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Breeding hygienic disease resistant bees

A report for the Rural Industries Research and Development Corporation

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Foreword

Chalkbrood is a highly infectious disease of honeybee brood. Since first being identified here in 1993, it has spread rapidly throughout Australia. Losses to honey production have proved significant. Chalkbrood cannot be effectively treated by the chemotherapeutic or comb sterilisation techniques often employed to treat other brood diseases.

The uncapping and removal of dead and diseased brood (hygienic behaviour) is a heritable behavioural trait of some honeybee strains. Hygienic behaviour is the primary mechanism of resistance to chalkbrood and other brood diseases, such as American foulbrood. Hygienic colonies also have some resistance to infestation by the parasitic mite *Varroa jacobsoni*.

Only approximately 20% of commercial honeybee stocks in Australia are hygienic. A reliable and efficient means of identifying hygienic stock for use in selective breeding of lines is desirable. This report presents an accurate determination of the number, location within the genome and relative level of influence of loci that directly influence hygienic behaviour in *Apis mellifera*. This not only provides a deeper genetic understanding of complex social behavioural traits in honeybees, but may also enable the development of molecular methods of identification of hygienic stock, thus having direct commercial application for the Australian honey industry.

This project was funded from industry revenue which is matched by funds provided by the Federal Government.

This report, a new addition to RIRDC's diverse range of over 800 research publications, forms part of our Honeybee R&D program, which aims to improve the productivity and profitability of the Australian beekeeping industry.

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Peter Core
Managing Director
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Abbreviations

AFB	American Foulbrood
cM	Centimorgan (unit of map distance)
CTAB	Hexadecyltrimethylammonium bromide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
MAS	Marker Assisted Selection
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RNA	Ribonucleic Acid
STS	Sequence Tagged Site

Contents

Executive Summary

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. 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*.

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 associated with 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% – 16% 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 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. Unfortunately, none have produced reliably diagnostic DNA profiles. Prospects for the development of marker-assisted selection systems for hygienic behaviour based on molecular techniques are therefore considered unlikely.

1. Introduction

1.1 Chalkbrood

The internal environment of a honeybee colony (characterised by constant warm temperatures, high humidity and extensive contact between individuals) is highly conducive to the spread of parasites and pathogens (Milne 1983). Chalkbrood, a highly infectious disease that affects honeybee brood, is caused by ingestion of the heterothallic fungus *Ascosphaera apis*. The disease is characterised by the presence of mummified larvae (Gilliam et al. 1978; Mehr et al. 1976). While the disease is most apparent in summer, *A. apis* spores can survive throughout the year in honey, in stored pollen and in the gut of adult bees. 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. However, chalkbrood is also stress related and certain predisposing physiological and environmental conditions are required for the development of infection (Gilliam 1978; Heath 1982). Further, it is clear that some stocks are far less affected by *A. apis* than others (Gilliam 1986).

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). Losses to honey production have proved significant, and may be as high as 10-15% (Kleinschmidt 1996), equating to $0.5M - $0.75M 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 (Gilliam 1990), and even if these methods were developed, problems with residues in honey might preclude the use of chemical treatments in the commercial sector.

Resistance to chalkbrood does occur in some colonies, and resistant bees can be selectively bred (Gilliam et al. 1983). Good nest cleaning - or “hygienic” behaviour, in which bees uncap and remove dead and diseased brood - inhibits the survival of the fungus *A. apis* (Gilliam et al. 1983), and is correlated with resistance to chalkbrood (Gilliam et al. 1988; Milne 1983; Palacio et al. 2000).

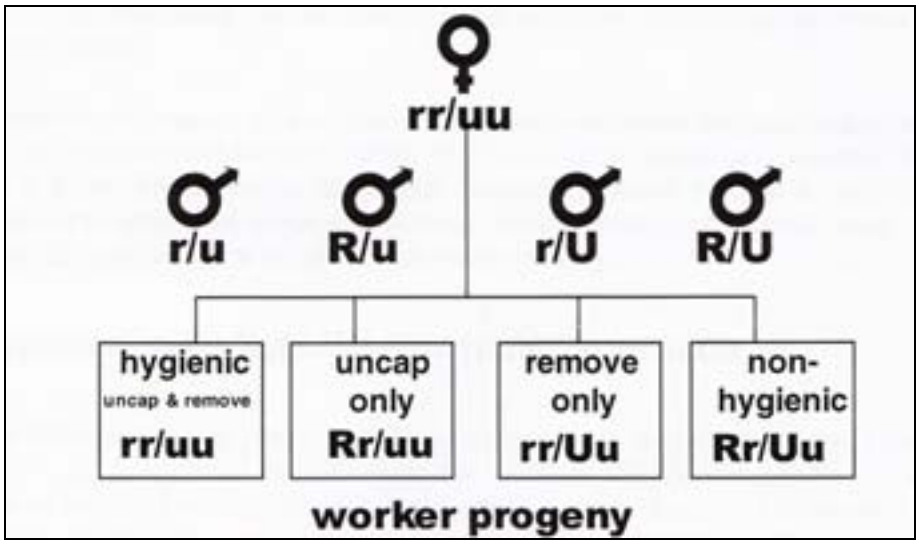
Although other mechanisms have been demonstrated, hygienic behaviour remains the primary mechanism of resistance to chalkbrood and other brood diseases, such as American foulbrood (Gilliam et al. 1988; Spivak and Gilliam 1993; Spivak and Gilliam 1998a; Woodrow and Holst 1942). Hygienic colonies also have some resistance to infestation by the parasitic mite *Varroa jacobsoni* (Boecking and Spivak 1999).

1.2 Hygienic behaviour

Honeybee hygienic behaviour first received attention as a potential mechanism for control of American foulbrood (AFB) (Park et al. 1937). In a series of elegant studies conducted during the 1960s, Walter Rothenbuhler and his colleagues investigated the genetics of AFB resistance and hygienic behaviour (reviewed in Spivak and Gilliam 1998b). These investigations demonstrated that the level of expression of hygienic behaviour was variable between colonies from different lines (Rothenbuhler 1964a), that hygienic behaviour was carried out by workers up to 28 days old (Thompson 1964), and that hygienic bees removed large numbers of dead pupae just as quickly as smaller numbers (Jones and Rothenbuhler 1964). It was also shown that the behaviour was innate to hygienic bees and not learned, and that mixed colonies composed of equal numbers of hygienic and non-hygienic workers still expressed the behavioural phenotype (Trump et al. 1967).

Rothenbuhler's studies did not only investigate the apicultural importance of hygienic behaviour but also the underlying genetic basis of this complex behavioural trait. Based on selective breeding experiments, he 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 1964a; Rothenbuhler 1964b). Rothenbuhler's two-gene model is considered a classic example of the Mendelian inheritance of a complex behavioural polymorphism (Figure 1.1).

