Sociogenetic investigation of the southern harvester termite,

*Microhodotermes viator*,
via genetic and behavioural bioassays.

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Department of Molecular and Cell Biology

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Declaration

This thesis reports the results of original research I conducted under the auspices of the Department of Molecular and Cell Biology, University of Cape Town, between 2009 and 2013. All the assistance I received has been acknowledged. This work has not been submitted for a degree at any other university.

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Natashia Muna
Abstract


This thesis presents the first investigation of the population structure of the southern harvester termite, *Microhodotermes viator* (Family: Hodotermitidae), by assessing the genetic state and behavioural interactions within and between twelve colonies, from four areas across the Western Cape of South Africa. This study also critically debates the relationship between *M. viator* and *heuweltjies* (small Mima-like earth mounds), with regards to their origins and age.

By critically analysing what is known, and debating the merits and shortcomings of various published hypotheses, this thesis concludes that *heuweltjies* are unequivocally attributable to the constructions and foraging activities of *M. viator*. However, the age and longevity of *heuweltjies* remains contentious. Several studies have attempted to ascertain age, using radiometric carbon dating on the basal calcrete layer found below mature *heuweltjies*, but there is disparity between results, primarily due to the challenges associated with dating calcrete. Therefore, an alternative method better equipped to mitigate these challenges, such as U-series isochron dating, is suggested for future research.

There are no published microsatellite loci for Hodotermitid species, therefore an enriched microsatellite library was constructed which identified seven polymorphic loci. Approximately 30 workers from each colony, and 17 workers comprising a background population (BGP), were screened for amplification at the seven loci: Mvit 4, Mvit 14, Mvit 17, Mvit 18, Mvit 21, Mvit 23 and Mvit 25. These loci yielded an average of 5.8 alleles per locus (range: 2 to 14) and an overall mean heterozygosity of 0.51 ± 0.3. At a population level, all loci were in Hardy-Weinberg equilibrium. Pairwise *F*<sub>CT</sub> values varied from 0.1 to 0.63, over a range of intercolonial distances (0.06 - 103 km), which was highly significant between all pairs of colonies (*p* < 0.0001 ± 0.00). Colonies were
grouped into four geographical populations, and pairwise $F_{ST}$ values ranged from 0.2 – 0.37 ($p < 0.0001 \pm 0.00$). These data indicate moderate to strong differentiation between all colonies and geographic sub-groups. The background level of genetic relatedness in the population was $r = 0.42$. While higher levels of average relatedness were found within geographic areas (range: $r = 0.59$ to 0.67), there is no clear relationship between relatedness and intercolonial distance. The overall measure of total inbreeding in all colonies, was not significantly different from zero ($F_{IT} = -0.18; [-0.41; 0.06] 95\% CI$), indicating that there is no excess or deficiency of heterozygotes. The colony inbreeding coefficient, $F_{IC}$, was determined as an average over all loci within each colony, with no colonies exhibiting significant inbreeding. The $F_{CT}, F_{ST}$ and relatedness averages within geographic subgroups cumulatively suggest mild population viscosity, likely attributable to limited alate dispersal distances, leading to genetic structuring at a local scale. In conjunction with the lack of inbreeding, the average level of intracolonial relatedness appears quite high.

Termite populations can exhibit variable family types, reflecting the ongoing processes of colony foundation and maturation. Based on the number of alleles and genotypes exhibited within a colony, $M$. *viator* has percentages of simple (33%) and extended (17%) families, well within the ranges exhibited by other termite species. Simple families had an average relatedness of $r = 0.5$, indicating a single pair of outbred reproductives, while extended families had low numbers of neotentics. Compared to other species, $M$. *viator* has a higher propensity for mixed family colonies (50%), attributable to either pleometrotic association or later adoption of an additional primary reproductive. The development of a mixed family requires a either a lack of intercolonial discrimination, or decreased rejection threshold and thus reduced levels of intercolonial aggression.

During the behavioural aggression trials, all control trials ended in acceptance, while 23% of the experimental trials ended in rejection. Colonies displayed variable levels of aggression, implying that colony state likely mediates defensive response, although this could not be wholly explained by family type. Additionally, variable outcomes were observed between trial replicates, implying that there may be division of labour among
workers, such that some are more inclined than others to engage in aggressive behaviour. Generally, *M. viator* is capable of discriminating between nestmates and non-nestmates, however as a species is only mildly aggressive or has a reduced rejection threshold, possible due to insufficient variation in discriminating cues. The discrimination that was observed could not be wholly attributed to either environmental or genetic cues. As such, it is likely that recognition occurs via a complex suite of cues, which may well include odour cues from symbiotic gut bacteria.
Acknowledgements

This thesis is dedicated to my husband, Marcel, and my daughter, Maelle. Over the last four years you have both made many sacrifices so that I could have the opportunity to do what I love and further my education. Without your understanding and support none of this would have been possible, so I thank you both from the bottom of my heart. I love you more than my feeble words are capable of expressing!

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### Acronyms and Abbreviations

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<tr>
<td>~</td>
<td>Approximately</td>
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<tr>
<td>°C</td>
<td>Degrees Centigrade</td>
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<tr>
<td>AMOVA</td>
<td>Analysis of Molecular Variance</td>
</tr>
<tr>
<td>BP</td>
<td>Before Present</td>
</tr>
<tr>
<td>bp</td>
<td>Base Pairs</td>
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<tr>
<td>BGP</td>
<td>Background Population</td>
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<tr>
<td>CI</td>
<td>Confidence Interval</td>
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<tr>
<td>CHC</td>
<td>Cuticular Hydrocarbon</td>
</tr>
<tr>
<td>DEH</td>
<td>Dorbank Erosion Hypothesis</td>
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<tr>
<td>d.f.</td>
<td>Degrees of freedom</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose Nucleic Acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>$F_{CT}$</td>
<td>Coefficient of inbreeding within a colony, relative to the total population</td>
</tr>
<tr>
<td>$F_{IT}$</td>
<td>Population inbreeding coefficient</td>
</tr>
<tr>
<td>$F_{ST}$</td>
<td>Genetic differentiation</td>
</tr>
<tr>
<td>FAM</td>
<td>Blue fluorescent dye used to visualise allele peaks when genotyping</td>
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<tr>
<td>GLMM</td>
<td>Generalised Linear Mixed Model</td>
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<tr>
<td>$H_e$</td>
<td>Expected Heterozygosity</td>
</tr>
<tr>
<td>$H_o$</td>
<td>Observed Heterozygosity</td>
</tr>
<tr>
<td>HEX</td>
<td>Green fluorescent dye used to visualise allele peaks when genotyping</td>
</tr>
<tr>
<td>IAM</td>
<td>Infinite Allele Model</td>
</tr>
<tr>
<td>LB Amp</td>
<td>Liquid Broth + Ampicillin</td>
</tr>
<tr>
<td>LFH</td>
<td>Limestone-Faulting Hypothesis</td>
</tr>
<tr>
<td>LGM</td>
<td>Last Glacial Maximum</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>Mya</td>
<td>Million years ago</td>
</tr>
<tr>
<td>$N_e$</td>
<td>Effective Population Size</td>
</tr>
<tr>
<td>NED</td>
<td>Yellow fluorescent dye used to visualise allele peaks when genotyping</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>$p$</td>
<td>Significance value</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>$r$</td>
<td>Genetic Relatedness</td>
</tr>
<tr>
<td>s.d.</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SMM</td>
<td>Stepwise Mutation Model</td>
</tr>
<tr>
<td>$T_a$</td>
<td>Annealing Temperature</td>
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<tr>
<td>TCH</td>
<td>Termite-Climate Hypothesis</td>
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<tr>
<td>TMH</td>
<td>Termite Molerat Hypothesis</td>
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<tr>
<td>TPM</td>
<td>Two-phase Model</td>
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<tr>
<td>TTH</td>
<td>Termite-Tree Hypothesis</td>
</tr>
<tr>
<td>UCT</td>
<td>University of Cape Town</td>
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<tr>
<td>UPGMA</td>
<td>Un-weighted pair-group method with arithmetic averaging</td>
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<td>WAH</td>
<td>Wind-Action Hypothesis</td>
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Chapter 1

General Introduction

Understanding the population genetic structure of a species is fundamental to developing a clear picture of its life history traits. Life history traits are secondarily mediated by the ecology of a species. Therefore, eusocial insects are an ideal model to evaluate how social behaviour impacts on population genetic structure, given that there are generally only a few breeding individuals within each colony (Vargo, 2003). Eusociality may be described as a cohabitation arrangement whereby adult members of a colony, who are derived from two or more overlapping generations (iteroparous reproduction), engage in cooperative care of the young and exhibit reproductive differentiation (Wilson & Holldobler, 2005). It is considered to be an evolutionarily advanced form of colonial existence, which is thought to have arisen because of the selective advantage that organized cooperative groups’ exhibit over solitary individuals and pre-social groups (Wilson & Holldobler, 2005). The most notable examples of eusociality are found in the Hymenoptera (ants, bees and wasps), and Isoptera (termites), although eusociality has also been found to occur sporadically within the Hemiptera, Thysanoptera, Coleoptera, Decapoda, and Rodentia (Trivers & Hare, 1976; Schmid-Hempel & Crozier, 1999; Wilson & Holldobler, 2005; Nowak et al., 2010; Herrera, 2013). Termites provide an important comparative system in which to study the evolution of eusociality, as they have evolved these traits independently of the Hymenoptera and in the absence of a haplodiploid sex-determination system (Reilly, 1987; Deheer & Vargo, 2004; Nowak et al., 2010). Although termites are a well-studied group in light of their prominent role in ecology and agriculture (Sobti et al., 2009), they have attracted relatively little attention with regards to genetic studies, when compared to the social Hymenoptera (Vargo, 2003).

Microhodotermes viator and Hodotermes mossambicus are the only two species of the termite family Hodotermitidae family found in South Africa (Coaton & Sheasby, 1974; Inward et al., 2007). M. viator, the southern harvester termite, which is the species of
focus for this thesis, has considerable ecological importance within the Western Cape Province of South Africa. Its importance derives from the fact that *M. viator* has various well-developed associations with other species, occupies a large area of land, has potentially very long-lived colonies and improves soil fertility within the local area around their nest, which develops a distinctive plant community and thus contributes to beta diversity (Midgley & Musil, 1990; Moore & Picker 1991).

There are a number of faunal associations with the nest system of *M. viator* and the associated locally-enriched soil and frass deposits, most of which remain undescribed. However it is known that *M. viator* nests provide the ideal habitat for the inquiline beetle *Hodoxenus sheasbyi*, only found in the Western Cape of South Africa, which exhibits a high degree of integration into the termite society (Ashe & Maus, 1998). *M. viator* also plays an important role as a prey item. The diet of the bat-eared fox, the only true insectivorous member of Canidae, is largely composed of termites, in particular *H. mossambicus* and *M. viator* (Kok & Hewitt, 1990; Nel & Mackie, 1990; Kuntzsch & Nel, 1992; Kok & Nel, 1992). At Tierberg in the Karoo, Dean (1989) found that the ant, *Ophthalmopone hottentota*, preyed exclusively on *M. viator*. Similarly, *M. viator* is considered to be the most important prey item for the lizard, *Cordylus cataphractus* (Shuttleworth et al., 2008). Aardvarks also commonly prey on *M. viator*, and Moore & Picker (1991) found that 53% of *M. viator* termitaria showed evidence of aardvark excavation. While little is known about *M. viator*, in comparison to *H. mossambicus*, Kok & Hewitt (1990) found that *H. mossambicus* was preyed upon by 65 bird and 19 mammal species and, by extension, it is likely that *M. viator* functions as a prey item for many more species than we are currently aware.

*M. viator* nests form localised areas of improved soil fertility compared to matrix soils. The enhanced soil condition is due to improved water infiltration as a result of termite burrowing activities, and significantly higher levels of macro- and micro-nutrients, due to termite foraging activities (Midgley & Musil, 1990, Milton, 1995, Corinna et al., 2005, Picker et al., 2007). These activities can be considered as ecological engineering effects, and alter local community structure, increasing species turnover and species richness (Picker et al., 2007). With improved soil moisture there is an increased rate of
humification, microbial degradation and mineralisation of imported and accumulated organic matter, thereby making essential nutrients more readily available for plant growth. Furthermore, burrowing activities improve aeration of the soil, which enhances the nitrification process (Midgley & Musil, 1990). A foliar analysis study by Midgley & Musil (1990), found that all plants growing on *M. viator* nest mounds had significantly higher nitrogen levels than inter-mound plants and, in particular species, higher levels of phosphorus, boron, magnesium and calcium. As such, mound soils are classified as agriculturally rich and the residual effects of zoogenic mounds on the performance of vine cultivars and wheat have been observed, even after several years of intensive cultivation (Midgley & Musil, 1990).

In conjunction with improved soil fertility, *M. viator* nest mounds support plant communities distinctive from the surrounding vegetation matrix (Midgley & Musil, 1990, Corinna *et al.*, 2005). In particular, seedlings of pioneer species such as *Augea capensis*, *Malephora lutea* and *Psilocaulon utile* (Milton, 1995), as well as deciduous species, are strongly associated with the mound habitat, while evergreens are predominantly found in the inter-mound areas (Midgley & Musil, 1990). Additionally, mound vegetation provides preferential grazing for herbivores, such as rodents and sheep (Dean, 1992; Corinna *et al.*, 2005; Levick *et al.*, 2010).

The strong association between *M. viator* nest density and rainfall suggests that the mounds have developed in response to current rainfall regimes. Picker *et al.* (2007) found that mound density increased along a rainfall gradient within succulent karoo vegetation. This trend remained constant for the entire *M. viator* range, as mound density generally increased from north to south and east to west, with increasing mean annual precipitation, which translates into greater vegetation cover.

Despite their position as a keystone species and likely vulnerability to climate change, very little research has been conducted on *M. viator*. Over the past 40 years, there have been several descriptive studies (Watson, 1973, Coaton & Sheasby, 1974; Dean, 1993) which focussed primarily on observations regarding castes and foraging behaviour. There is also a substantial body of literature debating the origins and nature of
heuweltjies (large Mima-like earth mounds) and their relationship with *M. viator* (Lovegrove & Siegfried, 1986; Cox *et al*., 1987; Lovegrove & Siegfried, 1989; Moore & Picker, 1991; Midgley *et al*., 2002; Picker *et al*., 2007; Potts *et al*., 2009; Cramer *et al*., 2012). Unfortunately, little consensus has been achieved. One of the primary sources of contention being that the original study describing the termitaria of *M. viator* (Coaton & Sheasby, 1974), was restricted to the eastern part of *M. viator’s* range where nests are subterranean, although small mounds (‘mounding-over’) may occur due to hardening of conical frass deposits about the nest. However, in the western part of their range, *M. viator* construction and foraging activities give rise to the formation of large earth mounds (*heuweltjies*), which Coaton & Sheasby (1974) did not described. This incongruence between the original nest description and what is now known, is critically analysed and debated in chapter 2. To date, no research has been done on the behavioural interaction between colonies or the genetic structure of colonies. This study is the first attempt to address these gaps.

The following literature review is intended to provide the reader with a foundation to the general evolution, biology and ecology of termites, in preparation for the more detailed and focussed literature reviews accompanying each chapter.

**The Origins of Termites and the Evolution of Eusociality within Termites**

Termites (Isoptera) fall into the super-order Dictyoptera, along with cockroaches (Blattodea) and praying mantids (Mantodea). Dictyoptera is a monophyletic assemblage on the basis of shared features in the genitalia, proventriculus and wings, as well as a perforation in the tentorium (skeletal part of the head) (Lo *et al*., 2000; Ware *et al*., 2008; Davis *et al*., 2009; Djernæs *et al*., 2012). Modern Dictyoptera date back to the Early Jurassic, approximately (~) 200 million years ago (mya) (Davis *et al*., 2009). The ancestor to all extant Dictyopterans, possibly an omnivorous detritus-feeder, is likely to have had a reproductive system which formed an internal oothecae and a significantly reduced ovipositor - a feature common to cockroaches and termites, but less so in the mantids (Lo *et al*., 2000). Although there is no firm consensus, the first appearance of cockroaches, termites and mantids appears to have been in the Late Jurassic to Early
Cretaceous (160 to 140 mya), however the record for termites and mantids is rather poor (Lo et al., 2000; Davis et al., 2009; Engel et al., 2009). Mantids are believed to have been the first to diverge and, following this split, the cockroaches are likely to have undergone a rapid radiation. Although termites have evolved many social structures similar to that of ants (Kaib et al., 2000), their origins predate ants by ~35 million years, and their evolution of eusociality, in the absence of a haplodiploid genetic system, has remained a matter of some interest (Lo et al., 2000; Davis et al., 2009; Engel et al., 2009). To shed some light on this issue, several phylogenetic studies are worth noting.

To resolve the position of Isoptera within Dictyoptera, Lo et al. (2000) looked at 16 taxa within the Dictyoptera, which were analysed on the basis of the majority of the 18S ribosomal DNA (rDNA) and COII genes, and then subjected, both individually and in combination, to maximum likelihood and maximum parsimony analysis. The analysis revealed strong support for a sister-group relationship between Isoptera and sub-social wood-feeding cockroaches of the genus Cryptocercus. Another study, by Inward et al. (2007), employed two mitochondrial (12S; COII), and three nuclear gene loci (28S; 18S; histone 3), to analyse 107 Dictyopteran and 11 out-group species. Both analyses generated very similar results, which also indicated that Isoptera nest within the cockroaches, with Cryptocercidae as their sister group. At some point, the ancestor to the termites and Cryptocercus species acquired cellulolytic flagellates, facilitating a shift to wood-nesting and feeding (Lo et al., 2000). This ancestor would have exhibited intraspecific degrees of coprophagy and gregariousness, to facilitate the transfer and co-evolution of symbiotic gut bacteria (Inward et al., 2007). The last common ancestor to Isoptera-Cryptocercidae was most likely winged and exhibited sub-social characteristics, such as semelparous reproduction – a single reproductive period per lifetime (Cole, 1954). There was also undoubtedly an extended period of parental care of offspring. This would have been necessitated as larvae are required to remain with their care-givers after each successive moult, to allow time for the transfer of flagellates via proctodeal trophallaxis, (Noirot, 1985). When these two lineages diverged, Cryptocercus remained sub-social, becoming wingless but retaining the hind-gut protozoa. On the other hand, the termite lineage developed characteristics which trended towards monogamy with an accompanying simplification of male genitalia, possibly
due to a decrease in sperm competition, and, as desiccation became less problematic due to the protective nest environment, a reduction and eventual loss of the oothecae, which is still retained in the most basal extant termite family, Mastotermitidae (Lo et al., 2000; Inward et al., 2007; Engel et al., 2009). Additionally, there was a shift to iteroparous reproduction and cooperative brood care. These characteristics are precursors to the evolution of permanent familial groups, in the form of colonies, which facilitated the evolution of a sterile worker and soldier caste to forage, provide alloparental care, nest maintenance and protection (Lo et al., 2000; Inward et al., 2007). Based on this, Cryptocercidae appears to be the link between solitary living Dictyopterans and the eusocial colonial structure of extant termites.

Recently, two more studies (Davis et al., 2009; Djernæs et al., 2012), have confirmed that Dictyoptera consists of two monophyletic orders: Mantodea and Blattodea. Blattodea is comprised of eight distinct clades: Blaberoidea, composed of two families; and six other clades of which Isoptera is one, now regarded as an epifamily. Within Isoptera, Mastotermitidae is sister to the remaining six termite families (Ware et al., 2008; Davis et al., 2009; Engel et al., 2009; Djernæs et al., 2012). The “lower” termites (Mastotermitidae, Kalotermitidae, Termopsidae, Hodotermitidae, Rhinotermitidae and Serritermitidae) still retain hindgut protozoa, while the “higher” termites, Termitidae, are now independent of this. Therefore, it is thought that the “lower” termites showed the first signs of eusocial evolution (Noirot, 1985, Legendre et al., 2008).

