993) reported that most colonies not selected for resistance to a particular disease are physiologically susceptible to that disease, regardless of their ability to perform hygienic behaviour. They speculate that in this case, hygienic behaviour may not be the appropriate response to diseased brood but may actually promote the spread of disease in the colony. Expression of hygienic behaviour at the colony level in response to disease would be expected to vary with variable levels of resistance and susceptibility to the pathogen, and it has been shown that most colonies do not fall easily into well defined phenotypic classes but show intermediate levels of hygienic behaviour (Spivak and Gilliam 1991; Spivak and Gilliam 1993).

The actual means of removal does not appear to be fixed in colonies selectively bred for hygienic behaviour. Milne found that AFB-killed larvae are removed by ingestion, chalkbrood mummies are removed by workers physically dragging whole mummies out of the hive, and freeze-killed brood are removed mostly by ingestion but also by physical removal (Milne 1983).

Heritability estimates for the uncapping and removal components of hygienic behaviour are also indicative of a large environmental effect on the trait, although these estimates have varied widely. Separate studies have produced estimates of $h^2 = 0.144 \pm 0.017$ for uncapping and $h^2 = 0.022 \pm 0.004$ for removal (Milne 1985b) and $h^2 = 0.65 \pm 0.61$ for hygienic behaviour incorporating both uncapping and removal behaviour (Harbo and Harris 1999). Milne's study

indicates that uncapping behaviour is heritable while removal is not. However, this study was carried out on caged bees and it is debatable whether the expression of hygienic behaviour in this setting has any relevance to the expression of the trait in the field. The estimate of Harbo and Harris is not a useful one due to the large standard error (0.61).

While hygienic behaviour has a strong genetic component, it is a complex social trait that is also influenced by environmental factors. Expression of the trait is facultative and depends not only on the genotype of individual workers but also on the age composition of workers within the colony; colony strength; resource conditions and availability; cell space requirements; presence of particular diseases or pathogens; and the degree of physiological resistance to the particular disease or pathogen in both adult bees and brood (Spivak and Gilliam 1993). All these factors illustrate a plasticity of the behavioural phenotype that is suggestive of a system more complex than simple Mendelian two-gene inheritance.

3.3 A more complex genetic basis?

Can expression of a complex, social behavioural trait such as hygienic behaviour be sufficiently explained by a simple Mendelian two-locus model of inheritance? Some researchers have challenged Rothenbuhlers' model, favouring a more complex genetic basis for hygienic behaviour, and it has been reported that Rothenbuhler himself was not certain that his model sufficiently explained the level of variability observed in the expression of the trait (Spivak

and Gilliam 1998b). Spivak and Reuter (1998) observed that the worker progeny of queens from a line selected for hygienic behaviour and naturally mated with multiple drones of unknown genotype still exhibited hygienic behaviour and subsequently suggested that hygienic behaviour may be controlled by more than two recessive loci (Spivak and Reuter 1998b). Palacio has also demonstrated that hygienic behaviour can be selected for using naturally mated queens from a hygienic line (Palacio *et al.* 2000). It is possible that the hygienic behaviour investigated in more recent studies may not reflect the expression of the same underlying mechanisms as that described in Rothenbuhler's cross. However, Rothenbuhler's studies did show that environmental factors influence the expression of the trait and it seems likely that his two-gene Mendelian model is over simplistic.

The strong influence of environmental and non-additive factors on the expression of hygienic behaviour prompted Moritz (1988) to re-evaluate Rothenbuhler's data, paying particular attention to classification of phenotype. He suggested that the subjective attribution of genotypes to discrete behavioural phenotypes as the major problem of Rothenbuhlers' original study. Incorporating larval susceptibility to AFB as well as hygienic behaviour of the workers, Moritz recalculated the behavioural phenotypes of Rothenbuhler's original data set. He found colonies with phenotypes intermediate between the hygienic and non-hygienic extremes in ratios that did not fit the two-locus model. Moritz suggested that Rothenbuhlers' model was an oversimplification of the genetic basis of the trait and proposed a non-epistatic three-locus model, involving a single recessive locus (*u*) for uncapping behaviour but two recessive

loci (r_1 and r_2) governing removal (Moritz 1988). Complete removal behaviour would only be exhibited by individuals homozygous at both removal loci, while homozygosity at only one of these loci would result in partial expression of removal behaviour. Individuals heterozygous at both loci would not remove dead larvae.

Milne estimated the genetic correlation between the uncapping and removal components of hygienic behaviour to be 0.215, concluding that the expression of the two traits is not genetically independent (Milne 1985b). Moritz also suggested that non-additive epistatic interactions might be likely between the loci of his three-gene model (Moritz 1988). While Moritz's model may more successfully explain the high level of variability observed in hygienic behaviour breeding experiments, there are cases not explained by either the Mendelian two- or three- locus models. Rothenbuhler even reported one completely hygienic colony arising from a backcross to the non-hygienic line in the original experiment on which he formulated the two-locus model (Moritz 1988; Rothenbuhler 1964b; Spivak and Gilliam 1998b).

Despite the evidence for a large environmental component and suggestions of a more complex genetic mechanism than that which Rothenbuhler originally proposed, it is still generally agreed that a small number (2-3) of unlinked genes produce a large effect on hygienic behaviour, that the desirable hygienic alleles are recessive and that they are inherited in a Mendelian manner.

4. field studies

4.1 Introduction: Breeding of hygienic bees in Australia

Expression of colony hygienic behaviour can be successfully increased by selective breeding by both artificial insemination and natural mating of hygienic line queens (Palacio *et al.* 2000; Spivak and Reuter 1998b).

In 1995, a survey of Australian commercial bee stock was undertaken to determine what proportion of genotypes were hygienic according to Spivak's (Spivak and Gilliam 1993) definition (Oldroyd 1996a). Key findings of that research were that most Australian bees (80%) are not hygienic, but that the use of hygienic breeding stock can confer hygienic behaviour on daughter colonies. It was also concluded that screening breeding stock by the traditional methods of inserting dead brood into colonies is too time consuming for commercial queen producers to contemplate routinely (Oldroyd 1996a; Oldroyd 1996b).

A more accurate determination of how many loci directly influence hygienic behaviour and their relative level of influence and location within the genome of *A. mellifera* is needed. This would not only provide a deeper understanding of variation in complex social behavioural traits in *A. mellifera*, but may also enable the development of molecular methods of identification of hygienic stock, thus having direct commercial application for the Australian honey industry.

4.2 Importation

Pure breeding hygienic and non-hygienic lines were established in 1993 by Dr. Marla Spivak at the University of Minnesota, St. Paul, USA. This was facilitated by a selective breeding programme involving the rearing of virgin queens from a hygienic (or non-hygienic) line and subsequent instrumental insemination with semen from drone offspring of the most hygienic (or non-hygienic) colonies from the previous generation. New queens were periodically included in the breeding programme to ensure sufficient genetic diversity (Spivak and Gilliam 1998a). Artificially inseminated queens from each of these lines were imported to Australia in 1996. These lines were propagated and maintained in an apiary located at the University of Western Sydney, Richmond NSW.

Many hundreds of queens were propagated through the Australian Quarantine Inspection Service (AQIS) facility at Wallgrove, NSW. Those that were not used in the research program were made available to industry free of charge. At the conclusion of the experiments, the most hygienic colonies were made available to local and interstate queen breeders, and were disseminated throughout the industry

4.3 Experimental Cross A

An F_1 backcross between the hygienic and non-hygienic lines was created as per Rothenbuhler's study (Rothenbuhler 1964b). The F_1 drones, which are expected to be segregating for hygienic behaviour, were backcrossed to virgin queens from the hygienic line (Experimental cross A, Figure 4.1). All matings were performed using single drone artificial insemination (Laidlaw 1977).

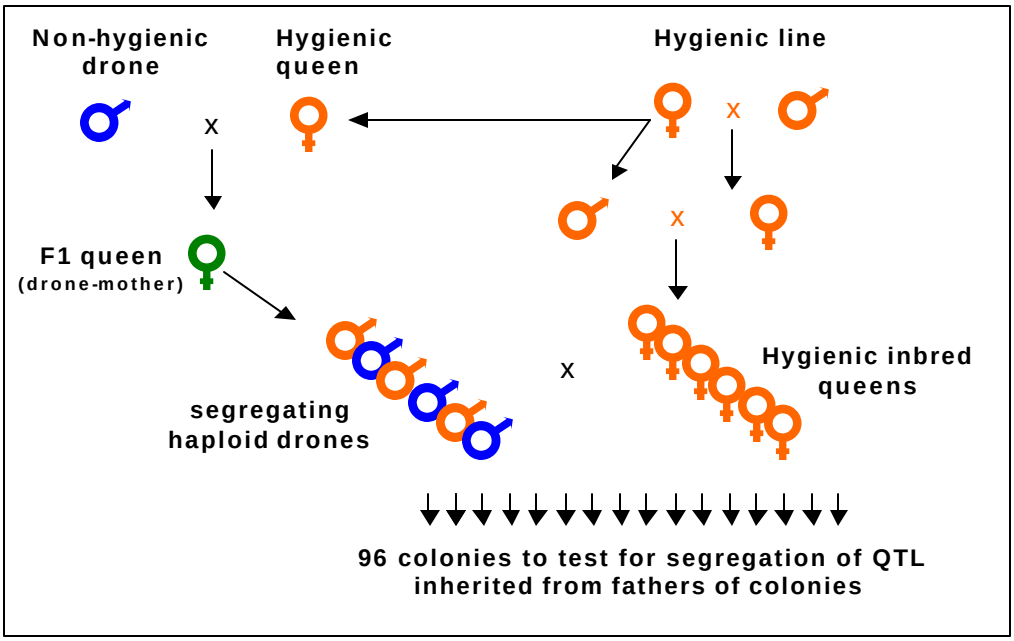


Figure 4.1 Experimental cross A: Schematic of F_1 backcross between hygienic and non-hygienic lines (after Rothenbuhler 1964b). An F_1 queen (**green**) was raised from a cross between a queen from the hygienic line (**orange**) and a drone from the non-hygienic line (**blue**). The segregating F_1 drones were backcrossed to virgin queens from the hygienic line using single drone insemination. A total of 96 resultant colonies were screened for expression of hygienic behaviour. The inseminating drones also constituted the mapping population for linkage analysis.

Experimental colonies were established in 4-6 frame nucleus hive boxes from approximately 130 inbred queens from the hygienic line during February and March 1997. Colonies were maintained over winter in several locations around Richmond, NSW. They were moved to a new location when necessary for food

supply. No evidence of sex allele homozygosity was observed so any reduced colony strength as a result of single drone insemination should not confound results. A total of 96 surviving backcross colonies, theoretically segregating for hygienic behaviour based on the genotype of the inseminating drones, were evaluated for hygienic behaviour during November - December 1997. All colonies were established at the same location a few weeks prior to field testing (Figure 4.2).

Figure 4.2: Nucleus colonies located near Richmond, NSW at the time of field evaluations for hygienic behaviour.

This cross exploits the haplo-diploid sex determination system of the honeybee. Whereas females develop from fertilized eggs and are diploid, males develop from unfertilized eggs and are haploid. As such, drones receive only maternal genes and pass on the same alleles to all their worker offspring. As all the queens heading the experimental colonies were raised from the same hygienic cross, it was expected that phenotypic segregation of hygienic behaviour in these colonies would be due to genotypic differences in the inseminating drones. The F_1 backcross could be expected to produce colonies that express the hygienic phenotype, others that do not, and colonies of intermediate

phenotype. The validity of the proposed two- and three- gene models can then be tested based on the observed phenotypic ratios. The expected genotypes and phenotypic ratios for Experimental backcross A as predicted by Rothenbuhler's two-gene and Moritz's three-gene models are given in Tables 4.1 and 4.2 respectively.

Table 4.1: Expected phenotypic ratios predicted by two-gene model

Phenotype	Hygienic	Uncap only	Remove only	Non-hygienic
Genotype	*uu rr*	*uu Rr*	*Uu rr*	*Uu Rr*
Ratio	1	1	1	1

Table 4.2: Expected phenotypic ratios predicted by three-gene model

Phenotype	Hygienic	Uncap only	Uncap + partial removal	Partial removal	Remove only	Non-hygienic
Genotype	*uu* r_1r_1 r_2r_2	*uu* R_1r_1 R_2r_2	*uu* R_1r_1 r_2r_2 *uu* r_1r_1 R_2r_2	*Uu* R_1r_1 r_2r_2 *Uu* r_1r_1 R_2r_2	*Uu* r_1r_1 r_2r_2	*Uu* R_1r_1 R_2r_2
Ratio	1	1	2	2	1	1

4.4 Experimental Cross B

A reciprocal backcross (Experimental cross B, Figure 4.3) between a hybrid F1 queen and a hygienic drone was also made and the resulting colony monitored

in an observation hive for several days during March 1998. Whereas cross A resulted in segregation of hygienic behaviour at the colony level, cross B resulted in segregation of hygienic behaviour at the individual level. Individually tagged bees that were observed performing hygienic behaviour when challenged with freeze-killed brood were collected for use in molecular investigations. These bees were used to evaluate markers identified as being tightly linked to hygienic alleles in cross A. Bees that were never observed performing the behaviour (highly non-hygienic bees) were also collected for molecular analysis. This analysis is discussed in detail in Chapter 5.

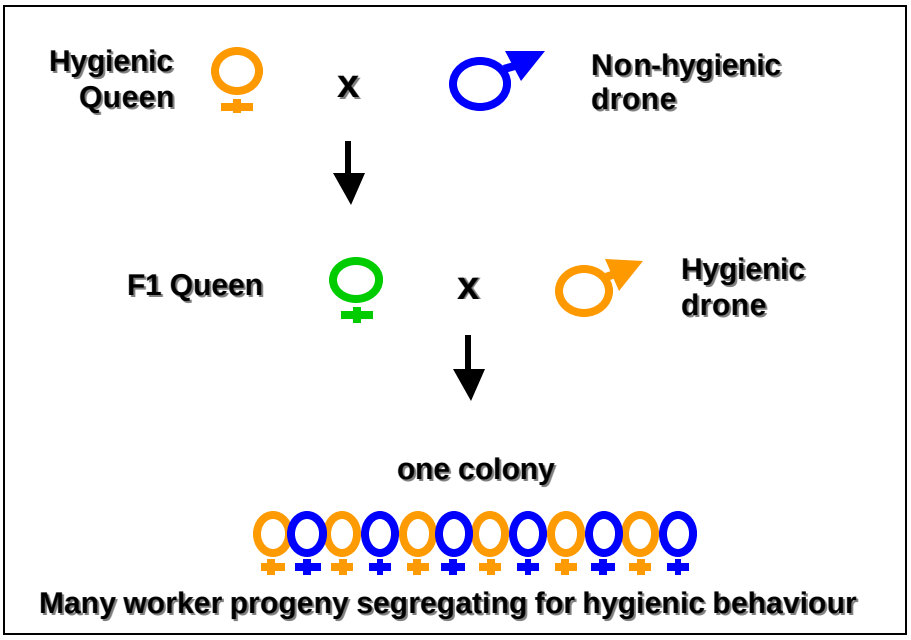


Figure 4.3 Experimental Cross B: Schematic of F_1 reciprocal backcross. An F_1 queen (**green**) was raised from a cross between a queen from the hygienic line (**orange**) and a drone from the non-hygienic line (**blue**). The F_1 queen was then backcrossed to a drone of the hygienic line. The single resultant colony comprised workers segregating for hygienic behaviour.

4.5 Field assays for segregating colonies

Many field assays have been developed to test for hygienic behaviour in honeybees (Spivak and Gilliam 1998a). Early studies on hygienic behaviour were carried out on colonies that had been selectively bred on the basis of their response to either brood treated with *Paeniacillus larvae larvae* bacteria – the pathogen which causes AFB (Rothenbuhler 1964b), or cyanide-killed brood (Jones and Rothenbuhler 1964). Other assays have been developed involving colony-level responses to pierced brood (Newton and Ostasiewski 1986), freeze-killed brood (Taber III 1982), and brood treated with macerated chalkbrood mummies (Taber III and Gilliam 1987). It has been determined that the most conservative and reliable screening method for hygienic behaviour is the freeze-killed brood assay (Spivak and Downey 1998), and that using liquid nitrogen is the best way to freeze brood (Spivak and Gilliam 1998a; Spivak and Reuter 1998a). The use of liquid nitrogen enables brood to be frozen within the comb, thus minimizing handling of and damage to brood comb. It should be noted however, that no study to date has repeated Rothenbuhlers' AFB assay to determine the correlation between the removal of spore inoculated larvae and the freeze-killed brood assay.

Testing for hygienic behaviour in this study employed an adapted liquid nitrogen field assay (Spivak and Gilliam 1998a; Spivak and Reuter 1998a). This involved challenging the colonies with freeze-killed brood and subsequent scoring for phenotypic variation in behavioural patterns (Figure 4.4).

Colonies were maintained in 4-6 frame nucleus hives. A single comb containing capped brood was removed from each colony and a cylindrical metal template covering 100 cells was inserted into the comb. Counts were made of the number of capped cells, uncapped cells and empty cells in the test region. Approximately 300 ml of liquid nitrogen was poured into the template and allowed to evaporate off to kill brood within this section (Figure 4.4).

Fig 4.4a

Fig 4.4b

Figure 4.4: Liquid nitrogen field assay for hygienic behaviour. A cylindrical metal template was inserted into capped brood comb covering 100 cells. **(a)** Brood in this section was freeze-killed by pouring 300ml liquid nitrogen into the template. **(b)** The liquid nitrogen was allowed to evaporate off and the comb allowed to thaw before removal of template. The number of capped cells, uncapped cells and empty cells in the test region were counted and recorded.

The comb was allowed to thaw before removal of the template (Figure 4.5). The location of the circular test section was marked on the frame with a thumbtack and the frame replaced in the hive of origin. The number of capped

cells, uncapped cells and empty cells were re-counted after 24 hours, and again after 48 hours. These treatments were conducted in triplicate on 23/10/1997, 13/11/1997 and 20/11/1997 with all colonies treated on the same day.

Figure 4.5: Liquid nitrogen field assay for hygienic behaviour. A treated frame ready to be replaced in the hive, with the freeze-killed region clearly visible.

A numerical score based on the average level of hygienic behaviour exhibited over the three tests was assigned to each colony. Each colony received two scores – one for expression of the uncapping phenotype only and one for expression of overall hygienic behaviour (uncapping + removal). Colonies that removed 95% or greater of capped, freeze-killed brood within 48 hours were arbitrarily classified as hygienic.

Numerical scores were assigned to each colony as a measurement of expression of the behavioural phenotypes for each of the three tests. These

were then averaged to obtain final colony scores for hygienic behaviour (uncapping + removal) and uncapping behaviour alone (see results).

Assessing removal behaviour in isolation from uncapping by manually uncapping cells following liquid nitrogen treatment was attempted. However, this procedure proved unusable. The large number of colonies to be tested simultaneously made it logistically impossible to do this rapidly enough without substantially damaging the comb. Under these conditions, it could not be determined whether any removal behaviour observed was the expression of hygienic behaviour or the result of workers repairing comb damage. Cryptic responses of selected hygienic and non-hygienic lines to manually uncapped cells containing freeze-killed brood have been reported by others indicating the unreliability of this technique. Spivak and Gilliam found that workers of non-hygienic lines tended to recap partially uncapped cells but removed dead brood from entirely uncapped cells (Spivak and Gilliam 1993).

4.6 Results

4.6.1 Segregating colonies

As expected, a range of phenotypes, from highly hygienic to completely non-hygienic, was observed in the experimental colony array. At the extremes, these phenotypic differences were marked (Figure 4.6).

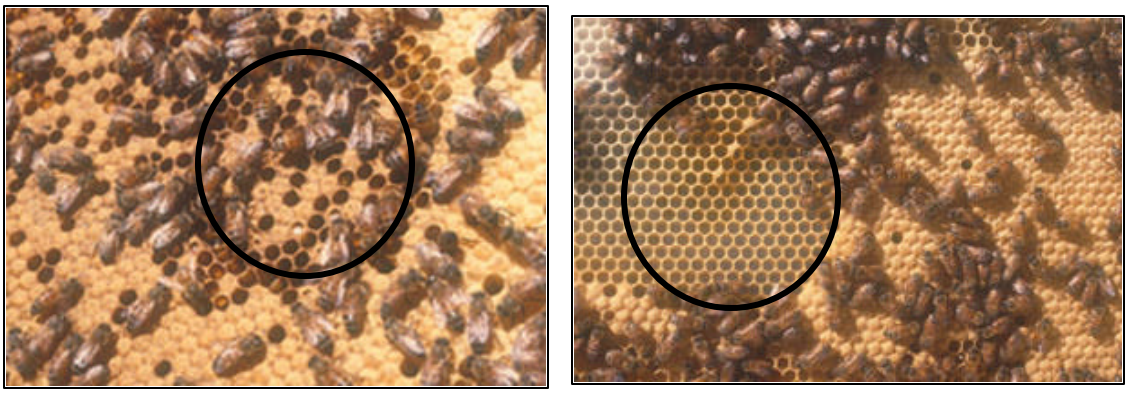


Fig 4.6a **Fig 4.6b**

Figure 4.6: Results of liquid nitrogen field assay for hygienic behaviour. The test combs of two colonies 48 hours post treatment are shown. In both cases the treatment region is circled. **A)** shows a colony considered to be non-hygienic and many capped cells are clearly visible within the treated area. **B)** illustrates the treated area of a non-hygienic colony. In this case, only three cells remain capped after 48 hours. Not all colonies were so obviously hygienic or non-hygienic. Many exhibited intermediate expression of the hygienic phenotype leaving between 50% and 95% of cells capped 48 hours after treatment.