Figure 1.1 Rothenbuhler's two-gene model for hygienic behaviour:



Expected phenotypic classes as predicted by Rothenbuhler's two-gene model for hygienic behaviour, based on the experimental backcross carried out in experiment A. 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. When backcrossed to a hygienic queen, the resulting worker progeny would all be of a distinct genotype. Only those that are homozygous recessive at both the removal and uncapping loci would express the hygienic phenotype.

Hygienic behaviour is a complex trait that has a strong environmental as well as genetic component. It has also been shown that factors such as worker age (Thompson 1964) and nectar flow (Momot and Rothenbuhler 1971) influence the expression of hygienic behaviour. A large environmental component for both uncapping and removal behaviour is also supported by estimates of their respective heritabilities, although these estimates have varied widely. Separate studies have produced estimates of h^2 = 0.144 and 0.022 for uncapping and removal respectively (Milne 1985) and h^2 = 0.65 for hygienic behaviour incorporating both uncapping and removal behaviour (Harbo 1995). Heritability estimates vary between 0 and 1. For a particular inherited trait, a high h^2 value (>0.6) indicates a greater influence of genetic factors while a lower value (<0.3) indicates a stronger influence of environmental factors.

Some researchers have challenged the two-locus model favouring a more complex genetic basis for hygienic behaviour. Re-evaluation of Rothenbuhler's data prompted Moritz to suggest a three-locus model, involving a single locus (*u*) for uncapping behaviour but two loci (r_1 and r_2) governing removal. (Moritz 1988). Milne also 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 1985). However, it is still generally agreed that a small number of unlinked genes produce a large effect on hygienic behaviour, that the desirable hygienic alleles are recessive and are inherited in a Mendelian manner.

In 1995, a RIRDC-funded survey of Australian commercial bee stock was undertaken to determine what proportion of genotypes were hygienic according to Spivak's definition (Spivak and Gilliam 1993). 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 will not only provide a deeper understanding of 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.

1.3 Behavioural genetics: the molecular approach

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 level of the gene. This has enabled the genetic study of complex behavioural traits to be opened up to a new level of scrutiny.

Quantitative traits

Characters that are controlled by single genes are readily studied by phenotypic analysis as the genotype can often reliably be predicted from observed phenotypic ratios. Such traits are inherited in a Mendelian manner and changes in the genes usually result in a qualitative change in phenotype. Examples of these include genes for eye colour, genes which confer antibiotic resistance, genes for dwarfism in plants, and of course, Mendel's classic experiments with garden peas.

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 and environmental effects which all influence phenotypic variance of characters (Falconer and Mackay 1996). This is especially true of complex behavioural traits.

Determination of the genetic basis of quantitative traits 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 population level linkage analysis of data generated by molecular tools (such as DNA-level markers) coupled with novel statistical techniques. Together, these techniques permit direct identification of all DNA regions associated with even very complex phenotypes such as hygienic behaviour.

Genetic markers

Mapping of loci that affect or control a quantitative trait requires allelic variation and linkage disequilibrium between alleles at marker loci and at the linked QTL. 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 (allelic forms of enzymes) – 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 (VNTR), 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).

Linkage Maps

A genetic map is an ordered array of genetic markers, usually arranged into 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) and a *Bracon* sp. near *hebetor* (Holloway et al. 2000).
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 genetic analyses. A linkage map based on RAPD markers has been published for *Apis mellifera* (Hunt and Page 1995) and economically important traits such as body size and alarm pheromone levels have been investigated using QTL analysis (Hunt et al. 1999; Hunt et al. 1998). 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. Previous studies have identified honeybee QTLs for traits relating to pollen and nectar foraging and defensive or stinging behaviour (Hunt et al. 1998; Hunt et al. 1995).

QTL Analysis

Quantitative Trait Loci (QTL) represent regions of the genome that have a measurable affect on a trait under investigation. QTL analysis essentially calculates a statistical association between the expression of a trait of interest and a group of linked 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 in mice (Gershenfeld and Paul 1997).

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 always associated with the hygienic genotype are expected to be part of, or very close to, gene(s) that influence hygienic behaviour.

2. Objectives

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 aims 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.

3. Methodology

3.1 Experimental Crosses

Importation

Pure breeding hygienic and non-hygienic lines were established by Dr. Marla Spivak at the University of Minnesota, St. Paul, USA. This was facilitated by a selective breeding program involving the rearing of virgin queens and subsequent instrumental insemination with semen from brother drones. 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.

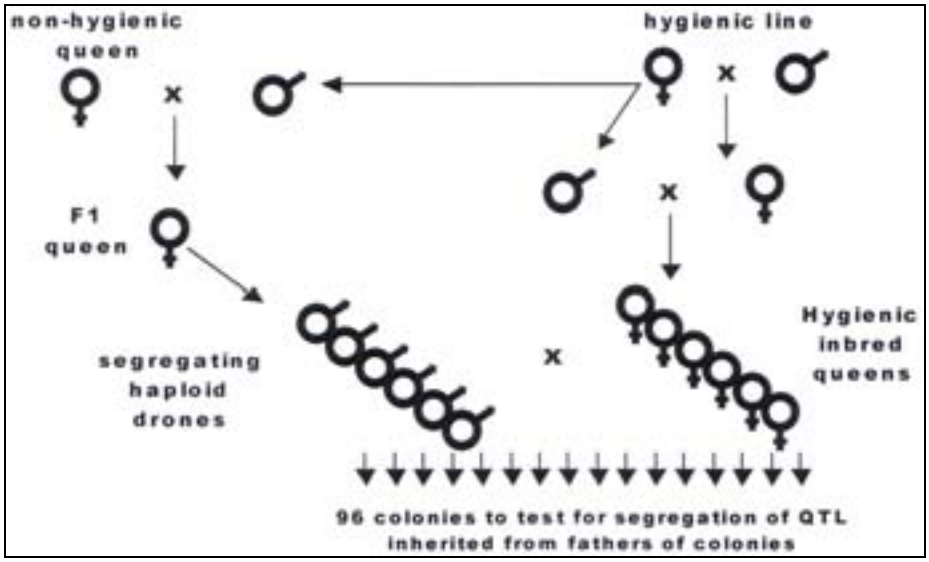
Many hundreds of queens were propagated through the quarantine facility at Wallgrove. Those that were not used in the research program were made available to industry free of charge.

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

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, segregating for hygienic behaviour, were backcrossed to virgin queens from the hygienic line (Experimental cross A, figure 3.1). It was expected that phenotypic segregation in these colonies would produce some colonies that express the hygienic phenotype, and others that do not. Experimental colonies were established from approximately 150 inbred queens from the hygienic line. All matings were performed using artificial insemination (Laidlaw 1977).

Figure 3.1 Experimental cross A:



A total of 96 backcross colonies, segregating for hygienic behaviour based on the genotype of the inseminating drones, were evaluated for hygienic behaviour during Nov-Dec 1997.