Despite their current ecological dominance, particularly in tropical and sub-tropical environments, prior to the divergence of the monophyletic Termitidae, 55 – 60 mya, termites only comprised ~1% of the fossil record for insects. With the rise of Termitidae, which now accounts for ~70% of all termites, there was an increase in colony size and variability of diet (Davis et al., 2009; Engel et al., 2009). Accordingly, foraging behaviour, which may or may not have developed alongside worker castes, seems to have evolved along a continuum, with all three distinct variations still exhibited today. Primitive ‘one-piece’ type foraging, is characterised by termites which nest and feed within a single piece of wood, with nest space increasing as wood is consumed. ‘Intermediate’ and ‘separate’ type species have uncoupled their foraging
sites from their nesting sites. Analysis suggests that the ancestral system was ‘one-piece’ type foragers. Although, as mentioned, the evolution from ‘one-piece’ to ‘separate’ is generally on a continuum, Legendre et al., (2008) have shown a single leap from ‘one-piece’ to ‘separate’ type for the clade containing *H. mossambicus* and *M. viator*. This evolution of foraging strategies may have been facilitated by the rapid spread of C₄ grasslands during the Miocene (5 – 24 mya), which afforded termites a competitive advantage and an opportunity for diversification (Engel et al., 2009).

**The Biology of Caste Development**

Social insects are characterised by reproductive division of labour, such that only a few individuals are responsible for reproduction, while the majority of the colony consists of sterile workers (Hayashi et al., 2007). Workers provide alloparental care of the offspring and are responsible for foraging, nest maintenance and protection, amongst other duties (Weil et al., 2007).

Termites exhibit complex coordinated group behaviours by differentiated castes (Table 1.1) (Legendre et al., 2008). It was thought that castes developed solely from a totipotent genome, of which gene expression was then environmentally mediated, due to highly plastic postembryonic development. However, evidence from a study by Hayashi et al., (2007) indicated that there is also a likely genetic component to caste determination. Under laboratory conditions, 99% of parthenogenetically produced offspring of *Reticulitermes seperatus* developed into nymphs. Also supporting genetic caste determination, Matsuura et al. (2009) found that for colonies of *R. seperatus*, secondary queens were produced almost exclusively via parthenogenesis, while workers and alates continued to be produced via sexual reproduction. Unfortunately, sex-determination within termites remains poorly understood. While females seem to always present symmetrical pairs of homologous chromosomes at meiosis, there is great variability among males with X0, XY, chains or rings all having been observed in various species. In particular, the formation of meiotic rings or chains facilitates sex-linked autosomal genes. The sex-linked portion of the genome has been estimated at around 10% in the Termitidae and as high as 50 - 60% in Kalotermitidae (Roisin, 2001).
Thus, although neither full maternal or paternal effects appear to be responsible, caste determination is almost certainly the result of a combination of genetic and environmental forces.

Table 1.1 Descriptive definitions of termite castes and developmental terminology (amended from Miller, 1969)

<table>
<thead>
<tr>
<th>Caste / Developmental State</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larva</td>
<td>An immature individual without any external signs of wing buds or specialised morphology.</td>
</tr>
<tr>
<td>Nymph</td>
<td>A post-larval individual with wing buds.</td>
</tr>
<tr>
<td>Worker</td>
<td>A permanently sterile individual, without visible differentiation towards either the alate or soldier caste.</td>
</tr>
<tr>
<td>Pseudergate</td>
<td>A regressed nymph who has lost its wing buds, or a larva which has undergone stationary moults. An individual which retains caste-differentiating ability.</td>
</tr>
<tr>
<td>Soldier</td>
<td>An individual which has defensive adaptation, such as large mandibles and a heavily sclerotised head.</td>
</tr>
<tr>
<td>Primary reproductives</td>
<td>Winged adult alates, colony founders – a.k.a. kings and queens</td>
</tr>
<tr>
<td>Nymphoid neotenic</td>
<td>A secondary, or replacement reproductive, derived from a nymph and retaining wing buds to some degree</td>
</tr>
<tr>
<td>Ergatoid neotenic</td>
<td>A secondary, or replacement reproductive, derived from a larvae</td>
</tr>
</tbody>
</table>

Much of the environmental mediation is pheromonal in nature and production of one caste may be stimulated by the presence of absence of another caste. For example, in *Kalotermes flavicollis* and *Prorhinotermes simplex*, soldier production is stimulated by the presence of reproductives, while in *Reticulitermes lucifugus*, soldier production increases when pseudergates are reared with nymphs (Bordereau, 1985). In *Kalotermes* and *Zootermopsis* species, alate nymph production is inhibited by the presence of primary reproductives and, as a corollary, in *Macrotermes bellicosus* the removal of the royal pair stimulates nymph production (Bordereau, 1985). In newly founded *Kalotermes, Reticulitermes* and *Zootermopsis* colonies, there is only one soldier for quite some time - if the soldier is removed, another one develops, thus the presence of a soldier prevents the development of additional soldiers (Bordereau, 1985).
A bifurcated caste pathway is believed to have developed from a linear pathway, which is still present in some basal species (Hayashi et al., 2007). For termites, the normal developmental pathway ends in alate production and all other castes are deviations from the norm - the earlier the deviation, the greater the differences (Noirot, 1985). In more than 80% of species, bifurcation occurs early in development. For example, in Hodotermitidae the alate and worker lines are clearly separate after the first few molts (Noirot, 1985). Most offspring will enter the functionally sterile, irreversibly wingless worker pathway, while the rest develop wing-buds and enter the nymph pathway which leads to the alate caste (Hayashi et al., 2007). Each caste requires a different number of molts: one for neotenics, two for soldiers and several for alates. However, individuals, particularly pseudergates, may also undergo stationary or even regressive molts, returning to morphological characters of an earlier instar (Weil et al., 2007).

Pseudergates have evolved convergently at least twice within the termites, while true workers may have evolved up to three times (Legendre et al., 2008). Neither caste was ancestrally present in the Isoptera and nor has it been determined which evolved first. True workers and pseudergates develop from different ontogenetic pathways and can coexist within a colony, as has been shown in Reticulitermes (Laine & Wright, 2003). True workers separate early and definitively from the imaginal line and constitute the primary work force of the colony. They have rudimentary sex organs, no wings and tend to be morphologically simple, although their head and mandibular muscle may be specialised (Noirot, 1985). Pseudergates separate late from the imaginal line following regressive and stationary molts, which lengthens the individual’s lifespan, improving the parent-offspring association and colony cooperation (Noirot, 1985). Pseudergates are no more active than other nymphs and thus do not represent the colony work force. They have no distinct morphological specialisations and may possess short wing buds or none at all, however, their genitals are similar in structure to those of nymphs of the same size (Legendre et al., 2008). As they arise from totipotent eggs they are able to develop into alates, neotenics or sterile soldiers (Weil et al., 2007).
Species with well constructed nests tend to be headed by a single pair of reproductives. The queens of such species are generally extremely physogastric, producing thousands of eggs a year. Additionally, the primary reproductives tend to be quite long-lived, with records of queens surviving upwards of 20 years (Thorne et al., 2002; Promislow, 2003). This increased longevity has been attributed to a combination of factors, including: the highly sheltered nest environment; low levels of sexual conflict between reproductives, particularly in species, such as termites, which tend towards monogamy; and age-specific fecundity (Keller & Genoud, 1997; Thorne et al., 2002; Promislow, 2003). In Zootermopsis neveadensis, there was no significant longevity differences between kings and queens, which were found to live between two and seven years (average 4.5 years) (Thorne et al., 2002). In R. seperatus, on the other hand, it is thought that the king is much longer-lived than the primary queen, and replacement appears to be rare (Matsuura et al., 2009). When the king and/or queen dies, replacement reproductives, known as neotenics, develop and assume the role of reproduction while retaining their juvenile characteristics.

Neotenics may develop from (adultoid) alates retained in the nest before a nuptial flight, or less frequently, from the nymphal pathway with wing buds (nymphoid) or the worker pathway without wing buds (ergatoid) (Thompson & Herbert, 1998; Hayashi et al., 2007). Some species, such as Macrotermes darwiniensis, Reticulitermes flavipes and R. lucifugus, constantly produce neotenics to allow for a rapid response to a change in colony condition (Lenz, 1985). When the role of reproduction has been filled, the excess neotenics, which are highly dependent on other colony members for the maturation of their gonads, are eliminated via the combined efforts of more dominant neotenics and the rest of the colony (Lenz, 1985). The number of neotenics maintained within a group is based on a combination of several factors influencing colony condition, including the size of the colony population, food availability and ambient temperature (Lenz, 1985). As neotenics cannot achieve the same level of ovarian development as primary queens, more than one neotenic is usually required to ensure optimal and continued colony growth (Lenz, 1985). In R. seperatus, it has been found that the mean number of active secondary queens in a colony is around 55 (Matsuura et al., 2009). The threshold at which elimination of secondary reproductives begins varies from one colony to the next.
Soldiers, which are a well defined caste, are a unique characteristic of Isoptera. They develop epigenetically via two successive moults: the first resulting in a totally dependent, non-functional soldier larvae; followed by the second, about 15 days later, which produces the fully sclerotized and pigmented adult (Noirot, 1985). They are quite short-lived, for example *Nasutitermes nigriceps* soldiers typically live for about three to six months, possibly due to the high risks of predation associated with foraging away from the nest (Thompson & Herbert, 1998). Soldiers are thought to have evolved early, as the first step towards polymorphism within Isoptera.

There is great variation among the lower termite families, from their specialisations to their developmental pathways, resulting in the presence or absence of various castes and variable gender skew within specific castes (Noirot, 1985). However, although comparatively little research has been conducted on *M. viator* itself, there does appear to be a fair amount of similarity among the harvester termites in general.

In *H. mossambicus* colonies, the un-pigmented sixth instar larvae work within the nest, caring for eggs, grooming reproductives, processing grass collected by foraging workers and feeding dependent castes, which includes the workers which are unable to adequately feed themselves (Nel et al., 1969). In both *M. viator* and *H. mossambicus* there are two worker castes, major and minor workers, which are both darkly pigmented, and are primarily involved in digging and foraging activities (Watson, 1973). As foraging occurs in the open during the day, their dark pigmentation provides protection against ultraviolet radiation (Watson, 1973; Dean, 1993). Duncan & Hewitt (1989) observed that in *H. mossambicus* the major workers are responsible for cutting vegetation, while minor workers are responsible for transporting the vegetation back to the nest. Among the harvesters, Watson (1973) found that minor workers out-number major workers by about four to one. Watson also showed that there is a distinct, significant difference in head capsule width between the two worker classes within any given colony. However, the absolute size of the two worker classes can vary widely from one colony to the next. In *H. mossambicus* all minor workers are female while all major workers are male and, as the soldiers are derived from the major workers, all
soldiers are male (Noirot, 1985). Caste gender bias has not been investigated in *M. viator* and provides an avenue for future research. However, given the similarities between the harvesters, one could hypothesise that *M. viator* may function similarly to *H. mossambicus* in this regard.

**The Ecology of Agonism and Spacing between Termite Colonies**

Among eusocial insects, colony integrity must be maintained through a strong and efficient defence strategy, which minimises the costs associated with reproduction with unrelated individuals and maximises protection of the brood and colony resources (Dornhaus *et al*., 2004; Neoh *et al*., 2012). However, initiating a defensive response is fundamentally contingent on the ability to discriminate between colony members and non-members. The underlying factor, upon which all recognition is based, is phenotypic variation (Husseneder *et al*., 1998), and two main categories of discriminating cues have been identified; chemical cues, having a genetic and/or environmental basis (Errard, 1994); and odour cues from symbiotic gut bacteria.

Hydrocarbon compounds are the major components of cuticular lipids, found on the cuticular surface of all terrestrial insects (Haverty *et al*., 1988; Dietemann *et al*., 2003; Howard & Blomquist, 2005). Cuticular hydrocarbons (CHC’s) play a central role in recognition (Haverty *et al*., 1988; Bagneres & Morgan, 1990; Jungnickel *et al*., 2004; Howard & Blomquist, 2005), and a high degree of chemical specificity within, and variability between, species and colonies has been documented (Lahav *et al*., 1999; Jungnickel *et al*., 2004).

Genetically determined hydrocarbons are internally synthesized and then transported to the insect cuticle (Howard & Blomquist, 2005). As nestmates tend to be highly related to one another (Buczkowski & Silverman, 2004), heritable genetic cues provide an important source of variation for recognition. Increased genetic variation within a colony, due to an increase in the number of reproductives, will extend the variation of the genetic component of recognition, thus lowering the rejection threshold by increasing the potential spectrum of similarity (Bagneres and Morgan, 1990; Errard,
Environmentally derived hydrocarbons, which are transferred via direct contact, are dynamic and differ with respect to season, diet, habitat and nesting material (Beye et al., 1998; Buczkowski & Silverman, 2004), and may even be capable of overriding more stable genetic cues (Beye et al., 1998; Buczkowski & Silverman, 2004). However, it is doubtful that CHC recognition cues act in isolation, and it is more plausible that they function in conjunction with odour cues from symbiotic gut bacteria (Adams, 1991; Olugbemi, 2013).

Symbiotic gut bacteria play an important role in recognition processes (Eutick et al., 1978; Kirchner & Minkley, 2003), and there appear to be termite family-specific assemblages of gut bacteria (Eutick et al., 1978). In support of this, Minkley et al. (2006) found that colonies of H. mossambicus possess distinctly different assemblages of gut bacteria. This is likely due to the fact that gut symbionts are heritable within colonies, as they are transferred from one individual to another via proctodeal trophallaxis (Kirchner & Minkley, 2003).

As noted, the primary purpose of recognition is the maintenance of an efficient defence strategy (Buczkowski & Silverman, 2004; Jungnickel et al., 2004), and thus recognition facilitates agonistic, or competitive, interactions between colonies. Agonism can be defined as the combination of offensive and defensive responses between competing individuals (Polizzi & Forschler, 1999), and generally occurs in the presence of non-nestmates and manifests as fighting, fleeing or submitting (Haverty & Thorne, 1989; Olugbemi, 2013). Agonism between colonies tends to vary with colony circumstance and environmental influences (Sundstrom, 1997; Dahbi and Lenoir, 1998; Lepage & Darlington, 2000). Colony circumstance includes genetic composition or family type, age, health and reproductive status of colonies, while environmental influences include season and geographical distribution of the population (Clement & Bagneres, 1998; Fuller et al., 2004; Korb & Foster, 2010; Yusuf et al., 2010; Neoh et al., 2012). As such, both the ecology and the environment mediate the relative costs and benefits of species interactions (Korb & Foster, 2010).
In order to limit expensive agonistic encounters, optimise resource utilisation and mitigate susceptibility to interspecific competition, established colonies are often regularly spatially distributed (Kaib et al., 2002; Korb & Foster, 2010). While this distribution is mediated by the spatial and temporal distribution of said resources, once established, termite territories tend to be stable and persistent (Kaib et al., 2002). For example, *M. viator* has a consistent intercolonial distance of approximately 45 to 50m across its range (Coaton & Sheasby, 1974; Lovegrove & Siegfried, 1989; Midgley & Musil, 1990; Lovegrove, 1991; Moore & Picker, 1991; Laurie, 2002; Cramer et al., 2012). This appears to be in keeping with observations of other subterranean termite species; Darlington (1982) found that the average intercolonial distance of *Macrotermes michaelseni* was approximately 50m.

Intercolonial distance is believed to play a key role in agonistic encounters; however the effects thereof appear to be variable. In some species or environments, neighbouring colonies are highly aggressive towards one another (Dunn & Messier, 1999), while in different species, or under a different set of ecological circumstances, neighbouring colonies may behave amicably (Temeles, 1994). However, due to the nature of the recognition cues utilised by termites, a common hypothesis and frequent observation is that intercolonial aggression is significantly correlated with intercolonial distance (Nowbahari et al., 1990).

**Theoretical Approach of this Thesis**

Behavioural ecology is an area of study which is integral to the contextual understanding of animal behaviour, however observations may be susceptible to subjective interpretation. Genetics, on the other hand, is a field often too far removed from the natural context of the species of interest, particularly when done is isolation in the laboratory. This study has therefore been designed under the umbrella of molecular ecology, in order to integrate both behavioural and genetic perspectives of colony composition and spatial organisation at various scales.
The organisation of eusocial societies can be defined at two levels; the breeding structure within colonies and the distinctness of each colony within the population (Thorne et al., 1999). Therefore, this thesis has several aims and hypotheses relating to the organisation of *M. viator* populations.

The first aim is to determine the genetic organisation of *M. viator* populations at various spatial scales, in terms of reproductive composition and family structure within colonies. The literature indicates that inbreeding avoidance is widespread in termites, principally during colony foundation (Vargo & Husseneder, 2011), and variable family types are common in populations of termite colonies, with extended families occurring particularly frequently among the ‘lower’ termites (Hacker et al., 2005; Vargo & Husseneder, 2011). Based on this, I predict that *M. viator* will likely exhibit inbreeding avoidance and that colony foundation will be attributable to an outbred pair of monogamous reproductives. Additionally, I expect to find evidence of variable family types among the study population.

The second aim is to understand the relationships between the same colonies, in terms of agonistic behaviour, which is the fundamental mechanism responsible for the observed under-dispersion between neighbouring colonies (Coaton & Sheasby, 1974; Lovegrove & Siegfried, 1989; Midgley & Musil, 1990; Lovegrove, 1991; Moore & Picker, 1991; Laurie, 2002; Cramer et al., 2012). Based on *M. viator*’s foraging pattern and very long-lived nest system, and given that agonism between colonies is the norm (Getty et al., 2000), it is likely that this species employs a high level of innate colony defence. Additionally, as many studies find that intercolonial aggression increases with intercolonial distance (Nowbahari et al., 1990), I would tentatively predict that the same may hold true in *M. viator*. I classify the prediction as tentative due to the fact that many variables, some of which have already been discussed, mediate agonism between colonies. However, should this prediction be accurate, I would anticipate a clear correlation between intercolonial distance and levels of genetic relatedness.

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1 See Chapter 4 for a full discussion on these topics.
2 See Chapter 2 for a full discussion on this topic.
3 See Chapter 5 for a full discussion on this topic.
From a theoretical perspective, this study aims to shed light on the debate surrounding the potential longevity of *M. viator* colonies. Generally, species with well-constructed nests which provide a sheltered environment, such as *M. viator*, tend to have long-lived primary reproductives (Thorne *et al.*, 2002; Promislow, 2003). Furthermore, due to the common termite strategy of reproductive replacement from within the colony, neotenics give a colony the possibility to be very long-lived (DeHeer & Vargo, 2006). As extended families (those headed by neotenics) are common among the ‘lower’ termites (Vargo & Husseneder, 2011), it is likely that *M. viator* colonies have considerable potential for longevity.

**Organisation of this Thesis**

Each chapter was developed around a particular theme within the broader focus of this thesis. Given the multidisciplinary approach of this research, and for the reader’s convenience, references are located at the end of each chapter. The following summaries are intended to provide the reader with a brief overview of the focus, aims and conclusions for each chapter.