As described previously, the number of capped cells, uncapped cells and empty cells were re-counted after 24 hours, and again after 48 hours for each colony. The two numerical scores assigned to each colony as a measurement of expression of the behavioural phenotypes were calculated as follows:

$$X_{hyg} = \frac{[(C + U)_{T=0}] - [(C + U)_{T=48}]}{(C + U)_{T=0}}$$

$$X_{uncap} = \frac{[C_{(T=0)} - C_{(T=48)}]}{C_{(T=0)}}$$

Where: X_{hyg} = the numerical score for overall hygienic behaviour (uncapping + removal) at T=48 hrs

X_{uncap} = the numerical score for uncapping only at T=48hrs

C = number of capped cells

U = number of uncapped cells (containing dead pupae)

T = 0: at the time of liquid nitrogen treatment

T = 48: 48 hours post liquid nitrogen treatment

Numerical scores were assigned to each colony for each of the three tests. These were then averaged to obtain final colony scores for hygienic behaviour (uncapping + removal) and uncapping behaviour alone. The three phenotypic tests were found to be highly correlated suggesting that the two scores are heritable (Figure 4.7). The raw phenotypic data is given in Appendix A.

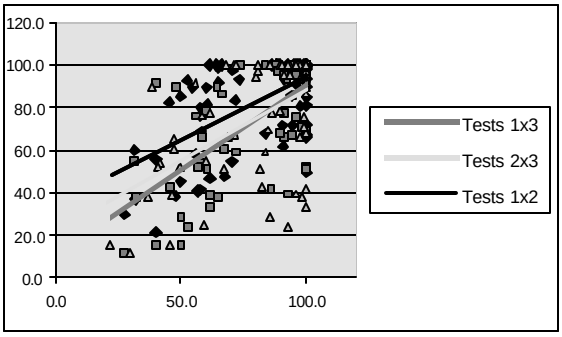


Fig 4.7a Uncapping + Removal correlation

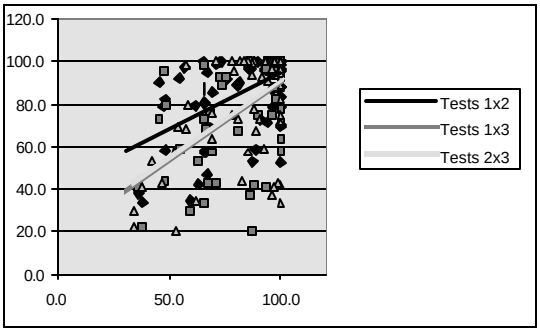


Fig 4.7b Uncapping only correlation

Figure 4.7: Pairwise correlations of phenotypic scores between the three tests
a) gives the correlation coefficients between each of the three tests for overall hygienic behaviour (*r* = 0.809 for tests 1 and 2; *r* = 0.787 for tests 1 and 3; *r* = 0.704 for tests 2 and 3). **b)** gives the corresponding values for the uncapping phenotype alone (*r* = 0.800 for tests 1 and 2; *r* = 0.743 for tests 1 and 3; *r* = 0.732 for tests 2 and 3).

Repeatability of uncapping and total hygienic behaviour (proportion of dead brood removed) was assessed across the three assays as a measure of the upper bound of heritability. The proportion data were first normalised using an arcsin x transformation. From this I calculated the intra-class correlation and the repeatability. The repeatability, *r*, (Falconer 1981) of uncapping was 0.54 ± 0.061 and the repeatability of overall hygienic behaviour was 0.57 ± 0.059. However, the studied 'population' was generated from a single cross of divergent lines. Thus the repeatability estimate should not be regarded as a 'selectability'.

Numerical scores for the two phenotypic types were also plotted against each other (Figure 4.8). For the average of the three tests, R^2 = 0.92. This high value indicates that the two phenotypic states are highly correlated and variation in one may be explained by variation in the other. This is expected, as removal cannot occur before uncapping.

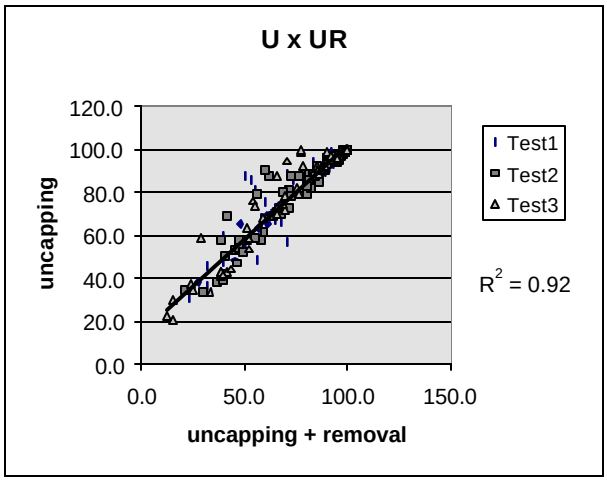


Figure 4.8: Uncapping plotted against overall hygienic behaviour across 3 tests

The observed phenotypic data (Figure 4.9) were analysed by a $\div^2$ test to determine if they differed statistically from the expected ratios predicted by Rothenbuhler's two-gene (Figure 3.1, Table 4.1) and Moritz's three gene (Table 4.2) models for hygienic behaviour (Moritz 1988; Rothenbuhler 1964b). As removal behaviour was unable to be assessed independently of uncapping, the expected 'remove only' class was pooled with the non-hygienic class. The results of this experiment were significantly different from those predicted by both models (for the two-gene model $\div^2 = 21.38$, $P = 8.77 \times 10^{-5}$, d.f. = 2 for the three-gene model $\div^2 = 20.13$, $P = 0.00016$, d.f. = 3. See Table 4.3).

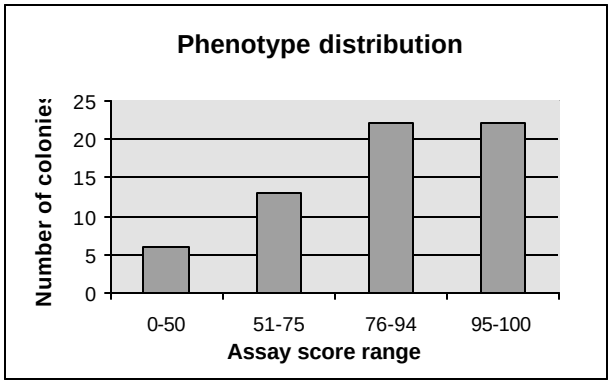


Figure 4.9: Distribution of hygienic behaviour phenotypes in experimental colonies. The number of colonies represented in four phenotypic ranges over the 48hr test period are shown. Colonies in the assay score range 95-100 are considered to be hygienic, those in the 0-50 range non-hygienic and the rest intermediate between the two.

Table 4.3: Values for $\div^2$ test of observed and expected phenotypic ratios

	Hygienic	Uncap only	Non-hygienic
Observed	19	0	44
Expected 2 gene model	15.75	15.75	31.5
Expected 3 gene model	9	9	45

4.7 Summary

Backcross colonies established from Experimental cross A were assayed for expression of the behavioural phenotype. Phenotypic segregation of hygienic behaviour in these colonies would be due to genotypic differences in the inseminating drones. This is essentially a repeat of Rothenbuhlers' original cross and is an effective test of the two- and three- gene hypotheses for Mendelian inheritance of hygienic behaviour (Moritz 1988; Rothenbuhler 1964b). The significant deviation of expected phenotypic proportions of colony behaviour under both models (for the two-gene model $\div^2 = 21.38$, $P < 0.001$, d.f. = 2 for the three-gene model $\div^2 = 20.13$, $P = 0.001$, d.f. = 3), together with the high measures of repeatability (for uncapping $r = 0.54 \pm 0.061$ and for overall hygienic behaviour $r = 0.57 \pm 0.059$) indicate that hygienic behaviour is highly heritable in the population tested, but that variance is quantitative and not controlled by Mendelian genes.

While it is obvious that hygienic behaviour is highly heritable and can be selected for, these results indicate that Rothenbuhler's two-gene model, and Mortiz's proposed three-gene model, are overly simplistic and the actual underlying genetic mechanism of hygienic behaviour is much more complex (ie. quantitative).

As suggested in chapter 3, it is possible that the hygienic behaviour investigated in more recent studies may not reflect the expression of the same underlying genetic mechanisms as that described in Rothenbuhler's cross. Alternatively,

observed variation in hygienic behaviour may be due to unknown differences between different field assays – for instance the freeze-killed assay employed in this case and Rothenbuhler's assay using *P. l. larvae* spore inoculated larvae. However, Rothenbuhler's original series of investigations using the hygienic Brown and non-hygienic Van Scoy lines also found that non-additive worker interactions and environmental factors have a measurable effect on colony level expression of hygienic behaviour. Older bees can be induced to express the behaviour during a nectar flow (Momot and Rothenbuhler 1971; Thompson 1964). Manipulation of colony strength and worker composition also influenced the level of expression of the trait (Trump *et al.* 1967). The field studies on which Rothenbuhler formulated his two-gene model also produced one colony that did not fit the model. He found that one colony established from a backcross of a F_1 drone to the non-hygienic Van Scoy line expressed the hygienic behaviour phenotype. Rothenbuhler had no explanation for this anomaly at the time, stating, "We cannot disregard this result, as much as we would like to, but we are basing the genetic hypothesis on the other data" (Rothenbuhler 1964b).

The results presented here, as well as those of many other more recent investigations, suggest that genotype-age-environment interactions are involved in the expression of hygienic behaviour and the variation observed cannot be solely attributed to Mendelian inheritance of two or three genes – even in Rothenbuhler's original crosses.

5. Quantitative traits and Linkage Mapping

5.1 Introduction

Recombinant DNA technology and molecular techniques such as the Polymerase Chain Reaction (PCR, Saiki *et al.* 1988) have revolutionised the study of behavioural genetics. Traditionally, investigations of inherited traits (such as Rothenbuhler's study of hygienic behaviour in honeybees) were limited to observations and analysis of morphological characters and phenotype. Today, patterns of inheritance and the underlying genetic mechanisms of traits can be investigated at the DNA level. This has enabled the genetic study of complex behavioural traits to be opened up to a new level of scrutiny.

5.1.1 Quantitative traits

Variation controlled by single genes is readily studied by phenotypic analysis as the genotype can often reliably be predicted from observed phenotypes. Such traits are inherited in a Mendelian manner and changes in the genes usually result in a qualitative change in phenotype.

The total phenotypic variance of most traits is not, however, determined by a single gene but rather by allelic variation at several loci. Collectively, the effect

of these quantitative loci on a trait is large, although their individual effects are often relatively small. In addition, there are often epistatic interactions between quantitative trait loci (QTL) and environmental effects which all influence phenotypic variance of characters (Falconer and Mackay 1996). This is especially true of complex behavioural traits. Expression of quantitative traits can be thought of as the outcome of multiple genetic loci interacting with each other and environmental factors (Beavis and Keim 1996).

Determination of the genetic basis of quantitative variation influenced by the action of several genes by phenotypic analysis is problematic. Individually these genes are inherited in the Mendelian manner but have small effects on variation of the trait so they do not usually exhibit Mendelian ratios and hence cannot be identified phenotypically. Modern quantitative genetics involves family level linkage analysis of data generated by molecular tools (such as DNA markers) coupled with novel statistical techniques. Together, these techniques permit direct identification of chromosomal regions associated with even very complex phenotypes such as hygienic behaviour.

5.1.2 Genetic markers

Mapping of loci that affect or control a quantitative trait requires allelic variation and linkage between alleles at marker loci and genetic loci which influence the trait in question (quantitative trait loci or QTL, see Chapter 6). When several genes influence the trait under investigation, markers need to be abundant throughout the genome as well as being highly polymorphic. Unlike

morphological or single gene markers, DNA-level markers are phenotypically neutral – allelic variation does not generally result in observable changes in phenotype. This decreases the chance that marker loci will have a larger phenotypic effect than the linked quantitative trait (Falconer and Mackay 1996; Tanksley 1993).

Mapping the loci underlying quantitative variation using traditional molecular markers - such as allozymes – is problematic as they usually exhibit low levels of polymorphism and are not abundant enough throughout the genome. DNA-based markers however, are highly polymorphic, abundant throughout the genome, are phenotypically neutral (as allelic variation is largely in non-coding regions), do not exhibit epistatic effects and are generally co-dominant. These include random amplified polymorphic DNA (RAPDs), restriction fragment length polymorphisms (RFLPs), variable number of tandem repeats (VNTRs), microsatellites, and amplified fragment length polymorphisms (AFLPs). The use of PCR-based markers (e.g. RAPDs, microsatellites, AFLPs) enables linkage maps to be constructed more rapidly than is possible using RFLP markers (Falconer and Mackay 1996; Ribaut and Hoisington 1998; Tanksley 1993).

5.1.3 Linkage Maps

A genetic map is an ordered array of genetic markers, usually arranged into linkage groups that reflect chromosomes. Linkage maps based predominantly on RAPD markers have been published for many plant and animal species including the Hymenopteran parasitoid wasp species *Trichogramma brassicae*

(Laurent *et al.* 1998), *Bracon* sp. near *hebetor* (Holloway *et al.* 2000), and the honeybee (Hunt and Page 1995).

Large family sizes, a very high rate of recombination (Hunt *et al.* 1995) and a haplo-diploid sex determination system make the honeybee well suited to QTL and genetic analyses. Honeybees are highly social insects with a complex behavioural repertoire and hierarchical structure. As a result, the honeybee is also a model organism for behavioural research at both an individual and colonial level.

Economically important traits such as body size; alarm pheromone levels; behaviour or stinging behaviour; and nectar and pollen foraging behaviour have been investigated using QTL analysis, and loci related to these characters mapped (Hunt *et al.* 1999; Hunt *et al.* 1998; Hunt *et al.* 1995).

5.2 Methodology

5.2.1 Generation of RAPD molecular data

Individual drones from the segregating population, which were the inseminating drones used to establish the experimental colonies in cross A (Figure 4.1), were sampled and total DNA extracted from each male. This group of males constitutes the mapping population and was screened with ten-nucleotide arbitrary primers purchased from Operon Technologies (USA) and the University of British Columbia (UBC, Vancouver, BC).

5.2.2 Extraction of genomic DNA

Bees were collected on dry ice and stored at -70°C. DNA was extracted using a modified CTAB lysis method (Hunt 1997b). The head and thorax section of individual frozen bees was placed in a 1.5ml microcentrifuge tube and 200µl of lysis solution (1% CTAB, 50mM Tris (pH 8.0), 10mM EDTA, 1.1M NaCl) and proteinase K (100µg/ml) added. Tissue was ground with a plastic pestle and incubated at 60°C for 1-5 hrs. Samples were extracted once by repeated gentle inversion after addition of 100µl phenol (equilibrated to pH 7.4) and 100µl chloroform (Sigma) followed by centrifugation at 15000 x g for 10 minutes. The upper aqueous layer was then transferred to a new tube. Samples were extracted a second time by addition of 200µl of chloroform (Sigma), repeated inversion and centrifugation for 2-3 minutes. DNA was precipitated in one tenth volume (20µl) 3 M sodium acetate (pH 5.2) and two volumes (400µl) cold 100%

ethanol. Samples were incubated for 10 minutes at -70°C then centrifuged for 20 minutes at 5000 x g. The resulting pellet was washed in cold 70% ethanol, vacuum dried and dissolved in 100µl $TE_{0.1}$ (10 mM Tris buffer (pH 7.6), 0.1 mM EDTA) by heating to 65°C for 10 minutes. Concentrated DNA stocks were diluted 1:200 in sterile H_2O for use in PCR.

5.2.3 Polymerase Chain Reactions

Generation of RAPD markers by PCR was carried out according to the methods of Williams et al. (1990). Each reaction contained 0.5µM primer (Operon Technologies, Alameda; University of British Columbia, Vancouver); 100µM each of dATP, dCTP, dGTP & dTTP (Biotech, Perth); 2.0mM $MgCl_2$ (Biotech, Perth); 1x Tth buffer (Biotech, Perth); 0.5U Tth plus polymerase (Biotech, Perth); and 2.0µl diluted genomic DNA in a total volume of 12µl. Amplifications were performed on a Hybaid Omnigene thermal cycler under the following conditions: 45 cycles of 94°C for 1 minute, 35°C for 1 minute, 2 minute ramp to 72°C, 72°C for 2 minutes (Hunt 1997a).

Amplification of microsatellite loci was performed as described by Estoup *et al.* (1995; 1994). Of seven microsatellite loci tested (A7, A8, A14, A28, A43, A88 and A107) only three (A8, A14, A43) were polymorphic in this cross and these were included in the linkage analysis.

5.2.4 Electrophoresis of PCR products

PCR products were diluted 1:5 with loading dye (5% Ficoll in 0.4 x TBE with bromophenol blue) and loaded onto a 4% non-denaturing polyacrylamide gel (4% bis-acrylamide, in 0.4x TBE). Samples were run on a GS2000 automated gel scanner (Corbett Research) at 700 V for 30-45 minutes, depending on the size range of the bands produced. Gels were stained with ethidium bromide (2.5µl of 10mg/ml EtBR solution added to the bottom buffer tank of the GS2000 apparatus during electrophoresis). The resulting scan was analysed using the One-D Scan program (Scanalytics).

5.3 Results and Analysis

Molecular data were generated from DNA samples from the mapping population using RAPD markers. DNA sequence variation is detected by PCR using arbitrary 10-base sequence primers (Williams *et al.* 1990). Scorable polymorphisms included presence / absence bands and size polymorphisms (Figure 5.1).

Linkage mapping using RAPD markers has some limitations as presence alleles are dominant to absence alleles, which may be masked in heterozygous individuals. RAPD generated polymorphisms also tend to be family specific with population based polymorphisms very difficult to identify. However, genotyping in this case was performed on haploid males, thus removing the problem of dominance in presence / absence alleles. RAPD markers have also been criticised for their lack of repeatability between PCR reactions. In this study, however, genotypic data were generated by two independent reactions for each marker, and only markers that produced consistent phenotypes were used. RAPD markers have also been successfully used in previous studies involving linkage mapping and QTL analyses on honeybees (Hunt 1997a; Hunt *et al.* 1999; Hunt *et al.* 1998; Hunt *et al.* 1995; Hunt and Page 1995).

The mapping population was screened with over 400 primers. Two hundred and sixteen of these yielded scorable polymorphic bands.

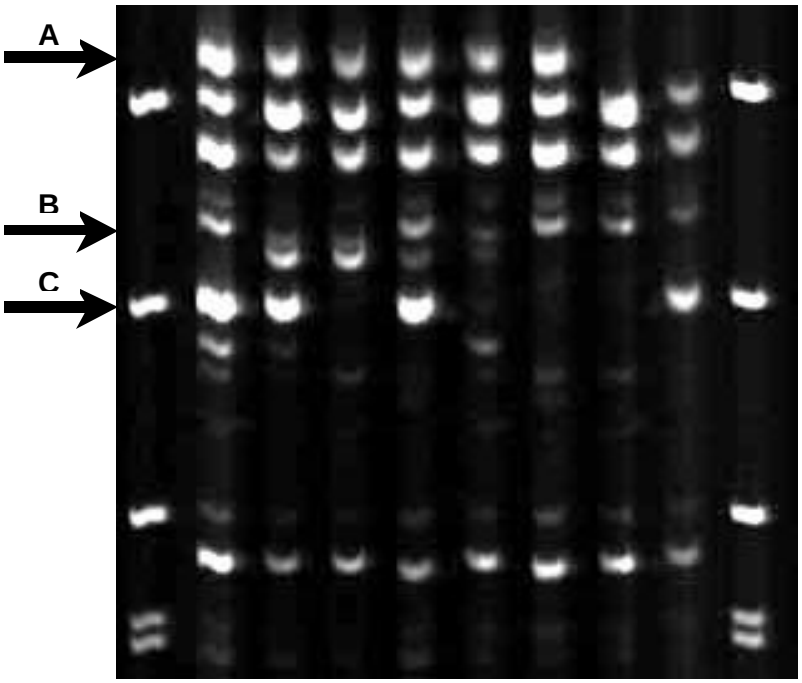


Figure 5.1: Electrophoresis of PCR products generated by RAPD primers. Each lane (2-9) represents the profile for an individual drone of the mapping population given by a single RAPD primer. Lanes 1 and 10 contain Probase 50 size standard (**Progen**). Presence / absence polymorphisms are illustrated by the bands at A and C. The band at B is representative of a size polymorphism.

Gels were scored once at initial data recording and rescored after linkage analysis had produced ordered linkage groups. Data that were difficult to score or which did not amplify were listed as missing data. The second scoring was necessary to double check bands which caused the linkage group to expand dramatically, which were problematic to assign to a linkage group, or which the Mapmaker programs' error detection option flagged as containing potential scoring errors (Lincoln and Lander 1992). Markers that were unclear or likely to have been misread were dropped from the analysis after rescoring.

The final data set consisted of 482 segregating marker loci (consisting of RAPDs, microsatellites, sts loci and the MDH allozyme) scored for 119

individual males. A linkage map was constructed based on segregation of RAPD markers and microsatellites in 119 haploid drones (the mapping population from experimental cross A), which were the progeny of the F1 queen in the backcross.

Linkage analyses were performed with MAPMAKER/EXP v3.0 software, PC version (Lander *et al.* 1987; Lincoln *et al.* 1992). As a requirement of the software, the data type was coded as "F2 backcross" with the allele inherited from the hygienic parent of the F1 queen listed as "H" (homozygous) and the allele from the non-hygienic parent coded "A" (heterozygous). Some markers were unable to be unambiguously assigned as either "A" or "H" as the non-hygienic parent of the imported F1 drone mother (Figure 4.1) was not available. In these cases, the band was scored as either one or the other then reassigned if the marker was not linked after two-point analysis (Hunt and Page 1995).