Experimental cross B:

A reciprocal backcross (Experimental cross B) between a hybrid 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 confirm the diagnostic use of markers identified as being tightly linked to hygienic alleles. Bees that were never observed performing the behaviour (highly non-hygienic bees) were also collected for molecular analysis.

3.2 Field experiments

Segregating colonies

Many field assays have been developed to test for hygienic behaviour in honeybees. 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 *Bacillus 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, and that using liquid nitrogen is the best way to freeze brood (Spivak and Downey 1998; Spivak and Reuter 1998).

Testing for hygienic behaviour in this study employed an adapted liquid nitrogen field assay (Spivak and Reuter 1998). This involved challenging the colonies with freeze-killed brood and subsequent scoring for phenotypic variation in behavioural patterns (Figure 3.2).

Figure 3.2a Liquid nitrogen field assay for hygienic behaviour

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. A brood comb with inserted cylindrical template just after treatment with liquid nitrogen is shown at right.

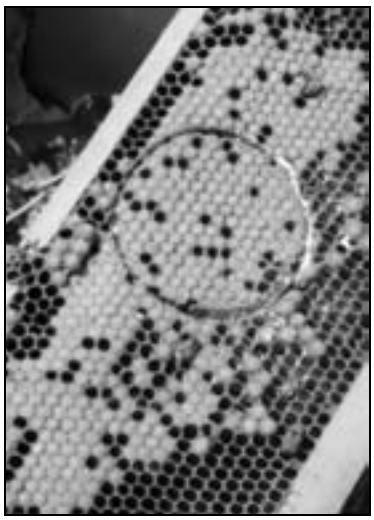


Figure 3.2b Liquid nitrogen field assay for hygienic behaviour

The comb was allowed to thaw before removal of the template. The location of the circular test section marked was marked on the frame and the frame replaced in the hive of origin. A treated frame ready to be replaced in the hive (with the freeze-killed region clearly visible) is shown at left. 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. Hygienic queens have been distributed to industry.

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).

Assessing removal behaviour in isolation from uncapping by manually uncapping cells following liquid nitrogen treatment was attempted. However, this procedure unfortunately proved unuseable. The large number of colonies to be tested simultaneously made it logistically impossible to do this rapidly enough without substantially damaging the comb. Colonies that removed 95% or greater of capped, freeze-killed brood within 48 hours were classified as hygienic.

Commercial stock

A field study on 32 colonies derived from various commercial lines was carried out in conjunction with Mr. Linton Briggs between 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
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 assay described above, to determine its behavioural phenotype. Samples of both drone and worker brood were taken from each of these colonies for testing of candidate markers identified by QTL analysis and statistical analysis of the molecular data set.

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 diagnostic markers. 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.

3.3 Molecular methodology

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

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 1997). 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 and centrifugation at 15000g 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, 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 5000g. 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.

Polymerase Chain Reactions

Generation of RAPD markers by PCR was carried out according to the methods of Williams et al. (Williams et al. 1990). Each reaction contained 0.5µM primer (Operon Technologies / UBC); 100µM each of dATP, dCTP, dGTP & dTTP (); 2.0mM MgCl2 (Biotech); 1x Tth buffer (Biotech); 0.5U Tth plus polymerase (Biotech); and 2.0µl 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 / 1 minute, 35°C / 1 minute, 2 minute ramp to 72°C, 72°C / 2 minutes.

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. 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).

Subtractive hybridisation

Recent advances in molecular biology techniques have produced new and powerful methods of locating genes which 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 which differ in a distinct, heritable manner.

A PCR-based cDNA subtraction 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). This method has the potential to hone directly in on genes governing hygienic behaviour. RNA was extracted from the heads of both hygienic and non-hygienic bees using the TRIzol reagent and a cDNA pool, representing expressed genes, made (Clontech kits). The technique essentially subtracts sequences common to both samples from the cDNA pool leaving just those genes which are differentially expressed in the hygienic background.

4. Detailed Results

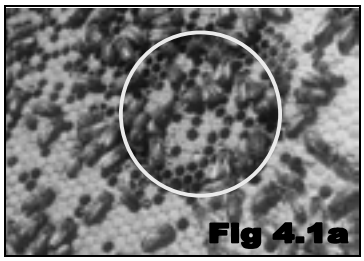
4.1 Field Experiments

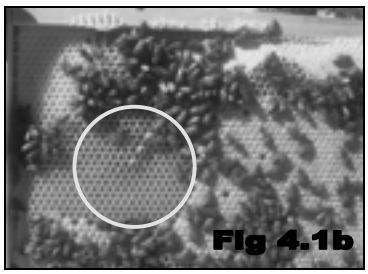
Segregating colonies

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 4.1).

Figure 4.1 Results of liquid nitrogen field assay for hygienic behaviour

The test combs of two colonies 48 hours post treatment are shown to the right. In both cases the treatment region is circled. Figure 4.1a shows a colony considered to be non-hygienic and many capped cells are clearly visible within the treated area. Figure 4.1b 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 4.2). Correlation values were as shown in figure 4.2.

Figure 4.2 Correlations of phenotypic scores between the three tests

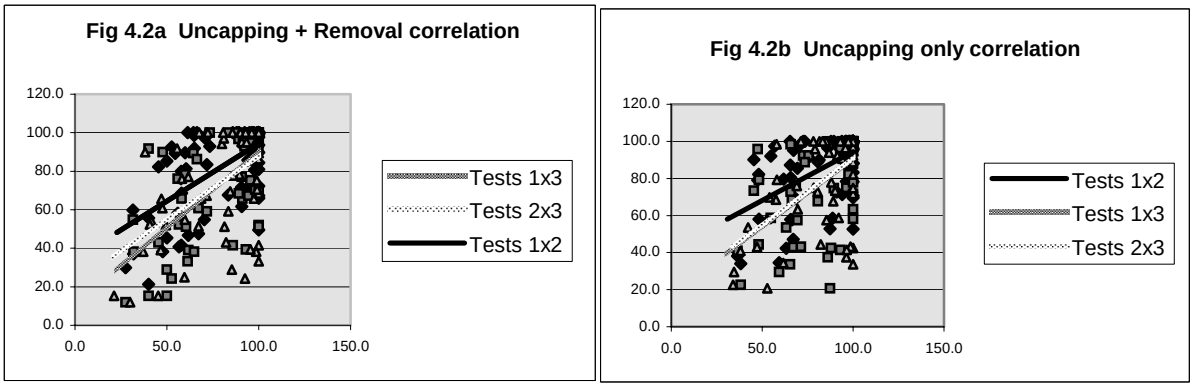
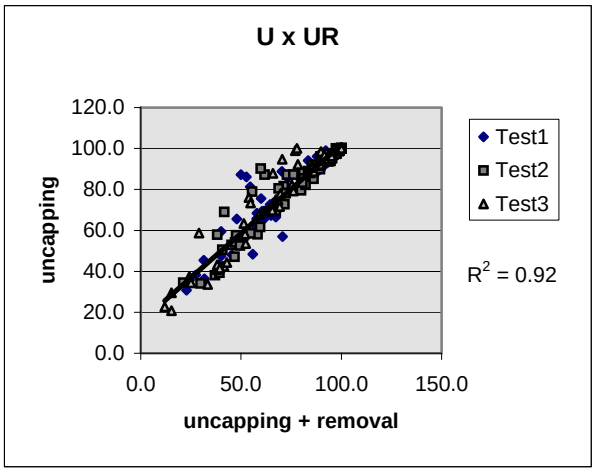


Fig. 4.2a gives the correlation coefficients between each of the three tests for overall hygienic behaviour (r = 0.809 for tests 1 and 2; r = 0.787 for tests 1 and 3; r = 0.704 for tests 2 and 3). Fig 4.2b gives the corresponding values for the uncapping phenotype alone (r = 0.800 for tests 1 and 2; r = 0743 for tests 1 and 3; r = 0.732 for tests 2 and 3).