*Chapter 2 – The origin and age of ‘heuweltjies’*

Attributing *heuweltjies* to the construction activities of *M. viator* has been an ongoing academic debate for almost 30 years. Therefore, the primary aim of this chapter is to critically address this debate and reach a clear conclusion. As such, this chapter provides an overview of the nest system of *M. viator* and argues for their congruence with ‘heuweltjies’. In addition to the origins, the longevity of *heuweltjies* is also unresolved, in spite of numerous papers addressing the age of *heuweltjie*. The studies that have attempted to ascertain the age of mature *heuweltjies* are critically reviewed, and an alternative to radiometric carbon dating is discussed.
Chapter 3 – Development of a microsatellite library for Microhodotermes viator

This chapter begins by discussing the importance of microsatellites as a genetic tool. The central aim of this chapter is the identification of microsatellite loci in *M. viator*, and the development and optimisation of novel PCR primers. As such, the enriched library making process that was followed is described in detail. Seven polymorphic loci were identified; Mvit 4, Mvit 14, Mvit 17, Mvit 18, Mvit 21, Mvit 23 and Mvit 25. Allele numbers ranged from 2 to 14, for an average of 5.8 alleles per locus. This represents a set of microsatellite loci in *M. viator*, sufficiently polymorphic for fine-scale population genetic studies. As this chapter describes the development of a genetic tool, utilised in Chapter 4, the structure deviates from standard scientific reporting and presents a combined methodology, results and discussion section.

Chapter 4 – The genetic structure of Microhodotermes viator colonies

This chapter describes the general process of colony foundation and the development of various family structures over time. It details alternative modes of colony foundation, the significance of inbreeding within colonies and the inferences which can be drawn from genetic structure regarding longevity of a colony. The primary aim of this chapter is to determine the genetic organisation of *M. viator* populations at various spatial scales, in terms of reproductive composition and family structure within colonies. Allele and genotype frequencies are described for each colony and the population as a whole, as are genetic differentiation and variance, genetic variation, family structure of colonies, relatedness and inbreeding coefficients.

Chapter 5 – Behavioural interactions between colonies of Microhodotermes viator

This chapter considers the importance of recognition in social insects and the various means by which recognition can be achieved, in particular using chemical cues, either genetically or environmentally derived, and odour cues. How these recognition cues are used to achieve recognition and agonism are then discussed. The main aim of this chapter is to understand the relationships between colonies of *M. viator*, in terms of
agonistic behaviour. Based on the behavioural bioassay results, the relationship between intercolonial distance, relatedness and trial outcome is then explored.

*Chapter 6 – Overview of population genetic structure and behavioural interaction in Microhodotermes viator*

This chapter draws on the results from both the genetic and behavioural investigations, as well as the debate regarding the relationship between *heuweltjies* and *M. viator*, to provide the reader with an holistic view of *M. viator* as a species. *M. viator* follows a standard mode of colony foundation by a monogamous pair of unrelated reproductives, giving rise to simple families. When the primary reproductives die they are replaced by low numbers of neotenics, giving rise to extended families. Additionally, mixed families are present in the population at a high frequency. This is interpreted as a potential system of periodic adoption of unrelated reproductives, which would likely increase colony longevity and may help to explain the processes supporting the formation and maintenance of extremely long-lived *heuweltjies*. The formation of mixed families, which requires a reduced rejection threshold, is congruent with the behavioural data which found that the overall level of intercolonial aggression was low. The highest levels of aggression were between colonies within the same geographic area, while nearest neighbours were not aggressive towards one another, which is interpreted as support for the Dear Enemy phenomenon (Temeles, 1994).
References


Lenz, M. 1985. Is Inter- and Intraspecific Variability of Lower Termite Neotenic Numbers due to Adaptive Thresholds for Neotenic Elimination? – Considerations from studies on Porotermes adamsoni (Froggatt) (Isoptera:


Chapter 2

In Support of a *Microhodotermes viator* Origin for *Heuweltjies*

Introduction

Large lenticular Mima-like earth mounds, locally referred to as *heuweltjies* (little hills), are a well-studied landscape phenomenon of the winter rainfall succulent karoo and fynbos biomes of South Africa. In 1986, Lovegrove and Siegfried first postulated that these *heuweltjies* are a product of the combined activities of the termite *Microhodotermes viator* and molerat *Cryptomys hottentotus*. However, both prior and subsequent to this assertion, *heuweltjies* have remained a contentious topic in the academic literature. This chapter first provides a description of *heuweltjie* dispersion, and associated soil structure and nutrient status, and then evaluates the various hypotheses that have been put forward regarding the origin and age of *heuweltjies* and their relationship with *M. viator*.

*Heuweltjies* are large earth mounds, typically about 25m in diameter (range: 5 – 35m) and 1-3m high (Lovegrove & Siegfried, 1986; Midgley & Hoffman, 1991; Moore & Picker, 1991). They have been estimated to occupy between 14 and 25% of the land surface area of the Western Cape (Lovegrove & Siegfried, 1989; Picker et al., 2007), and are restricted to lower mountain slopes and valleys, inland flats and coastal lowlands (Lovegrove & Siegfried, 1986). *Heuweltjie* distribution ranges from the Orange River in the north, to Somerset Strand in the south and Dysseldorp in the east (Fig 2.1) (Lovegrove & Siegfried, 1986). Across their range, *heuweltjies* are non-randomly distributed, with consistent inter-mound distances of 45 to 50m, with an increasing density with increasing rainfall from north to south and east to west (Lovegrove & Siegfried, 1989; Midgley & Musil, 1990; Lovegrove, 1991; Moore & Picker, 1991; Laurie, 2002; Picker et al., 2007; Cramer et al., 2012). This
underdispersed\textsuperscript{4} spatial pattern is reflected in an R value (Clark & Evans, 1954) of 1.7 (Lovegrove & Siegfried, 1989).

![Fig. 2.1 Distribution of heuweltjies (stippled areas) across the south western part of South Africa (Lovegrove & Siegfried, 1986, Fig. 1).](image)

Like similar earth mounds, which have been observed in various environments around the globe, heuweltjies tend to occur on poorly drained shallow soils with an underlying basement layer (Lovegrove & Siegfried, 1986; Lovegrove, 1991). Heuweltjies are made up of sandy to clayey soils (Cox et al., 1987; Knight et al., 1989), with approximately 80% of the soil having a particle size of less than 2mm (Cramer et al., 2012), and a higher clay and silt fraction than inter-mound soils (Midgley & Musil, 1990; Francis et al., 2012). Francis et al. (2012) found that the clay faction of the soil tends to be concentrated in the centre of the heuweltjies with a clear increase in coarse particles from the centre to the periphery. In terms of small stone content, gravel (8 – 15mm) and

\textsuperscript{4} Here the word ‘underdispersed’ is being used in the statistical sense, to refer to the non-random, highly regular spatial distribution of colonies.
Pebbles (15 - 50mm) are ubiquitous components and are distributed throughout the *heuweltjie* profile, each accounting for approximately 10% of the soil volume. These also show a significant increase in size and concentration with increasing depth into the mound (Cox *et al*., 1987; Potts *et al*., 2009; Cramer *et al*., 2012). Both soil particle size and small stone content are significantly reduced on *heuweltjies* when compared to the inter-mound soils (Cox *et al*., 1987; Cramer *et al*., 2012). Many *heuweltjies* also have exposed rocks (< 2kg) on their surface (Cramer *et al*., 2012), which has been interpreted by Cramer *et al.* (2012) as evidence against the formation of *heuweltjies* by termites. However, the activity of small burrowing and large digging mammals, such as mole rats and aardvarks (Moore & Picker, 1991) on *heuweltjies*, could account for their presence on surface soils.

*Heuweltjies* give the landscape a ‘pock-marked’ appearance (Fig. 2.2) as they support plant communities distinctive from the surrounding vegetation matrix (Midgley & Musil, 1990; Lovegrove, 1991; Corinna *et al*., 2005).

**Fig. 2.2** Satellite image of heuweltjie field just north of Nuwerus, Western Cape, South Africa (31° 8'5.33" S, 18°21'21.22" E)(Image source: Google™ Earth). Elevation approximately 400 m.

*Heuweltjies* are strongly favoured by deciduous plants, while slower-growing, stress-tolerant and evergreen forms dominate in the inter-mound areas - a feature which has been predominantly attributed to the superior nutrient status of *heuweltjie* soils, which have significantly higher levels of macro and micro nutrients (with the exception of sodium), and a significantly higher water infiltration than inter-mound soils (Knight *et al*., 1989; Midgley & Musil, 1990; Lovegrove, 1991). As such, *heuweltjie* soils are
considered to be agriculturally rich. This characterisation is further supported by observations of the long term positive effects of *heuweltjie* soil on vine cultivars and wheat, even after several years of intensive farming (Midgley & Musil, 1990).

Additionally, *heuweltjie* soils are weakly acidic or alkaline, while inter-mound soils are strongly acidic (Midgley & Musil, 1990; Francis *et al*., 2012). The higher pH is attributed to the greater nutrient concentration in *heuweltjie* soil, particularly of basic cations such as calcium and magnesium (Francis *et al*., 2012). Moreover, this higher pH is implicated in the formation of a calcrete layer immediately below *heuweltjies* as alkaline *heuweltjie* soils, in conjunction with rainfall, ground water and evaporation, facilitate calcrete precipitation at their base (Midgley & Hoffman, 1991; Francis *et al*., 2012). The effect is to stabilise the soil structure and thus improve resistance to erosion (Francis *et al*., 2012). Over time, the calcrete coalesces forming a hardened layer, significantly increasing *heuweltjie* volume (Midgley & Hoffman, 1991; Candy *et al*., 2004; Candy *et al*., 2005, Francis *et al*., 2012).

**Origins of Heuweltjies**

In spite of many years of examination, the origin of *heuweltjies* remains unresolved. Since the 1940s there have been seven competing hypotheses concerning the origin of *heuweltjies*. Three of these hypotheses are geologically-based - Limestone-Faulting, Wind-Action and Dorbank Erosion – with a subsequent extension of the Dorbank Erosion hypothesis by Cramer *et al.* (2012), to include a precursor of self-patterning vegetation. The remaining four hypotheses are all faunal-based - Termite-Tree, Termite-Climate, Termite-Molerat and Termite.

The Limestone-Faulting hypothesis (LFH) (Van der Merwe, 1940, as cited in Lovegrove & Siegfried, 1989), suggests that the presence of a calcrete layer below *heuweltjies* could be best explained by past faulting or folding of the sedimentary shales. The Wind-Action hypothesis (WAH) (Ten Cate, 1966, as cited in Lovegrove & Siegfried, 1989), suggests that wind-blown sand builds up around the base of vegetation, over time the vegetation dies, leaving only the *heuweltjie* mound behind. On
the other hand, the Dorbank Erosion hypothesis (DEH) (Slabber, 1945, as cited in Lovegrove & Siegfried, 1989), suggests that during a past semi-arid climate, the ground was covered with a very dry limestone-rich layer which was eroded due to climate change, exposing circular limestone-banded mounds (*heuweltjies*), with plant communities protecting the *heuweltjies* from further erosion.

The postulates of the LFH are not supported by any data. Moore & Picker (1991) found *heuweltjies* to be uniformly distributed across the landscape and not concentrated over areas of geological faults. Furthermore, the calcrete layer below *heuweltjies* is now attributed to various processes related to, and resulting from, their higher nutrient status compared to that of inter-mound soils (Midgley & Hoffman, 1991; Francis *et al*., 2012). The WAH is also not supported. Moore & Picker (1991) found no evidence of wind-blown sand accumulating around the base of any vegetation and nor were the shape and slope of *heuweltjies* aligned with the general wind direction, although both Moore & Picker (1991) and Cramer *et al*. (2012), did find evidence of down-slope slumping (visible on Fig. 2.2). That said, the LFH, WAH and DEH are all geological models based on natural processes, but given that *heuweltjies* are under dispersed (Coaton & Sheasby, 1974; Lovegrove & Siegfried, 1989; Moore & Picker, 1991; Laurie, 2002), it is highly unlikely that random processes could have resulted in such a consistently regular spatial array.

Recently, Cramer *et al*. (2012) extended the DEH to include the biological component of plant communities, using regular spatial distribution (self-organising patterning) of vegetation that has been observed in many water-limited environments (e.g. Rietkerk & van de Koppel, 2008). As such, they suggest that positive feedback mechanisms within, and competition between, vegetation clumps may have resulted in a non-random array of vegetation clumps in an ancient environment, and that this may underlie the non-random distribution pattern of *heuweltjies* evident in the landscape today. Typically, the initial establishment of vegetation clumps is associated with positive feedback mechanisms, such as higher levels of soil moisture and greater nutrient availability (Kefi *et al*., 2007; Scanlon *et al*., 2007). However, although *heuweltjies* are currently sites of local enrichment, prior to their formation there is no evidence for distinctly
nutrient-rich sites within the environment required to facilitate the initiation of vegetation clumps. Furthermore, the driving force behind the transition from a random to non-random spatial distribution of vegetation clumps relies heavily on long-distance negative feedback mechanisms - in other words, competition (Rietkerk & van de Koppel, 2008). This competition acts between superficial root systems competing for water and nutrients (Cramer et al., 2012). Thus, while short-distance positive feedback continues to act and enhance the distinctness of the resulting pattern, it is not an essential driver of the process (Rietkerk & van de Koppel, 2008). However, given the large inter-mound distances between heuweltjies (45 – 50m), it is highly unlikely that even an extensive root system (Kambatuku et al., 2011) could produce a zone of competitive exclusion of this magnitude. Essentially, the pervasive association of the termite M. viator with heuweltjies was interpreted by Cramer et al. (2012) as a secondary colonisation of resource-rich sites. Nevertheless, even with the additional biological component postulated by Cramer et al. (2012), the DEH remains an unconvincing hypothesis for the origin of heuweltjies.

The four zoogenic hypotheses for the origin of heuweltjies all include termites. The Termite-Tree hypothesis (TTH) (Van der Merwe, 1940, as cited in Lovegrove & Siegfried, 1989), suggests that wind-blown sand and organic matter built up around the base of trees, providing an ideal nesting site for termites, which then assimilate the organic matter into the earth. Over time the trees died, leaving only the mounds behind. This hypothesis is an extension of the WAH and, as mentioned above, cannot explain the regular distribution of mounds, even with the suggested zoogenic aspect. Additionally, although the natural spacing of trees may at times not be random, they are often distributed non-randomly with regards to a particular resource. Caylor et al. (2003) found that of the largest 25% of all trees analysed, those which could conceivably be responsible for such large mounds were randomly distributed.

The Termite-Climate hypothesis (TCH) (Burgers, 1975) proposes that during the Last Glacial Maximum (LGM), 20 000 – 25 000 yr BP (before present), when the Western Cape vegetation was thought to be predominantly grassland (Sealy, 1996; Chase & Meadows, 2007), it would have favoured inhabitation by Macrotermes spp., which
currently occupy African savannahs and construct termitaria up to 10m high, with a basal diameter of up to 30m. The TCH goes on to suggest, that during the Holocene the termites occupying these termitaria were eliminated due to climate change and erosive forces began to take their toll, with associated plant communities protecting the mounds from complete erosion. At some point *M. viator* colonised the vacant termitaria and are responsible for all the modern physical features of mound soil (Burgers, 1975). However, this theory cannot account for the fact that the development from immature to mature mounds, all inhabited by *M. viator*, can be observed along a size continuum (Moore & Picker, 1991). Furthermore, in the eastern part of the range of *M. viator* where, likely due to differing soil structure, *heuweltjies* are not formed, there is no evidence of nuptial pairs taking advantage of other abandoned nest structures as a colony foundation strategy (Coaton & Sheasby, 1974; Picker *et al.*, 2007). Additionally, in *Hodotermes mossambicus*, the probable sister taxon of *M. viator*, colony foundation occurs via nuptial pairs of alates digging into bare soil, rather than preferentially choosing established vegetation clumps (Darlington *et al.*, 1977). As such, evidence for the TCH is lacking.

The Termite-Molerat hypothesis (TMH) (Lovegrove & Siegfried, 1986) suggests that molerats, *Cryptomys hottentotus*, take advantage of *heuweltjies* primarily to avoid winter flooding, and are responsible for the enlargement and maintenance of mounds. Generally, *C. hottentotus* will construct their own nests, but are also known to nest within small termite and ant mounds. Lovegrove and Siegfried (1986) suggest that, through moving excavated soil backwards towards the mound, *C. hottentotus* could be responsible for the massive enlargement of small mounds created by *M. viator* to the large *heuweltjies* evident in the landscape. However, although they found evidence of activity in close proximity to *heuweltjies*, in the form of mole heaps, they did not actually observe *C. hottentotus* in occupation of a *heuweltjie*. Similarly, Moore and Picker (1991) found no evidence of annexure of *heuweltjies* by mole rats and when evidence of mole rat tunnelling was observed in conjunction with a *heuweltjie*, it was found to have a dispersive rather than concentrative effect; exposing the upper mound layers to erosion by both wind and rain (Midgley & Hoffman, 1991). Lastly, the average
heuweltjies size is also far smaller than the average home range of *C. hottentotus*, which is in the region of 1.6km\(^2\) (Moore & Picker, 1991).

The final Termite hypothesis is that heuweltjies are formed as the direct result of nest construction, and related activities of *M. viator* (Moore & Picker, 1991). This hypothesis proposes that the central-based foraging activities and subsequent frass output of the termites enriches heuweltjie soils, and improves aeration and water infiltration and retention. As described above, the higher nutrient levels result in an increased soil pH, leading to the precipitation of calcrete, which improves soil stability and mitigates erosion (Midgley & Hoffman, 1991; Francis *et al*., 2012). Over time, the calcrete coalesces forming a hardened layer, significantly increasing heuweltjie volume and necessitating the upward movement of termite construction activities (Midgley & Hoffman, 1991; Candy *et al*., 2004; Candy *et al*., 2005; Francis *et al*., 2012). An ecological engineering effect of the higher nutrient status and improved water infiltration is that heuweltjies become ideal sites for seedling recruitment and hence the formation of distinctive vegetation communities, with colonisation and establishment of species that prefer enriched and disturbed soils (Knight *et al*., 1989; Midgley & Musil, 1990; Midgley & Hoffman, 1991). The non-random spacing of heuweltjies is attributed to intercolonial competition, as the average inter-mound distance (45m) (Lovegrove & Siegfried, 1989; Midgley & Musil, 1990; Lovegrove, 1991; Moore & Picker, 1991; Laurie, 2002; Cramer *et al*., 2012), is in direct agreement with average colony foraging distances, which radiate out about 45m from the central hive (Coaton & Sheasby, 1974). Interestingly, this foraging radius was determined in the eastern part of *M. viator’s* range, where heuweltjies are absent, suggesting that this distance is independent of the heuweltjies and is rather attributable to the *M. viator* foraging strategy.

Cramer *et al*., (2012) have challenged the termite hypothesis as they only found evidence of *M. viator* on some heuweltjies examined at the same site where Moore & Picker (1991) did their study. However, Cox *et al*., (1987) found evidence of *M. viator* on 24 out of 26 heuweltjies and Moore & Picker (1991), found a 78.9% occupation of intact mounds by *M. viator* in the same area. Furthermore, Moore & Picker (1991), using a naturally water-eroded field of heuweltjies on the floor of the Clanwilliam dam (Fig.
2.3), found that *heuweltjies* have an internal structure that indicates progressive concentric growth, due to the deposition of successive ferruginised sand layers over a very long period of time. The lower layers contain fossilised termite constructs, such as tunnels and storage chambers, while the upper layers are still inhabited by termites and contain the central hive as well as further tunnels, storage chambers and frass deposits. Similarly, Francis *et al.* (2012) also found clear evidence of *M. viator* construction within *heuweltjies* in the form of tunnels and storage chambers, lined with organic matter, and frass deposits.

![Image](image.jpg)

*Fig. 2.3* John Moore and eroded *heuweltjie* on the floor of the Clanwilliam dam after retreat of the water in late summer, April 1986. The sand capping of the mound has been removed, revealing the intricate termite tunnelling (Image: M. Picker).