The MAPMAKER/EXP program uses two-point analysis to calculate the recombination fraction between pairs of marker loci, and tests for linkage between the two using a LOD score statistic (Lander *et al.* 1987; Lincoln *et al.* 1992). The LOD score is Log_{10} of the odds ratio of the likelihood of obtaining the estimated recombination fraction given linkage over the likelihood of obtaining the recombination fraction without linkage. A LOD score of 3.0 and recombination fraction of 0.34 were used as the default linkage criteria throughout most of the MAPMAKER/EXP analysis. A LOD score of 3.0 for linkage means that the estimated recombination fraction between two marker loci is 1000 times more likely if the loci are linked than if they are not. The

Kosambi mapping function, which converts recombination fractions to map distances, was also used throughout the analysis (Kosambi 1944).

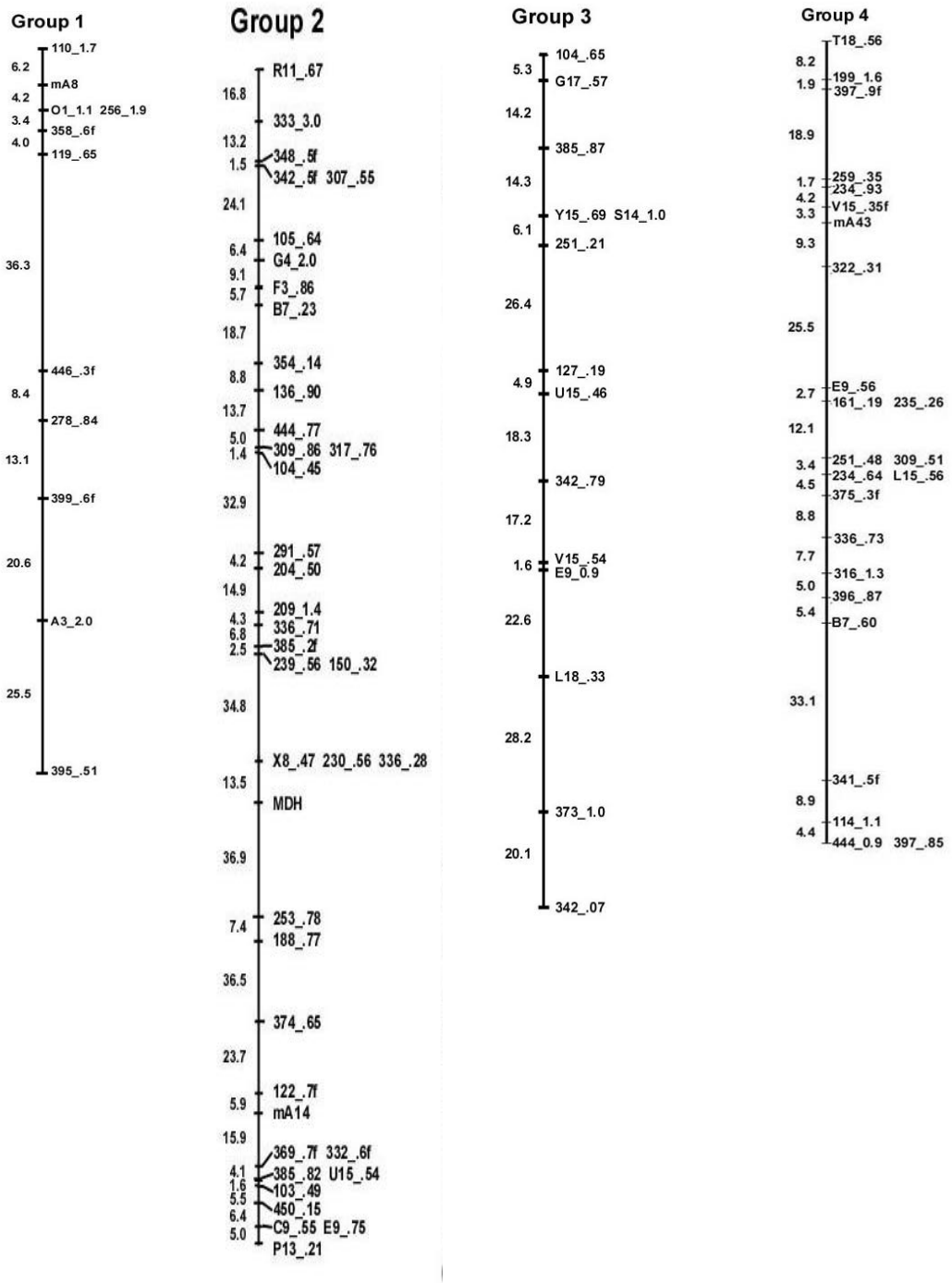
The "group" command was first used on the entire data set to determine putative linkage groups by two-point comparisons of markers. Each linkage group was then separately subjected to full multipoint analysis (essentially the simultaneous estimation of all recombination fractions in the primary data set) to find the most likely order of markers in a single linkage group, which was then tested using the "ripple" command.

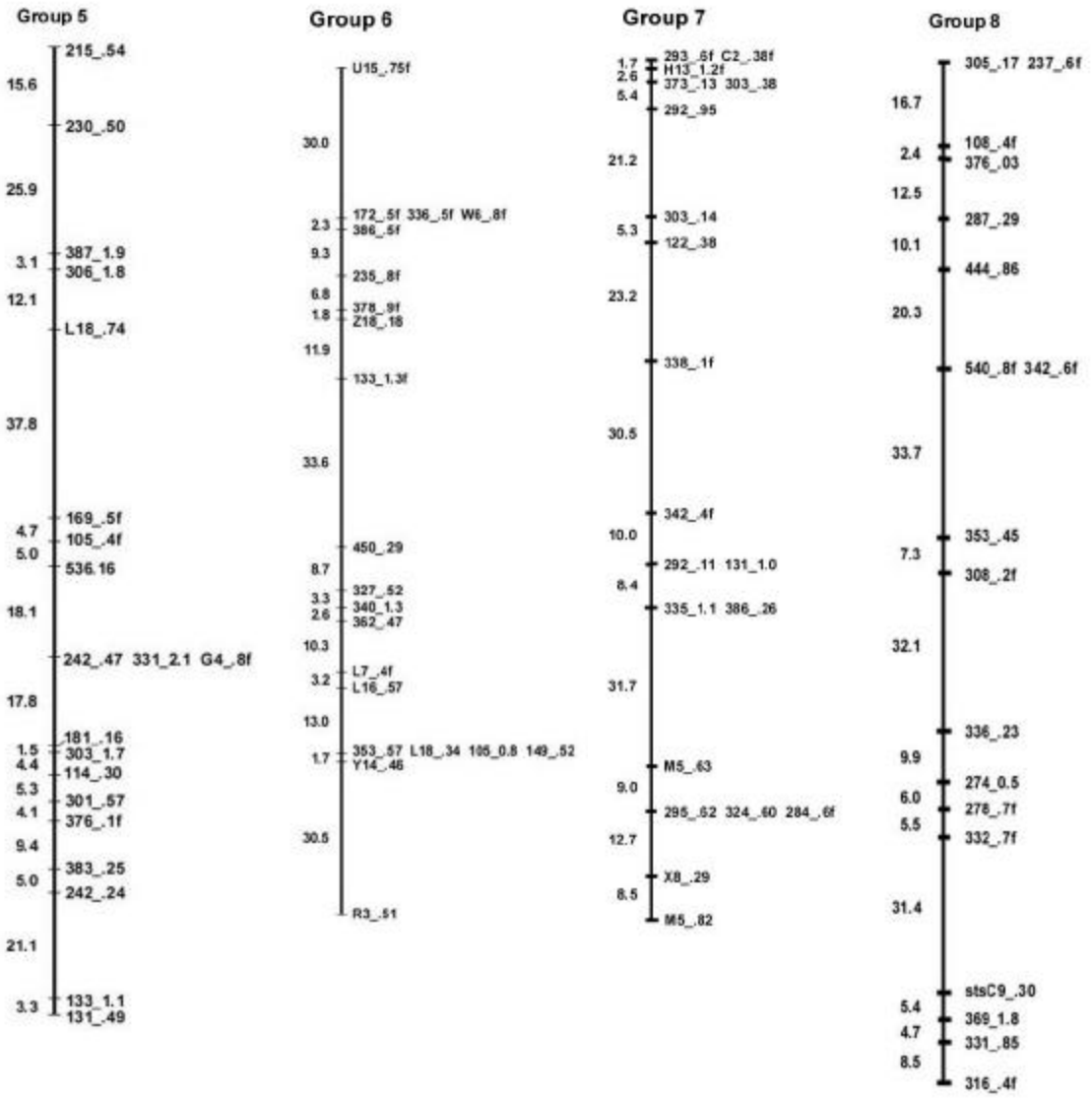
Each linkage group was analysed for possible additional weak linkages at minimum LOD 2.0 and maximum recombination distance of 50 cM. This was to identify potential linkage between separate linkage groups. If several marker loci at the end of one linkage group were related to loci that belonged to another linkage group or were unlinked at LOD values of >2.0 but <3.0 then it is likely that those groups are linked.

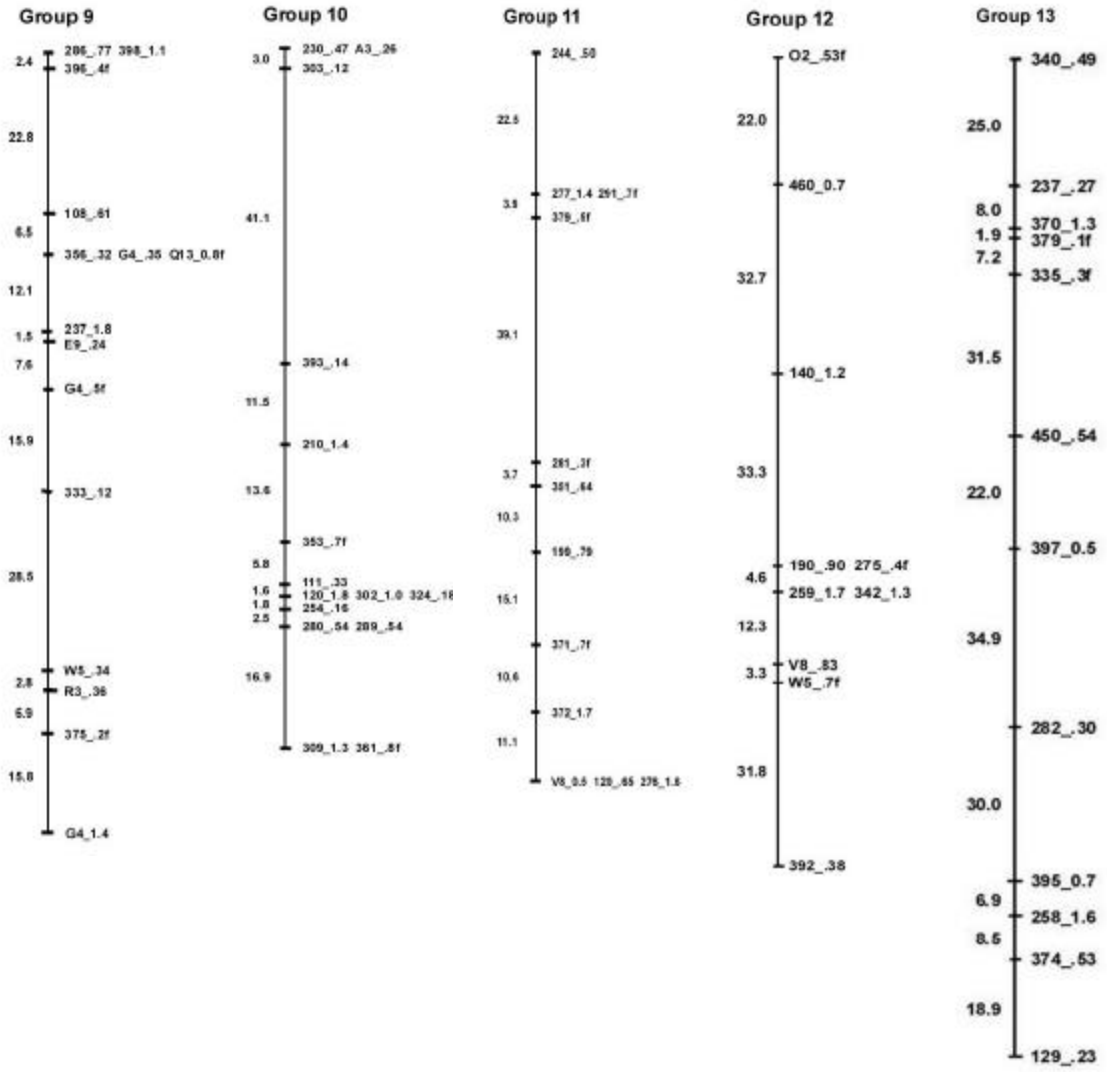
Linkage analysis by Mapmaker v3.0 produced 358 marker loci ordered over 25 linkage groups spanning a total distance of 3406.2cM. The average distance between each marker was 9.5cM (Figure 5.2).

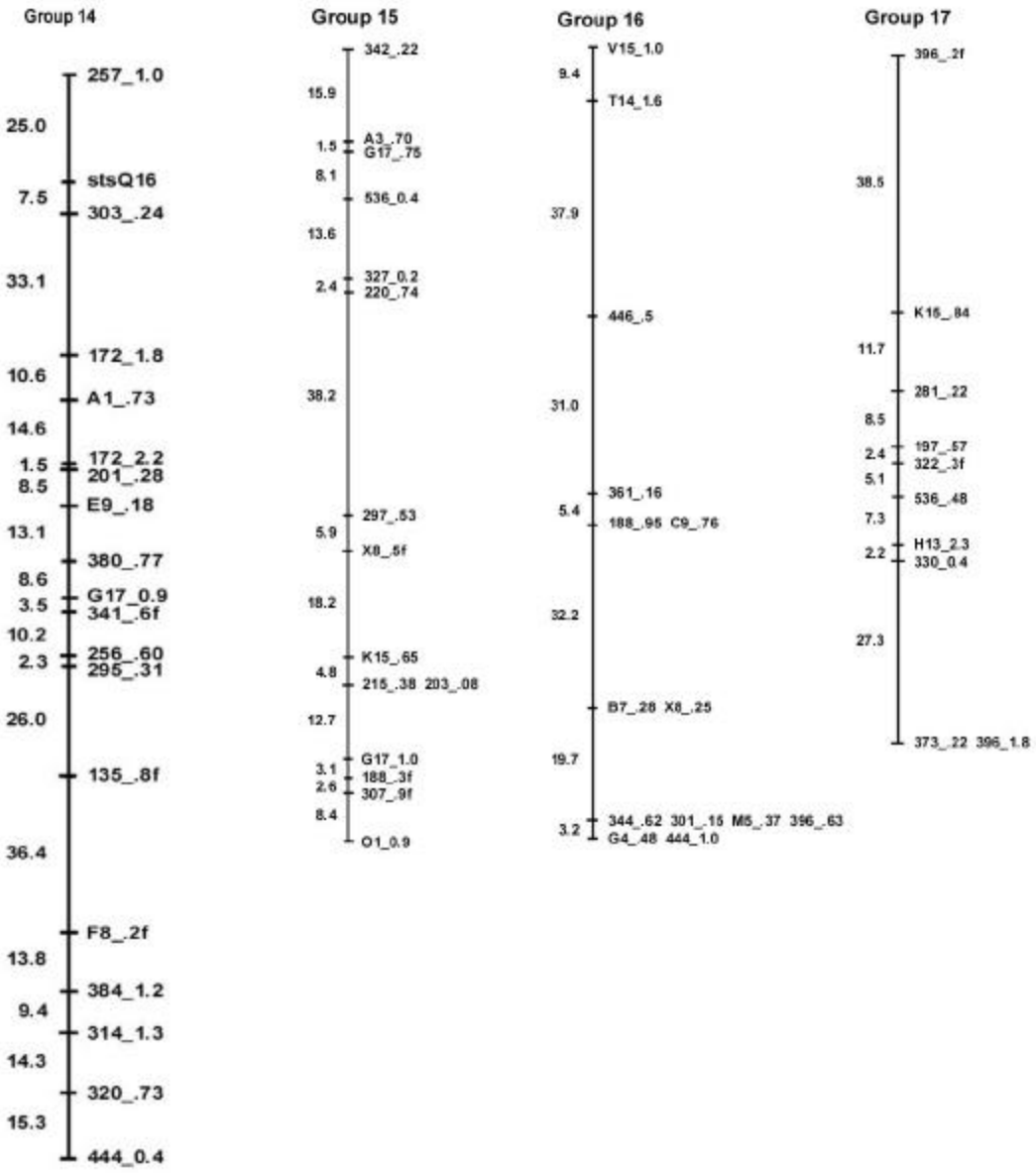
Figure 5.2 Linkage map of the honeybee.

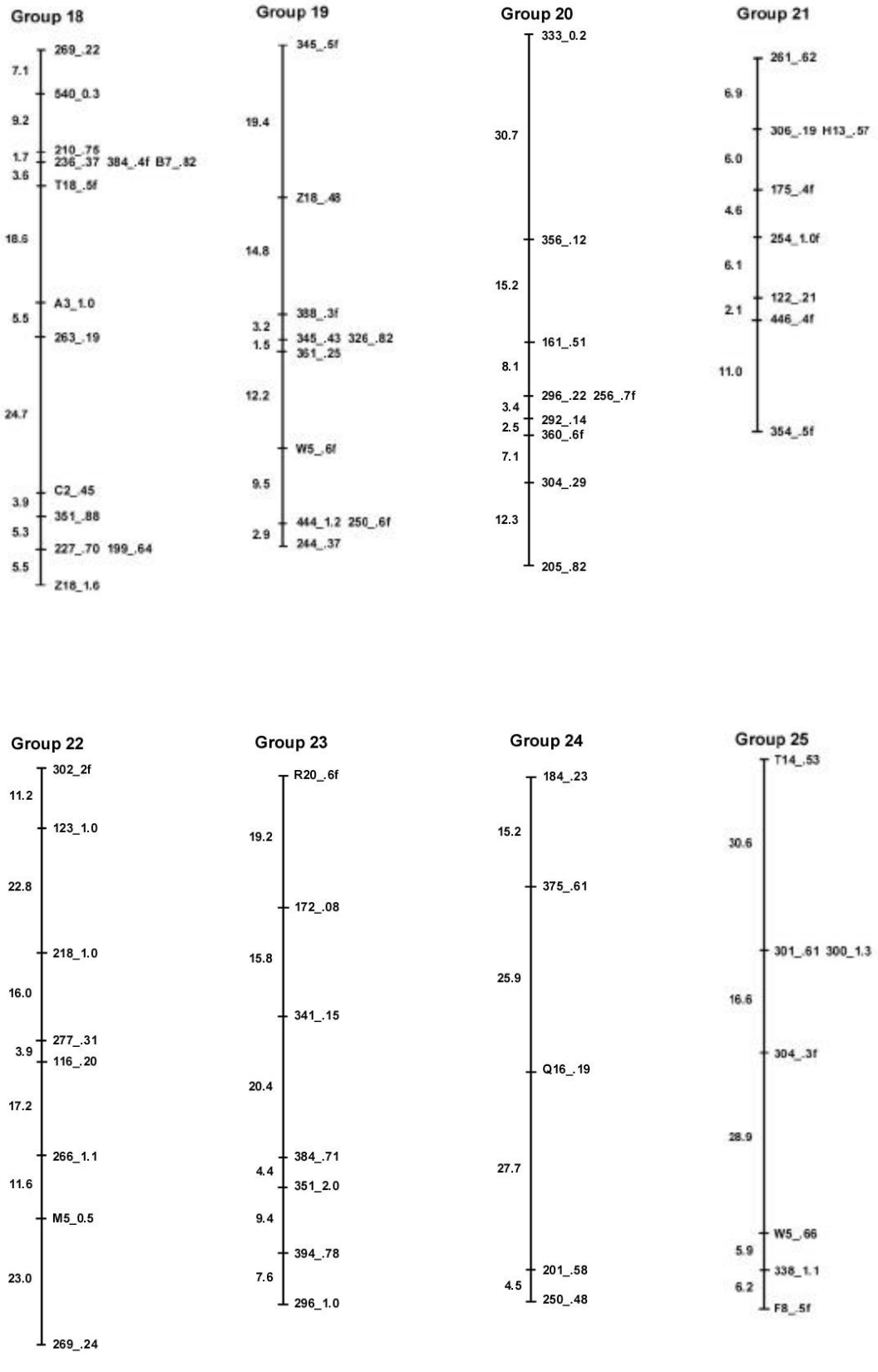
The 25 linkage groups identified are shown. Numbers on the left refer to map distances between marker loci in centimorgans (cM). Marker loci positions are given on the right and are named according to the primer which generated it, followed by an underscore and the size of the band produced (kB). Loci names falling within the range 100-500 were generated by RAPD primer kits obtained from the University of British Columbia. Those starting with a capital letter followed by a number were generated by RAPD primer kits obtained from Operon Technologies. Loci designated mA followed by a number refer to microsatellites, loci with the prefix sts refer to sequence tagged sites. Size polymorphisms are indicated by an "f" following the marker name. All other markers are presence/absence polymorphisms.