Each colony was scored in two different ways – first for expression of the uncapping phenotype and second for expression of uncapping and removal phenotypes combined. Colonies that removed 95% or greater of capped, freeze-killed brood within 48 hours were classified as hygienic. Numerical scores for the two phenotypic types were also plotted against each other (Figure 4.3). Although 96 colonies were assayed for expression of hygienic behaviour, only 63 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.

Figure 4.3 Uncapping plotted against overall hygienic behaviour across 3 tests

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.



The observed phenotypic data (fig. 4.4) was analysed by a χ^2 test to determine if they differed statistically from the expected ratios predicted by Rothenbuhler's two-gene (Fig. 1.1) and Moritz's three gene models for hygienic behaviour (Moritz 1988; Rothenbuhler 1964b). The results of this experiment were significantly different from those predicted by both models (for the two-gene model $\chi^2 = 21.38$, $P = 8.77 \times 10^{-5}$, for the three-gene model $\chi^2 = 20.13$, $P = 0.00016$).

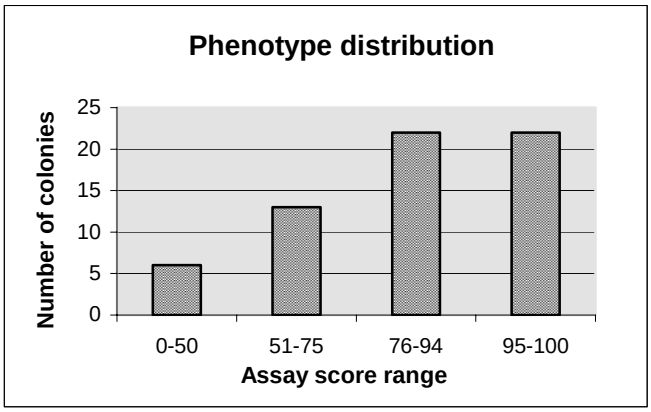


Figure 4.4 Distribution of hygienic behaviour phenotypes in experimental colonies.

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.

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.

Commercial colonies

The 32 colonies derived from various commercial lines kindly supplied by Mr. Linton Briggs were also tested for expression of hygienic behaviour using the liquid nitrogen field assay. 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 4.5). 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.

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.

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.

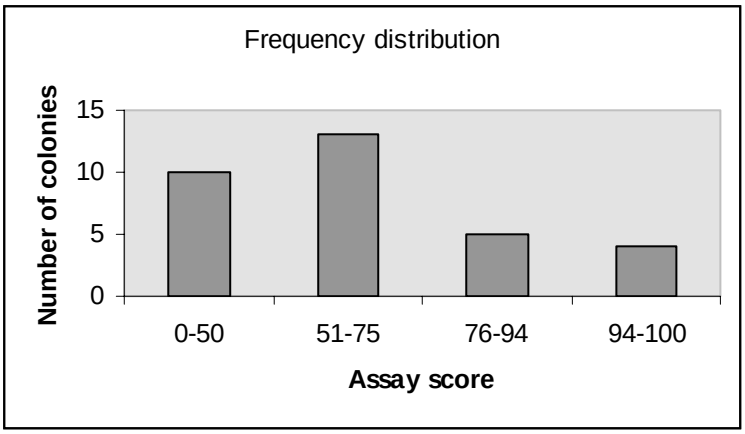


Figure 4.4 Distribution of hygienic behaviour phenotypes in commercial colonies.

Reciprocal backcross colonies

Bees observed actually engaged in hygienic behaviour were studied for the presence or absence of candidate markers.

4.2 Generation of molecular data

Molecular data were generated from DNA samples from the mapping population using RAPD (randomly amplified polymorphic DNA) markers as described in section 3.3. 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. The mapping population was screened with over 400 primers. Two hundred and sixteen of these yielded scorable polymorphic bands. 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 individuals.

Subtractive hybridisation

Several fragments were purified from the amplified subtracted DNA and sequenced. A 600bp region of the *Apis mellifera* α*-glucosidase* gene was identified in cDNA from hygienic bee. This product was not present in the non-hygienic sample. It has previously been shown that α-glucosidase is synthesised in the hypopharyngeal gland and that the gene is differentially expressed based on age and/or role of the bee (Kubo *et. al.* 1996). At the time the reciprocal backcross B experiment was carried out we were not planning to try the cDNA subtraction technique and did not control tightly enough for age of the bees. As such, we are unable to ascertain (without a substantial amount of further field and lab work) whether differential expression of the α*-glucosidase* gene is due to role- or age- dependent differences in this case. We have however shown 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 the genetic basis of learning behaviours in honeybees (Kucharski et al. 1998; Maleszka 1998).

4.3 Data analysis

Linkage mapping

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. The colonies assayed for expression of the hygienic behaviour phenotype were also established from artificial inseminations of sister hygienic queens with the drones of the mapping population (figure 3.1).

Linkage analyses were performed with MAPMAKER/EXP v3.0 software, PC version (Lander et al. 1987; Lincoln et al. 1992). 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). We were unable to unambiguously assigned some markers as either "A" or "H" as we did not have the non-hygienic parent of the American F1 drone mother. 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.

The MAPMAKER/EXP program uses two-point analysis to calculate the recombination fraction between two marker loci and tests for linkage between the two using the LOD score statistic (Lander et al. 1987; Lincoln et al. 1992). The LOD score is the 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 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. 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 then 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. All linkage groups are presented in Appendix 7.1.

QTL analysis

QTL analysis combines the 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 the introduction, such genes cannot be investigated individually using classical Mendelian genetics as their effects are usually small or non-existent in isolation 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 genotype classes.

QTL analysis was performed using MapQTL v3.0 software for the Macintosh (Van Ooijen and Maliepaard 1996). 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). 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 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 the others (tables 4.1 & 4.2). 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. Analyses were performed on both the overall hygienic behaviour phenotypic data and the uncapping only data.