Another concern that Cramer *et al.* (2012) raise, is that the above-ground (epigeal) *M. viator* nest size is significantly smaller that the volume of the average *heuweltjie*. However, *M. viator* nests are always partially or fully subterranean (Coaton & Sheasby, 1974; Midgley *et al*., 2002; Picker *et al*., 2002). As such, the above ground “nests” to which Cramer *et al.* (2012) is referring, are examples of the rare ‘mounding over’
phenomenon described by Coaton & Sheasby (1974). Coaton & Sheasby (1974) never described *M. viator* termitaria in the western part of *M. viator’s* range. However, in the eastern part of *M. viator’s* range, they observed that in sheltered environments with high clay-content soils, conical frass deposits above *M. viator’s* subterranean nests may harden into sharp conical mounds, up to 120cm high (Fig 2.4). So, while Cramer *et al.* (2012) are correct that there is a significant difference in size between these mounds and *heuweltjies*, they are in fact comparing two different things. Furthermore, the large volume of *heuweltjies* has been attributed to the formation of a basal calcrete layer (Francis *et al.*, 2012).

![Fig. 2.4 A hardened conical dome above a subterranean *M. viator* nest in Beaufort West, South Africa (Coaton & Sheasby, 1974, Plate 4).](image)

Cramer *et al.* (2012) also raise concern regarding the presence of stones on and within the *heuweltjies* they examined, which they cite as evidence against a termite role for the building up of the epigeal component of *heuweltjies*. They also observed small rocks on the surface of the *heuweltjies* examined, although significantly less than in the inter-mound area, and large rocks on the surface of several *heuweltjies*. The ground at the Clanwilliam site is particularly rocky, predominantly due to the presence of weathered fragments of the superficial underlying sandstone (Cox *et al.*, 1987; Cramer *et al.*, 2012). However, anthropogenic portage, or translocation by small burrowing and large digging mammals, seems to provide the most likely explanations for the presence of this
rock (Cox et al., 1987). In support of this, Moore & Picker (1991) found that 53% of heuweltjies examined showed evidence of aardvark excavation, which can be extensive - bringing pebbles, boulders and basal calcrete to the surface.

Cramer et al. (2012) proposed that the presence of small stones distributed throughout heuweltjies profiles was also inconsistent with termite activity, as the stones were too large to have been transported by termites. Cox et al. (1987) similarly found that gravel (8 – 15mm) and pebbles (15 - 50mm) were ubiquitous throughout the profiles of all heuweltjies they examined in both Robertson and Clanwilliam. Additionally, Cox et al., (1987), found that both the number and mass of stones significantly increased with increasing depth into the mound. Potts et al. (2009) also observed an accumulation of rocks and stones (larger than 3cm) below heuweltjies. Both Cox et al. (1987) and Potts et al. (2009) interpret this as clear evidence of termite activity, due to the fact that termite building causes the larger stones to sink and accumulate within the mound. It should additionally be noted that Cox et al. (1987) also found significant differences in stone number and size, not only between their two study sites, but also between mounds within each study site. Thus, their results highlight the fact that inferences relating to stone content within mounds should be made with caution and results cannot be broadly extrapolated, nor do they provide indisputable proof for, or against, any particular hypothesis as purported by Cramer et al. (2012).

Many termite species are known to preferentially select clay particles for nest construction. Nutting et al. (1987) found that Heterotermes aureus and Gnathamitermes perplexus cumulatively bring 21kg/ha/year of clay particles to the surface. Likewise, termitaria of Macrotermes bellicosus are predominantly composed of clay, while termiteria of Cubitermes niokoloensis have a three to ten times greater clay fraction than reference soils (Fall et al., 2001). Similarly, Psuedacanthotermes spiniger preferentially selects clay particles for construction when provided with a mixture of sand and clay (Jouquet et al., 2007). As such, Cramer et al., (2012) question the fact that they found no difference in clay fraction between heuweltjies and inter-mound soils or any evidence of particle sorting across the heuweltjies profile. However, Francis et al., (2012) found that heuweltjies do have a higher clay mineral fraction than inter-mound
soils, which is concentrated in the centre of the *heuweltjies*, and that the proportion of coarse particle fragments increases from the centre to the periphery. Likewise, a study by Midgley & Musil (1990), found approximately double the amount of clay and silt on *heuweltjies* versus off them. In line with this, Cramer *et al.* (2012) did note higher silt content on *heuweltjies*. In fact, it is entirely possible that the ‘silt’ Cramer *et al.*, (2012) observed is actually a microaggregate, known as pseudo-silt (Jungerius *et al.*, 1999). These microaggregates (0.03 – 1mm), which are extremely stable and persistent, are formed during termite construction activities when soil is mixed with saliva (Jungerius *et al.*, 1999). If this is true, Cramer *et al.*’s (2012) grain-size analysis may well provide evidence for, rather than against, termite activity.

The best supported hypothesis for *heuweltjie* formation is attributable to a *M. viator* origin. The distribution patterns of *M. viator* and *heuweltjies* are also congruent. *M. viator*’s range fully overlaps that of *heuweltjies*, although *heuweltjies* are most evident in the western part of *M. viator*’s range, where soil structure is conducive to their development (Picker *et al.*, 2007). Most importantly, no *heuweltjie* formation has been observed outside of the distribution range of *M. viator*.

**Age of Heuweltjies**

As described above, under suitable conditions, and over time, biogenic processes occurring on and within *heuweltjies* result in the formation of a calcrete layer below mature *heuweltjies* (Potts *et al.*, 2009). Three studies - Moore and Picker (1991), Midgley *et al.* (2002) and Potts *et al.* (2009) - have attempted to ascertain the age of *heuweltjies*. All three studies employed radiometric carbon dating on the basal calcrete layer found below mature *heuweltjies*.

Moore and Picker (1991) sampled calcrete from two *heuweltjies*, one from Kleinfontein and the other from the Clanwilliam dam (Table 2.1), and returned the youngest ages recorded for mature *heuweltjies*. Midgley *et al.* (2002) sampled calcrete from three *heuweltjies* in the same general area and returned ages of approximately 25 000 yrs BP, significantly greater than those recorded by Moore and Picker considering the similarity
in the size, structure and source material from the two studies. It is also worth noting, as a comparison, that Coaton (1981) aged fossilised non-*heuweltjie* termite hives from the same areas, at approximately 30 000 yrs BP.

Table 2.1 Results of radiometric carbon dating of termitaria *calretes*.

<table>
<thead>
<tr>
<th>Pta number</th>
<th>Sample</th>
<th>Locality</th>
<th>Source Material</th>
<th>Estimated Age (yrs BP)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5215</td>
<td>1</td>
<td>Kleinfontein</td>
<td>Basal Calcrete</td>
<td>5590</td>
<td>Moore &amp; Ricker, 1991</td>
</tr>
<tr>
<td>5227</td>
<td>2</td>
<td>Clanwilliam Dam</td>
<td>Basal Calcrete</td>
<td>3970</td>
<td></td>
</tr>
<tr>
<td>7906</td>
<td>1</td>
<td>Clanwilliam Dam</td>
<td>Basal Calcrete</td>
<td>24 600 380</td>
<td></td>
</tr>
<tr>
<td>7931</td>
<td>2</td>
<td>Elands Bay</td>
<td>Basal Calcrete</td>
<td>29 800 640</td>
<td>Midgley et al., 2002</td>
</tr>
<tr>
<td>7930</td>
<td>3a</td>
<td>Elands Bay</td>
<td>Basal Calcrete</td>
<td>25 400 270</td>
<td></td>
</tr>
<tr>
<td>7932</td>
<td>3b</td>
<td>Elands Bay</td>
<td>Basal Calcrete</td>
<td>25 500 460</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1a</td>
<td>Worcester</td>
<td>Powdery Matrix</td>
<td>30 083 – 32 035</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1b</td>
<td>Worcester</td>
<td>Upper Nodule</td>
<td>33 780 – 37 524</td>
<td>Potts et al., 2009</td>
</tr>
<tr>
<td></td>
<td>1c</td>
<td>Worcester</td>
<td>Lower Nodule</td>
<td>33 629 – 36 709</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1d</td>
<td>Worcester</td>
<td>Laminar Crust</td>
<td>21 676 – 23 256</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1e</td>
<td>Worcester</td>
<td>Basal Calrete</td>
<td>29 174 – 31 066</td>
<td></td>
</tr>
<tr>
<td>6102</td>
<td>1</td>
<td>Clanwilliam/ Vredendal</td>
<td>Fossilised non-<em>heuweltjie</em> hive</td>
<td>27 700</td>
<td>Coaton, 1981 in Midgley et al., 2002</td>
</tr>
<tr>
<td>6103</td>
<td>2</td>
<td>Clanwilliam/ Vredendal</td>
<td>Fossilised non-<em>heuweltjie</em> hive</td>
<td>35 400</td>
<td></td>
</tr>
</tbody>
</table>
Both of Moore and Picker’s (1991) samples are very young, given the source material of basal calcrete. The formation of calcrete is both progressive and predictable, over hundreds to hundreds of thousands of years, and has been well documented (Candy et al., 2004; Candy et al., 2005). Although the rate of formation may vary, generally rapid formation of immature calcretes may occur within 1000 years, while mature calcrete formation requires 10 000 to 250 000 years (Candy et al., 2005). Initially, carbonate accumulates on the undersides of rock fragments, which continue to grow forming immature calcrete nodules one to two centimetres in diameter. Over time these nodules coalesce creating a hardened horizon of mature calcrete known as the basal layer, which may become brecciated. Finally, a super-mature laminated crust, representing the youngest layer, is precipitated on top of the basal layer (Candy et al., 2004; Candy et al., 2005). Moore and Picker (1991) provide a detailed description of mature heuweltjie structure, which includes both a hardened and brecciated basal layer and laminar crust. As such, the ages returned on their samples are not in agreement with the rate of mature calcrete formation and are quite likely too young. A plausible explanation for this error is the effect of re-crystallisation in calcretes. When runoff water, which may be highly unsaturated in bicarbonate, reaches the calcrete it dissolves the carbonate. As this excess moisture evaporates during dry seasons, the carbonate is re-precipitated thus introducing new carbon into the calcrete, which lowers the apparent age of the sample (Vogel & Geyh, 2008).

While Midgley et al., (2002) dated their samples to approximately 25 000 yrs BP (Table 2.1), which appears to be in better agreement with the formation of mature calcrete, their results also contain some anomalies. Two of their Elands Bay samples (3a & 3b in Table 2.1) were derived from a single heuweltjie. One sample came from the top of the basal plate and the other from the bottom of the plate, 7cm below it. However, there is scarcely any difference in age between the two samples. Midgley et al., (2002) suggest that perhaps the calcrete layer formed from the inside out, which would explain why the top and bottom of the plate have such similar ages. Yet, as discussed, calcrete forms predictably from the bottom up, and it is therefore highly improbable that the basal plate could have thickened by 7cm in such a short space of time. Both Elands Bay heuweltjies sampled were 3 to 4m above present-day sea level, but had previously been eroded by
the mid-Holocene high sea level. As both heuweltjies had already developed mature calcrete prior to their erosion, it suggests that their origins significantly predate the mid-Holocene (± 6000 yrs BP). This observation casts further doubt on the ages of heuweltjies reported by Moore & Picker’s study (1991).

In addition to radiometric dating, Midgley et al. (2002) also sampled organic and inorganic δ¹³C down the soil profile of the heuweltjies, which provides some particularly illuminating results. As δ¹³C fractionates differently under different photosynthetic pathways, it is possible to determine whether the major carbon contribution was derived from C₃ woody plants, or C₄/CAM grasses and succulents. Although there was a slight trend towards C₄/CAM derived carbon down the soil profile, their results indicate that the major significant carbon contribution was always derived from C₃ plants. A similar trend was noted by Potts et al. (2009).

Potts et al. (2009) found that the δ¹³C content of extant heuweltjie vegetation and fresh frass was analogous. Based on this direct relationship, they analysed 53 calcrete samples from a single heuweltjie, in order to examine historical levels of δ¹³C and δ¹⁸O. In addition, they performed radiocarbon dating on five calcrete samples from different parts and depths of the same heuweltjie (Table 2.1). Their results indicated that the heuweltjie calcrete ranged in age from 21 676 yrs BP, in the laminar crust, to 37 524 yrs BP in a large calcrete nodule. These dates are in general accordance with those reported by Midgley et al. (2002), and with the period of climatic conditions favorable for calcrete formation. However, it is surprising that the date range for the basal calcrete sample, at a depth of 200cm, is younger than the range reported for a calcrete nodule, at a depth 30cm. This anomaly aside, the main focus of the study was the analysis of historical δ¹³C and δ¹⁸O levels. Potts et al. (2009) found that that was a decline, from 35 to 15%, in the contribution of C₄/CAM plants over the period from 36 700 to 21 700 yrs BP, and a corresponding increase in C₃ plants. The change in δ¹⁸O, over the same period, indicated a shift to a colder, wetter environment.

If the calcrete dating, by Midgely et al. (2002) and Potts et al. (2009) is accurate at 25 000 – 35 000 yr BP, this would place the heuweltjie origins before or during the
LGM, when the Western Cape would have been both wetter and 5 - 7°C cooler than present-day temperatures (Partridge et al., 1999; Baker et al., 2001). This was a time of rapid C₄ dominated ecosystem expansion (Ehleringer et al., 1997), not decline as reported by Potts et al. (2009). The reduced levels of atmospheric CO₂ would have placed C₃ plants at a significant disadvantage compared to C₄/CAM plants, regardless of the low temperatures and winter rainfall in the western and southern Cape (Ehleringer et al., 1997). This is supported by data from the Boomplaas Cave in the southern Cape, which shows archaeological evidence of large grazing mammals from 80 000 to 25 000 yr BP (Chase & Meadows, 2007). Similarly, a study by Sealy (1996), which sampled fossilized bones and teeth of large grazers for stable isotope analysis, found that C₄ grasses accounted for a significant dietary component during the LGM. It is therefore likely that the dating of these heuweltjies is inaccurate and that the calcrete is actually younger than reported.

During the LGM, the Western Cape would likely have favoured grassland termite species such as *H. mossambicus*. There is some support for this provided by Coaton’s (1981) study, which dated subterranean fossilised non-heuweltjie hives at around 30 000 yrs BP. It is possible that these were *H. mossambicus* termitaria, as they also construct subterranean nests (Picker et al., 2002). During deglaciation, around 12 000 to 15 000 yrs BP, at the onset of the Holocene there was a shift to a warmer, more arid environment in the Western Cape (Baker et al., 2001). In accordance with this, there was a decline in C₄/CAM plants and an increased dominance of C₃ plants (Ehleringer et al., 1997). Grassland species, such as *H. mossambicus*, were possibly excluded to the eastern summer rainfall regions where they occur today, and the area became available for colonisation by shrubland species, such as *M. viator* (Duncan & Hewitt, 1989). Furthermore, the environment would have favoured the formation of calcretes, particularly during the more arid thermal maxima 6000 to 8000 yrs BP (Netterberg, 1969; Partridge et al., 1999; Chase & Meadows, 2007).
Dating *Heuweltjies*

Accurately dating *heuweltjies* however, still poses a challenge. While the age range for *heuweltjies* is within the upper limits of radiocarbon dating (60,000 yr BP) (Woods Hole Oceanographic Institute, 2013), it is the source material of basal calcrete which creates problems. Calcretes typically contain large amounts of detrital contamination, in the form of isotopic signals of different rocks (Candy *et al*., 2005), and are also susceptible to recrystallisation (Vogel & Geyh, 2008). As they form over many thousands of years, each phase may also contain a wide range of ages and, in order to increase accuracy, sub-samples of coeval carbonate fractions which have crystallised in situ, during the same period, must be analysed (Candy *et al*., 2004). This presents a further problem, as calcretes often contain no morphological evidence to indicate where contemporary phases occur (Candy *et al*., 2005). Radiometric carbon dating is not able to accommodate for these challenges and thus produces disparate results. Therefore an alternative method of dating needs to be employed.

U-series isochron techniques seem to provide the most suitable means by which to accurately date calcrete. The isochron technique is best able to correct for detrital contamination as well as assessing the reliability of the derived data (Candy *et al*., 2005). At the time of rock formation, Uranium, the ‘parent’ element, is present in abundance. Over time and at a known rate, the parent decays into the “daughter”, Thorium, which was not initially present (Faure, 1977). In order to construct an isochron, multiple samples must be extracted from a single horizon. Each sample is then totally dissolved and the isotopic ratios analysed. The ratio between the parent and daughter, $^{230}\text{Th}/^{238}\text{U}$, and the parent and another stable isotope of the same element, $^{234}\text{U}/^{238}\text{U}$, are plotted against the XYZ data, ($^{238}\text{U}/^{232}\text{Th}$, $^{230}\text{Th}/^{232}\text{Th}$, $^{234}\text{U}/^{232}\text{Th}$), the slope and shape of which can be used to correct for detrital contamination (Kelly *et al*., 2000). The reliability of the derived age can be established, by statistically determining the probability of fit and mean sum of weighted deviations of the data points to the isochron (Candy *et al*., 2005). If the fit is poor, it most likely indicates that the sample has undergone some diagenic alteration. In order to avoid this, very careful sampling needs to be performed. To precisely date mature calcrete, contemporary sub-samples must still be extracted, which is problematic. Therefore, as the isochron technique
assumes that sub-samples are contemporary, mature calcretes will tend to produce a less precise linear trend. The counter to this, is that as immature calcretes consist of an horizon with a limited age range, it is fairly straightforward to obtain highly accurate results for them (Candy et al., 2005).

**Conclusions**

Despite the on-going debate and concerns raised in recent research regarding the origins of *heuweltjies*, it is clear that the overwhelming evidence supports *M. viator* colonies as the architects of *heuweltjies*. From a biological perspective, it is challenging to reconcile the current range of possible *heuweltjie* ages with the potential longevity of a colony. However, primary reproductives tend to be quite long-lived, with records of queens surviving upwards of 20 years (Thorne et al., 2002; Promislow, 2003). When the king and/or queen dies, replacement reproductives, known as neotenics, develop and assume the role of reproduction, giving rise to what are known as ‘extended families’ (Vargo et al., 2003). Based on this strategy of reproductive replacement from within the colony, neotenics do afford a colony the potential to be very long-lived (DeHeer & Vargo, 2006). However, until *heuweltjies* have been more appropriately dated, the relationship between their age and colony longevity will remain something of a moot point. Therefore, I suggest that future attempts to date *heuweltjies* employ U-series isochron techniques. Since only fossilised tunnels and storage chambers, probably previously extending down from the central hive, are found within the basal layer (Moore & Picker, 1991), these would be the most accurate sites from which to sample the calcrete of mature *heuweltjies*, as they are likely to represent the oldest fossilised termitaria structures. U-series isochron dating would also provide an excellent means by which to date immature *heuweltjies* very accurately. Since *heuweltjies* less than 5m in diameter do not have a calcrete base (Moore & Picker, 1991), dating would allow us to track the rate of *heuweltjie* growth in accordance with age. Accurately determining the ages of *heuweltjies* would allow for a better understanding of biologically-initiated calcrete formation within South Africa, as well as of the life histories of their occupants, *M. viator*. 
References


Thorne, B. L., Breisch, N. L. & Haverty, M. I. 2002. Longevity of kings and queens and first time of production of fertile progeny in dampwood termite (Isoptera;

Van der Merwe, C.R. 1940. Soil groups and sub-groups of South Africa. *Scientific Bulletin* 231, Department of Agriculture, Pretoria.


Chapter 3

Construction of a Microsatellite Library for *Microhodotermes viator*

Introduction

Population structure depends upon numerous features in social insects, such as the sex-determination system, the number of reproductives and their mode of dispersal (Sanetra & Crozier, 2003). Furthermore, the amount of genetic differentiation observed among colonies is strongly associated with sociogenetic organization of a colony and the way in which new colonies are founded (Macaranas *et al.*, 2001; Viginier *et al.*, 2004; Bolton *et al.*, 2006; Zinck *et al.*, 2007). Quantification of gene flow and genetic variation, within and between species, is important to infer a biologically accurate image of population structure (Behura, 2006), thus genetic tools are useful in elucidating these mechanisms.