Group 5
215_.54
15.6
230_.50
25.9
387_1.9
3.1
306_1.8
12.1
L18_.74
37.8
169_.5f
4.7
105_.4f
5.0
536.16
18.1
242_.47 331_2.1 G4_.8f
17.8
181_.16
1.5
303_1.7
4.4
114_.30
5.3
301_.57
4.1
376_.1f
9.4
383_.25
5.0
242_.24
21.1
133_1.1
3.3
131_.49
Group 6
U15_.75f
30.0
172_.5f 336_.5f W6_.8f
2.3
386_.5f
9.3
235_.8f
6.8
378_.9f
1.8
Z16_.16
11.9
133_1.3f
33.6
450_.29
8.7
327_.52
3.3
340_1.3
2.6
362_.47
10.3
L7_.4f
3.2
L16_.57
13.0
353_.57 L18_.34 166_0.8 149_.52
1.7
Y14_.46
30.5
R3_.51
Group 7
293_.6f C2_.38f
1.7
H13_1.2f
2.6
373_.13 303_.38
5.4
292_.95
21.2
303_.14
5.3
122_.38
23.2
338_.1f
30.5
342_.4f
10.0
292_.11 131_1.0
8.4
335_1.1 386_.26
31.7
M5_.63
9.0
295_.62 324_.60 284_.6f
12.7
X8_.29
8.5
M5_.82
Group 8
305_.17 237_.6f
16.7
108_.4f
2.4
376_.03
12.5
287_.29
10.1
444_.86
20.3
540_.8f 342_.6f
33.7
353_.45
7.3
308_.2f
32.1
336_.23
9.9
274_0.5
6.0
278_.7f
5.5
332_.7f
31.4
stsC9_.30
5.4
369_1.8
4.7
331_.85
8.5
316_.4f


Group 9
2.4
286_.77 398_1.1
396_.4f
22.8
108_.61
6.5
356_.32 G4_.35 Q13_0.8f
12.1
1.5
237_1.8
E9_.24
7.6
G4_.5f
15.9
333_.12
28.5
W5_.34
2.8
R3_.36
5.9
375_.2f
15.8
G4_1.4
Group 10
3.0
230_.47 A3_.26
303_.12
41.1
393_.14
11.5
210_1.4
13.6
353_.7f
5.8
111_.33
1.6
120_1.8 302_1.0 324_.18
1.8
254_.16
2.5
280_.54 289_.54
16.9
309_1.3 361_.8f
Group 11
244_.50
22.5
277_1.4 291_.7f
3.8
376_.5f
39.1
281_.3f
3.7
361_.64
10.3
166_.79
15.1
371_.7f
10.6
372_1.7
11.1
V8_0.5 128_.65 276_1.6
Group 12
O2_.53f
22.0
460_0.7
32.7
140_1.2
33.3
190_.90 275_.4f
4.6
259_1.7 342_1.3
12.3
3.3
V8_.83
W5_.7f
31.8
392_.38
Group 13
340_.49
25.0
237_.27
8.0
370_1.3
1.9
379_.1f
7.2
335_.3f
31.5
450_.54
22.0
397_0.5
34.9
282_.30
30.0
395_0.7
6.9
258_1.6
8.5
374_.53
18.9
129_.23


Group 14
257_1.0
25.0
stsQ16
7.5
303_.24
33.1
172_1.8
10.6
A1_.73
14.6
172_2.2
1.5
201_.28
8.5
E9_.18
13.1
380_.77
8.6
G17_0.9
3.5
341_.6f
10.2
256_.60
2.3
295_.31
26.0
135_.8f
36.4
F8_.2f
13.8
384_1.2
9.4
314_1.3
14.3
320_.73
15.3
444_0.4
Group 15
342_.22
15.9
A3_.70
1.5
G17_.75
8.1
536_0.4
13.6
327_0.2
2.4
220_.74
38.2
297_.53
5.9
X8_.5f
18.2
K15_.65
4.8
215_.38 203_.08
12.7
G17_1.0
3.1
188_.3f
2.6
307_.9f
8.4
O1_0.9
Group 16
V15_1.0
9.4
T14_1.6
37.9
446_.5
31.0
361_.16
5.4
188_.95 C9_.76
32.2
B7_.28 X8_.25
19.7
344_.62 301_.15 M5_.37 396_.63
3.2
G4_.48 444_1.0
Group 17
396_.2f
38.5
K15_.84
11.7
281_.22
8.5
197_.57
2.4
322_.3f
5.1
536_.48
7.3
H13_2.3
2.2
330_0.4
27.3
373_.22 396_1.8


Group 18
269_.22
7.1
540_0.3
9.2
210_.75
1.7
236_.37 384_.4f B7_.82
3.6
T18_.5f
18.6
A3_1.0
5.5
263_.19
24.7
C2_.45
3.9
351_.88
5.3
227_.70 199_.64
5.5
Z16_1.6
Group 19
345_.5f
19.4
Z18_.48
14.8
388_.3f
3.2
345_.43 326_.82
1.5
351_.25
12.2
W5_.6f
9.5
444_1.2 250_.6f
2.9
244_.37
Group 20
333_0.2
30.7
356_.12
15.2
161_.51
8.1
296_.22 256_.7f
3.4
292_.14
2.5
360_.6f
7.1
304_.29
12.3
205_.82
Group 21
261_.62
6.9
306_.19 H13_.57
6.0
175_.4f
4.6
254_1.0f
6.1
122_.21
2.1
446_.4f
11.0
354_.5f
Group 22
302_2f
11.2
123_1.0
22.8
218_1.0
16.0
277_.31
3.9
116_.20
17.2
266_1.1
11.6
M5_0.5
23.0
269_.24
Group 23
R20_.6f
19.2
172_.08
15.8
341_.15
20.4
384_.71
4.4
351_2.0
9.4
394_.78
7.6
296_1.0
Group 24
184_.23
15.2
375_.61
25.9
Q16_.19
27.7
201_.58
4.5
250_.48
Group 25
T14_.53
30.6
301_.61 300_1.3
16.6
304_.3f
28.9
W5_.66
5.9
338_1.1
6.2
F8_.5f

5.4 Summary

A genetic map of the honeybee genome was constructed by full multipoint linkage analysis of 358 segregating marker loci. Loci consisted of predominantly RAPD markers with 2 sts loci (stsQ16 and stsC9, Hunt *et al.* 1998)), 3 microsatellite loci (A8, A14 and A43, Estoup *et al.* 1995; Estoup *et al.* 1994)), and the MDH locus also included. The 25 linkage groups cover a total map distance of 3406 cM.

The number of linkage groups in a saturated map should theoretically match the number of haploid chromosomes in the organism (Heckel 1993). The haploid chromosome number of the honeybee is 16, so this map contains 9 gaps, which are likely to span distances greater than that equating to the maximum recombination fraction of 0.34 used in the analysis. The map published by Hunt and Page in 1995, also based on segregating RAPD markers, covered 3110cM on 26 linkage groups. Taking into account the 10 gaps in their map and a total physical size of 178,000 kb (Jordan and Brosemer 1974), they estimated the total genome size to be 3450cM with a ratio of physical size to recombinational size of 52 kb/cM (Hunt and Page 1995). If it is similarly assumed that the 9 gaps in the map presented here are at least 34cM each, then the estimated genome size based on this map is 3712cM with a ratio of physical size to recombinational size of 48 kb/cM. This is in keeping with the previously reported high rate of recombination in the honeybee (Hunt and Page 1995).

The current map is approximately 7.5% larger in recombination units than that previously published by Hunt and Page (1995). Scoring errors will introduce spurious crossovers and increase the interval size between markers (Lincoln and Lander 1992). However, Mapmaker's built in error detection option was used for all analyses to identify problematic data. This function gives a LOD_{error} value for each pair of markers and is representative of the likelihood that a particular data point is an error (Lincoln *et al.* 1992; Lincoln and Lander 1992). Data points flagged by the error detection function were rechecked. Any that were not clear upon rechecking were either dropped from the data set or rescored as missing data.

Although many of the same RAPD primers used in the previous study (Hunt and Page 1995) were also used in this map, it was virtually impossible to identify individual linkage groups in common between the two maps. This is not unexpected as RAPD markers will yield different bands and show differing levels of polymorphism in separate crosses. For example, sequence-tagged sites (STS) are often used to confirm linkage group identity between independent maps. Fourteen STS loci have been previously described and included on the previously published map (Hunt *et al.* 1998). These were tested in my mapping population, but only three were polymorphic in this cross. Theoretically, these should be able to be used as anchor loci to identify common linkage groups between the two maps. However, loci flanking the STS markers were not common between the maps. Neither were the sizes of the bands produced by the STS primers consistent between the maps.

6. qtl analysis

6.1 Introduction

Quantitative Trait Loci (QTL) represent regions of the genome that have a measurable affect on variation in a particular trait. QTL analysis essentially calculates a statistical association between the phenotypic trait of interest and segregating genetic markers. QTL mapping requires a linkage map of the genome based on polymorphic marker loci and measurable variation for the trait under investigation within or between populations or strains (Falconer and Mackay 1996).

QTL analysis has proven to be very useful in identifying the genetic components of important economic traits such as milk production in dairy cattle (Georges *et al.* 1995; Zhang *et al.* 1998); climatic adaptation in pine trees (Hurme *et al.* 2000); fruit mass, yield, and pH in tomatoes (Paterson *et al.* 1990); floral morphology (Bradshaw *et al.* 1998); wood quality in eucalyptus (Grattapaglia *et al.* 1996); and drought responses in maize (Lebreton *et al.* 1995). Identification of QTLs for economically important traits is desirable for implementation into breeding schemes facilitated by marker assisted selection (MAS).

QTL methods also enable study of the genetic basis of complex behavioural patterns. They have been successfully utilized in investigations of behaviour as

diverse as alcohol consumption (Buck 1995; Gehle and Erwin 1998; Plomin and McClearn 1993); IQ and cognitive ability (Plomin *et al.* 1994); anxiety (Gray *et al.* 1999); obesity and anorexia (Schalling *et al.* 1999); and fear-like behaviour responses in mice (Gershenfeld and Paul 1997).

QTL analysis combines phenotypic and molecular data and attempts to find a statistical association between genetic marker(s) and the expression of a trait. It is a powerful method of studying the effects of individual genes (such as gene frequencies and magnitude of effect) that contribute to the expression of a quantitative trait. As discussed in Chapter 5, such genes cannot be investigated individually using classical Mendelian genetics as their effects are usually individually small and get lost in the background of all other variation, whether it be genetic or environmental (Falconer and Mackay 1996). QTL analysis requires genotypic data at each marker locus and phenotypic data for each quantitative trait, for each individual in the mapping population. Linkage of a QTL for a trait to a marker is inferred if there is a difference in mean phenotype of the trait among marker genotype classes.

A more accurate determination of the number, location within the genome and relative level of influence of loci that directly influence hygienic behaviour in *A. mellifera* is achievable with molecular techniques. Markers that are associated with the hygienic genotype are expected to be part of, or very close to, gene(s) that influence hygienic behaviour.

6.2 Methodology

6.2.1 Generation of phenotypic data

Phenotypic data were obtained as described in Chapter 4.6.1 and consisted of numerical scores assigned to each colony for each of the triplicate field tests. The three phenotypic tests were found to be highly correlated (Figure 4.7), so the scores were averaged to obtain final colony scores for overall hygienic behaviour (uncapping + removal) and uncapping behaviour alone (Table 6.1). These scores provide a measurable numerical value of the phenotypic performance of each colony with respect to overall hygienic behaviour and uncapping behaviour in response to freeze-killed brood over a 48 hour time period. Raw phenotypic data is given in Appendix A.

Table 6.1: *Numerical scores assigned to each of 63 test colonies for overall hygienic behaviour (**H**) and uncapping behaviour only (**U**).*

Colony	*H*	U	Colony	H	U	Colony	H	U	Colony	H	U
1	84.8	95.3	**17**	62.5	94.9	**33**	35.7	38.5	**49**	98.2	95.5
2	87.2	90.4	**18**	96.0	98.7	**34**	96.6	98.7	**50**	98.2	99.2
3	89.9	93.2	**19**	100.0	100.0	**35**	95.7	95.7	**51**	63.6	69.3
4	75.8	76.8	**20**	51.2	71.1	**36**	63.4	61.9	**52**	23.1	31.6
5	92.7	96.1	**21**	85.9	93.0	**37**	64.3	70.7	**53**	76.5	94.2
6	58.5	64.2	**22**	85.4	92.2	**38**	84.6	88.3	**54**	95.1	97.9
7	99.5	99.5	**23**	100.0	100.0	**39**	64.9	66.3	**55**	94.7	95.3
8	85.7	90.8	**24**	100.0	100.0	**40**	76.0	86.4	**56**	90.2	92.3
9	77.8	83.2	**25**	72.0	85.6	**41**	84.2	87.4	**57**	49.1	52.4
10	89.8	90.5	**26**	99.7	100.0	**42**	99.7	99.7	**58**	83.0	85.4
11	85.9	88.1	**27**	64.2	73.0	**43**	36.9	53.6	**59**	100.0	100.0
12	97.9	98.5	**28**	49.9	53.0	**44**	99.3	99.6	**60**	67.2	71.0
13	100.0	100.0	**29**	99.3	99.6	**45**	98.4	98.7	**61**	81.2	81.9
14	58.6	74.0	**30**	100.0	100.0	**46**	95.3	97.9	**62**	90.8	92.7
15	83.7	88.5	**31**	71.5	79.1	**47**	40.7	50.5	**63**	62.6	73.7
16	93.3	94.9	**32**	100.0	100.0	**48**	94.4	95.5			

Although 96 colonies were assayed for expression of hygienic behaviour (Chapter 4), only 63 of these were included in the QTL analysis. This was necessary because the last 33 drones used in the inseminations to establish the experimental colonies were obtained from a sister queen to the original F1 drone mother. This occurred as the drones in the original F1 colony died at the end of the 1997 season and no new ones were produced. While the other drones were related enough for use in the linkage mapping analysis, they were omitted from any of the quantitative trait analyses to avoid any dampening of the phenotypic signal on the genotypic data.

6.2.2 Generation of molecular data

Molecular data were generated from screening of RAPD markers on the mapping population as described previously (refer to Chapter 5). Again the data set was limited to the 63 individual colonies described above.

6.3 Results and Analysis

6.3.1 Identification of QTLs

QTL analysis was performed using MapQTL v3.0 software for the Macintosh (Van Ooijen and Maliepaard 1996). Three types of data files are required by the program. Molecular data were provided to the program in the form of a locus genotype file and the data coded as BC1 to indicate a backcross. The quantitative data file contained the numerical scores for each individual (Table 6.1). The map file lists the marker loci and their map positions for each linkage group (Chapter 5).

Primary analysis identified potential QTLs by standard interval mapping (single-QTL model) which determines the likelihood for the presence of a segregating QTL at every position in the genome, calculating a "QTL likelihood map" (Lander and Botstein 1989; Van Ooijen and Maliepaard 1996). Maximum likelihood interval mapping uses the genotype information for each adjacent pair of markers to maximize the likelihood function in order to best explain the distribution of the quantitative phenotypic data (Beavis and Keim 1996). Again, the LOD score is the test statistic, which this time represents the Log_{10} of the ratio of the likelihood that a segregating QTL is present to the likelihood that there is no segregating QTL. The higher the calculated LOD score at any given point on the linkage map, the more likely t is that there is a segregating QTL for the quantitative trait being tested at that position.

The restricted multiple-MQM model (MQM) was then used as a means to simultaneously fit more than one QTL on the map. This uses the markers closest to the putative QTLs identified in the interval mapping as cofactors while fitting other QTLs (Tables 6.2 & 6.3). MQM analysis enhances the power of QTL mapping by reducing genetic background noise (Jansen and Stam 1994; Jansen 1993). Detection of a QTL is indicated when the LOD score exceeds a significance threshold. Following Hunt *et. al.* (1999) I used a LOD score of 1.5 to be suggestive of a QTL. A QTL with this LOD score should explain 7-8% of the total phenotypic variance in this population. Analyses were performed on both the overall hygienic behaviour phenotypic data and the uncapping only data.

Table 6.2: *Putative QTLs for overall hygienic behaviour in honeybees*

Linkage Group	Nearest marker	LOD score	% Variance explained	Cofactors*
2	336_.71	2.91	13.1%	336_.71
4	397_.9f	3.04	14.5%	397_.9f
5	242_.24	2.07	8.9%	-
6	172_.5f	1.72	9.3%	172_.5f
13	395_0.7	2.14	9.2%	-
15	G17_1.0	2.17	9.2%	G17_1.0
22	123_1.0	3.37	15.2%	123_1.0

*No markers were used as cofactors for QTLs associated with linkage groups 5 and 13 in the MQM analysis. When cofactors from these groups were included in the analysis, the QTLs could no longer be detected. This may be due to epistasis between the two loci.

Table 6.3: *Putative QTLs for the uncapping aspect of hygienic behaviour in honeybees*

Linkage Group	Nearest marker	LOD score	% Variance expl	Cofactors
2	336_.71	2.68	12.0%	336_.71
4	397_.9f	3.08	15.8%	397_.9f
6	U15_.75f	1.90	10.4%	U15_.75f
22	123_1.0	2.95	13.6%	123_1.0

A total of 7 QTLs were identified from the experimental cross A data. QTLs associated with linkage groups 2, 4, 6 and 22 were detected for both overall hygienic behaviour and uncapping only data. Additional QTLs located on linkage groups 5, 13 and 15 were associated with the total hygienic behaviour data (Figure 6.1, Tables 6.2 and 6.3). Scans of all linkage groups for overall hygienic behaviour and uncapping behaviour QTL are given in Appendix B.

Figure 6.1: LOD score profiles for putative QTLs for expression of the hygienic behaviour a nd uncapping phenotypes

LOD score profiles for linkage groups associated with putative QTLs are shown. In each case profiles for both overall hygienic behaviour and uncapping only behaviour are shown. LOD profiles for groups 13 and 15 (Figures 6.1e and 6.1f respectively) are for hygienic behaviour only as QTLs were not segregating for uncapping behaviour. The percentage of the genetic variance of each trait that is explained by each QTL is also given.