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 4.5, tables 4.1 and 4.2).

Figure 4.5 LOD score profiles for putative QTLs for expression of the hygienic behaviour and 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 (figs. 4.5e and 4.5f 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.

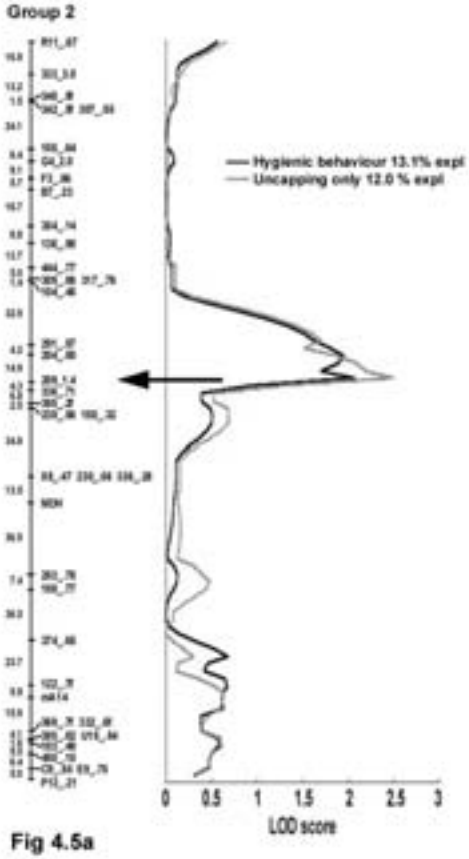


Fig 4.5a

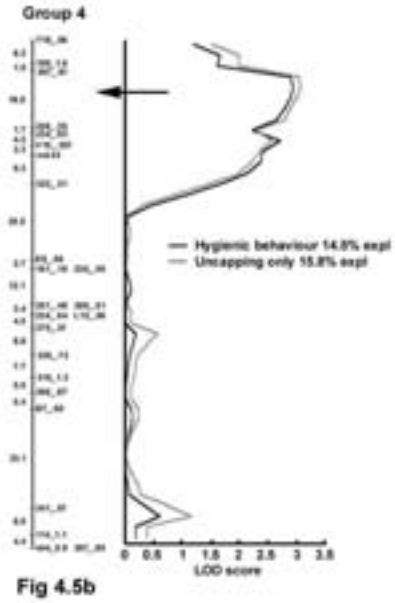


Fig 4.5b

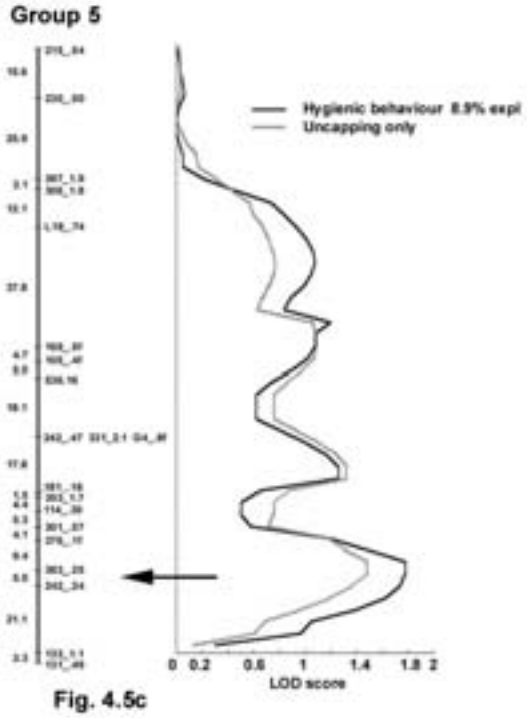


Fig. 4.5c

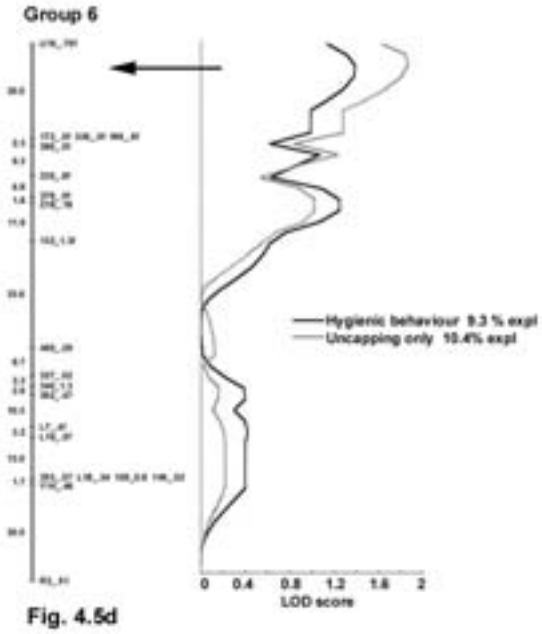


Fig. 4.5d

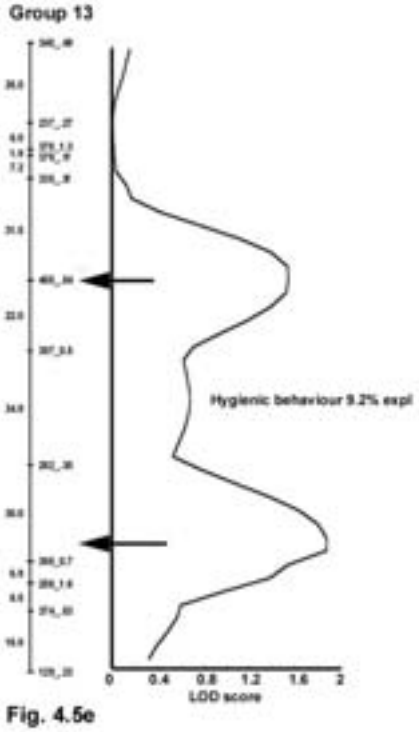


Fig. 4.5e

Fig. 4.5f

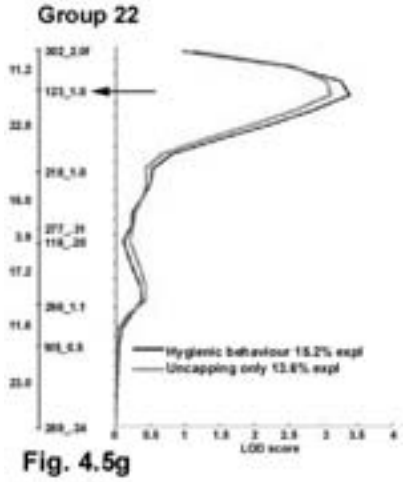


Fig. 4.5g

Table 4.1 Putative QTLs for overall hygienic behaviour in honeybees

Linkage Group	Nearest marker	LOD score	% Variance expl	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 4.2 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

Testing of candidate markers

Several 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 classses 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 diagnostic markers. DNA was extracted from the brood samples and from individual bees identified as either highly hygienic or non-hygienic in an earlier backcross experiment and from samples taken from the commercial colonies of Mr. Linton Briggs. These samples were then screened with the candidate markers.