Microsatellite markers are considered an excellent approach to examining genetic structure and population history, and they are particularly well suited to studies of fine-scale structure (Vargo, 2000). They are easy to use, extremely prevalent in eukaryote genomes - up to 45% in Coleoptera (Caterino *et al.*, 2000) - generally neutral, have high variation and mutation rates and inform on many distinguishable loci with co-dominant alleles that can be unambiguously scored (Queller *et al.*, 1993; Luikart & England, 1999; Pedersen & Boomsma, 1999; Behura, 2006). Microsatellites are tandem repeats of short nucleotide sequences, on average one to six base pairs (bp) long. They tend to have high heterozygosity and many alleles per locus due to their high mutation rate (Queller *et al.*, 1993). The primary source of microsatellite polymorphism lies in the variation in the number of tandem repeats, resulting in allelic variation (Queller *et al.*, 1993; Gertsch *et al.*, 1995). Additionally, microsatellites can provide genetic data from very small amounts of biological sample, by using locus-specific primers and exponentially increasing the amount of target DNA by PCR amplification.
Microsatellite loci are particularly useful for studies of insect populations, which have relatively short generation times (Caterino et al., 2000). Within populations, they can be used to assign workers to specific colonies, establish pedigree and detect the presence of multiple unrelated reproductives within a colony (Vargo, 2003). They are also invaluable in elucidating colony structure, for example with regards to population size, and have been shown to be applicable in studies of fine-scale differentiation and local gene flow, via spatial distribution of alleles (Queller et al., 1993; Macaranas et al., 2001). Additionally, microsatellite loci can be used to investigate relationships between colonies such as kinship and levels of inbreeding. However, the high mutation rate of microsatellites may result in potentially misleading genetic information. For example, diploid males tend to exhibit higher mutation rates than diploid females of the same species, which has been attributed to the greater number of germline cells generated in males (Behura, 2006). Furthermore, microsatellite loci mutation rates vary widely based on repeat units, repeat length and flanking sequences (Behura, 2006); alleles with a greater number of sequence repeats will generally have a higher mutation rate than short alleles (Queller et al., 1993; Crozier et al., 1999). Despite these caveats, microsatellites remain one of the most well utilised tools for studies of population genetics.

Unfortunately, there are relatively few published microsatellite loci for termite species (Table 3.1) - one of the primary challenges being that appropriately variable microsatellite loci must first be identified and primers developed for them, before they can be utilised as a population genetics tool. However, with new and improved techniques and protocols, this process is becoming increasingly expedient and cost-effective (Glenn & Schable, 2005). Furthermore, primers developed for one species will often work for other species within the same genus (Pamilo et al., 1997; Vargo & Husseneder, 2011). For example, microsatellites identified in Zootermopsis nevadensis, were successfully amplified in both Z. augusticolis and Z. n. nuttingi (Aldrich & Kambhampati, 2004; Booth et al., 2012). Similarly, microsatellites developed for Reticulitermes flavipes, were successfully amplified in R. seperatus (Matsuura et al., 2004). It has even been noted that occasionally amplification is possible across different families. Goodisman et al. (2001) was able to successfully amplify loci Mdar2 and
Table 3.1 Polymorphic microsatellite loci identified in various termite species (amended from Vargo & Husseneder, 2011)

<table>
<thead>
<tr>
<th>Family/Species</th>
<th>No. of loci</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mastotermitidae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mastotermes darwinensis</td>
<td>10</td>
<td>Goodisman et al., 2001</td>
</tr>
<tr>
<td><strong>Termopsidae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zootermopsis nevadensis</td>
<td>10</td>
<td>Aldrich &amp; Kambhampati, 2004</td>
</tr>
<tr>
<td>Z. augusticolis &amp; Z. n. nuttingi</td>
<td>5</td>
<td>Booth et al., 2012</td>
</tr>
<tr>
<td><strong>Kalotermitidae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incisitermes minor</td>
<td>10</td>
<td>Indrayani et al., 2006</td>
</tr>
<tr>
<td>I. minor</td>
<td>15</td>
<td>Booth et al., 2008</td>
</tr>
<tr>
<td><strong>Cryotermes secundus</strong></td>
<td>6</td>
<td>Fuchs et al., 2003</td>
</tr>
<tr>
<td><strong>Rhinotermitidae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reticulitermes flavipes</td>
<td>9</td>
<td>Vargo, 2000</td>
</tr>
<tr>
<td>R. flavipes</td>
<td>11</td>
<td>Dronnet et al., 2004</td>
</tr>
<tr>
<td>R. speratus</td>
<td>7</td>
<td>Hayashi et al., 2002</td>
</tr>
<tr>
<td>R. hesperus</td>
<td>3</td>
<td>Copren, 2007</td>
</tr>
<tr>
<td>Coptotermes formosanus</td>
<td>12</td>
<td>Vargo &amp; Henderson, 2000</td>
</tr>
<tr>
<td>C. lacteus</td>
<td>6</td>
<td>Thompson et al., 2000</td>
</tr>
<tr>
<td>C. gestroi</td>
<td>11</td>
<td>Yeap et al., 2009</td>
</tr>
<tr>
<td><strong>Termitidae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amitermes meridionalis</td>
<td>10</td>
<td>Schmidt et al., 2007</td>
</tr>
<tr>
<td>Cubitermes subarquartus</td>
<td>7</td>
<td>Harry et al., 2001</td>
</tr>
<tr>
<td>Labiotermes labralis</td>
<td>6</td>
<td>Harry et al., 2007</td>
</tr>
<tr>
<td>Macrotermes michaelseni</td>
<td>4</td>
<td>Kaib et al., 2000</td>
</tr>
<tr>
<td>M. gilvus</td>
<td>11</td>
<td>Singham et al., 2012</td>
</tr>
<tr>
<td>Nasutitermes corniger</td>
<td>8</td>
<td>Atkinson et al., 2007</td>
</tr>
<tr>
<td>Odontotermes srinakarinensis sp. nov.</td>
<td>29</td>
<td>Cheng et al., 2013</td>
</tr>
</tbody>
</table>
Mdar4, developed for *Macrotermes darwinensis*, in *Coptotermes lacteus, Cryptotermes dudleyi, Neotermes insolaris, Porotermes adamsoni* and *Hodotermes mossambicus*.

To date, no specific microsatellite loci have been identified in any Hodotermitid species, although, as mentioned, two *M. darwinensis* loci were successfully amplified in *H. mossambicus* (Goodisman *et al.*, 2001). Therefore, the aim of this investigation was to first test several microsatellite loci developed for other species in *Microhodotermes viator* and, if these failed to amplify successfully, to develop microsatellite loci specifically for *M. viator*. This chapter details the development of a genetic tool, the implementation of which is described in Chapter 4. As such, the structure of this chapter deviates from standard scientific reporting and presents a combined methodology, results and discussion section.

**Materials, Methods, Results and Discussion**

*Testing of microsatellite loci developed for other termite species*

Primers developed for two other primitive termite families, Mastotermitidae (Goodisman *et al.*, 2001) and Termopsidae (Aldrich & Kambhampati, 2004), were tested for positive and correct amplification in *M. viator*. Primers were tested over a range of annealing temperatures (T_a) and magnesium chloride (MgCl_2) and primer concentrations (Table 3.2), on between five and ten individuals from different colonies. When bands in the correct size range were amplified, they were excised and sent for sequencing at the Central Analytical Facility at Stellenbosch University. The sequences were examined using Chromas Lite 2.1.1 by Technelysium Pty Ltd. Unfortunately, none of the bands were correct. Therefore, the decision was made to rather develop a microsatellite library specifically for *M. viator*.
### Table 3.2 Primers and PCR conditions tested for amplification in *M. viator*.

<table>
<thead>
<tr>
<th>Locus</th>
<th>( T_a (^\circC) )</th>
<th>( \text{MgCl}_2 ) (mM)</th>
<th>Primer concentration (( \mu \text{mol} / \mu l ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mdar 2</td>
<td>58</td>
<td>1.5</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>60.5</td>
<td>1.5, 2.25, 3</td>
<td>0.3, 0.3, 0.5</td>
</tr>
<tr>
<td></td>
<td>61</td>
<td>1.5, 2.25, 3</td>
<td></td>
</tr>
<tr>
<td>Mdar 4</td>
<td>67</td>
<td>1.5, 2.25, 3</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>67.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Zoot 28</td>
<td>45</td>
<td>1, 1.5, 2.25, 3, 3.5</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1, 1.5, 2</td>
<td>0.3, 0.5</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60.5</td>
<td>1.5, 2.25, 3</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>61</td>
<td>1.5, 2.25, 3</td>
<td>0.2, 0.3, 0.5</td>
</tr>
<tr>
<td>Zoot 29</td>
<td>67</td>
<td>1.5, 2.25, 3</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>67.5</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Zoot 73</td>
<td>45</td>
<td>1, 1.5, 2.25, 3, 3.5, 4</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>3</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>48.5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1, 1.5, 2, 3</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>60.5</td>
<td>1.5, 2.25, 3</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>61</td>
<td>1.5, 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>62</td>
<td>1.5, 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>63</td>
<td>1.5, 2.25, 3</td>
<td>0.3</td>
</tr>
<tr>
<td>Zoot 101</td>
<td>65</td>
<td>1.5, 2.25, 3</td>
<td>0.3</td>
</tr>
<tr>
<td>Zoot 117</td>
<td>65</td>
<td>1.5, 2.25, 3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

**Microsatellite library construction**

The microsatellite library construction was repeated twice, as an insufficient number of polymorphic microsatellite loci were identified after the first round. The first round was performed using the combined DNA of seven individuals, one from each of seven different colonies. The second round was performed using the DNA of a single individual, from an eighth colony. All procedures followed were the same for both rounds, and will therefore be discussed as a single process where appropriate.

(i) **Total genomic DNA extraction**

Total genomic DNA was extracted from eight individuals in total. Prior to extraction the head and gaster of each termite were removed and discarded, and the legs and thorax macerated using a sterile razor blade. The blade, forceps and glass work surface were all sterilized with 96% ethanol between each dissection. In round 1, the macerated tissue from the seven individuals was combined. Total genomic DNA was extracted using a
universal salt-extraction (Aljanabi & Martinez, 1997), with the inclusion of a chloroform/isoamyl alcohol step prior to the first centrifugation. The crude DNA extraction was then purified, using the Promega Wizard® SV Gel and PCR Clean-Up System, following the manufacturer’s instructions. The DNA yield for the combined sample was 732.9ng/µl, measured using a NanoDrop® ND-1000 Spectrophotometer and the associated software, NanoDrop Version 3.1.0.

(ii) Library construction

Microsatellite library construction followed the protocol of Glenn & Schable (2005). Total genomic DNA (20µl) was digested using 3µl of the restriction enzyme, Rsa I (Fig. 3.1a). The digest product was then ligated to double-stranded SuperSNX24 linkers in triplicate (Fig. 3.1b; Table 3.3). The linker-ligated DNA was then enriched using a mix of 3’ labelled Biotinylated oligonucleotide probes (Table 3.3). The Biotinylated labelled DNA fragments of interest were then captured using Streptavidin M-280 Dynabeads (Invirtogen™) and a Magnetic Particle Concentrator (Invirtogen™). The DNA was then released from the Dynabeads, captured and precipitated using 70% ethanol (EtOH). The pellet was re-suspended in Tris low-EDTA buffer (TLE). The enrichment process was then repeated using the final product from the first enrichment. The final product from this process is referred to as “pure Gold”. In order to increase the amount of “pure Gold” DNA, a PCR was done using the SuperSNX-24 primer (Fig. 3.1c).

![Fig. 3.1 Agarose gel (1.5%) electrophoresis of DNA stained with Ethidium Bromide. (a) Restriction enzyme digest product, against a 100bp ladder. (b) Linker ligation product, against a 100bp ladder. (c) Final product from the “Gold-enrichment” PCR, against a 100bp ladder.](image-url)
Table 3.3 Primers and 3’ labelled Biotinylated Oligonucleotides used for the construction of a microsatellite library.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence: 5’ – 3’</th>
<th>Number of base pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>SuperSNX24 (F)</td>
<td>GGT TAA GGC CTA GCT AGC AGA ATC</td>
<td>24</td>
</tr>
<tr>
<td>SuperSNX24+4P (R)</td>
<td>GAT TCT GCT AGC TAG GCC TTA AAC AAA A</td>
<td>28</td>
</tr>
<tr>
<td>3’ labelled Biotinylated</td>
<td>AG                     24</td>
<td></td>
</tr>
<tr>
<td>Oligonucleotides</td>
<td>TG                     24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AAC                    18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AAG                    24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AAT                    36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACT                    36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATC                    24</td>
<td></td>
</tr>
</tbody>
</table>

The product from the “Gold-enrichment” PCR was then ligated into a plasmid, using the Promega pGEM®–T Easy Vector System, following the manufacturer’s instructions. The plasmid DNA was then transformed into *E. coli* JM109 competent cells, using the standard heat-shock method, which were spread on LB Ampicillin + agar plates, spread with X-gal and IPTG (Promega, 2010). The plates were then incubated at 37°C for 24 hours. Positive white colonies were then picked from the plates and each colony was placed in a separate well on a 96 well microtitre plate and grown in a medium of LB + Amp at 37°C for 24 hours, with vigorous shaking. A colony PCR was done using M13 primers, following the protocol of Glenn & Schable (2005). The PCR product was then run on an agarose gel (Fig. 3.2) and bands in the appropriate size range (300 – 1000bp) were cut out. The excised bands were then run through the Promega Wizard® SV Gel and PCR Clean-Up System, following the manufacturer’s instructions.
The purified colony PCR products (102 in total) were sent for sequencing at the Central Analytical Facility at Stellenbosch University. The sequences were edited using Chromas Lite 2.1.1 by Technelysium Pty Ltd. In total, 27 unique microsatellite loci were identified using WebSat (Martins et al., 2009).

(iii) Primer design and testing

Appropriate primer sequences were identified using WebSat (Martins et al., 2009). Non-fluorescently labelled primer pairs were ordered from the Synthetic DNA Laboratory at the University of Cape Town. The primers were tested for positive and correct amplification, by PCR, and I was able to successfully optimise amplification of 21 of the 27 pairs. The forward primer of each of the 21 pairs was then remade with either a HEX, FAM or NED fluorescent label, by the Synthetic DNA Laboratory at the University of Cape Town (UCT).

The PCR’s were repeated using the fluorescently labled primers and DNA extracted from 17 individuals\(^5\), one from each of 17 discrete colonies (5 from Worcester, 6 from Tygerberg and 6 from Darling) across the geographical distribution, not previously sampled in this study. The PCR products were genotyped to identify polymorphisms, on an ABI PRISM® 3100 Genetic Analyser. The genotyping results were visualised using

\(^5\) These 17 individuals form the Background Population described in Chapter 4

Fig. 3.2 Agarose gel (1.5%) of colony PCR product stained with Ethidium Bromide, against a 1Kb ladder (very faint). The double white lines indicate the desired product size range, from 500 to 1200bp.
ABI Peak Scanner™ v1.0 Software. Of the 21 loci tested, seven were found to be polymorphic (Table 3.4).

Table 3.4 Characteristics of seven polymorphic microsatellite loci and their primer sequences, for Microhodotermes viator.

<table>
<thead>
<tr>
<th>Locus</th>
<th>No. of alleles amplified</th>
<th>Repeat Motif</th>
<th>Forward Primer (5’ – 3’)</th>
<th>Reverse Primer (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mt4</td>
<td>2</td>
<td>(AC)7</td>
<td>TCCTTCAAACCACTATCTTGAGC</td>
<td>CTTATCAGCCCATATCCAGTCA</td>
</tr>
<tr>
<td>Mt14</td>
<td>2</td>
<td>(AAC)4</td>
<td>AAGGAATTGACAACCGCTCAA</td>
<td>TCATCAACCAATCAAGTAGGC</td>
</tr>
<tr>
<td>Mt17</td>
<td>12</td>
<td>(TG)12</td>
<td>CAATTCCTCCAGTTTCTGAT</td>
<td>AAGTCCCTTAGCGCTTTCTGC</td>
</tr>
<tr>
<td>Mt18</td>
<td>14</td>
<td>(AC)10</td>
<td>AGCCAGTCGTATTCGCTGTC</td>
<td>AGGAACTCTTATAAATGCAA</td>
</tr>
<tr>
<td>Mt21</td>
<td>2</td>
<td>(AC)11</td>
<td>TGTCCTGAAATAAGCTTCCAC</td>
<td>ATCTGCTTGTGTGGGTTTCTC</td>
</tr>
<tr>
<td>Mt23</td>
<td>2</td>
<td>(AGA)5</td>
<td>GGAAGACGAGATGTTGCAAGAT</td>
<td>ACAAGTGTTAGACGCTGGAAT</td>
</tr>
<tr>
<td>Mt25</td>
<td>7</td>
<td>(TAC)14</td>
<td>CATACACACTGAGGGACAAACA</td>
<td>GTTTGGAACCGGCAACAGATA</td>
</tr>
</tbody>
</table>

The seven microsatellites identified are polymorphic, with allele numbers ranging from 2 to 14, for an average of 5.8 alleles per locus. This present set of microsatellites in *M. viator* is sufficiently polymorphic for fine-scale population genetic studies to be performed.

Unfortunately, due to time and budget constraints, it was not possible to test for cross-amplification in other Hodotermitid species, which is a worthwhile avenue for future research. Should these microsatellites prove to be mutable across the Hodotermits, they will be extremely valuable in aiding our understanding of this poorly studied primitive family of harvester termites.
References


Chapter 4

Colony and Population Genetic Structure of Microhodotermes viator

Introduction

Sociogenetic studies of eusocial insects are generally based on the premise that a colony functions as a discrete entity within the population (Thorne et al., 1999). However, as individuals form colonies and colonies form populations, the genetic structure of a colony is closely linked to that of the population (Vargo & Husseneder, 2011). As such, the purpose of population genetics studies is to quantify and explain the features and processes generating variation in natural populations (Vargo & Husseneder, 2011). In terms of eusocial insects, these features and processes include; the number of reproductives and their mode of dispersal, colony population size, the spatial extent of colonies and genetic variation within and between colonies and historical features, such as selection and drift (Hussender et al., 1998; Thorne et al., 1999; Sanetra & Crozier, 2003; Vargo & Husseneder, 2011).

Given their system of secondary reproductives, a termite colony is a long-lived group of individuals sharing various degrees of kinship. Thus, colonies can be considered to be small populations of related, interbreeding individuals, genetically isolated from neighbouring colonies (Reilly, 1987). Owing to the sedentary nature of insect colonies, gene flow tends to be restricted to the period of dispersal prior to colony founding. The amount of differentiation observed is strongly associated with the sociogenetic organization of a colony, making the mode of colony foundation of utmost importance in determining the population structure (Pamilo et al., 1997; Macaranas et al., 2001; Viginier et al., 2004; Bolton et al., 2006; Zinck et al., 2007).

Little is known about M. viator and there are no data on the population structure of this species. Therefore, the following section presents a general review of the modes of
colony foundation, family structure, inbreeding, colony growth and longevity in termites.

**Colony founding**

Most termite colonies follow the same general mode of colony foundation. Primary winged reproductives, known as alates, are released as a swarm from their natal colonies for a nuptial flight. Swarming is the primary means of dispersal for most termite species, the timing of which is typically dependent on both seasonal and internal factors (Nutting, 1969; Bordereau & Pasteels, 2011). Kok & Hewitt (1990) found evidence indicating that in *H. mossambicus* alate release occurs primarily in October. Similarly, Coaton & Sheasby (1974) observed that in *M. viator*, alate release also occurs primarily in October, but can extend to May, with nuptial flights taking place within 24 hours after a rainfall event.