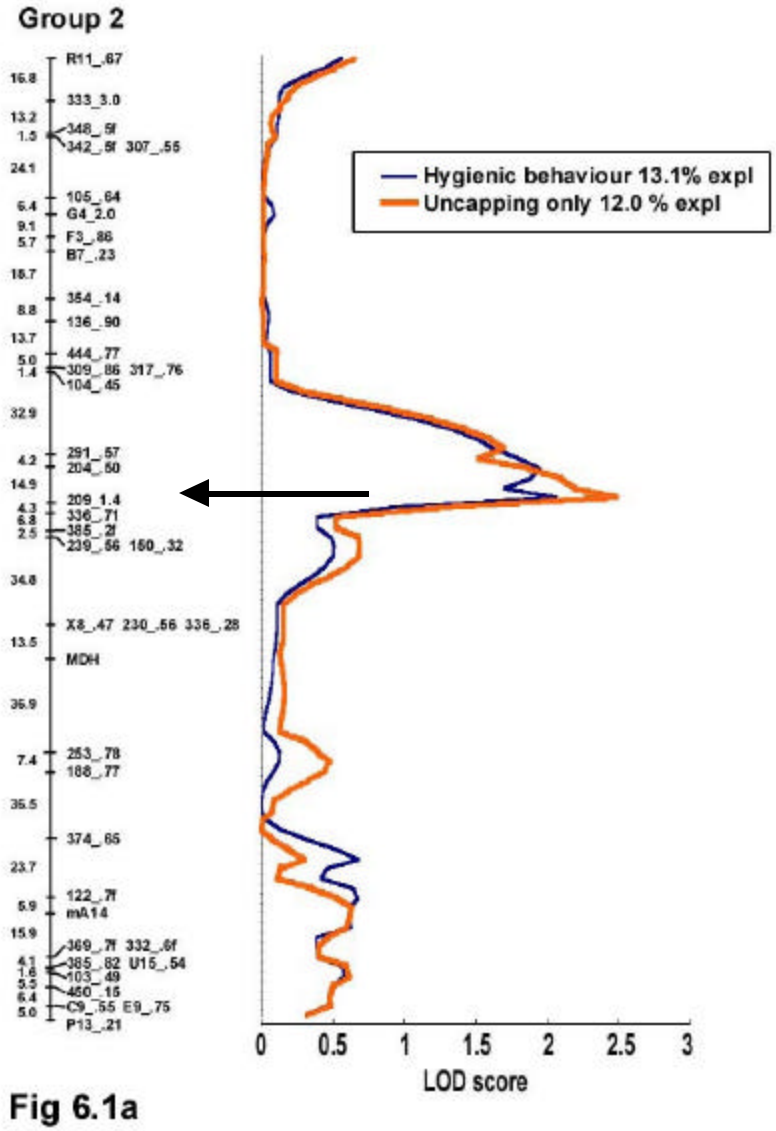


Fig 6.1a

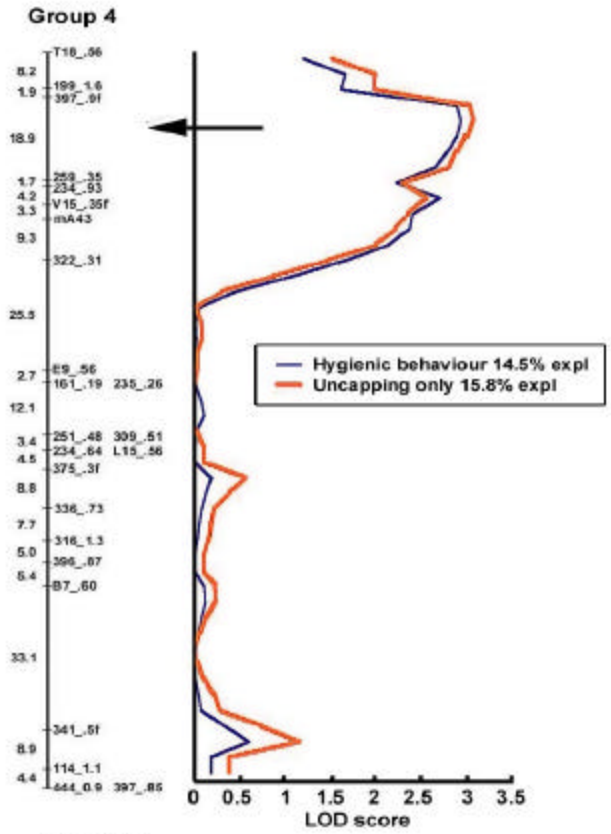


Fig 6.1b

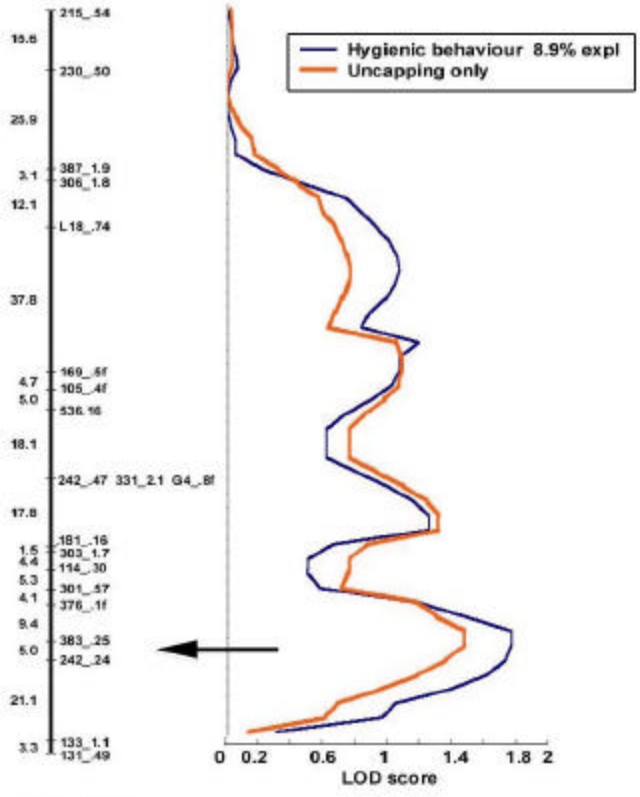


Fig 6.1c

Group 6

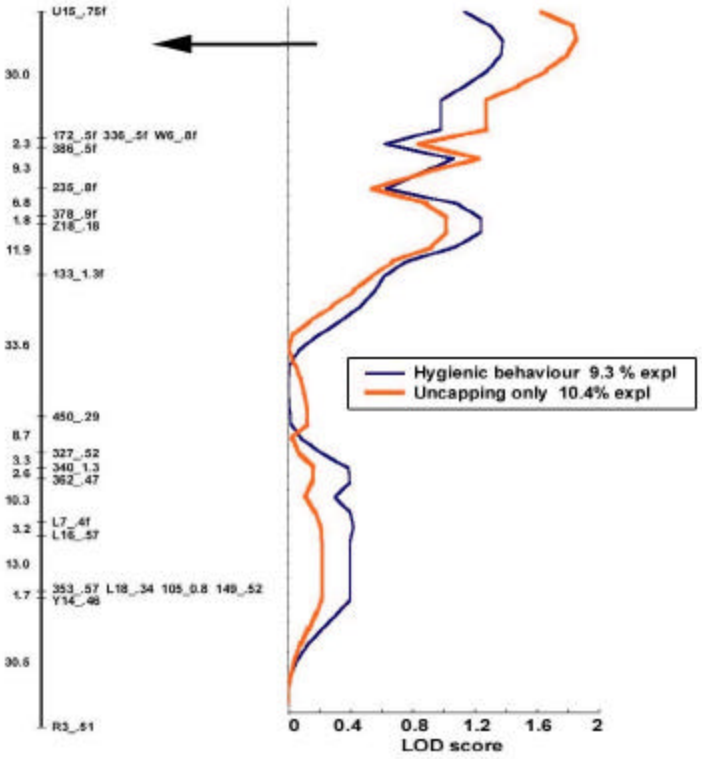


Fig 6.1d

Group 13

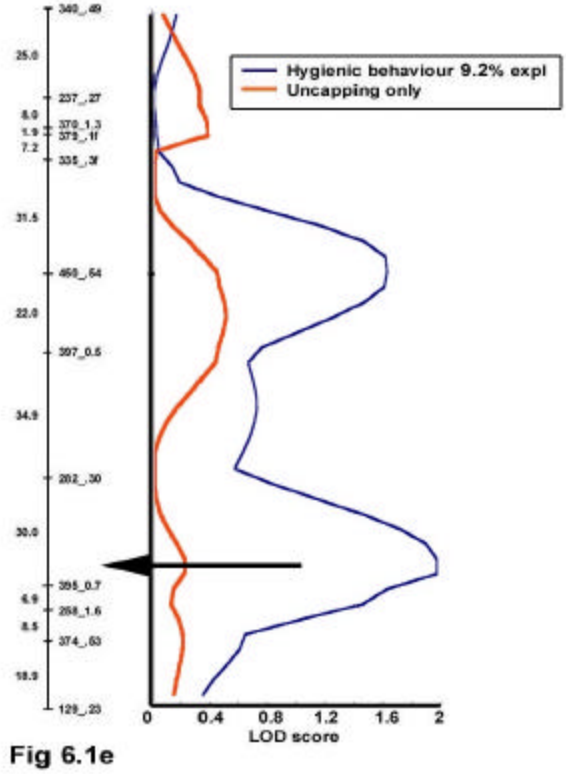


Fig 6.1e

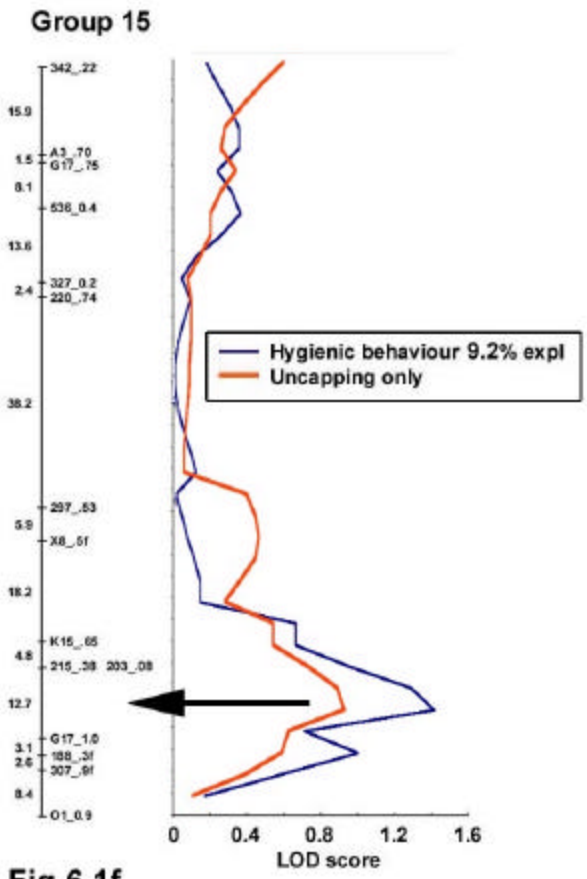


Fig 6.1f

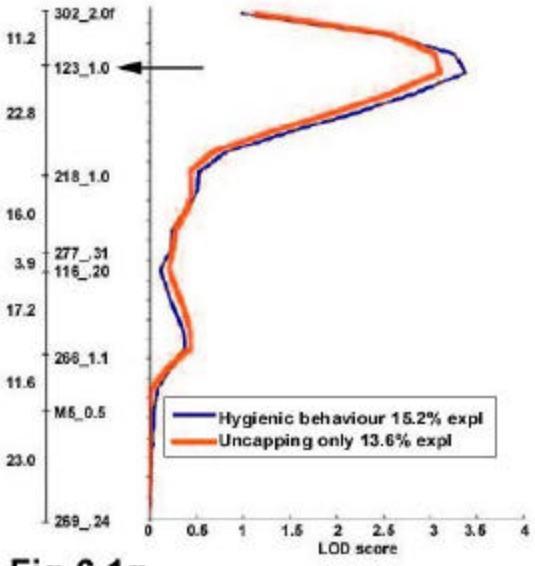


Fig 6.1g

6.3.2 Testing of candidate markers

A field study on 32 colonies derived from various commercial lines was carried out in conjunction with Mr. Linton Briggs from December 15-17, 1999 at Glenrowan, Victoria. This study included colonies from various sources of Australian commercial stocks:

- 2 colonies from the Western Australia Bee Improvement Program.
- 9 colonies derived from hygienic stock imported from the University of Minnesota.
- 2 colonies derived from the La Trobe university hygienic research program (Oldroyd 1996a).
- 8 colonies from lines from the closed population Australian Honey Bee Improvement Program.
- 11 colonies from a population of Caucasian stock established by Mr. Linton Briggs.

Each colony was challenged with freeze-killed brood, using the liquid nitrogen field assay previously described (Chapter 4.5), to determine its behavioural phenotype. Samples of both drone and worker brood were taken from the most hygienic and the most non-hygienic of these colonies for molecular testing of candidate markers identified by QTL analysis and statistical analysis of the molecular data set.

Of these colonies, 4 (12.5%) exhibited distinct hygienic behaviour, 13 (41%) were distinctly non-hygienic and the remaining 15 (46.5%) intermediate between the two (Figure 6.2). This is in agreement with the findings of Oldroyd

that only approximately 20% of commercial honeybee stock in Australia expresses the hygienic behavioural phenotype (Oldroyd 1996a). It is very surprising that there has been no improvement in the frequency of the hygienic trait since 1995, as industry has made considerable efforts to distribute hygienic stock.

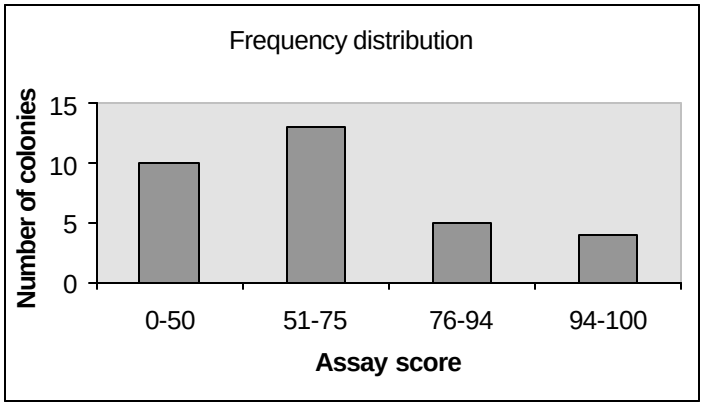


Figure 6.2: Distribution of hygienic behaviour phenotypes in commercial colonies.

Several RAPD marker loci were identified as potential markers associated with QTLs for hygienic behaviour. *t*-tests were performed on behavioural data using genotype of each marker as grouping variable. Loci with significantly different phenotypic scores between the "A" and "H" genotypic classes tended to group together on the linkage map. Identified QTLs tended to be centered around these groups of markers.

Those markers that were both statistically significant and associated with LOD scores above a threshold of 1.5 on the genetic map were considered to be good candidates for evaluation in field tests. DNA was extracted from the brood

samples and from individual bees identified as either highly hygienic or non-hygienic. These samples were then screened with the candidate markers to determine if any marker was reliably associated with field-based hygienic behaviour. Although it is often difficult to transfer RAPD markers between populations or even families, this can be remedied by cloning and sequencing the marker then converting it into a sequence tagged site (STS) by designing longer, more specific primers.

Markers shown to have the strongest statistical association with hygienic behaviour in the backcross colonies were assessed for their potential as markers of hygienic behaviour in field colonies. Twelve worker bees were assessed from each of theses colonies with 5-10 of the most promising markers (Table 6.4).

Table 6.4: *Frequency (%) of workers (n = 11-12) carrying the putative hygienic allele of candidate markers in the most and least hygienic colonies from a commercial apiary.*

	Hygienic colonies				Non-hygienic colonies	
Locus	**Colony 1**	**Colony 2**	**Colony 3**	**Colony 4**	**Colony 1**	**Colony 2**
204	82	83	80	-	100	100
234	100	16	0	0	0	50
X8	73	42	50	91	100	83
536	58	67	58	67	-	16
Q9	100	100	100	100	100	100
301	0	30	50	0	0	58
320	30	30	67	30	100	100
335	50	100	58	100	100	100

Table 6.4 shows that no candidate locus was unambiguously associated with hygienic behaviour. However, there appear to be differences in allele frequencies between the 4 hygienic colonies and the two non-hygienic colonies for loci. To test this association, I compared the number of workers carrying the

marker with the number of workers not carrying the marker. If associations between marker and phenotype are random, then I would expect there to be no significant association in band frequency between the two classes (hygienic and non-hygienic). I tested the null hypothesis that the proportion of bees in the four most hygienic colonies carrying the putative markers was equivalent to the proportion of bees carrying the equivalent marker in the non-hygienic colonies using a two-way contingency table for each marker (d.f. = 2). Significant differences were found for markers 204_0.5 (χ^2 = 4.8 P = 0.027), 234_.94 (χ^2 = 5.5, P = 0.019), X8_.29 (χ^2 = 6.43, P = 0.004); 536_.35 (χ^2 = 8.1, P = 0.004); 320_.96 (χ^2 = 13.8 P < 0.001) and 335_.9f (χ^2 = 7.6, P = 0.006). These loci are strong candidates as useful markers for hygienic behaviour. Note that whether the band is present or absent in the hygienic or non-hygienic class does not indicate the presence or absence of the hygienic allele but is merely indicative of the presence or absence of the linked marker. The other loci tested did not show significant differences between the hygienic and non-hygienic colonies studied.

These candidate loci were also studied in the reciprocal backcross colony. In that colony, the non-hygienic and the hygienic alleles are expected to be at equal frequency (0.5). We studied the frequency of the putative hygienic allele in a sample of six workers observed in the act of removing a dead pupa from a cell in an observation hive. If these loci had no effect on hygienic behaviour we would expect 3 bees to carry the hygienic allele and 3 the non-hygienic allele.

The hygienic allele was present in 6 of 6 bees observed in the act of uncapping or removal of dead pupae in loci 204, 234, 351, 536, 301 and 335. This is a statistically significant result ($\chi^2 = 9.0$, $P < 0.05$).

6.4 Summary

QTL analysis of the experimental cross A data has identified seven putative genetic markers associated with hygienic behaviour in honeybees (Figure 5.1). QTLs associated with linkage groups 2, 4, 6 and 22 were detected for both overall hygienic behaviour and uncapping only data. This is expected, as we were unable to experimentally assay for expression of removal behaviour in the absence of uncapping behaviour, which is necessary for identification of QTLs that influence uncapping behaviour alone. Additional QTLs located on linkage groups 5, 13 and 15 were associated with the total hygienic behaviour data. This suggests that these QTLs influence the removal aspect of hygienic behaviour but not uncapping.

Individually, each QTL is of relatively small effect with each explaining only 9% – 16% of the variance observed. Collectively, the putative QTLs identified here explain 79.4% of the observed variance in the expression of hygienic behaviour. These results indicate that there are many genes of low to moderate effect rather than few genes of large effect involved in this complex behavioural trait. This is typical of inherited quantitative traits that do not exhibit Mendelian phenotypic ratios (see Chapter 5.1).

The number of QTLs detected by linkage with markers, such as the case here, will usually be an underestimate of the actual number of loci (Falconer and Mackay 1996). Two or more QTLs closely linked to each other will often appear as only one locus.

DNA samples from the brood samples taken during testing of commercial stock lines, and from individual bees identified as either highly hygienic or non-hygienic in a reciprocal backcross experiment, were screened with candidate markers associated with putative QTLs to test their diagnostic power. Several of these show great promise as markers of hygienic behaviour, but this needs to be confirmed with a larger sample size.

7. subtractive hybridisation

7.1 Introduction

It seems likely that olfactory perception and processing of stimuli are important in determining whether an individual bee will express the hygienic behaviour phenotype. It has been shown that bees of hygienic genotype and individuals from a non-hygienic genotype show differential discrimination towards healthy and diseased brood odors, suggesting that the two lines have different response thresholds to brood odors (Masterman *et al.* 2000).

Evidence also suggests that biogenic amines are involved in the regulation of age polyethism and task specialization in honeybees. Differences in brain levels of dopamine, serotonin and octopamine have been demonstrated in worker bees of different ages and behavioural castes. Differential levels of octopamine are observed in foragers and nurse bees, independent of age differences (Wagener-Hulme *et al.* 1999). Octopamine has also been shown to enhance behavioural responses of bees to olfactory stimuli (Hildebrandt and Muller 1995; Mercer and Menzel 1982), and preliminary studies involving immunofluorescence staining for octopamine suggest differential levels of the compound are present in the brains of hygienic and non-hygienic bees (Melton *et al.* 1994). It has therefore been postulated that expression of the hygienic

response to dead or diseased brood may be facilitated by octopamine (Melton *et al.* 1994; Spivak and Gilliam 1998a).

Recent advances in molecular biology techniques have produced new and powerful methods of locating genes that are differentially expressed in sub-species or different tissue types. Such techniques would also be useful in identifying differences in gene expression in individual organisms from the same species that differ in a distinct, heritable manner.

A PCR-based cDNA subtractive hybridisation method was employed to differentially screen gene expression messages from individual hygienic bees against non-hygienics (identified and collected from a reciprocal backcross B observation hive, Figure 4.3). Messenger RNA from heads of bees were used, as it seems likely that the genes involved in behavioural processing of olfactory stimuli would be expressed in the brain. Only bees actually seen performing the behaviour (both uncapping and removal tasks) were chosen for the hygienic sample.

This method has the potential to home in directly on genes governing hygienic behaviour by selectively amplifying sequences that are present in one sample but not the other. Fundamentally, mRNA is extracted from the hygienic and non-hygienic workers and reverse transcribed into two separate cDNA pools. These are hybridized and any common sequences removed. Any unhybridised cDNA remaining represents sequences expressed in one sample but not the other.

7.2 Methodology

7.2.1 Preparation of equipment and RNase-free water

Due to it's single stranded structure, RNA is highly vulnerable to degradation and care must be taken to eliminate potential RNase contamination when extracting or working with RNA. All equipment and utensils such as plastic pestles and glassware were soaked in 2M NaOH for 10 minutes then rinsed thoroughly with dH_2O and sterilized by autoclaving for 40 minutes. RNase-free water was prepared by drawing MQ-H_2O into a glass flask that had been rinsed with 2M NaOH then MQ-H_2O. In a fume hood diethylpyrocarbonate (DEPC) was added to a concentration of 0.01% (v/v). The flask was then covered, left to stand overnight, then autoclaved.

7.2.2 Extraction of mRNA

Frozen tissue from the heads of two hygienic workers and two non-hygienic workers from experimental cross B were homogenized in 500 µl TRIzol Reagent (1.0 ml per 50-100mg tissue; Life Technologies, cat.# 15596) and incubated for 5 minutes at room temperature. Only two individuals were used for each population in order to obtain relatively pure samples and minimize any unrelated differences in gene expression. RNA was extracted by addition of 0.1 ml chloroform (Sigma) and vigorous mixing for 15 seconds. Samples were

incubated for 2-3 minutes at room temperature and centrifuged at no more than 12000 x g for 15 minutes at 4°C. The upper aqueous phase containing RNA was transferred to a new microcentrifuge tube. Precipitation of RNA from solution was achieved by addition of 0.25 ml isopropanol (Sigma), incubation at room temperature for 10 minutes and centrifugation at no more than 12000 x g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet washed once with 75% ethanol. Following washing, the pellet was briefly vacuum dried then resuspended in 100 µl RNase-free water by heating for 10 minutes at 55°C-60°C. This was distributed into 10 µl aliquots and stored at -70°C.

7.2.3 cDNA Synthesis

Two cDNA pools, representing expressed genes from the hygienic and non-hygienic samples, were made using the SMART™ PCR cDNA Synthesis Kit as described in the manufacturers directions (Clontech, cat.# K1052-1, Figure 7.1). This kit was used in order to obtain a high-quality pool of full-length cDNA molecules from the relatively small mRNA samples obtained in 7.2.2.

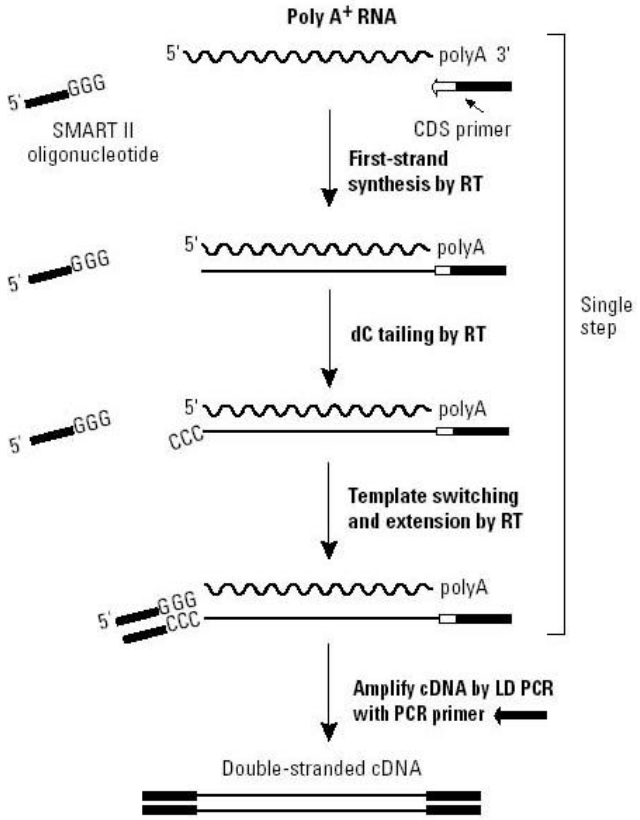


Figure 7.1: cDNA synthesis using the Clontech SMART™ PCR kit (Clontech, cat.# K1052-1). This method selectively amplifies full length mRNA from total extracted RNA. First strand synthesis is primed by an oligo(dT) primer (CDS primer) which exploits the presence of the polyA tail to target mRNA. The reverse transcriptase enzyme used in the kit has a terminal transferase activity, which adds deoxycytidine nucleotides to the 3' end of the newly synthesized cDNA strand. The SMART™ oligonucleotide binds to the 3' terminus of the cDNA strand, providing a primer for PCR amplification of the full length cDNA molecule in the opposite direction. (Reproduced from SMART™ PCR cDNA Synthesis Kit product manual [Clontech, cat.# K1052-1])

7.2.4 cDNA Subtraction

This technique essentially subtracts sequences common to both samples from the cDNA pool leaving just those genes that are differentially expressed in the hygienic background. The PCR-Select cDNA Subtraction Kit was used and all reactions carried out as specified by the manufacturer (Clontech, cat.# K1804-1, Figure 7.2).