Table 4.3 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 4.3 shows that no candidate locus was unambiguously associated with hygienic behaviour. However, there highly significant differences in allele frequencies between the 4 hygienic colonies and the two non hygienic colonies for loci 204 ($\chi^2 = 4.8$ $P = 0.027$), 234 ($\chi^2 = 5.5$, $P = 0.019$), X8 ($\chi^2 = 6.43$, $P = 0.004$); 536 ($\chi^2 = 8.1$, $P = 0.004$); 320 ($\chi^2 = 13.8$ $P < 0.001$) and 335 ($\chi^2 = 7.6$, $P = 0.006$). These loci are strong candidates as useful markers for hygienic behaviour. The other loci 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 allele 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 ($\chi^2 = 9.0$, $P < 0.05$).

5. Discussion of results

This study has utilised molecular techniques to elucidate the genetic mechanisms involved in honeybee hygienic behaviour. Experimental backcross colonies were established and assayed for expression of the behavioural phenotype. Triplicate assays were found to be highly correlated confirming that hygienic behaviour is highly heritable. 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 (Moritz 1988; Rothenbuhler 1964b).

A genetic map of the honeybee genome was constructed by full multipoint linkage analysis of 358 segregating marker loci. The 25 linkage groups cover a total map distance of 3406 cM. This is similar in size to the map published by Hunt and Page in 1995. This map, also based on segregating RAPD markers, covered 3110cM on 26 linkage groups, though they estimated the total genome size to be ~3450cM (Hunt and Page 1995). Although many of the same RAPD primers were used in our map, we found it virtually impossible to identify individual linkage groups in common between the two maps. This is not unexpected as marker loci 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. Of the fourteen STS loci previously described (Hunt et al. 1998) that were tested in our mapping population, only 3 were polymorphic in our cross.

QTL analysis of the experimental cross A data has identified seven putative genetic markers associated with hygienic behaviour in honeybees. 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 (figure 4.5). 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 which do not exhibit Mendelian phenotypic ratios (see Introduction, section 1.3).

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 or may not be detected at all.

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.

6. Implications and recommendations

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. MAS 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. Individual genes influencing quantitative traits generally have small phenotypic effects so MAS of these traits usually means that several QTLs have to be manipulated at the same time in order to produce any detectable effect.

Our 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 we wish to confirm our finding with larger sample sizes, we believe we have uncovered genuine QTLs and markers that are associated with hygienic behaviour.

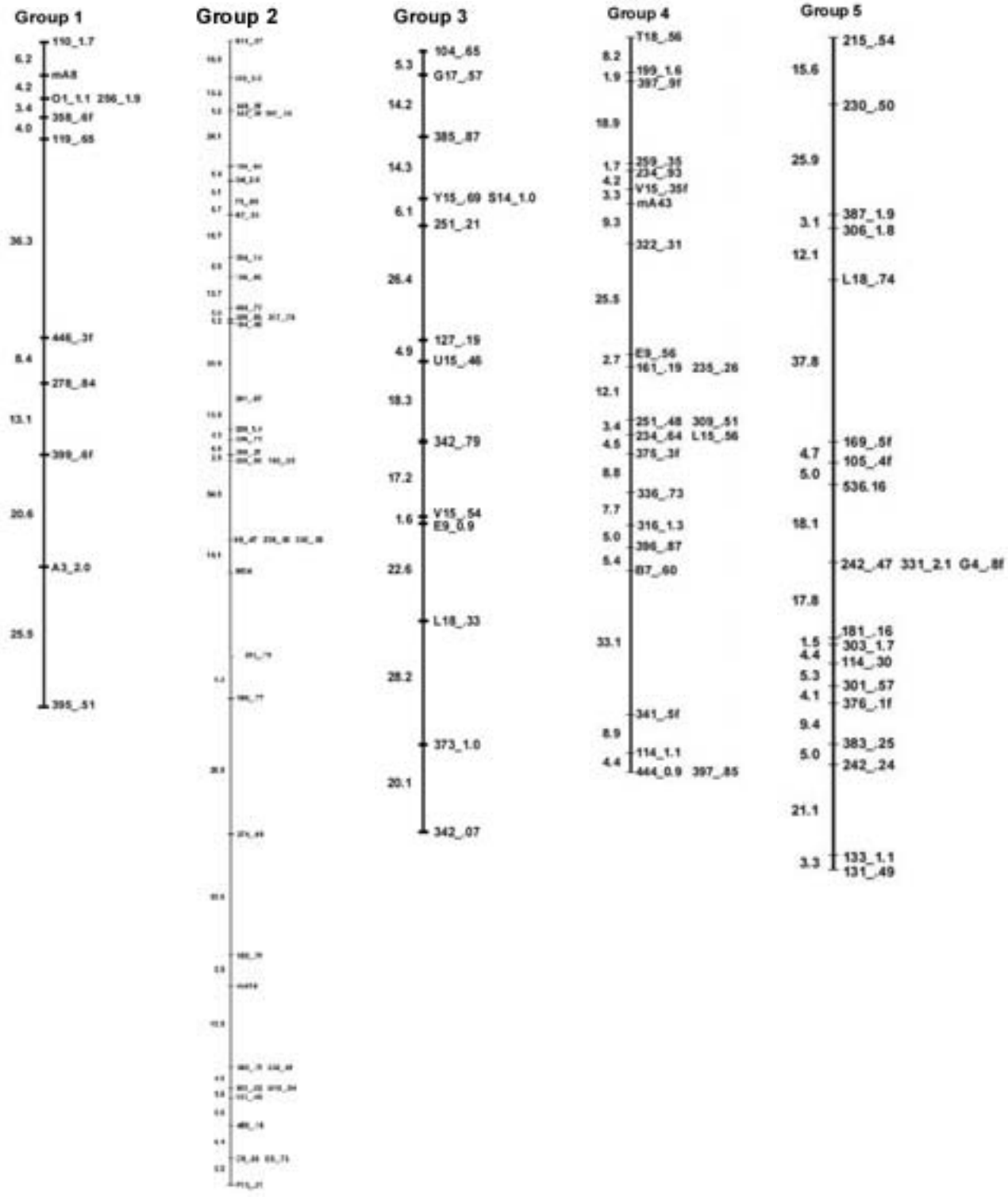
Can these markers be used successfully to select for hygienic behaviour in honeybee stocks? This study has shown that hygienic behaviour is a polygenic, quantitative trait. As each identified QTL is responsible for only a small proportion of the total phenotypic variance for hygienic behaviour, many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