Dispersal distance is somewhat variable as alates are generally thought to be weak fliers, and active flight seems to be limited to around 500m (Vargo *et al*., 2003; DeHeer & Vargo, 2006; Vargo & Husseneder, 2011). However, factors such as prevailing winds may significantly increase the dispersal distances - a study of *Macrotermes michaelseni* found alates could travel up to 50 kilometres from their natal nest by passive drift alone (Brandl *et al*., 2005). Subsequent to a nuptial flight, male and female alates, which are usually unrelated, join up to form primary reproductive pairs (Thorne *et al*., 1999; Vargo, 2003; Vargo *et al*., 2003; Deheer & Vargo, 2004; Brandl *et al*., 2005; Vargo & Husseneder, 2011). Hacker *et al.* (2005) found that sterile offspring from a monogamous *M. michaelseni* pair showed relatedness close to $r = 0.5$, and the mean relatedness sampled between kings and queens of the species was zero. Similarly, in a study on *Reticulitermes flavipes*, it was found that simple family colonies showed no significant inbreeding, indicating that that the primary pair of reproductives was outbred (Deheer & Vargo, 2004). Once the pair has established themselves at their chosen nest site, copulation commences (Nutting, 1969). This mode of colony foundation gives rise to simple families.
Simple families

Simple families tend to be young colonies, where all the workers are the offspring of the primary reproductive pair. Consequently, the colony population exhibits no more than four different alleles or genotypes at any microsatellite locus (Thorne et al., 1999; Vargo, 2003; Deheer & Vargo, 2004; Vargo & Husseneder, 2011). In a South Carolina population of *R. flavipes*, simple families accounted for 85% of the colonies (Vargo & Husseneder, 2011). Over time, one or both primary reproductives will die and be replaced by non-alate derived secondary reproductives, known as neotenics (Vargo, 2003). Accordingly, inhibition of secondary reproductives is generally thought to be due to the presence of primary reproductives (Thorne et al., 1999). As neotenics are non-winged, they do not disperse, but instead mate at the nest (Leniaud et al., 2010), giving rise to extended families.

Extended families

Extended families are those headed by neotenics, which are descended from the original pair of monogamous reproductives (Vargo et al., 2003). Consequently, the colony population still exhibits no more than four alleles at any microsatellite locus, but, due to inbreeding, may exhibit more than four genotypes per locus (Vargo, 2003; Vargo & Husseneder, 2011). Alternatively, if only four genotypes are expressed, they may be at frequencies inconsistent with simple Mendelian segregation of alleles (Deheer & Vargo, 2004). In a study on a North Carolina population of *R. flavipes*, Vargo (2003) found that 23% of colonies were extended families, generally headed by three neotenics – two females and a male. Microsatellite analysis, and the presence of a single mitochondrial haplotype per a colony, confirmed that the neotenics were the direct decedents of the primary reproductive pair. Neotenics are the most common form of reproductives and mature colonies tend to be headed by a variable number, generally ranging from a few to a few dozen, but in rare cases up to several hundred (Thompson & Herbert, 1998; Thorne et al., 1999; Vargo et al., 2003; Deheer & Vargo, 2004). Primary reproductive females are generally very physogastric, capable of producing thousands of eggs a year, however when the queen dies and is replaced, no neotenic can achieve the same level of
ovarian development and therefore often more than one neotenic is required to ensure optimal and continued colony growth (Lenz, 1985). For example, in the genus *Nasutitermes*, where colonies may consist of up to 500,000 individuals, there can be hundreds of neotenics present simultaneously (Thompson & Herbert, 1998). When the need for neotenics arises within a colony, they are generally produced in greater numbers than they are required. Excess neotenics are then eliminated via the combined efforts of more dominant neotenics and the rest of the colony (Lenz, 1985). The threshold at which elimination of neotenics begins varies from one colony to another, and depends on colony size and condition, resource availability and ambient temperature (Lenz, 1985). Additionally, these factors also seem to determine the final number of neotenics which the colony will support and maintain (Lenz, 1985). Based on this strategy of reproductive replacement from within the colony, neotenics give a colony the potential to be very long-lived (DeHeer & Vargo, 2006). However, it must be noted that while common, not all species will produce neotenics, and extended families occur far more frequent among the lower termites and have been documented in *Schedorhinotermes*, *Reticulitermes*, *Mastotermes* and *Coptotermes* (Hacker et al., 2005; Vargo & Husseneder, 2011). In a study by Brandl et al., (2005) on Kenyan populations of *M. michaelseni*, no neotenics were found to be present in any of the colonies sampled.

*Mixed families*

In addition to simple and extended families, a third strategy of mixed families may also be employed by termite colonies. Mixed families are those headed by three or more unrelated reproductives and, consequently, the colony population exhibits more than four different alleles per a microsatellite locus (Deheer & Vargo, 2004; Vargo & Husseneder, 2011). Mixed families are generally rare, but have been documented in several species such as *Reticulitermes grassei* (Clement et al., 2001), *Nasutitermes corniger* (Atkinson & Adams, 1997) and *Mastotermes darwiniensis* (Goodisman & Crozier, 2002), via the presence of multiple mitochondrial haplotypes, indicating that females who currently serve, or have previously served, as reproductives may not be closely related (Deheer & Vargo, 2004). Furthermore, although rare, mixed families
may occur at high frequencies in particular populations or species (Deheer & Vargo, 2004; Vargo & Husseneder, 2011). For example, the presence of mixed families has been found at 33% in Zootermopsis nevadensis nuttingi (Aldrich & Kambhampati, 2007), 27% in M. darwiniensis and 20% in M. michaelseni (Brandl et al., 2005). There are three main mechanisms, which may function individually or collectively, leading to the formation of mixed families, namely; pleometrotic associations, adoption of unrelated reproductives or colony fusion (Thorne et al., 1999; Vargo & Husseneder, 2011).

Pleometrosis is the association between multiple primary reproductives, which cooperatively found and reproduce within a colony (Nobre et al., 2008). Pleometrotic associations are generally due to polygyny (Roy et al., 2010), and have been found in some termitids (Vargo & Husseneder, 2011). In M. michaelseni, 20% of the colonies had an additional female primary reproductive (Brandl et al., 2005). Although unrelated queens can stably coexist, not all queens may contribute equally to reproduction (Hacker et al., 2005). In the case of adoption, immediately after swarming, lone alates may infiltrate and be accepted by mature colonies (Reilly, 1987; Vargo et al., 2003; Deheer & Vargo, 2004). In a study of R. grassei, all mixed families were consistent with formation via either pleometrosis or adoption (Nobre et al., 2008). Alternatively, unrelated reproductives may be united through colony fusion, which has been shown in both Reticulitermes seperatus and R. flavipes (Vargo & Husseneder, 2011). However, in order for adoption to occur or for two colonies to fuse, the process would quite likely need to be facilitated by a breakdown in the ability to discriminate between nestmates and non-nestmates, and/or a lack of intercolonial aggression, as was demonstrated in R. flavipes (Reilly, 1987; Vargo et al., 2003; Deheer & Vargo, 2004). Within mixed families, the reduced average level of intracolonial relatedness results in a decrease in accrued fitness benefits by workers (Vargo & Husseneder, 2011), and although relatedness may still be sufficiently high enough to maintain colony integrity (Deheer & Vargo, 2004), the motivation behind the development of mixed families warrants some consideration.
Sociobiological theory postulates that insect societies are generally closed, however the cost of defending resources or engaging in agonistic behaviour may be greater than the costs incurred by sharing, thus facilitating potential mixing of colonies (Nobre et al., 2008). Some authors have proposed that abiotic factors may play a role in facilitating mixed family formation. For example, a study conducted on *M. michaelseni* in Kenya found that polygyny increased with decreasing rainfall (Brandl et al., 2004). As such, increased genetic variation may mitigate the effects of extreme environmental conditions (Brandl et al., 2005). Another theory explaining the development of mixed families is the ‘parasite hypothesis’. This hypothesis proposes that susceptibility to parasites is genetically variable and therefore more genetically heterogeneous colonies should be less susceptible to parasites (Liersch & Schmid-Hempel, 1998; Schmid-Hempel & Crozier, 1999; Brandl et al., 2005; Calleri et al., 2006). Improved parasite resistance is important as the negative effects of parasitism have been shown to include increased worker mortality, decreased ovary size and a reduction in early colonial growth (Liersch & Schmid-Hempel, 1998). The parasite hypothesis has been well-documented in ants, honey bees and bumble bees, where it has been shown that for a wide range of parasite types, irrespective of their mode of transmission, average relatedness between individuals within the population correlate positively with parasite number (Liersch & Schmid-Hempel, 1998). A study by Calleri et al., (2006), reared laboratory colonies of *Zootermopsis angusticollis* headed by either inbred siblings pairs or outbred pairs of primary reproductives. When the two colonies types (inbred & outbred) were exposed to a high pathogen dosage, outbred termites survived significantly longer than inbred termites, and their cuticular microbial load was approximately half that of termites from inbred colonies. However, when the cellular immune response of individuals from both colony types was tested using a nylon implant, the results indicated that there was no significant difference in their physiological responses. Calleri et al. therefore postulated that, as levels of both cuticular and internal pathogens have been shown to decrease after bouts of mutual grooming, a reduction in heterozygosity may have impacted on the efficacy of social mechanisms of disease resistance. Additionally, within ants, a diverse array of glands is known to produce anti-bacterial and/or anti-fungal secretions and variation in the size of the glands, and volume of secretion, has been shown to have a genetic basis (Schmid-
Hempel & Crozier, 1999). Therefore, it is likely that increased genetic variation may impact on several factors which collectively function to improve pathogen resistance. However, despite the mitigation against environmental conditions and improved pathogen resistance, mixed family colonies do not appear to be stable over long periods of time. Studies seem to indicate that they may be short-lived and colonies could revert back to separate family groups or reproductives from one of the colonies may be executed (Vargo & Husseneder, 2011). DeHeer & Vargo (2008) found that in the majority of fused colonies of R. flavipes there was no inbreeding between the reproductives of the previously separate colonies. This trend continued over a two year period and led them to postulate that the colonies may have been orphaned. Although, whether this occurred prior to fusion – thus facilitating a breakdown in colony integrity – or subsequent to the fusion, is impossible to say. While mixed families may be generated via colony fusion, independent colonies may be generated by colony fission. Thus the converse process, budding, maybe important in the genesis of colonies.

**Budding**

Budding is an alternative mode of colony foundation, which has been well documented in ants (Boomsma et al., 1995; Hussender et al., 1998; Sanetra & Crozier, 2003; Viginier et al., 2004; Zinck et al., 2007), but is not common among termites (Brandl et al., 2005; Husseneder et al., 2005; Roy et al., 2006; Vargo & Husseneder, 2011), although it may occur more frequently in multiple-site nesting termites, such as subterranean species (Vargo, 2003). As a termite colony grows and expands its foraging range, groups of foragers and satellite nests can become separated from the central nest. Consequently, primary reproductives may not be able to suppress the development of neotenics in spatially distant nests, thus resulting in new, reproductively-active colonies (Hussender et al., 1998; Vargo et al., 2003; Vargo, 2003; Husseneder et al., 2005). In a species or population where budding is common, one would expect to find a relatively high proportion of extended families and high population viscosity (Vargo, 2003; Brandl et al., 2005). Schedorhinotermes lamanianus have polygynous, polycalic colonies (where multiple nests comprise a single colony) with a dispersed nest structure, which may extend for up to 100m. In colonies of this size, subsidiary nests often have
their own distinct genetic profile (Hussender et al., 1998). For the Formosan subterranean termite, *Coptotermes formosanus*, colonies tend to begin as simple families and, as the colony grows, they can expand their subterranean foraging area up to 50m, with tunnels and satellite nests. In their 2005 study, Husseneder et al. found evidence of genetic structuring across these large extended family colonies. Therefore, it seems that among termite species, budding is less of an explicit strategy of colony foundation, and rather occurs more as a secondary result of extensive colonial growth.

**Inbreeding**

The life history of many termite species is characterised by having cycles of outbreeding and inbreeding (Calleri et al., 2006). Inbreeding is thought to promote the evolution of eusociality and altruism, as it increases within-family relatedness and between-family genetic variance, relative to within-family variance (Reilly, 1987; Hacker et al., 2005; DeHeer & Vargo, 2006). Inbreeding may occur at the time of colony foundation and/or due to neotenics reproduction (Reilly, 1987; Thompson & Herbert, 1998; DeHeer & Vargo, 2006; Roy et al., 2010). At the time of colony foundation, inbreeding may be due to asynchronous swarming, non-random assortment of flown alates or limited dispersal distances (Reilly, 1987; Thompson & Herbert, 1998; DeHeer & Vargo, 2006). When asynchronous swarming occurs, there may be a drastically reduced number of unrelated mates available, leaving alates little option but to inbreed (DeHeer & Vargo, 2006). Likewise, as dispersal distances affect the likelihood that closely related individuals will encounter one another during the nuptial flight, restricted dispersal can lead to non-random assortment of flown alates and local mate competition (Pamilo et al., 1997; DeHeer & Vargo, 2006; Vargo & Husseneder, 2011). Therefore, limited dispersal distances can lead to clear genetic structuring at the population level (Vargo & Husseneder, 2011). Inbreeding, due to neotenic reproduction, is a standard reproductive strategy employed by many termite species, resulting in increased levels of relatedness, which may in turn promote cooperation within colonies (Roy et al., 2010). However, many of the assumptions about the ubiquitous employment of inbreeding as a reproductive strategy may have been premature.
Recent evidence suggests that inbreeding may not be as extensively utilised by termite as previously thought, and in fact inbreeding depression may occur (Vargo & Husseneder, 2011). Colony breeding structures are likely sensitive to environmental conditions, and as such there may be ecological factors selecting against inbreeding (Brandl et al., 2004; Vargo & Husseneder, 2011). Several studies indicate that mate-seeking alates actively avoid pairing with nestmates (Bordereau & Pasteels, 2011). For example, kin discrimination during mate choice has been observed in Z. nevadensis, with active inbreeding avoidance (Vargo & Husseneder, 2011). Alternatively, when sibling alates do pair up, their union and/or colony may not be long-lived. Deheer and Vargo (2006) found that the likelihood of Reticulitermes virginicus and R. flavipes alates pairing with siblings was inversely proportional to the dispersal abilities of these two species. Furthermore, when they compared the proportion of sibling pairs among newly released alates, relative to the number of mature colonies headed by a primary sibling pair, they found a significant decrease. This suggests that some mechanism may be acting on inbred pairs and/or their colonies early during establishment to eliminate them from the population. Additionally, many studies show no clear fitness advantage in outbred versus inbred offspring, although advantages may exist, such improved parasite resistance (Calleri et al., 2006), which only become apparent under stressful conditions (DeHeer & Vargo, 2006). It appears therefore that inbreeding, while common, should not be assumed and ecological factors may be acting to facilitate inbreeding depression.

Colony growth and longevity

In monogamous species males and females tend to have concordant strategies to maximise fitness, with low levels of sexual conflict, resulting in increased longevity and lower rates of aging (Promislow, 2003). In eusocial species queens may be very long-lived, with survival of upwards of 20 years being documented in some ant species, which has been attributed to the highly sheltered nest environment (Promislow, 2003). The average lifespan of termite queens is unknown, however in Reticulitermes species it has been estimated at between six and ten years (Thorne et al., 1999; Grube & Forshler, 2004). Interestingly, Matsuura et al., (2009) found that in 19 out of 21 R.
seperatus colonies, while the primary queen had been replaced, the primary king was still retained, suggesting that kings may have an even longer lifespan than queens. However, given the relatively short lifespan of reproductives, and the rate of reproduction, it raises questions as to how very large colonies, of tens or hundreds of thousands of workers, are produced. In a study of 103 R. flavipes colonies, it was found that growth rates, over a two year period, were similar for colonies headed by either a pair or multiple reproductives (Grube & Forschler, 2004). Another study, using laboratory colonies of R. flavipes, found that over a period of two years, census population size reached between 50 and 1000 individuals (Thorne et al., 1999). Naturally, growth rates within a colony are likely to increase exponentially over time, as the increasing number of workers is able to facilitate increased egg production. However, there is almost certainly some upper limit on egg production, probably as a function of queen fecundity (Thorne et al., 1999). While primary reproductive females are thought to be very physogastric, neotenics cannot achieve the same level of ovarian development, therefore multiple neotenics are usually required to ensure optimal and continued colony growth (Lenz, 1985).

Another reproductive strategy which may be employed to increase colony growth is that of asexual queen succession. This phenomenon has been well documented in R. seperatus where secondary queens, or neotenics, are related to the primary queen by a value of $r = 0.99$, and yet are completely unrelated to the primary king (Matsuura et al., 2009). The benefits of this system appear to be two-fold. Firstly, the primary queen is able to dramatically increase her reproductive output, while incurring minimal costs to her fitness. Secondly, matings between the primary king and the asexually produced neotenics are essentially equivalent in the level of fitness and inbreeding to offspring produced by mating between the primary king and queen. This was confirmed by the fact that within these colonies, all workers and alate nymphs were still produced by sexual reproduction and that heterozygosity among workers remained high (Matsuura et al., 2009). The evolution of a strategy such as asexual queen succession lends further credence to the idea that in order to produce very large colony populations, primary reproductives and, by extension, termite colonies must be long-lived, and furthermore that neotenics play a fundamental role in ensuring colony longevity.
Population genetics of Microhodotermes viator

There is a dearth of information on colony founding and population genetic structure of *M. viator*, and hence no published studies regarding these important life history traits in this species. Given that *M. viator* is a keystone species in the winter rainfall succulent karoo and fynbos biomes of South Africa and given the potential longevity and ecological importance of *heuweltjies*, this chapter aims to elucidate a general understanding of colony and population genetics of *M. viator*.

Using microsatellites, I will examine family structure, genetic variation, diversity, variance and differentiation, inbreeding coefficients and relatedness within and between colonies. As termite colonies are generally closed (Reilly, 1987), it is likely that I will find moderate to strong differentiation between colonies. However, as alates tend to be weak fliers (Vargo *et al*., 2003; DeHeer & Vargo, 2006; Vargo & Husseneder, 2011), I also anticipate evidence of mild population viscosity and moderate to strong differentiation between geographical areas.

Recent data indicate that many termite species actively avoid inbreeding (Vargo & Husseneder, 2011) and populations of colonies often synchronise alate release (DeHeer & Vargo, 2006), evidence of which has been found in *H. mossambicus* (Kok & Hewitt, 1990). As such, I anticipate that the primary reproductives in *M. viator* will likely be outbred and there should be little evidence of inbreeding within colonies, particularly those which are simple families.

Variable family types tend to reflect the process of colony foundation, growth and maturation over time (Vargo & Husseneder, 2011), and extended families are common among the ‘lower’ termites (Lenz, 1985). Therefore, based on the allele and genotype frequencies within colonies, I expect to find a mix of family types.

Finally, as budding is uncommon among termite species (Brandl *et al*., 2005; Husseneder *et al*., 2005; Roy *et al*., 2006; Vargo & Husseneder, 2011), and *M. viator* has well-defined territories with a consistent intercolonial distance (Coaton & Sheasby,
Lovegrove & Siegfried, 1989; Midgley & Musil, 1990; Lovegrove, 1991; Moore & Picker, 1991; Laurie, 2002; Picker et al., 2007; Cramer et al., 2012), it is unlikely that I will find any evidence of budding occurring in *M. viator*.

**Materials and Methods**

**Sample collection**

The collection of *M. viator* samples is challenging for several reasons. Firstly, although nests are readily identifiable in the field due to the change in vegetation which occurs above the termitaria (Midgley & Musil, 1990; Lovegrove, 1991; Corinna et al., 2005), there are no surface structures to indicate where the main hive is located. Additionally, any disturbances, such as an attempt to dig into the hive, only result in sending the termites deeper into the nest structure, which extends 1-3m below the surface (Moore & Picker, 1991). Furthermore, given the volume of mature termitaria, attempts to excavate them using earth-moving equipment have unfortunately resulted in the complete destruction of the colony (Picker, pers. comm.). Therefore, the only opportunity to sample a large number of individuals arises when workers engage in *en masse* foraging activities. However, emergence of *M. viator* foragers is known to be unpredictable (Dean, 1993), as such foraging colonies were opportunistically identified by walking through renosterveld vegetation rich areas on days when the climatic conditions were suitable for foraging. By this method, twelve discrete colonies were identified and sampled from across four distinct geographical areas - Tygerberg, Malmesbury, Darling and Worcester - in the Western Cape of South Africa, from May to July 2011 (Table 4.1 and Fig. 4.1). Colonies T1 and T2 were located within the Tygerberg Nature Reserve and a permit was obtained in order to sample them. Twenty to thirty individuals from each colony were returned to the laboratory and stored at -20°C prior to genetic analysis.