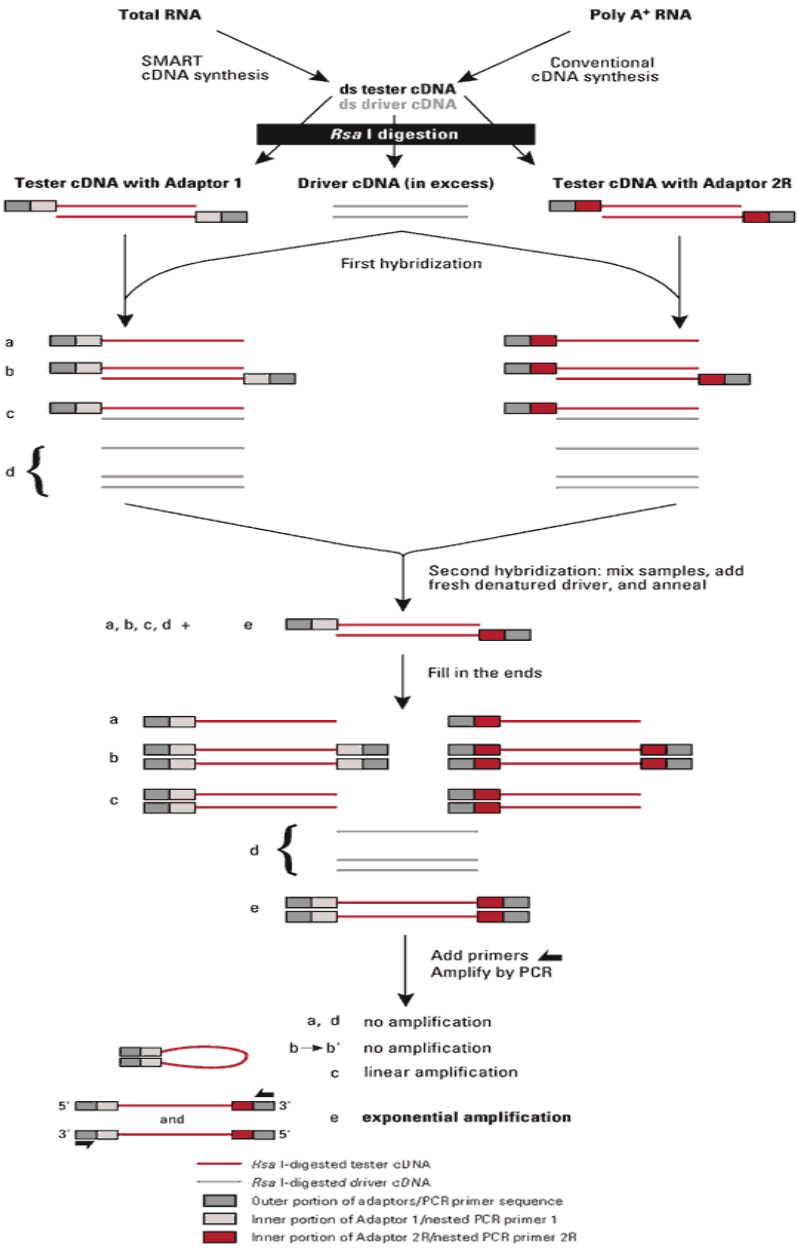


Figure 7.2: cDNA subtraction using the Clontech PCR-Select™ kit (Clontech, cat.# K1804-1). Tester and driver cDNA from the two mRNA samples under comparison (ie. cDNA prepared from hygienic and non-hygienic samples) are separately digested with *Rsa I* endonuclease. Different adaptor primers are ligated to two tester populations. Each sample of tester is heat denatured in the presence of excess driver cDNA and allowed to anneal. A second hybridisation is carried out in which the two primary hybridisation samples are mixed without denaturing, generating templates for PCR amplification from differentially expressed sequences. Supression PCR selectively amplifies differentially expressed sequences (type e) exponentially. (Reproduced from PCR-Select cDNA Subtraction Kit product manual [Clontech, cat.# K1804-1])

cDNA from the hygienic sample was used as the tester and the non-hygienic sample the driver. The first hybridisation results in common sequences between the tester and driver annealing to each other and effectively being subtracted from the pool. The second hybridisation enables sequences unique to the tester (hygienic) sample to rehybridise. These fragments are then selectively and exponentially amplified by PCR. Amplification products were visualized by electrophoresis on a 1% agarose (Progen), 0.5 x TBE gel.

7.2.5 Cloning and sequencing of cDNA subtraction products

Amplification fragments of interest from the cDNA subtraction were extracted from the electrophoresis gel using the CONCERT Rapid Gel Extraction Kit according to the manufacturers directions (Life Technologies, cat#: 11456019). Purified fragments were incubated in a 3:1 ratio with ddT-tailed pBS vector at 16°C for 2 hours in a 36µl reaction mixture containing 15mM Tris-HCl (pH 7.6), 1mM $MgCl_2$, 4x ligation solution A and 1x ligation solution B (Amersham Ligation kit).

200µl of competent DH5α cells were overlaid with the 18µl of the reaction mixture and left on ice for 30 mins. Transformation was achieved by heat shock at 42°C for 45 seconds followed by addition of 800µl of SOC media (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 10mM $MgCl_2$, 2.5mM KCl, 20mM glucose, pH 7.0) and incubation at 37°C for 1.5 hours. Cell suspension was then plated onto LAXI plates (LB containing 1.5% agar, 40µg/ml IPTG,

100µg/ml ampicillin sodium salt, 50µg/ml X-gal) and incubated overnight at 37°C.

Positive colonies were selected, patched onto a new plate and eluted in 75µl H_2O by boiling for 5 minutes. Presence of the DNA insert was confirmed by PCR using 1µl of the eluate as template for the T3 and T7 bacterial primers (which flank the *Eco*RV cloning site of the pBS plasmid vector). Isolation of plasmid DNA from transformed bacterial cells was achieved by the alkaline lysis method described by Sambrook *et al.* (1989).

Purified DNA was sent to the Australian Genome Research Facility (AGRF, the University of Queensland) for sequencing.

7.3 Results and Analysis

Several fragments were purified from the amplified subtracted DNA, cloned and sequenced. The vector sequence flanking the cloning site was first identified and removed from the full sequence obtained. Sequences were analysed using BioNavigator (Entigen Corp, http://www.bionavigator.com/).

Open reading frames (ORFs) were identified and translated into protein sequence using the "Frames" and "flipORFs" programs. These protein sequences were then compared to protein databases using the "blastp" program. DNA sequences were not directly compared to nucleotide databases

as sequences were cDNA and thus represented only exon regions of expressed genes. Matches between our sequences and characterised sequences in the database may not be found if the database sequences included introns or other non-transcribed or translated regions.

A 600bp region of the *Apis mellifera α-glucosidase* gene was identified in cDNA from the hygienic bee sample. This product was identified in 3 out of 8 clones and was not present in any of the clones resulting from cDNA subtraction of the non-hygienic sample. Most other sequenced fragments yielded no matches with the protein database or vector only matches. A couple of sequences matched short regions of the *A. mellifera* ribosomal subunits.

7.4 Summary

α-Glucosidase is a protein expressed in the hypopharyngeal gland of *A.* mellifera (Kubo *et al.* 1996; Ohashi *et al.* 1996). The hypopharyngeal gland is differentiated into two distinct states by the synthesis of different major proteins, depending on age-dependent role change in the worker (Kubo *et al.* 1996). It is well developed in nurse bees and synthesizes bee-milk proteins, then shrinks in foragers and produces enzymes involved in the hydrolysis of sucrose in nectar into glucose and fructose (Ohashi *et al.* 1997; Ohashi *et al.* 1999; Ohashi *et al.* 1996). Expression of *amylase* and *glucose oxidase* genes have been identified in addition to that of *α-glucosidase* in the hypopharyngeal gland of foragers (Ohashi *et al.* 1999).

The *α-glucosidase* gene is differentially expressed based on age and/or role of the bee. It is expressed specifically in the hypopharyngeal gland of foragers and is not expressed in nurse bees (Kubo *et al.* 1996; Ohashi *et al.* 1996). Given this, it is unusual that this study has identified expression of *α-glucosidase* in hygienic bees, as hygienic behaviour is usually exhibited by middle-aged workers who have not yet begun foraging (Arathi *et al.* 2000).

It is difficult to speculate on explanations for this. Limited knowledge is available regarding functional mechanisms of the honeybee hypopharyngeal gland proteins and their role in age polyethism and behavioural differences. Only a small number of clones were analysed in the preliminary subtractive hybridisation carried out in this study, and further protein and expression profile experiments would need to be carried out in order to determine more fully any differences in *α-glucosidase* gene expression between hygienic and non-hygienic bees.

Further, it seems that the function of the hypopharyngeal gland in the worker possesses a certain amount of plasticity depending on colony conditions. For instance, foragers in a dequeened colony revert to synthesizing the brood-milk proteins characteristic of nurse bees (Ohashi *et al.* 2000). The expression of hypopharyngeal proteins characteristic of foragers by nurse bees has not been reported however. Perhaps conditions in an observation hive are artificial enough to effect the normal partitioning of hypopharyngeal activity in the worker population. At the time the reciprocal backcross B experiment was carried out, I

was not planning to try the cDNA subtraction technique and did not stringently control for age of the bees. It is not known if the worker population in the observation hive was representative of the normal age composition present in a field colony.

As such, it is impossible to ascertain (without a substantial amount of further field and lab work) whether the differential expression of the *α-glucosidase* gene observed in this case is due to role-, age- or behaviour-dependent differences. However, these results show that this technique does work in honeybees and can potentially be very useful in the study of the genetic basis of behavioural traits.

This method has the potential to produce markers (to be used in commercial marker-assisted selection in bee breeding) of a greater sensitivity and reliability than those arising from linkage and QTL analyses alone. Similar techniques have recently been applied successfully to the investigation of differential gene expression in developing queens and workers and of the genetic basis of learning behaviours in honeybees (Evans and Wheeler 1999; Kucharski *et al.* 1998; Maleszka 1998).

8. conclusions & implications

This study has provided a better understanding of the genetic basis of honeybee hygienic behaviour. It is clear that this trait is not governed by Mendelian inheritance of two or even three genes, but rather is a complex quantitative trait with an underlying genetic basis of at least seven loci, each of which individually have a relatively small effect.

One goal of this work was to ultimately develop reliable, diagnostic molecular markers that could be used to quickly and efficiently identify strains that expressed the hygienic phenotype without the need for complicated and time consuming breeding experiments and field assays. Such strains could then be used in selective breeding programmes to increase or introduce the expression of hygienic behaviour in commercial apiaries as a natural and effective mechanism for controlling brood diseases such as Chalkbrood. Such use of molecular technology in breeding programmes is also known as marker assisted selection (MAS) and is being utilised widely in both plant and livestock agriculture worldwide (Kumar 1999; Lande and Thompson 1990; Mackill *et al.* 1999; Spelman and Garrick 1997).

The honeybee is an economically important species that is theoretically ideally suited to the use of genetic technology in breeding. Unlike most livestock, the

honeybee has a short generation time with large numbers of progeny. Lines are readily propagated and artificial insemination is a relatively straightforward procedure. Marker assisted selection is most useful when the desired trait is regulated by a single gene, or when a single gene is responsible for a large proportion of the phenotypic variance (Ribaut and Hoisington 1998). Polygenic, quantitative traits are more problematic due to their genetic complexity, such as greater numbers of genes involved and the effect of epistatic interactions between loci. Individual genes influencing quantitative traits generally have small phenotypic effects so marker assisted selection of these traits usually means that several QTLs have to be manipulated at the same time in order to produce any detectable effect.

The analysis has shown that several of the candidate markers investigated in this study have a strong association with the hygienic phenotype. (It is not surprising that the putative hygienic allele was not present in every worker sampled from hygienic colonies. Colonies can express the hygienic phenotype with less than half the workers carrying the hygienic allele). Thus, although these findings need to be confirmed with larger sample sizes, it is probable that this study has uncovered genuine QTLs and markers that are associated with hygienic behaviour.

Can these markers be used successfully to select for hygienic behaviour in honeybee stocks? This study has shown that hygienic behaviour is a polygenic, quantitative trait. As each identified QTL is responsible for only a small proportion of the total phenotypic variance for hygienic behaviour, many

markers would have to be used, which reduces their usefulness. Unfortunately, there appears to be no prospect of getting a molecular marker for each of Rothenbuhler's 'uncapping' and 'removal' genes.

At this point, marker assisted selection of hygienic behaviour in honeybees does not appear to be feasible, especially in the context of identifying hygienic colonies from commercial colonies headed by naturally-mated queens, as was my objective when this project commenced. However, it is possible that within the context of a selection program using artificial insemination and single drone inseminations, some of the candidate loci identified in this study may prove very useful.

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Appendices

Appendix A: Phenotype data used in QTL analysis

Key: **Nuc =** Nucleus colony identifier
%Removed = percentage of cells uncapped and dead larvae removed in 48 hours
%Uncapped = percentage of cells uncapped only in 48 hours
*** =** indicates data missing due to insufficient brood at time of test.

	Replicate 1		Replicate 2		Replicate 3		Average	
Nuc	**%Removed**	**%Uncapped**	**%Removed**	**%Uncapped**	**%Removed**	**%Uncapped**	**Removed**	**Uncapped**
A 224	91.1	98.0	97.3	100.0	65.9	87.8	84.8	95.3
A 211	*	*	80.0	86.8	94.3	94.0	87.2	90.4
A 246	95.5	97.5	99.0	100.0	75.3	82.2	89.9	93.2
A 292	85.9	87.9	100.0	100.0	41.5	42.5	75.8	76.8
A 280	100.0	100.0	81.0	88.2	97.1	100.0	92.7	96.1
A 201	67.3	66.4	47.5	57.6	60.8	68.6	58.5	64.2
A 210	*	*	99.0	99.0	100.0	100.0	99.5	99.5
A 297	*	*	71.4	81.6	100.0	100.0	85.7	90.8
A 247	94.4	96.3	71.6	78.4	67.3	75.0	77.8	83.2
A 213	100.0	100.0	100.0	100.0	69.3	71.6	89.8	90.5
A 236	89.2	89.5	100.0	100.0	68.5	74.7	85.9	88.1
A 206	100.0	100.0	93.6	95.4	100.0	100.0	97.9	98.5
A 298	*	*	100.0	100.0	100.0	100.0	100.0	100.0
A 214	47.8	65.6	38.2	57.9	89.9	98.5	58.6	74.0
A 288	83.5	94.1	67.6	71.3	100.0	100.0	83.7	88.5
A 268	94.1	97.1	85.8	87.7	100.0	100.0	93.3	94.9
A 231	40.0	47.5	55.6	79.0	91.8	95.9	62.5	74.1
A 218	87.9	96.2	100.0	100.0	100.0	100.0	96.0	98.7
A (35)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
A 254	57.9	68.4	41.6	69.0	54.0	76.0	51.2	71.1
A 258	98.8	98.7	73.0	87.3	*	*	85.9	93.0
A 251	*	*	*	*	85.4	92.2	85.4	92.2
A (39)	100.0	100.0	100.0	100.0	*	*	100.0	100.0
A 251	*	*	100.0	100.0	*	*	100.0	100.0
B 238	54.6	81.3	89.3	89.9	*	*	72.0	85.6
B 204	100.0	100.0	99.0	100.0	100.0	100.0	99.7	100.0
B 353	58.1	65.7	68.7	80.5	65.9	72.8	64.2	73.0
B 272	56.6	63.2	40.8	42.3	52.4	53.6	49.9	53.0
B 84	100.0	100.0	97.8	98.9	100.0	100.0	99.3	99.6
B 60	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
B 376	71.8	80.6	83.5	89.0	59.1	67.7	71.5	79.1
B 59	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
B 396	31.8	36.4	37.1	38.1	38.2	40.9	35.7	38.5
B 331	100.0	100.0	89.7	96.2	100.0	100.0	96.6	98.7
B9	*	*	97.6	97.6	93.8	93.8	95.7	95.7
B 391	55.9	48.3	58.2	58.1	76.1	79.3	63.4	61.9
B 326	60.4	69.3	81.3	85.4	51.2	57.5	64.3	70.7
B 358	64.4	72.6	100.0	100.0	89.4	92.3	84.6	88.3
B 20	61.3	65.4	100.0	100.0	33.3	33.6	64.9	66.3
B 327	60.0	75.6	89.6	91.4	78.3	92.3	76.0	86.4

	Replicate 1		Replicate 2		Replicate 3		Average	
Nuc	%Removed	%Uncapped	%Removed	%Uncapped	%Removed	%Uncapped	Removed	Uncapped
B 65	66.3	73.5	100.0	100.0	86.3	88.8	84.2	87.4
B 395	100.0	100.0	100.0	100.0	99.1	99.1	99.7	99.7
B 14	50.0	87.3	45.3	52.9	15.3	20.7	36.9	53.6
C 40	100.0	100.0	100.0	100.0	97.8	98.9	99.3	99.6
C 329	99.1	99.1	96.2	97.1	100.0	100.0	98.4	98.7
C 367	89.1	93.8	100.0	100.0	96.7	100.0	95.3	97.9
C 232	*	*	40.7	50.5	*	*	40.7	50.5
C 263	93.3	95.5	95.5	95.4	*	*	94.4	95.5
C 225	100.0	100.0	100.0	100.0	94.5	97.7	98.2	99.2
C 316	*	*	63.6	69.3	*	*	63.6	69.3
C 337	27.5	38.2	29.9	34.0	12.0	22.7	23.1	31.6
C 310	98.0	100.0	99.1	100.0	70.5	94.7	89.2	98.2
C 67	90.7	96.3	61.7	87.2	77.1	99.0	76.5	94.2
C 235	92.3	98.9	100.0	100.0	92.9	94.9	95.1	97.9
C 11	97.1	96.6	92.3	93.9	*	*	94.7	95.3
C 202	*	*	94.7	94.0	85.6	90.6	90.2	92.3
C 264	61.6	67.0	46.7	47.1	39.0	43.0	49.1	52.4
C 379	100.0	100.0	66.0	70.7	*	*	83.0	85.4
C 300	*	*	100.0	100.0	100.0	100.0	100.0	100.0
C 256	64.8	71.3	98.6	98.6	38.2	43.2	67.2	71.0
C 217	90.6	91.0	71.7	72.7	*	*	81.2	81.9
C 269	100.0	100.0	72.3	78.2	100.0	100.0	90.8	92.7
C 325	70.4	88.8	54.8	58.5	*	*	62.6	73.7

Appendix B: Linkage group scans for hygienic behaviour and uncapping behaviour QTL.