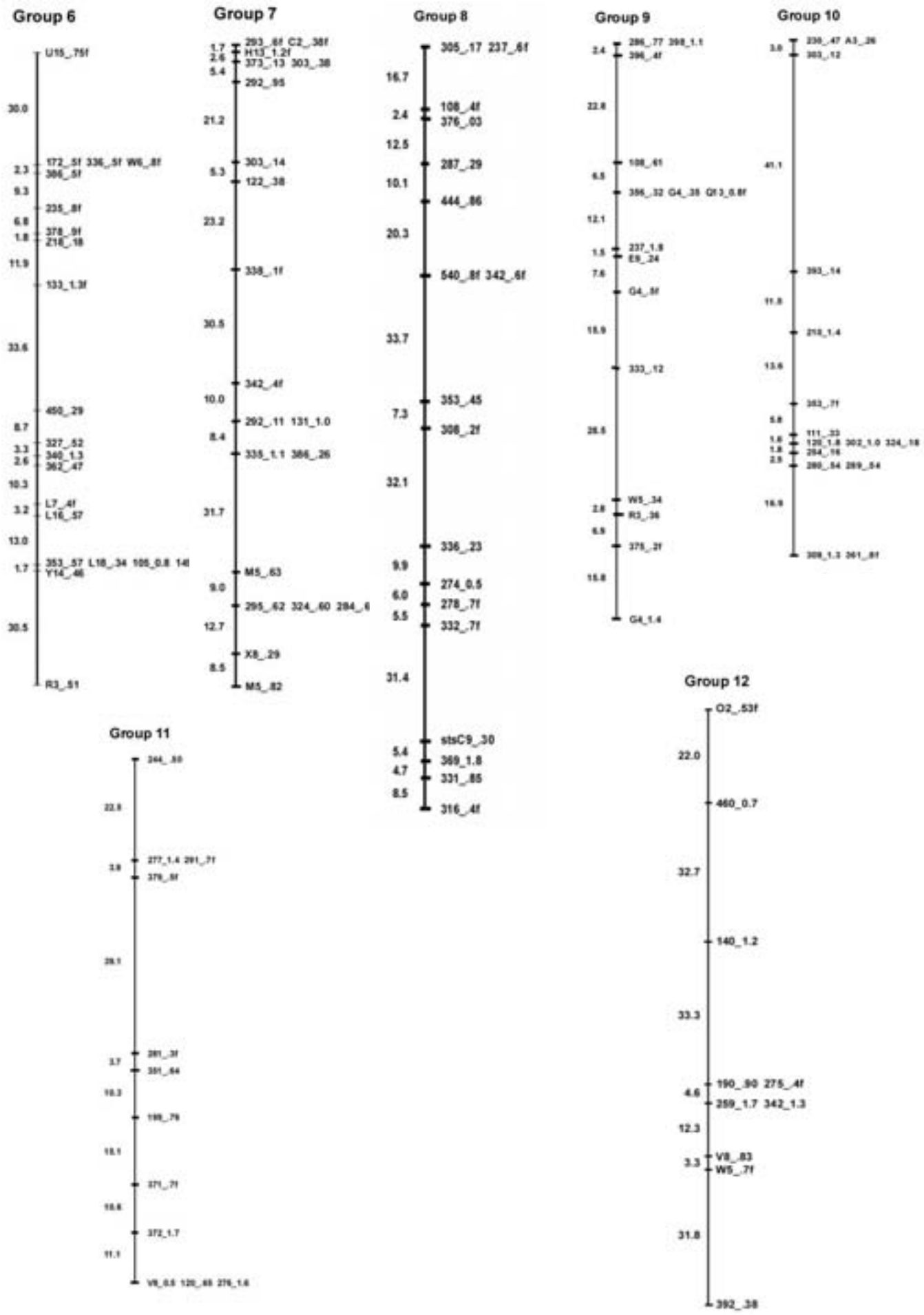
At this point, marker assisted selection of hygienic behaviour in honeybees does not appear to be a feasible option, especially in the context of identifying hygienic colonies from commercial colonies headed by naturally-mated queens, as was our objective when this project commenced. However, we believe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

This laboratory will undertake to screen the candidate loci identified on a large population of bees from hygienic and non-hygienic colonies before December 2001. If any locus appears to be consistently associated with hygienic behaviour, we will advise RIRDC in a supplementary report.