For purposes of genetic comparison and to increase the sample sizes for the population-level statistical analyses, a Background Population (BGP) was also collected. The BGP was comprised of 17 individuals, one from each of 17 discrete colonies (5 from
Worcester, 6 from Tygerberg and 6 from Darling) across the geographical distribution, not previously sampled in this study.

Table 4.1 Geographic distribution of twelve *M. viator* colonies, sampled for genetic and behavioural analysis.

<table>
<thead>
<tr>
<th>Area</th>
<th>Colony Name</th>
<th>GPS coordinates (decimal degrees &amp; minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tygerberg</td>
<td>T1</td>
<td>33.87471°S 18.5960°E</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>33.87827°S 18.59659°E</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>33.85188°S 18.60552°E</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>33.85479°S 18.59888°E</td>
</tr>
<tr>
<td>Malmesbury</td>
<td>M5</td>
<td>33.45976°S 18.74372°E</td>
</tr>
<tr>
<td></td>
<td>M6</td>
<td>33.46712°S 18.74000°E</td>
</tr>
<tr>
<td>Darling</td>
<td>D8</td>
<td>33.37391°S 18.37657°E</td>
</tr>
<tr>
<td></td>
<td>D9</td>
<td>33.38763°S 18.38231°E</td>
</tr>
<tr>
<td></td>
<td>D10</td>
<td>33.38926°S 18.38229°E</td>
</tr>
<tr>
<td>Worcester</td>
<td>W7</td>
<td>33.60986°S 19.44442°E</td>
</tr>
<tr>
<td></td>
<td>W11</td>
<td>33.62693°S 19.43371°E</td>
</tr>
<tr>
<td></td>
<td>W12</td>
<td>33.62689°S 19.43435°E</td>
</tr>
</tbody>
</table>

Fig. 4.1 Satellite image of the Western Cape of South Africa showing the location of twelve colonies of *M. viator* (Image source: Google™ Earth). Elevation approximately 172 km.
Nucleic acid extraction

Total genomic DNA was extracted from each individual, following the manufacturer’s instructions for the ‘Dilution Protocol’, from the Phire® Animal Tissue Direct PCR Kit. Two modifications were made to the Dilution Protocol. Firstly, the extraction was performed using one macerated leg from each individual. Secondly, the sample was incubation at the elevated temperature of 65°C for five minutes, before continuing to the standard denaturation step of 98°C for two minutes. It was not possible to accurately quantify the DNA concentrations yielded by the extraction procedure, as particular reagents in kit’s extraction buffer interfered with the NanoDrop® ND-1000 Spectrophotometer readings.

Microsatellite amplification

(i) PCR

Approximately 30 workers from each of the 12 colonies, as well as the 17 workers comprising the BGP, were screened for microsatellite amplification at seven microsatellite loci: Mvit 4, Mvit 14, Mvit 17, Mvit 18, Mvit 21, Mvit 23 and Mvit 25 (Table 4.2).

The forward primer of each locus was fluorescently labeled with HEX, FAM or NED. DNA amplification reactions were performed in 0.2ml PCR tubes in 20µl reaction volumes containing the following reagents: 0.4µl Phire® Hot Start ll DNA Polymerase, 10µl 2X Phire® Animal Tissue PCR Buffer (which includes dNTP’s and MgCl₂), 0.12µl each of fluorescently labeled forward primer & reverse primer (to a final concentration of 0.3µmol/µl), 8.36µl Millipore water and 1µl of template DNA. For each PCR performed the master mix cocktail was assembled and aliquotted out into individual sample reaction tubes in a DNA-free environment, prior to addition of template DNA to each reaction volume. Two blank reactions, containing no DNA, were included in each PCR performed, in order to control for any possible DNA contamination during either the assembly or amplification process. The first blank was
not opened once it had been brought from the DNA-free environment into the laboratory. The second blank was opened in the laboratory, during addition of DNA to the sample tubes. This protocol allowed me to immediately identify the source of contamination, should it have occurred, as either my reagents or acquired from the laboratory environment.

Thermal cycling was performed on an ABI GeneAmp® PCR System 2700. The cycling profile consisted of an initial denaturizing step for 5 minutes at 98°C followed by 40 cycles composed of 98°C for 5 seconds, $T_a$ for 5 seconds (Table 4.2) and extension at 72°C for 20 seconds, followed by a final extension step at 72°C for 1 minute. Each PCR reaction was stopped with a rapid cool-down to 4°C. Samples were maintained at 4°C prior to electrophoresis.

Table 4.2 Characteristics of microsatellite loci amplified in *Microhodotermes viator*. Allelic size range based on the genotypes of 369 individuals

<table>
<thead>
<tr>
<th>Locus</th>
<th>Fluorescent Label</th>
<th>$T_a$ (°C)</th>
<th>Size Range (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mvit 4</td>
<td>HEX</td>
<td>54</td>
<td>129 - 131</td>
</tr>
<tr>
<td>Mvit 14</td>
<td>NED</td>
<td>53</td>
<td>370 - 373</td>
</tr>
<tr>
<td>Mvit 17</td>
<td>HEX</td>
<td>55</td>
<td>156 – 198</td>
</tr>
<tr>
<td>Mvit 18</td>
<td>HEX</td>
<td>55</td>
<td>302 - 346</td>
</tr>
<tr>
<td>Mvit 21</td>
<td>FAM</td>
<td>55</td>
<td>119 - 121</td>
</tr>
<tr>
<td>Mvit 23</td>
<td>FAM</td>
<td>55</td>
<td>145 - 148</td>
</tr>
<tr>
<td>Mvit 25</td>
<td>HEX</td>
<td>55</td>
<td>209 - 239</td>
</tr>
</tbody>
</table>

(ii) Agarose gel electrophoresis

All PCR products were electrophoresed on 1.5 to 2% agarose gels, and visualised after ethidium bromide staining. A 100bp DNA standard was also electrophoresed on each gel to determine that both positive and correct amplification had occurred and the appropriate dilution for each sample in the following genotyping step (Sambrook *et al*., 1989).
(iii) Genotyping

The appropriate concentration of each PCR product to use was determined from the intensity of the DNA, when visualized on an agarose gel. Based on this, 1 - 3µl of each sample was loaded into a well on a 96-well plate, with 8µl Hi-Di™ Formamide (ABI) and 0.25µl of GeneScan™ 500 ROX™ Size Standard (ABI). Genotyping was then performed on an ABI PRISM® 3100 Genetic Analyser. The genotyping results were visualised using ABI Peak Scanner™ v1.0 Software.

Data analysis

(i) Sample structure

For the purposes of population-level tests, namely the presence of null alleles, Hardy Weinberg equilibrium, linkage disequilibrium and observed and expected heterozygosity, each geographical area was identified as a distinct population. The samples for each population were comprised of a single, randomly-chosen individual from each colony within the area and the individuals from the BGP which were collected within that particular area. Based on this approach, the sample sizes for each population are as follows: Tygerberg – 10, Malmesbury – 2, Darling – 9, Worcester – 8. As these sample sizes are quite small, particularly for the Malmesbury population, interpretation of the analyses will be appropriately cautious.

For the purposes of colony-level tests, such as the determination of family structure, each colony will be identified as a distinct population.

(ii) Statistical significance

Statistical significance was set to \( \alpha = 0.05 \) for all statistical tests. Significance values, \( p \), generated via multiple applications of the same test were corrected for Type I errors, erroneous rejection of a true null hypothesis, using the Bonferroni correction. The Bonferroni correction functions by adjusting the individual significance level of each
test, relative to the number of tests to be performed and the overall desired significance level (Weir, 1996).

(iii) Micro-checker

Genotyping errors can potentially bias subsequent analysis, and therefore it is important to be aware of any possible genotyping errors in the data. The scoring of genotypes was checked for the presence of typographical errors, stuttering, large-allele dropout and null alleles, using the program MICRO-CHECKER (version 2.2.3) (van Oosterhout et al., 2004). Stuttering, which is common at dinucleotide loci, occurs due to slippage during PCR amplification and creates difficulty in discriminating between homo- and heterozygotes when scoring genotypes. Large-allele dropout can occur due to low template DNA concentrations or preferential amplification of smaller alleles. Finally, null alleles can occur when there are mutations at primer sites, such that one of the alleles does not amplify and the individual is erroneously identified as a homozygote. However, the latter possibility is unlikely to be the case here, as primers were developed as part of this study, specifically for *M. viator*.

(iv) Allele and genotype frequencies

Allele and genotype frequencies were calculated for each population and locus, using the web implementation of GENEPOP (version 4.2) (Raymond and Rousset, 1995). Although observed frequencies can be used as estimates of true allele frequencies (Weir, 1996), because all individuals within my colony population groups are highly likely to be related, the problem of bias needs to be addressed. Therefore, the true global frequencies can be estimated from the BGP, as it is comprised of a group of 17 unrelated individuals.

(v) Hardy-Weinberg equilibrium and linkage disequilibrium

Population-level departures of allele frequencies, from those expected under Hardy-Weinberg equilibrium, were tested for in the web implementation of the GENEPOP
(version 4.2) (Raymond and Rousset, 1995), on each geographical population, under the null hypothesis of random union of gametes. In all instances the probability test, synonymous with the ‘exact Hardy-Weinberg test’ of Weir (1996) was performed.

Where possible (four or less alleles per locus), complete enumeration were performed. When the number of alleles at any particular locus equaled or exceeded five, the Markov Chain Method was used with the following parameters: 1000 dememorization steps; 100 batches and 1000 iterations per batch.

To test for linkage disequilibrium, all combinations of loci were tested within each of the four geographical populations, as well as with all samples combined over all populations, using a log likelihood ratio statistic with no assumption of Hardy Weinberg equilibrium, and Markov Chain parameters of 1000 dememorization steps; 100 batches and 1000 iterations per batch, in the web implementation of GENEPOP (version 4.2) (Raymond and Rousset, 1995). In both instances, the tests were performed against the null hypothesis that genotypes at one locus are independent of genotypes at another locus.

(vi) Genetic differentiation and variance

Genetic differentiation, $F_{ST}$, is a conservative correlation coefficient, which quantifies the relative level of differentiation between subdivisions within a population, based on allele frequency (Weir, 1996; Hedrick, 2000). $F_{ST}$ is a positive value, ranging from 0 to 1, with a high value indicative of a high level of differentiation. Genetic differentiation was computed using version 3.5.1.2 of the program Arlequin (Schneider et al., 2000), under the null hypothesis that alleles are drawn from the same distribution in all populations.

An Analysis of Molecular Variance (AMOVA) was performed in version 3.5.1.2 of the program Arlequin (Schneider et al., 2000) with 1000 permutations. This particular method, while similar to other approaches, also evaluates and takes into account the number of mutations between molecular haplotypes.
The AMOVA was performed on colony populations T1 – W12, where each colony was considered as a single population, as well as within their geographic sub-groups (Tygerberg: T1, T2, T3 & T4; Malmesbury: M5 & M6; Darling: D8, D9 & D10; Worcester: W7, W11 & W12) and at the within-individual level.

In order to examine pairwise genetic distances between all colonies, a matrix of distances was generated using the unweighted pair-group method with arithmetic averaging (UPGMA), with ‘coancestry identity’ above the diagonal and ‘coancestry distance’ below. The analysis was performed in version 1.0 of the program Genetic Data Analysis (GDA) (Lewis & Zaykin, 2001). The UPGMA is a simple method of constructing a phenetic tree, by using a clustering algorithm whereby relationships between groups are identified in order of pairwise similarity of a relevant descriptor, and upon which the tree is built in a hierarchical manner. The distance between two groups or clusters, is taken as the average distance between all pairs of objects in each group or cluster.

(vii) Genetic variation and diversity

Observed and expected heterozygosity (H_0 and H_e respectively) were determined for each locus within each population, using the web implementation of GENEPOP (version 4.2) (Raymond and Rousset, 1995). H_e was calculated based on observed allelic counts, under Hardy-Weinberg equilibrium. The associated p values were calculated in version 3.5.1.2 of the program Arlequin (Schneider et al., 2000) using 1000 000 steps in the Markov Chain Method and 100 000 permutations.

Within each population, genetic diversity was calculated as an average over all loci, using version 3.5.1.2 of the program Arlequin (Schneider et al., 2000).
(viii) Family structure

Once the number of alleles present and genotypes expressed by each colony has been determined, this data can be used to ascertain the type of family that is represented by each colony, namely; simple, extended or mixed. Simple families are defined as being headed by a monogamous pair of outbred reproducitives and, as such, exhibit no more than four alleles or genotypes at any locus (Thorne et al., 1999; Vargo, 2003; Deheer & Vargo, 2004; Vargo & Husseneder, 2011). Extended families are defined as colonies headed by secondary reproducitives, neotenics, from within the colony. As such, they still only exhibit a maximum of four alleles at any locus, but more than four genotypes may be expressed (Vargo, 2003; Vargo & Husseneder, 2011). Mixed families are defined as colonies headed by more than two unrelated reproducitives. Consequently, these colonies should exhibit more than four alleles and genotypes at each locus (Deheer & Vargo, 2004; Vargo & Husseneder, 2011).

(ix) Relatedness and Inbreeding Coefficients

Genetic relatedness ($r$) was calculated using the program IDENTIX 1.1.5 (Belkhir et al., 2002). Relatedness is a positive value, ranging from 0 to 1, with a high value indicative of a high level of relatedness. Here, relatedness is considered in terms of the proportion of homozygous individuals. This falls under the ‘identity’ function of the program and, given the potential for inbreeding within $M.\ viator$ colonies, is appropriate for determining relatedness where alleles are likely to be identical by decent. Given the fact that colonies are highly likely to deviate from Hardy-Weinberg equilibrium, the analysis was carried out at the genotypic level with five permutations. Relatedness was calculated as a mean value within the BGP, within all colonies combined and within each individual colony. Furthermore, mean relatedness values were calculated for all pairwise colony combinations. The standard deviations were obtained from the estimates of sample variance.

The overall inbreeding coefficient, $F_{IT}$, the coefficient of inbreeding within a colony relative to the total population, $F_{CT}$ (analogous to genetic differentiation, $F_{ST}$), and the
coefficient of inbreeding within a colony, $F_{IC}$, were determined for the population as a whole, within each individual colony ($F_{IC}$ only) and overall for each family type (simple, extended or mixed). Confidence intervals, at $\alpha = 0.05$, were determined for each estimate by bootstrapping over loci with 1000 replicates. The coefficients of inbreeding can range from $-1.0$, indicating that all individuals are heterozygous, to $+1.0$, indicating that all individuals are homozygous. The analysis was performed in version 1.0 of the program GDA (Lewis & Zaykin, 2001).

**Results and Discussion**

*Micro-checker*

The genotype scoring was checked for the presence of stochastic effects, such as stuttering, large-allele dropout and, at a population level, null alleles, using the program MICRO-CHECKER (version 2.2.3) (van Oosterhout et al., 2004). While there was no evidence of large allele dropout or null alleles, some loci exhibited stuttering. See Appendix A for full details.

*Allele and genotype frequencies*

(i) Loci with two alleles

Four loci had two alleles each; Mvit 4, Mvit 14, Mvit 21 and Mvit 23, with one allele present at low frequencies in some populations. Locus Mvit 4 has the two alleles; 129 and 131 (Fig. 4.2). In the BGP, the frequencies of the alleles are 0.24 and 0.76 respectively. In the Tygerberg colonies (T1 – T4) allele 131 is only present at very low frequencies, and similarly so for allele 129 in the Worcester colonies (W7, W11 & W12). Similar trends are evident in the genotype frequencies (Appendix B, Fig. B.1), with an apparent overabundance of homozygotes.

Locus Mvit 14 has the two alleles, 370 and 373 (Fig. 4.3). In the BGP, the frequencies of the alleles are 0.88 and 0.12 respectively. Allele 373 is either absent or only
represented at very low frequencies in the colony populations (T1 - 0.02; M5 - 0.07). However, the exception to this trend is observed within the Worcester colonies, which all exhibit allele 373 at higher frequencies, ranging from 0.18 to 0.5. Again, similar trends are present in the genotype frequencies (Appendix B, Fig. B.2), with most colonies being almost exclusively homozygous for genotype 370/370, while W11 remains exceptional, as the only colony to have all offspring as heterozygotes.

Locus Mvit 21 has the two alleles, 119 and 121 (Fig. 4.4). In the BGP, the frequencies of the alleles are 0.85 and 0.15 respectively. Similarly to locus Mvit 14, allele 121 is either absent or only present at low frequencies within the colony populations (M5 – 0.22; D8 – 0.05; D10 – 0.03; W12 – 0.03). However, the converse to this trend is evident in the Tygerberg population, where allele 121 is present in all colonies, at frequencies ranging from 0.3 to 0.72. In addition, the Tygerberg colonies also appear to represent the most variation in terms of genotypes, while the remaining colonies are predominantly homozygous for the genotype 119/119 (Appendix B, Fig. B.3).

Locus Mvit 23 has the two alleles, 145 and 148 (Appendix B, Fig. B.4). In the BGP, the frequencies of the alleles are 0.97 and 0.03 respectively. Allele 148 is only present in six colonies but, interestingly, in five out of the six colonies it is at a higher frequency than in the BGP (T1 – 0.2; T3 – 0.02; M5 - 0.15; M6 - 0.26; W7 - 0.18; W11 - 0.22). This pattern of frequency distribution remains similarly consistent for the genotype frequencies (Appendix B, Fig. B.5), with W7 being the only colony to exhibit the homozygous genotype for allele 148.

For all the loci with two alleles, the variation in frequency between the two alleles appears to be geographically distributed. Generally, the Tygerberg and Worcester populations display contrary allele frequencies, with the Malmesbury and Darling populations being more intermediate and a closer representation of what is observed in the BGP. These results are suggestive of a geographical cline in the distribution of genetic variance.
(ii) Loci with three or more alleles

Three loci exhibited more than three alleles; Mvit 17, Mvit 18 and Mvit 25, with numbers ranging from seven (Mvit 25) to 14 (Mvit 18). Locus Mvit 17 had twelve alleles; 156, 164, 166, 168, 170, 174, 176, 180, 186, 188, 192 and 198 (Fig. 4.5).
However, only eight alleles are represented in the BGP, with alleles 170, 176, 188 and 198 absent. Despite this absence, allele 170 is still represented in at least one colony population from each of the four geographical areas. Allele 176 is only represented in two Tygerberg populations, at a frequency of 0.02 in each. Allele 188 is only found in T4, at a frequency of 0.39, and in D10 at 0.02. Finally, allele 198 is only found in W7 at a frequency of 0.02. Interestingly, colony T4 has nine alleles – two more than in the BGP. In total, there were 29 different genotypes identified at locus Mvit 17, which is relatively few given the number of different alleles (Appendix B, Fig. B.6). Similarly to Mvit 14, so here too W11 remain the only colony to express a single heterozygous genotype.

Locus Mvit 18 has fourteen alleles; 302, 308, 316, 318, 322, 324, 328, 330, 332, 334, 336, 340, 344 and 346 (Appendix B, Fig. B.7). Similarly to Mvit 17, five of these alleles (316, 330, 340, 344 & 346) are not represented in the BGP. Alleles 316 and 344 are only found in the Tygerberg and Malmesbury colonies. Allele 330 is only found in one of each of the Malmesbury and Worcester colony populations. Allele 340 is only represented in the Worcester colonies and allele 346 is only represented in Malmesbury. There were 38 genotypes observed across the 13 populations, 11 of which were in the BGP and 13 in M5 (Appendix B, Fig. B.8).