The map position (cM) and associated LOD score for likelihood of the presence of a segregating QTL are given for each marker (or at intervals of 5 cM) in each linkage group. Scans were performed using MapQTL v 3.0 software (Van Ooijen and Maliepaard 1996).

linkage group 1

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.27	110_1.7	0	0.52	110_1.7
5	0.1		5	0.59	
6.2	0.07	mA8	6.2	0.59	mA8
10.4	0.09	O1_1.1	10.4	0.23	O1_1.1
10.4	0.08	256_1.9	10.4	0.2	256_1.9
13.8	0.07	358_.6f	13.8	0.25	358_.6f
17.8	0.06	119_.65f	17.8	0	119_.65f
22.8	0.1		22.8	0.01	
27.8	0.16		27.8	0.03	
32.8	0.24		32.8	0.06	
37.8	0.31		37.8	0.1	
42.8	0.37		42.8	0.14	
47.8	0.42		47.8	0.17	
52.8	0.45		52.8	0.2	
54.1	0.45	446_.3f	54.1	0.2	446_.3f
59.1	0.33		59.1	0.16	
62.5	0.23	278_.84	62.5	0.13	278_.84
67.5	0.24		67.5	0.22	
72.5	0.17		72.5	0.21	
75.6	0.12	399_.6f	75.6	0.18	399_.6f
80.6	0.17		80.6	0.22	
85.6	0.23		85.6	0.26	
90.6	0.3		90.6	0.29	
95.6	0.36		95.6	0.33	
96.2	0.37	A3_2.0	96.2	0.33	A3_2.0
101.2	0.53		101.2	0.5	
106.2	0.73		106.2	0.71	
111.2	0.92		111.2	0.93	
116.2	1.08		116.2	1.15	
121.2	1.19		121.2	1.32	
121.7	1.19	395_.51	121.7	1.34	395_.51

linkage group 2

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.29	R11_.67	0	0.59	R11_.67
5	0.2		5	0.45	
10	0.11		10	0.3	
15	0.05		15	0.17	
16.8	0.03	339_3.0	16.8	0.13	339_3.0
21.8	0.05		21.8	0.09	
26.8	0.07		26.8	0.05	
30	0.08	348_.5f	30	0.04	348_.5f
31.5	0.09	342_.5f	31.5	0.05	342_.5f
31.5	0.05	307_.55	31.5	0.02	307_.55
36.5	0.03		36.5	0.01	
41.5	0.01		41.5	0	
46.5	0		46.5	0	
51.5	0		51.5	0	

linkage group 11

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.02	244_.50	0	0.13	244_.50
5	0.01		5	0.14	
10	0		10	0.14	
15	0		15	0.14	
20	0		20	0.14	
22.5	0	277_1.4	22.5	0.14	277_1.4
22.5	0	291_.7f	22.5	0.14	291_.7f
26.4	0.08	379_.5f	26.4	0.04	379_.5f
31.4	0.1		31.4	0.04	
36.4	0.13		36.4	0.04	
41.4	0.16		41.4	0.04	
46.4	0.16		46.4	0.04	
51.4	0.14		51.4	0.03	
56.4	0.12		56.4	0.02	
61.4	0.09		61.4	0.01	
65.5	0.08	281_.3f	65.5	0.01	281_.3f
69.2	0.01	351_.64	69.2	0.24	351_.64
74.2	0.07		74.2	0.09	
79.2	0.17		79.2	0.01	
79.5	0.17	199_.79	79.5	0.01	199_.79
84.5	0.25		84.5	0	
89.5	0.32		89.5	0.01	
94.5	0.38		94.5	0.05	
94.6	0.38	371_.7f	94.6	0.05	371_.7f
99.6	0.62		99.6	0.11	
104.6	0.77		104.6	0.19	
105.2	0.78	372_1.7	105.2	0.2	372_1.7
110.2	0.63		110.2	0.09	
115.2	0.33		115.2	0.02	
116.3	0.26	V8_0.5	116.3	0.01	V8_0.5
116.3	0.26	120_.65	116.3	0.01	120_.65
117.2	0.26	276_1.6	117.2	0.07	276_1.6

linkage group 12

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.51	O2_.53f	0	1.14	O2_.53f
5	0.42		5	1.02	
10	0.34		10	0.84	
15	0.26		15	0.63	
20	0.19		20	0.43	
22	0.17	460_0.7	22	0.37	460_0.7
27	0.16		27	0.27	
32	0.15		32	0.16	
37	0.12		37	0.06	
42	0.1		42	0	
47	0.07		47	0.02	
52	0.04		52	0.08	
54.7	0.03	140_1.2	54.7	0.12	140_1.2

60.5	0.03		60.5	0.06		64.7	0.03		64.7	0.03	
61.9	0.03	G4_2.0	61.9	0.08	G4_2.0	69.7	0.03		69.7	0.01	
66.9	0.03		66.9	0.03		74.7	0.03		74.7	0	
71	0.02	F3_.86	71	0.01	F3_.86	79.7	0.03		79.7	0.01	
76	0		76	0.02		84.7	0.03		84.7	0.02	
76.7	0	B7_.23	76.7	0.02	B7_.23	88	0.03	190_.90	88	0.04	190_.90
81.7	0		81.7	0.02		88.4	0.04	275_.4f	88.4	0.07	275_.4f
86.7	0		86.7	0.02		92.6	0.06	259_1.7	92.6	0.45	259_1.7
91.7	0.01		91.7	0.02		92.6	0.06	342_1.3	92.6	0.45	342_1.3
95.4	0.02	354_.14	95.4	0.02	354_.14	97.6	0.03		97.6	0.37	
100.4	0		100.4	0		102.6	0.02		102.6	0.25	
104.2	0.01	136_.90	104.2	0	136_.90	104.8	0.01	V8_.83	104.8	0.19	V8_.83
109.2	0.03		109.2	0		108.1	0.18	W5_.7f	108.1	0.01	W5_.7f
114.2	0.06		114.2	0		113.1	0.17		113.1	0.01	
117.9	0.09	444_.77	117.9	0	444_.77	118.1	0.15		118.1	0.02	
122.9	0.19		122.9	0.08		123.1	0.12		123.1	0.03	
122.9	0.19	309_.86	122.9	0.08	309_.86	128.1	0.1		128.1	0.04	
122.9	0.19	317_.76	122.9	0.08	317_.76	133.1	0.07		133.1	0.04	
124.2	0.19	104_.45	124.2	0.08	104_.45	138.1	0.05		138.1	0.04	
129.2	0.41		129.2	0.24		139.9	0.04	392_.38	139.9	0.04	392_.38
134.2	0.78		134.2	0.56							
139.2	1.18		139.2	0.92		**linkage group 13**					
144.2	1.5		144.2	1.25		Overall hygienic behaviour			Uncapping behaviour only		
149.2	1.72		149.2	1.51		map	lod	locus	map	lod	locus
154.2	1.87		154.2	1.71		0	0	340_.49	0	0.06	340_.49
158.1	1.95	291_.57	158.1	1.82	291_.57	5	0		5	0.11	
161.3	2.14	204_.50	161.3	1.75	204_.50	10	0.01		10	0.16	
166.3	2.54		166.3	2.13		15	0.02		15	0.22	
171.3	2.8		171.3	2.39		20	0.02		20	0.27	
176.2	2.91	209_1.4	176.2	2.54	209_1.4	25	0.03		25	0.3	
180.5	2.8	336_.71 !	180.5	2.68	336_.71 !	25	0.03	237_.27	25	0.3	237_.27
185.5	1.93		185.5	1.5		30	0.02		30	0.34	
187.3	1.24	385_.2f	187.3	0.67	385_.2f	33	0.02	370_1.3	33	0.35	370_1.3
189.8	1.24	239_.56	189.8	0.67	239_.56	34.9	0.07	379_.1f	34.9	0.01	379_.1f
190.3	1.15	150_.32	190.3	0.8	150_.32	39.9	0.03		39.9	0	
195.3	1.22		195.3	0.85		42.1	0.02	335_.3f	42.1	0	335_.3f
200.3	1.22		200.3	0.86		47.1	0.11		47.1	0	
205.3	1.14		205.3	0.79		52.1	0.31		52.1	0.04	
210.3	0.97		210.3	0.63		57.1	0.56		57.1	0.12	
215.3	0.73		215.3	0.44		62.1	0.78		62.1	0.23	
220.3	0.49		220.3	0.29		67.1	0.92		67.1	0.32	
224.6	0.34	X8_.47	224.6	0.2	X8_.47	72.1	0.96		72.1	0.41	
224.6	0.34	230_.56	224.6	0.2	230_.56	73.6	0.96	450_.54	73.6	0.43	450_.54
224.6	0.34	336_.28	224.6	0.2	336_.28	78.6	0.96		78.6	0.46	
229.6	0.41		229.6	0.2		83.6	0.87		83.6	0.47	
234.6	0.44		234.6	0.18		88.6	0.73		88.6	0.45	
238	0.43	MDH	238	0.16	MDH	93.6	0.58		93.6	0.42	
243	0.45		243	0.16		95.6	0.52	397_0.5	95.6	0.4	397_0.5
248	0.47		248	0.16		100.6	0.55		100.6	0.32	
253	0.46		253	0.15		105.6	0.58		105.6	0.24	
258	0.44		258	0.14		110.6	0.59		110.6	0.15	
263	0.4		263	0.12		115.6	0.59		115.6	0.08	
268	0.35		268	0.11		120.6	0.56		120.6	0.03	
273	0.3		273	0.09		125.6	0.52		125.6	0	
274.9	0.28	253_.78	274.9	0.08	253_.78	130.5	0.47	282_.30	130.5	0	282_.30
279.9	0.38		279.9	0.32		135.5	0.79		135.5	0	
282.3	0.41	188_.77	282.3	0.45	188_.77	140.5	1.2		140.5	0.01	
287.3	0.37		287.3	0.53		145.5	1.6		145.5	0.04	
292.3	0.27		292.3	0.46		150.5	1.92		150.5	0.09	
297.3	0.14		297.3	0.31		155.5	2.14		155.5	0.15	
302.3	0.05		302.3	0.16		160.5	2.27		160.5	0.2	

307.3	0.01		307.3	0.06	
308.8	0	374_.65	308.8	0.05	374_.65
313.8	0		313.8	0	
318.8	0.03		318.8	0.01	
323.8	0.08		323.8	0.08	
328.8	0.15		328.8	0.2	
332.5	0.21	122_.7f	332.5	0.3	122_.7f
337.5	0.16		337.5	0.16	
338.4	0.15	mA14	338.4	0.13	mA14
343.4	0.37		343.4	0.41	
348.4	0.6		348.4	0.62	
353.4	0.76		353.4	0.71	
354.3	0.78	369_.7f	354.3	0.72	369_.7f
355	0.86	332_.6f	355	0.65	332_.6f
358.4	0.53	385_.82	358.4	0.54	385_.82
359.2	0.43	U15_.54	359.2	0.45	U15_.54
360	0.43	103_.49	360	0.45	103_.49
365	0.79		365	0.55	
365.5	0.82	450_.15	365.5	0.56	450_.15
370.5	0.7		370.5	0.46	
371.9	0.67	C9_.55	371.9	0.43	C9_.55
372.7	0.67	E9_.75	372.7	0.43	E9_.75
377	0.34	P13_.21	377	0.29	P13_.21

linkage group 3

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.26	104_.65	0	0.26	104_.65
5	0.47		5	0.52	
5.3	0.48	G17_.57	5.3	0.54	G17_.57
10.3	0.56		10.3	0.41	
15.3	0.61		15.3	0.27	
19.5	0.63	385_.87	19.5	0.17	385_.87
24.5	0.52		24.5	0.32	
29.5	0.4		29.5	0.49	
33.8	0.29	Y15_.69	33.8	0.6	Y15_.69
34.7	0.3	S14_1.0	34.7	0.55	S14_1.0
39.7	0.13		39.7	0.49	
39.9	0.13	251_.21	39.9	0.49	251_.21
44.9	0.07		44.9	0.39	
49.9	0.02		49.9	0.27	
54.9	0		54.9	0.16	
59.9	0.01		59.9	0.08	
64.9	0.04		64.9	0.03	
66.3	0.05	127_.19	66.3	0.02	127_.19
71.2	0.07	U15_.46	71.2	0.02	U15_.46
76.2	0.01		76.2	0	
81.2	0.02		81.2	0.09	
86.2	0.13		86.2	0.24	
89.5	0.23	342_.79	89.5	0.35	342_.79
94.5	0.38		94.5	0.34	
99.5	0.56		99.5	0.32	
104.5	0.73		104.5	0.29	
106.7	0.79	V15_.54	106.7	0.28	V15_.54
108.3	0.71	E9_0.9	108.3	0.19	E9_0.9
113.3	0.56		113.3	0.16	
118.3	0.38		118.3	0.11	
123.3	0.2		123.3	0.05	
128.3	0.06		128.3	0.01	
130.9	0.02	L18_.33	130.9	0	L18_.33
135.9	0.04		135.9	0.01	
160.5	2.27	395_0.7	160.5	0.2	395_0.7
165.5	2.04		165.5	0.13	
167.4	1.94	258_1.6	167.4	0.11	258_1.6
172.4	1.71		172.4	0.16	
175.9	1.42	374_.53	175.9	0.19	374_.53
180.9	1.32		180.9	0.19	
185.9	1.22		185.9	0.17	
190.9	1.11		190.9	0.15	
194.8	1.02	129_.23	194.8	0.13	129_.23

linkage group 14

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.01	257_1.0	0	0.14	257_1.0
5	0.01		5	0.17	
10	0		10	0.17	
15	0		15	0.14	
20	0		20	0.09	
25	0.01		25	0.05	
25	0.01	stsQ16_.6f	25	0.05	stsQ16_.6f
30	0		30	0.02	
32.5	0.01	303_.24	32.5	0.01	303_.24
37.5	0.01		37.5	0.03	
42.5	0.02		42.5	0.07	
47.5	0.02		47.5	0.12	
52.5	0.03		52.5	0.18	
57.5	0.03		57.5	0.23	
62.5	0.04		62.5	0.27	
65.6	0.04	172_1.8	65.6	0.29	172_1.8
70.6	0.03		70.6	0.22	
75.6	0.02		75.6	0.16	
76.2	0.02	A1_.73	76.2	0.15	A1_.73
81.2	0		81.2	0.16	
86.2	0		86.2	0.17	
90.8	0	172_2.2	90.8	0.16	172_2.2
92.3	0	201_.28	92.3	0.16	201_.28
97.3	0		97.3	0.15	
100.8	0.02	E9_.18	100.8	0.13	E9_.18
105.8	0.07		105.8	0.21	
110.8	0.15		110.8	0.29	
113.9	0.21	380_.77	113.9	0.33	380_.77
118.9	0.27		118.9	0.43	
122.5	0.27	G17_0.9	122.5	0.5	G17_0.9
126	0.22	341_.6f	126	0.43	341_.6f
131	0.17		131	0.33	
136	0.11		136	0.22	
136.2	0.1	256_.60	136.2	0.21	256_.60
138.5	0.04	295_.31	138.5	0.24	295_.31
143.5	0.01		143.5	0.14	
148.5	0.01		148.5	0.04	
153.5	0.11		153.5	0	
158.5	0.22		158.5	0.08	
163.5	0.28		163.5	0.17	
164.5	0.29	135_.8f	164.5	0.19	135_.8f
169.5	0.22		169.5	0.19	
174.5	0.15		174.5	0.19	
179.5	0.08		179.5	0.18	
184.5	0.02		184.5	0.16	
189.5	0		189.5	0.14	
194.5	0.01		194.5	0.1	
199.5	0.03		199.5	0.07	

140.9	0.05		140.9	0.01	
145.9	0.07		145.9	0.02	
150.9	0.09		150.9	0.03	
155.9	0.11		155.9	0.04	
159.1	0.13	373_1.0	159.1	0.04	373_1.0
164.1	0.06		164.1	0.01	
169.1	0		169.1	0.01	
174.1	0.03		174.1	0.07	
179.1	0.08		179.1	0.11	
179.2	0.08	342_.07	179.2	0.12	342_.07

linkage group 4

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	1.25	T18_.56	0	1.52	T18_.56
5	1.56		5	1.99	
8.2	1.43	199_1.6	8.2	1.99	199_1.6
10.1	3.23	397_.9f !	10.1	3.03	397_.9f !
15.1	3.04		15.1	3.08	
20.1	2.7		20.1	3	
25.1	2.24		25.1	2.89	
29	1.85	259_.35	29	2.79	259_.35 !
30.7	1.27	234_.93	30.7	2.3	234_.93
34.9	1.52	V15_.35f	34.9	2.55	V15_.35f
38.2	1.24	mA43	38.2	2.35	mA43
43.2	1.04		43.2	2.21	
47.5	0.81	322_.31	47.5	2	322_.31
52.5	0.57		52.5	1.47	
57.5	0.29		57.5	0.87	
62.5	0.06		62.5	0.3	
67.5	0		67.5	0.01	
72.5	0.08		72.5	0.07	
73	0.1	E9_.56	73	0.09	E9_.56
75.7	0.07	161_.19	75.7	0.03	161_.19
75.7	0.07	235_.26	75.7	0.03	235_.26
80.7	0.01		80.7	0.01	
85.7	0		85.7	0	
87.7	0.01	251_.48	87.7	0	251_.48
87.7	0.01	309_.51	87.7	0	309_.51
91	0.08	234_.64	91	0.11	234_.64
91	0.05	L15_.56	91	0.1	L15_.56
95.4	0.42	375_.3f	95.4	0.56	375_.3f
100.4	0.54		100.4	0.38	
104.2	0.55	336_.73	104.2	0.22	336_.73
109.2	0.54		109.2	0.18	
111.9	0.52	316_1.3	111.9	0.16	316_1.3
116.9	0.39		116.9	0.1	
116.9	0.39	396_.87	116.9	0.1	396_.87
121.9	0.71		121.9	0.22	
122.3	0.73	B7_.60	122.3	0.23	B7_.60
127.3	0.57		127.3	0.13	
132.3	0.39		132.3	0.05	
137.3	0.21		137.3	0	
142.3	0.08		142.3	0.02	
147.3	0.01		147.3	0.1	
152.3	0		152.3	0.21	
155.4	0.02	341_.5f	155.4	0.28	341_.5f
160.4	0.17		160.4	0.73	
164.3	0.38	114_1.1	164.3	1.14	114_1.1
168.7	0.18	444_0.9	168.7	0.38	444_0.9
168.7	0.18	397_.85	168.7	0.38	397_.85
200.9	0.04	F8_.2f	200.9	0.07	F8_.2f
205.9	0.05		205.9	0.51	
210.9	0.46		210.9	1.12	
214.7	0.87	384_1.2	214.7	1.53	384_1.2
219.7	0.59		219.7	0.85	
224.1	0.24	314_1.3	224.1	0.18	314_1.3
229.1	0.06		229.1	0.04	
234.1	0.01		234.1	0.01	
238.4	0.1	320_.73	238.4	0.09	320_.73
243.4	0.25		243.4	0.14	
248.4	0.44		248.4	0.19	
253.4	0.62		253.4	0.21	
253.7	0.63	444_0.4	253.7	0.22	444_0.4

linkage group 15

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.35	342_.22	0	0.61	342_.22
5	0.41		5	0.49	
10	0.45		10	0.38	
15	0.48		15	0.28	
15.9	0.49	A3_.70	15.9	0.26	A3_.70
17.4	0.39	G17_.75	17.4	0.34	G17_.75
22.4	0.49		22.4	0.26	
25.5	0.54	536_0.4	25.5	0.2	536_0.4
30.5	0.39		30.5	0.2	
35.5	0.2		35.5	0.14	
39.1	0.08	327_0.2	39.1	0.07	327_0.2
41.5	0.16	220_.74	41.5	0.09	220_.74
46.5	0.12		46.5	0.09	
51.5	0.08		51.5	0.09	
56.5	0.04		56.5	0.08	
61.5	0.02		61.5	0.08	
66.5	0		66.5	0.07	
71.5	0		71.5	0.06	
76.5	0.01		76.5	0.05	
79.7	0.02	297_.53	79.7	0.05	297_.53
84.7	0.07		84.7	0.4	
85.6	0.11	X8_.5f	85.6	0.45	X8_.5f
90.6	0.17		90.6	0.47	
95.6	0.25		95.6	0.45	
100.6	0.29		100.6	0.37	
103.8	0.29	K15_.65	103.8	0.28	K15_.65
108.6	1.14	215_.38	108.6	0.55	215_.38
109.4	1.14	203_.08	109.4	0.55	203_.08
114.4	1.6		114.4	0.75	
119.4	2.03		119.4	0.91	
121.3	2.17	G17_1.0 !	121.3	0.95	G17_1.0
124.4	1.57	188_.3f	124.4	0.64	188_.3f
127	1.62	307_.9f	127	0.6	307_.9f
132	1.32		132	0.39	
135.4	0.55	O1_0.9	135.4	0.1	O1_0.9

linkage group 16

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.35	V15_1.0	0	0.01	V15_1.0
5	0.12		5	0.02	
9.4	0	T14_1.6	9.4	0.11	T14_1.6
14.4	0		14.4	0.12	
19.4	0		19.4	0.12	

linkage group 5

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.11	215_.54	0	0.03	215_.54
5	0.12		5	0.04	
10	0.11		10	0.05	
15	0.09		15	0.05	
15.6	0.09	230_.50	15.6	0.05	230_.50
20.6	0.04		20.6	0.01	
25.6	0.01		25.6	0	
30.6	0		30.6	0.01	
35.6	0.01		35.6	0.06	
40.6	0.03		40.6	0.12	
41.5	0.04	387_1.9	41.5	0.13	387_1.9
44.6	0.21	306_1.8	44.6	0.27	306_1.8
49.6	0.3		49.6	0.36	
54.6	0.38		54.6	0.43	
56.7	0.41	L18_.74	56.7	0.46	L18_.74
61.7	0.47		61.7	0.49	
66.7	0.53		66.7	0.52	
71.7	0.58		71.7	0.54	
76.7	0.6		76.7	0.53	
81.7	0.59		81.7	0.51	
86.7	0.56		86.7	0.48	
91.7	0.51		91.7	0.43	
94.5	0.48	169_.5f	94.5	0.41	169_.5f
99.2	0.69	105_.4f	99.2	0.81	105_.4f
104.2	0.76		104.2	0.8	
104.2	0.76	536_.16	104.2	0.8	536_.16
109.2	0.77		109.2	0.83	
114.2	0.71		114.2	0.81	
119.2	0.6		119.2	0.73	
122.3	0.52	242_.47	122.3	0.67	242_.47
122.3	0.52	331_2.1	122.3	0.67	331_2.1
122.3	0.52	G4_.8f	122.3	0.67	G4_.8f
127.3	0.62		127.3	0.84	
132.3	0.69		132.3	0.98	
137.3	0.7		137.3	1.1	
140	0.69	181_.16	140	1.15	181_.16
141.5	0.69	303_1.7	141.5	1.15	303_1.7
145.9	0.38	114_.30	145.9	0.78	114_.30
150.9	0.38		150.9	0.72	
151.2	0.37	301_.57	151.2	0.71	301_.57
155.3	0.42	376_.1f	155.3	0.68	376_.1f
160.3	1.14		160.3	1.18	
164.7	1.66	383_.25	164.7	1.39	383_.25
169.7	1.94		169.7	1.4	
169.7	1.94	242_.24	169.7	1.4	242_.24
174.7	2.07		174.7	1.2	
179.7	2		179.7	0.97	
184.7	1.77		184.7	0.72	
189.7	1.45		189.7	0.46	
190.8	1.37	133_1.1	190.8	0.41	133_1.1
194.1	0.81	131_.49	194.1	0.13	131_.49

linkage group 6

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.93	U15_.75f	0	1.75	U15_.75f !
5	1.3		5	1.9	
24.4	0		24.4	0.13	
29.4	0		29.4	0.13	
34.4	0		34.4	0.13	
39.4	0.01		39.4	0.12	
44.4	0.01		44.4	0.12	
47.3	0.01	446_0.5	47.3	0.11	446_0.5
52.3	0		52.3	0.12	
57.3	0		57.3	0.12	
62.3	0		62.3	0.1	
67.3	0.01		67.3	0.08	
72.3	0.03		72.3	0.05	
77.3	0.04		77.3	0.03	
78.3	0.04	361_.16	78.3	0.02	361_.16
83.3	0.01		83.3	0.14	
83.7	0.02	188_.95	83.7	0.15	188_.95
84.1	0.02	C9_.76	84.1	0.15	C9_.76
89.1	0.02		89.1	0.15	
94.1	0.02		94.1	0.14	
99.1	0.02		99.1	0.13	
104.1	0.02		104.1	0.11	
109.1	0.02		109.1	0.1	
114.1	0.02		114.1	0.08	
115.9	0.02	B7_.28	115.9	0.08	B7_.28
115.9	0.02	X8_.25	115.9	0.08	X8_.25
120.9	0		120.9	0.07	
125.9	0		125.9	0.06	
130.9	0.01		130.9	0.04	
135.6	0.03	344_.62	135.6	0.03	344_.62
136.2	0.01	301_.15	136.2	0.07	301_.15
136.2	0.01	M5_.37	136.2	0.07	M5_.37
136.2	0.01	396_.63	136.2	0.07	396_.63
138.7	0	G4_.48	138.7	0.08	G4_.48
139.5	0	444_1.0	139.5	0.08	444_1.0

linkage group 17

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.32	396_.2f	0	0.3	396_.2f
5	0.22		5	0.28	
10	0.12		10	0.24	
15	0.04		15	0.2	
20	0		20	0.15	
25	0.02		25	0.1	
30	0.08		30	0.06	
35	0.17		35	0.03	
38.5	0.23	K15_.84	38.5	0.02	K15_.84
43.5	0.24		43.5	0.05	
48.5	0.24		48.5	0.09	
50.2	0.24	281_.22	50.2	0.1	281_.22
55.2	0.15		55.2	0.09	
58.7	0.09	197_.57	58.7	0.08	197_.57
61.1	0.25	322_.3f	61.1	0.03	322_.3f
66.1	0.23		66.1	0.03	
66.2	0.23	536_.48	66.2	0.03	536_.48
71.2	0.25		71.2	0	
73.5	0.25	H13_2.3	73.5	0	H13_2.3
75.7	0.34	330_0.4	75.7	0.04	330_0.4
80.7	0.19		80.7	0.02	
85.7	0.06		85.7	0	
90.7	0		90.7	0	
95.7	0.05		95.7	0.01	