7. Appendices

7.1 Linkage map of the honeybee




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
216_.18
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_.34 105_0.8 14
1.7
Y14_.46
30.5
R3_.51
Group 7
1.7
293_.6f C2_.38f
2.6
H13_1.2f
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_.6
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
386_.32 G4_.38 Gt13_0.8f
12.1
1.5
237_1.8
E9_.24
7.6
G4_.5f
15.9
333_.12
26.5
W5_.34
2.8
R3_.36
6.9
375_.2f
15.8
G4_1.4
Group 10
3.0
336_.47 A3_.26
303_.12
41.1
393_.14
11.8
218_1.4
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353_.7f
5.8
111_.33
1.6
120_1.8 302_1.0 324_.18
1.8
284_.16
2.5
280_.54 289_.54
16.9
309_1.3 361_.8f
Group 11
344_.80
22.8
277_1.4 291_.7f
3.8
376_.5f
29.1
281_.3f
3.7
351_.64
10.3
199_.79
13.1
371_.7f
10.6
372_1.7
11.1
V8_0.5 120_.65 276_1.6
Group 12
O2_.53f
22.0
460_0.7
32.7
140_1.2
33.3
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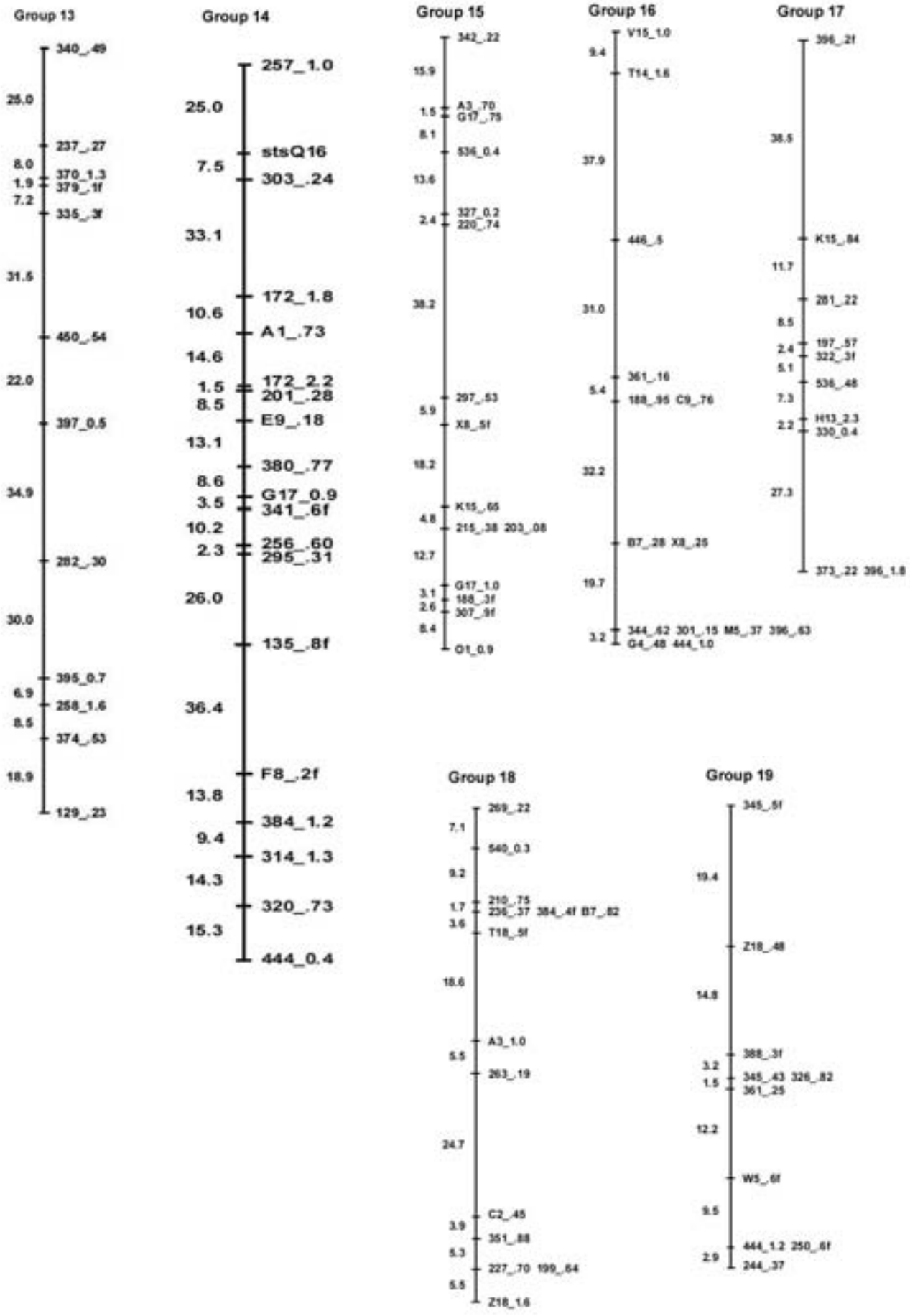
V8_.83
3.3
W5_.7f
31.8
392_.38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22.0
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34.9
282_.30
30.0
395_0.7
6.9
258_1.6
8.5
374_.53
18.9
129_.23
Group 14
257_1.0
25.0
stsQ16
7.5
303_.24
33.1
172_1.8
10.6
A1_.73
14.6
172_2.2
1.5
201_.28
8.5
E9_.18
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
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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
Z18_1.6
Group 19
345_.5f
19.4
Z18_.48
14.8
388_.3f
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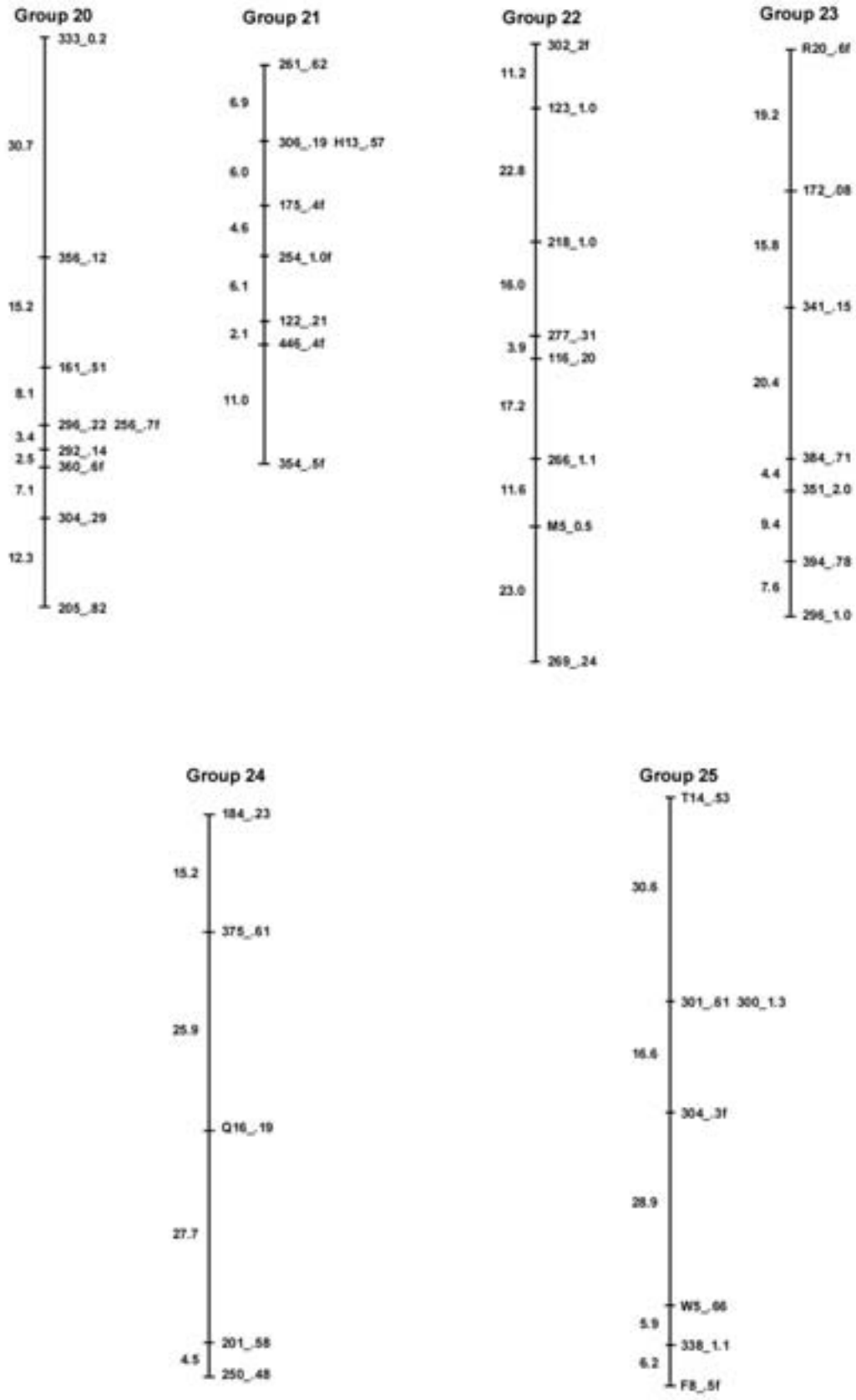
244_.37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7.1
304_.29
12.3
205_.82
Group 21
261_.62
6.9
306_.19 H13_.57
6.0
175_.4f
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302_.2f
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3.9
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11.6
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R20_.6f
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Group 24
184_.23
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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

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