10	1.55		10	1.85	
15	1.68		15	1.7	
20	1.72		20	1.5	
25	1.67		25	1.26	
30	1.55		30	1.03	
30	1.55	172_.5f !	30	1.03	172_.5f
30.5	1.55	336_.5f	30.5	1.03	336_.5f
30.5	1.09	W6_.8f	30.5	0.65	W6_.8f
32.2	1.48	386_.5f	32.2	0.98	386_.5f
37.2	1.13		37.2	0.65	
41.5	0.73	235_.8f	41.5	0.38	235_.8f
46.5	0.91		46.5	0.66	
48.3	0.98	378_.9f	48.3	0.77	378_.9f
50.1	0.98	Z18_.18	50.1	0.77	Z18_.18
55.1	0.93		55.1	0.7	
60.1	0.75		60.1	0.5	
62	0.65	133_1.3f	62	0.41	133_1.3f
67	0.62		67	0.34	
72	0.55		72	0.24	
77	0.43		77	0.14	
82	0.28		82	0.04	
87	0.14		87	0	
92	0.05		92	0.01	
95.6	0.02	450_.29	95.6	0.03	450_.29
100.6	0		100.6	0.05	
104.3	0	327_.52	104.3	0.08	327_.52
107.6	0	340_1.3	107.6	0.07	340_1.3
110.2	0.1	362_.47	110.2	0.05	362_.47
115.2	0.27		115.2	0.14	
120.2	0.48		120.2	0.27	
120.5	0.49	L7_.4f	120.5	0.28	L7_.4f
123.7	0.39	L16_.57	123.7	0.19	L16_.57
128.7	0.51		128.7	0.25	
133.7	0.57		133.7	0.26	
136.7	0.57	353_.57	136.7	0.25	353_.57
136.7	0.57	L18_.34	136.7	0.25	L18_.34
136.7	0.57	105_0.8	136.7	0.25	105_0.8
137.5	0.57	149_.52	137.5	0.25	149_.52
138.3	0.57	Y14_.46	138.3	0.25	Y14_.46
143.3	0.52		143.3	0.19	
148.3	0.43		148.3	0.12	
153.3	0.33		153.3	0.07	
158.3	0.21		158.3	0.02	
163.3	0.11		163.3	0	
168.3	0.05		168.3	0	
168.8	0.04	R3_.51	168.8	0	R3_.51

linkage group 7

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.02	293_.6f	0	0	293_.6f
0	0.11	C2_.38f	0	0.02	C2_.38f
1.7	0.04	H13_1.2f	1.7	0.02	H13_1.2f
4.3	0.22	373_.13	4.3	0.03	373_.13
4.3	0.07	303_.38	4.3	0.01	303_.38
9.3	0.12		9.3	0.02	
9.7	0.12	292_.95	9.7	0.02	292_.95
14.7	0.1		14.7	0.01	
19.7	0.07		19.7	0	
24.7	0.04		24.7	0	
29.7	0.02		29.7	0.01	
100.7	0.16		100.7	0.03	
103	0.22	373_.22	103	0.04	373_.22
103	0.22	396_1.8	103	0.04	396_1.8

linkage group 18

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.07	269_.22	0	0.1	269_.22
5	0.05		5	0.14	
7.1	0.04	540_0.3	7.1	0.16	540_0.3
12.1	0		12.1	0.09	
16.3	0.05	210_.75	16.3	0.04	210_.75
18	0.01	236_.37	18	0.02	236_.37
18.8	0.02	384_.4f	18.8	0	384_.4f
18.8	0.02	B7_.82	18.8	0	B7_.82
21.5	0.15	T18_.5f	21.5	0.02	T18_.5f
26.5	0.15		26.5	0.05	
31.5	0.14		31.5	0.11	
36.5	0.11		36.5	0.15	
40.1	0.09	A3_1.0	40.1	0.15	A3_1.0
45.1	0		45.1	0.04	
45.6	0.01	263_.19	45.6	0.04	263_.19
50.6	0.01		50.6	0.05	
55.6	0		55.6	0.06	
60.6	0		60.6	0.06	
65.6	0		65.6	0.05	
70.3	0	C2_.45	70.3	0.05	C2_.45
74.2	0.23	351_.88	74.2	0.17	351_.88
79.2	0.14		79.2	0.04	
79.5	0.14	227_.70	79.5	0.04	227_.70
79.5	0.14	199_.64	79.5	0.04	199_.64
84.5	0.33		84.5	0.13	
85	0.34	Z18_1.6	85	0.14	Z18_1.6

linkage group 19

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.03	345_.5f	0	0.05	345_.5f
5	0.1		5	0.01	
10	0.21		10	0	
15	0.37		15	0.04	
19.4	0.52	Z18_.48	19.4	0.11	Z18_.48
24.4	0.38		24.4	0.05	
29.4	0.2		29.4	0.01	
34.2	0.07	388_.3f	34.2	0	388_.3f
37.4	0.13	345_.43	37.4	0	345_.43
38.2	0.08	326_.82	38.2	0.01	326_.82
38.9	0.08	361_.25	38.9	0.05	361_.25
43.9	0.06		43.9	0.03	
48.9	0.05		48.9	0.02	
51.1	0.04	W5_.6f	51.1	0.01	W5_.6f
56.1	0.02		56.1	0.03	
60.6	0	444_1.2	60.6	0.07	444_1.2
60.6	0	250_.6f	60.6	0.07	250_.6f
63.4	0.01	244_.37	63.4	0.13	244_.37

linkage group 20

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.19	333_0.2	0	0	333_0.2
5	0.3		5	0.04	

30.9	0.02	303_.14	30.9	0.01	303_.14
35.9	0.31		35.9	0	
36.2	0.34	122_.38	36.2	0	122_.38
41.2	0.39		41.2	0.01	
46.2	0.43		46.2	0.03	
51.2	0.44		51.2	0.07	
56.2	0.44		56.2	0.13	
59.4	0.42	338_.1f	59.4	0.16	338_.1f
64.4	0.45		64.4	0.17	
69.4	0.45		69.4	0.17	
74.4	0.4		74.4	0.16	
79.4	0.32		79.4	0.13	
84.4	0.22		84.4	0.08	
89.4	0.14		89.4	0.05	
89.9	0.13	342_.4f	89.9	0.05	342_.4f
94.9	0.04		94.9	0	
99.9	0		99.9	0.01	
99.9	0	292_.11	99.9	0.01	292_.11
100.6	0	131_1.0	100.6	0.01	131_1.0
105.6	0.05		105.6	0.1	
108.3	0.13	335_1.1	108.3	0.19	335_1.1
108.3	0.13	386_.26	108.3	0.19	386_.26
113.3	0.19		113.3	0.16	
118.3	0.27		118.3	0.12	
123.3	0.35		123.3	0.07	
128.3	0.42		128.3	0.02	
133.3	0.43		133.3	0	
138.3	0.41		138.3	0	
139.9	0.39	M5_.63	139.9	0	M5_.63
144.9	0.48		144.9	0	
148.9	0.44	295_.62	148.9	0	295_.62
148.9	0.68	324_.60	148.9	0.01	324_.60
148.9	0.44	284_.6f	148.9	0	284_.6f
153.9	0.3		153.9	0	
158.9	0.17		158.9	0.02	
161.6	0.11	X8_.29	161.6	0.03	X8_.29
166.6	0.12		166.6	0.18	
170.1	0.08	M5_.82	170.1	0.25	M5_.82

linkage group 8

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.05	305_.17	0	0	305_.17
0	0.05	237_.6f	0	0	237_.6f
5	0.05		5	0	
10	0.05		10	0	
15	0.04		15	0	
16.7	0.04	108_.4f	16.7	0	108_.4f
19.1	0.04	376_.03	19.1	0.17	376_.03
24.1	0		24.1	0.1	
29.1	0		29.1	0.04	
31.6	0.02	287_.29	31.6	0.02	287_.29
36.6	0.01		36.6	0.01	
41.6	0.01		41.6	0	
41.7	0.01	444_.86	41.7	0	444_.86
46.7	0		46.7	0	
51.7	0		51.7	0.01	
56.7	0.01		56.7	0.01	
61.7	0.02		61.7	0.02	
62	0.02	540_.8f	62	0.02	540_.8f
62.3	0.02	342_.6f	62.3	0.02	342_.6f
10	0.43		10	0.16	
15	0.51		15	0.34	
20	0.54		20	0.52	
25	0.54		25	0.69	
30	0.51		30	0.82	
30.7	0.5	356_.12	30.7	0.84	356_.12
35.7	0.58		35.7	0.97	
40.7	0.62		40.7	0.98	
45.7	0.62		45.7	0.9	
45.9	0.62	161_.51	45.9	0.89	161_.51
50.9	0.38		50.9	0.81	
54	0.2	296_.22	54	0.39	296_.22
54	0.18	256_.7f	54	0.39	256_.7f
57.4	0.11	292_.14	57.4	0.16	292_.14
59.9	0.11	360_.6f	59.9	0.16	360_.6f
64.9	0.36		64.9	0.47	
67	0.48	304_.29	67	0.6	304_.29
72	0.7		72	1.06	
77	0.68		77	0.85	
79.3	0.6	205_.82	79.3	0.65	205_.82

linkage group 21

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.12	261_.62	0	0.23	261_.62
5	0.05		5	0.16	
6.9	0.03	306_.19	6.9	0.14	306_.19
6.9	0.03	H13_.57	6.9	0.14	H13_.57
11.9	0.05		11.9	0.43	
12.8	0.05	175_.4f	12.8	0.48	175_.4f
17.4	0.08	254_1.0f	17.4	0.61	254_1.0f
22.4	0.03		22.4	0.16	
23.5	0.07	122_.21	23.5	0.06	122_.21
25.6	0.15	446_.4f	25.6	0.08	446_.4f
30.6	0.08		30.6	0.02	
35.6	0.03		35.6	0	
36.6	0.02	354_.5f	36.6	0	354_.5f

linkage group 22

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	1.16	302_2.0f	0	0.95	302_2.0f
5	2.7		5	2.35	
10	3.3		10	2.87	
11.2	3.37	123_1.0 !	11.2	2.95	123_1.0 !
16.2	2.75		16.2	2.47	
21.2	1.99		21.2	1.89	
26.2	1.14		26.2	1.24	
31.2	0.42		31.2	0.66	
34	0.2	218_1.0	34	0.43	218_1.0
39	0.17		39	0.46	
44	0.12		44	0.42	
49	0.07		49	0.32	
50	0.06	277_.31	50	0.3	277_.31
53.9	0.01	116_.20	53.9	0.23	116_.20
58.9	0.02		58.9	0.33	
63.9	0.04		63.9	0.45	
68.9	0.06		68.9	0.54	
71.1	0.07	266_1.1	71.1	0.57	266_1.1
76.1	0		76.1	0.26	
81.1	0.02		81.1	0.04	

67.3	0.02		67.3	0.02	
72.3	0.02		72.3	0.01	
77.3	0.02		77.3	0.01	
82.3	0.03		82.3	0.01	
87.3	0.03		87.3	0	
92.3	0.03		92.3	0	
95.7	0.03	353_.45	95.7	0	353_.45
100.7	0.02		100.7	0	
103	0.01	308_.2f	103	0.01	308_.2f
108	0.03		108	0.01	
113	0.04		113	0.01	
118	0.06		118	0.02	
123	0.07		123	0.02	
128	0.08		128	0.02	
133	0.09		133	0.02	
135.1	0.08	336_.23	135.1	0.02	336_.23
140.1	0.01		140.1	0.02	
145	0.02	274_0.5	145	0.02	274_0.5
150	0.26		150	0.07	
151	0.32	278_.7f	151	0.08	278_.7f
156	0.03		156	0.05	
156.5	0.01	332_.7f	156.5	0.07	332_.7f
161.5	0.06		161.5	0.05	
166.5	0.15		166.5	0.02	
171.5	0.29		171.5	0.01	
176.5	0.44		176.5	0	
181.5	0.59		181.5	0.01	
186.5	0.72		186.5	0.02	
187.9	0.75	stsC9_.35	187.9	0.02	stsC9_.35
192.9	0.21		192.9	0.01	
193.3	0.18	369_1.8	193.3	0.01	369_1.8
198	0.51	331_.85	198	0.17	331_.85
203	0.28		203	0.16	
206.5	0.14	316_.4f	206.5	0.15	316_.4f

linkage group 9

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.05	286_.77	0	0.17	286_.77
0	0.02	398_1.1	0	0.11	398_1.1
2.3	0.11	396_.4f	2.3	0.17	396_.4f
7.3	0.12		7.3	0.12	
12.3	0.12		12.3	0.06	
17.3	0.12		17.3	0.02	
22.3	0.11		22.3	0	
25.1	0.1	108_.61	25.1	0	108_.61
30.1	0.21		30.1	0.02	
31.6	0.23	356_.32	31.6	0.04	356_.32
32.4	0.23	G4_.35	32.4	0.04	G4_.35
32.4	0.23	Q13_0.8f	32.4	0.04	Q13_0.8f
37.4	0.29		37.4	0.02	
42.4	0.32		42.4	0.01	
43.6	0.31	237_1.8	43.6	0	237_1.8
45.1	0.31	E9_.24	45.1	0	E9_.24
50.1	0.04		50.1	0.12	
52.7	0	G4_.5f	52.7	0.18	G4_.5f
57.7	0.01		57.7	0.42	
62.7	0.04		62.7	0.57	
67.7	0.06		67.7	0.61	
68.6	0.07	333_.12	68.6	0.61	333_.12
73.6	0.05		73.6	0.52	
82.7	0.04	M5_0.5	82.7	0.01	M5_0.5
87.7	0.05		87.7	0	
92.7	0.05		92.7	0	
97.7	0.05		97.7	0	
102.7	0.05		102.7	0.01	
105.7	0.05	269_.24	105.7	0.02	269_.24

linkage group 23

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.44	R20_.6f	0	0.19	R20_.6f
5	0.34		5	0.2	
10	0.21		10	0.18	
15	0.09		15	0.14	
19.2	0.03	172_.08	19.2	0.09	172_.08
24.2	0.03		24.2	0.05	
29.2	0.02		29.2	0.02	
34.2	0.01		34.2	0	
35	0.01	341_.15	35	0	b 341_.15
40	0.02		40	0	
45	0.03		45	0	
50	0.04		50	0	
55	0.04		55	0	
55.4	0.05	384_.71	55.4	0	384_.71
59.8	0	351_2.0	59.8	0.05	351_2.0
64.8	0.01		64.8	0	
69.2	0.03	394_.78	69.2	0.01	394_.78
74.2	0.01		74.2	0.01	
76.8	0	296_1.0	76.8	0	296_1.0

linkage group 24

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.12	184_.23	0	0.11	184_.23
5	0.16		5	0.05	
10	0.16		10	0	
15	0.1		15	0.01	
15.2	0.1	375_.61	15.2	0.01	375_.61
20.2	0.03		20.2	0.05	
25.2	0		25.2	0.12	
30.2	0.03		30.2	0.21	
35.2	0.1		35.2	0.29	
40.2	0.19		40.2	0.34	
41.1	0.2	Q16_.19	41.1	0.35	Q16_.19
46.1	0.15		46.1	0.34	
51.1	0.1		51.1	0.33	
56.1	0.05		56.1	0.3	
61.1	0.02		61.1	0.27	
66.1	0		66.1	0.23	
68.8	0	201_.58	68.8	0.21	201_.58
73.3	0	250_.48	73.3	0.16	250_.48

linkage group 25

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.38	T14_.53	0	0	T14_.53
5	0.41		5	0.03	
10	0.42		10	0.08	
15	0.4		15	0.16	
20	0.35		20	0.27	
25	0.27		25	0.37	

78.6	0.03		78.6	0.4	
83.6	0.02		83.6	0.26	
88.6	0		88.6	0.12	
93.6	0		93.6	0.03	
97.1	0	W5_.34	97.1	0.01	W5_.34
99.9	0.45	R3_.36	99.9	0.24	R3_.36
104.9	0.24		104.9	0.04	
106.8	0.14	375_.2f	106.8	0	375_.2f
111.8	0.15		111.8	0.01	
116.8	0.17		116.8	0.02	
121.8	0.18		121.8	0.03	
122.6	0.18	G4_1.4	122.6	0.03	G4_1.4

linkage group 10

Overall hygienic behaviour			Uncapping behaviour only		
map	lod	locus	map	lod	locus
0	0.03	230_.47	0	0.04	230_.47
0.9	0	A3_.26	0.9	0.05	A3_.26
3	0.03	303_.12	3	0.08	303_.12
8	0.04		8	0.08	
13	0.04		13	0.07	
18	0.05		18	0.07	
23	0.06		23	0.07	
28	0.07		28	0.06	
33	0.08		33	0.06	
38	0.09		38	0.05	
43	0.09		43	0.04	
44.1	0.09	393_.14	44.1	0.04	393_.14
49.1	0.07		49.1	0.15	
54.1	0.03		54.1	0.22	
55.6	0.02	210_1.4	55.6	0.23	210_1.4
60.6	0.03		60.6	0.22	
65.6	0.03		65.6	0.16	
69.2	0.03	353_.7f	69.2	0.09	353_.7f
74.2	0.01		74.2	0.15	
75	0.01	111_.33	75	0.16	111_.33
76.6	0.01	120_1.8	76.6	0.16	120_1.8
76.6	0.01	302_1.0	76.6	0.16	302_1.0
76.6	0.01	324_.18	76.6	0.16	324_.18
78.3	0.02	254_.16	78.3	0.13	254_.16
80.8	0.08	280_.54	80.8	0	280_.54
80.8	0.1	289_.54	80.8	0.02	289_.54
85.8	0.09		85.8	0.06	
90.8	0.05		90.8	0.1	
95.8	0.02		95.8	0.14	
97.7	0.01	309_1.3	97.7	0.16	309_1.3
98.5	0	361_.8f	98.5	0.05	361_.8f
30	0.2		30	0.46	
30.6	0.19	301_.61	30.6	0.47	301_.61
30.9	0.19	300_1.3	30.9	0.31	300_1.3
35.9	0.12		35.9	0.25	
40.9	0.05		40.9	0.15	
45.9	0		45.9	0.06	
47.2	0	304_.3f	47.2	0.04	304_.3f
52.2	0		52.2	0.03	
57.2	0		57.2	0.01	
62.2	0		62.2	0	
67.2	0		67.2	0.01	
72.2	0.01		72.2	0.03	
76.1	0.01	W5_.66	76.1	0.04	W5_.66
81.1	0		81.1	0.03	
82	0	338_1.1	82	0.03	338_1.1
87	0.04		87	0.09	
88.2	0.07	F8_.5f	88.2	0.1	F8_.5f