Locus Mvit 25 has seven alleles; 209, 212, 218, 221, 230, 236 and 239 (Fig. 4.6). All alleles are represented in the BGP except for 139, which in only found in M5 at a frequency of 0.05. There are 16 genotypes observed across the 13 populations (Fig. 4.7), 10 of which are seen in the BGP. Greater variability is expressed in W11 with four genotypes, compared to at other loci.

At locus Mvit 17, and slightly less so at Mvit 25, geographic structuring is clearly observable, with clustering in the Tygerberg, Malmesbury, Darling and Worcester colonies. However, as the number of alleles is greater at Mvit18, distinct patterns of gene flow become more challenging to observe.
Fig. 4.5 Allele frequencies at locus Mvit 17, across all populations

Fig. 4.6 Allele frequencies at locus Mvit 25, across all populations
Hardy-Weinberg equilibrium and linkage disequilibrium

Within each of the four geographical populations (Tygerberg, Malmesbury, Darling and Worcester) all loci were found to be in Hardy-Weinberg equilibrium, after applying a Bonferroni correction ($p = 0.0039$), with the exception of locus Mvit18 in the Darling population.

No significant linkage disequilibrium was observed within any of the four geographical populations, nor was any linkage found when pairwise combinations of loci were considered over all populations. As such, all loci are assumed to assort independently.
Genetic differentiation and variance

The interpretation of results from genetic investigations relies heavily on the ability to define the population. In studies of eusocial species the colony is generally considered to be a discrete entity within a population. It is therefore important to be able to physically define the nest/s of a single colony in order to be able to analyse the spatial dynamics and interactions between individuals and colonies within a population (Thorne et al., 1999). However, in subterranean and mound building species colony boundaries may be difficult to determine (Thorne et al., 1999). Therefore, examining genetic differentiation, distance and variance within and between nests is a useful approach to confirming independence.

Genetic differentiation was determined for each colony pair ($F_{CT}$), across all loci (Husseneder et al., 2005). Pairwise $F_{CT}$ values varied from 0.1 to 0.63, over a range of distances from 0.06 to 103km, indicating moderate to strong differentiation, which was highly significant between all pairs of colonies ($p < 0.0001 \pm 0.00$). An AMOVA was also performed on all colonies, with each colony being considered as a single population (Table 4.3). Similarly, the results indicate that there is little variance and no significant differentiation among individuals within any given population, while approximately 37% of the total variation is distributed between populations. Therefore, as predicted, each colony can be considered to be a discrete entity.

<table>
<thead>
<tr>
<th>Level of analysis</th>
<th>df*</th>
<th>% variation</th>
<th>Fixation Index</th>
<th>$p \pm s.d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among populations</td>
<td>11</td>
<td>37.1</td>
<td>0.371</td>
<td>$&lt;0.0001 \pm 0.00$</td>
</tr>
<tr>
<td>Among individuals within populations</td>
<td>340</td>
<td>-12.3</td>
<td>-0.195</td>
<td>1.00 $\pm 0.00$</td>
</tr>
<tr>
<td>Within Individuals</td>
<td>352</td>
<td>75.2</td>
<td>0.248</td>
<td>$&lt;0.0001 \pm 0.00$</td>
</tr>
</tbody>
</table>

Colonies were then grouped into four geographical populations; Tygerberg (T1 – T4), Malmesbury (M5 & M6), Darling (D8 – D10) and Worcester (W7, W11 & W12). Pairwise $F_{ST}$ values ranged from 0.2 – 0.37, all of which were highly significant ($p <
An AMOVA was also performed on the four geographic populations (Table 4.4). From the results, we can see that 15% of the variation is ascribed between colonies within a geographic population, while 25% of the variation of is distributed between geographic populations. In other words, both the individual colonies and the geographic sub-groups and are significantly differentiated from one another and from the global population. These results are corroborated by the \( F_{ST} \) values, and together indicate moderate to strong differentiation, suggesting low to moderate population viscosity, likely attributable to alate dispersal distances.

**Table 4.4 Analysis of molecular variance, where the colony populations were first structured according to the geographic sub-groups.**

<table>
<thead>
<tr>
<th>Level of analysis</th>
<th>df*</th>
<th>% variation</th>
<th>Fixation Index</th>
<th>( p \pm \text{s.d} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among geographic groups</td>
<td>3</td>
<td>25.4</td>
<td>0.253</td>
<td>&lt;0.0001 ± 0.00</td>
</tr>
<tr>
<td>Among populations within groups</td>
<td>8</td>
<td>14.9</td>
<td>0.199</td>
<td>&lt;0.0001 ± 0.00</td>
</tr>
<tr>
<td>Among individuals within populations</td>
<td>340</td>
<td>-11.7</td>
<td>-0.195</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>Within Individuals</td>
<td>352</td>
<td>71.5</td>
<td>0.285</td>
<td>&lt;0.0001 ± 0.00</td>
</tr>
</tbody>
</table>

The AMOVA results are further corroborated when we examine the UPGMA phenetic tree (Fig. 4.8), produced using pairwise genetic distances between all colonies in GDA, version 1.0 (Lewis & Zaykin, 2001). The genetic distance at each node, between two groups or clusters, is taken as the average distance between all pairs of objects in each group or cluster.
From a phylogenetic perspective, UPGMA is very sensitive to unequal rates of evolution. However, given that in this study all groups in the analysis are colonies from a single species, one can assume equal rates of evolution. Small population sizes can also alter rates of evolution, but again, effective population size varies little between the 12 colonies examined. The only caveat to UPGMA analysis is that the accumulation of new mutations is, at least in part, a function of the number of generations, which does vary from one colony to the next. However, this variable is unknown, but unlikely to confound the result of the phenetic tree given the life-history traits of my population.

From the topology of the tree, clear geographic clustering is observed, consistent with the results from other analyses. This further supports the hypothesis that the effect of variation in the number of generations does not significantly affect the topology of the tree. Interestingly, none of the ‘most similar’ groups in each geographic population are nearest neighbours, which supports the prediction that budding is unlikely to be occurring in M. viator.

Most termite species tend to show low levels of differentiation at 30 to 50km and moderate to strong levels of differentiation ($F_{ST} = 0.16 – 0.25$) at distances greater than...
100km (Vargo & Husseneder, 2011) (Table 4.5). Therefore, the results for *M. viator* are in keeping with those reported in the literature for other termite species, over similar distances.

### Table 4.5 Levels of genetic differentiation among termite populations (amended from Vargo & Husseneder, 2011).

<table>
<thead>
<tr>
<th>Family/species</th>
<th>Number of Populations</th>
<th>$F_{ST}$</th>
<th>Spatial Scale (km)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hodotermitidae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Microhodotermes viator</em></td>
<td>4</td>
<td>0.2 – 0.37</td>
<td>34 - 103</td>
<td>This Study</td>
</tr>
<tr>
<td><strong>Mastotermitidae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mastotermes darwiniensis</em></td>
<td>4</td>
<td>0.42 – 0.62</td>
<td>2 – 400</td>
<td>Goodisman &amp; Crozier, 2002</td>
</tr>
<tr>
<td><strong>Rhinotermitidae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Coptotermes lacteus</em></td>
<td>6</td>
<td>0.01</td>
<td>3 – 11</td>
<td>Thompson et al., 2007</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Reticulitermes flavipes</em></td>
<td>8</td>
<td>0.0 – 0.17</td>
<td>1 - 450</td>
<td>Vargo &amp; Carlson, 2006;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DeHeer &amp; Vargo, 2004;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bulmer et al., 2001</td>
</tr>
<tr>
<td><em>R. grassei</em></td>
<td>3</td>
<td>0.0 - 0.14</td>
<td>30 - 120</td>
<td>DeHeer et al., 2005</td>
</tr>
<tr>
<td><em>R. lucifugus spp.</em></td>
<td>3</td>
<td>0.26 – 0.54</td>
<td>150 - 300</td>
<td>Lefebvre et al., 2008</td>
</tr>
<tr>
<td><strong>Termitidae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Laboitermes labralis</em></td>
<td>3</td>
<td>0.16 – 0.25</td>
<td>150 - 300</td>
<td>Dupont et al., 2009</td>
</tr>
<tr>
<td><em>Macrotermes michaeïseni</em></td>
<td>7</td>
<td>0.017</td>
<td>0 - 50</td>
<td>Brandl et al., 2006</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0.02 – 0.21</td>
<td>50 - 500</td>
<td></td>
</tr>
<tr>
<td><em>Nasutitermes nigriceps</em></td>
<td>3</td>
<td>0.03 – 0.11</td>
<td>100 - 200</td>
<td>Thompson &amp; Hebert, 1998a</td>
</tr>
</tbody>
</table>

**Genetic variation**

Observed and expected heterozygosity ($H_0$ & $H_e$) were determined at each locus within each geographical population (Table 4.6). A value of one indicates a completely heterozygote locus, and zero indicates a completely homozygous locus. Generally there was no significant heterozygote excess or deficiency at any of the loci in any of the populations, with the exception of Mvit 4 in the Tygerberg population ($p = 0.047$) and Mvit18 in the Darling population ($p = 0.001$). Both of these instances are examples of heterozygote deficiency, although in the Tygerberg population the deficiency was no
longer significant after the application of a Bonferroni correction (adjusted significance level \( p = 0.008 \)).

While the most likely cause for heterozygote deficiency at an individual locus is the presence of null alleles (Husseneder et al., 2005), this is unlikely given that the loci are species specific (See Appendix A for further discussion regarding null alleles). In this instance, the significant deficit at Mvit18 in the Darling population corresponds to the only locus not in Hardy-Weinberg equilibrium. Furthermore, the heterozygote deficits observed in the geographical populations are possibly attributable to the Wahlund effect - that is a reduction in \( H_0 \) due to the pooling of individuals from discrete colonies, or subpopulations, into a single population which may not actually represent a randomly interbreeding unit (Dharmarajan et al., 2013).

Table 4.6 Observed and expected heterozygosity (\( H_0 \) & \( H_e \)) and average gene diversity within each geographical population. Bold values are significant at \( p = 0.05 \), while highlighted values indicate those differences which remained significant after a Bonferroni correction.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Tygerberg</th>
<th>Malmesbury</th>
<th>Darling</th>
<th>Worcester</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( H_0 )</td>
<td>( H_e )</td>
<td>( H_0 )</td>
<td>( H_e )</td>
</tr>
<tr>
<td>Mvit 4</td>
<td>0.1</td>
<td>0.39</td>
<td>0</td>
<td>0.66</td>
</tr>
<tr>
<td>Mvit 14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mvit 17</td>
<td>0.8</td>
<td>0.78</td>
<td>0.5</td>
<td>0.83</td>
</tr>
<tr>
<td>Mvit 18</td>
<td>0.6</td>
<td>0.76</td>
<td>0.5</td>
<td>0.83</td>
</tr>
<tr>
<td>Mvit 21</td>
<td>0.2</td>
<td>0.53</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mvit 23</td>
<td>0.2</td>
<td>0.19</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Mvit 25</td>
<td>0.7</td>
<td>0.74</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Gene Diversity</td>
<td>0.484 ± 0.289</td>
<td>0.476 ± 0.367</td>
<td>0.393 ± 0.217</td>
<td>0.396 ± 0.247</td>
</tr>
</tbody>
</table>
Family structure

Termite colonies are generally thought to be closed populations, originally founded by a single primary reproductive pair \( (N_e = 2) \), generating simple families, and then subsequently propagated by neotenics from within the colony, generating extended families. However, additional reproductives, unrelated to the primary pair, may be accepted into the colony or colonies may fuse, giving rise to mixed families (Deheer & Vargo, 2004). In order to determine the family structure of a particular colony, we need to examine the number of alleles and genotypes expressed at each locus (Table 4.7).

<table>
<thead>
<tr>
<th>Locus</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>M5</th>
<th>M6</th>
<th>D8</th>
<th>D9</th>
<th>D10</th>
<th>W7</th>
<th>W11</th>
<th>W12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mvit 4</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mvit 14</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mvit 17</td>
<td>3</td>
<td>6</td>
<td>4</td>
<td>9</td>
<td>5</td>
<td>9</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Mvit 18</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>8</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>7</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Mvit 21</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mvit 23</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Mvit 25</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

Colonies T1, D8, D9 and W11 all represent simple families, with no more than four alleles present or genotypes expressed at any one locus. From this we can conclude that these are quite likely younger colonies and that reproduction is still being performed by the primary pair. Colonies T3 and M6 represent extended families. As such, neither colony has more than four alleles at any particular locus, but due to inbreeding via
neotenic reproduction, more than four genotypes are expressed at some loci. Colonies T2, T4, M5, W7, D10, W12 represent mixed families, with more than four alleles and genotypes at some loci.

It is common for populations to exhibit variable family types among different colonies, and examples of this have been noted in *R. grassei* (Nobre *et al.*, 2008), *R. flavipes* (Deheer & Vargo, 2004) and *C. formosanus* (Husseneder *et al.*, 2005), to mention but a few. It appears that, at least partially, the composition of family types within a population is a function of the maturity of individual colonies. For example, a prevalence of simple families suggests a relatively young population (Husseneder *et al.*, 2005). As such, a variety of family types reflects the continuing processes of colony foundation and maturation overtime.

Overall, *M. viator* appears to have percentages of simple and extended families, well within the ranges exhibited by many other termite species (Table 4.8). However, *M. viator* also seems to have a higher propensity for mixed family colonies, when compared to other species. While the sex of the additional reproductives are unknown, based on the literature (Hacker *et al.*, 2005), they are quite likely to be females and are clearly contributing to colony reproduction. The development of a mixed family requires a either a lack of discrimination between colony members and non-members, or at least a decreased rejection threshold. As such, we may expect to find reduced levels of intercolonial aggression (see Chapter 5). Additionally, the formation of mixed family colonies may be a strategy to mitigate the effects of stressful environmental conditions (Brandl *et al.*, 2005). The distribution of *M. viator heuweltjies* has been shown to be sensitive to environmental conditions, with density increasing with rainfall gradients from north to south and east to west across their range (Picker *et al.*, 2007). As such, there may be some unidentified environmental pressure facilitating the formation of mixed families. Finally, periodic acceptance of new unrelated reproductives may be a system to increase colony longevity, which may be linked to the extreme longevity of *heuweltjies*. 
Table 4.8 Colony family structure across various termite species, using molecular markers (amended from Vargo & Husseneder, 2011).

<table>
<thead>
<tr>
<th>Family/species</th>
<th>No. of pops</th>
<th>Simple</th>
<th>Extended</th>
<th>Mixed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hodotermitidae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microhodotermes viator</td>
<td>4</td>
<td>0 - 33%</td>
<td>25 - 66%</td>
<td>33 - 66%</td>
<td>This Study</td>
</tr>
<tr>
<td><strong>Mastotermitidae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mastotermes darwiniensis</td>
<td>3</td>
<td>26%</td>
<td>47%</td>
<td>27%</td>
<td>Goodisman &amp; Crozier, 2002</td>
</tr>
<tr>
<td><strong>Termopsidae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zootermopsis nevadensis</td>
<td>1</td>
<td>0%</td>
<td>100%</td>
<td>0%</td>
<td>Aldrich &amp; Kambhampati, 2007</td>
</tr>
<tr>
<td>Z. nevadensis nuttingi</td>
<td>1</td>
<td>45%</td>
<td>22%</td>
<td>33%</td>
<td></td>
</tr>
<tr>
<td><strong>Rhinotermitidae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coptotermes lacteus</td>
<td>6</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>Thompson et al., 2007</td>
</tr>
<tr>
<td>C. formosanus</td>
<td>8</td>
<td>14 – 90%</td>
<td>10 – 89%</td>
<td>0%</td>
<td>Husseneder et al., 2012; Husseneder et al., 2005; Vargo et al., 2003</td>
</tr>
<tr>
<td>Reticulitermes flavipes</td>
<td>4</td>
<td>65 – 89%</td>
<td>10 – 56%</td>
<td>0 - 22%</td>
<td>Vargo &amp; Carlson, 2008; DeHeer &amp; Vargo, 2004; Vargo, 2003</td>
</tr>
<tr>
<td>R. hageni</td>
<td>2</td>
<td>54 – 100%</td>
<td>0 – 46%</td>
<td>0</td>
<td>Vargo &amp; Carlson, 2006</td>
</tr>
<tr>
<td>R. grassei</td>
<td>4</td>
<td>0 – 45%</td>
<td>53 – 100%</td>
<td>13%</td>
<td>Nobre et al., 2008; DeHeer et al., 2005</td>
</tr>
<tr>
<td>R. malletei</td>
<td>1</td>
<td>54%</td>
<td>46%</td>
<td>0%</td>
<td>Vargo &amp; Carlson, 2006</td>
</tr>
<tr>
<td><strong>Termintidae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laboitermes labralis</td>
<td>3</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>Dupont et al., 2009</td>
</tr>
<tr>
<td>Nasutitermes nigriceps</td>
<td>3</td>
<td>95%</td>
<td>5%</td>
<td>0%</td>
<td>Thompson &amp; Hebert, 1998a</td>
</tr>
<tr>
<td>N. corniger</td>
<td>1</td>
<td>0%</td>
<td>11%</td>
<td>89%</td>
<td>Atkinson et al., 2008</td>
</tr>
</tbody>
</table>
Relatedness and inbreeding coefficients

Average genetic relatedness within a colony is a function of both the reproductive strategy of the colony and the relatedness of the primary reproductives. For some questions of colony genetic structure, such as nestmate relatedness, workers are the preferred sampling unit, while for other questions, such as sex-biased dispersal tendencies, reproductive individuals are far more informative (DeHeer & Vargo, 2006). Relatedness between primary reproductives is a function of dispersal distances, sex-biased alate production within colonies and possible kin discrimination during mating (Vargo & Husseneder, 2011), which, in conjunction with reproductive strategies, affect inbreeding coefficients. However, in the lower termites, relatedness between reproductives generally has to be inferred by relatedness between offspring, due to the inaccessibility of reproductives (Roy et al., 2010).

Genetic relatedness was calculated using the program IDENTIX 1.1.5 (Belkhir et al., 2002), as a mean value within each individual colony (Table 4.9), within the BGP, within all colonies combined and within each geographic area and among family types (Table 4.10). Standard deviations were obtained from the estimates of sampling variance. The overall inbreeding coefficient, $F_{IT}$, the coefficient of inbreeding within a colony relative to the total population, $F_{CT}$, and the coefficient of inbreeding for individuals within a colony, $F_{IC}$, were determined for the population as a whole, within each individual colony ($F_{IC}$ only) (Table 4.9) and overall for each family type (Table 4.10).

The background level of relatedness in the population, as estimated from the BGP ($r = 0.42$), was not significantly different ($p = 1.0$) from the average level of relatedness estimated across all colonies ($r = 0.44$). While this appears to be a rather high value for relatedness, it is important to bear in mind the limited number of polymorphic loci, low average numbers of alleles per locus (5.8) and relatively small sample sizes. The higher levels of average relatedness within geographic areas (range: $r = 0.59 – 0.67$) is likely a function of limited alate dispersal distances, leading to mild population viscosity and genetic structuring at a local scale. The overall measure of total inbreeding in all
colonies, was not significantly different from zero ($F_{IT} = -0.18; [-0.41; 0.06]$ 95% CI), indicating that overall there is no excess or deficiency of heterozygotes. The coefficient of inbreeding for colonies relative to the total population, analogous to genetic differentiation, was significantly greater than zero ($F_{CT} = 0.37; [0.27; 0.51]$ 95% CI), indicating that colonies are genetically isolated from one another.

The colony inbreeding coefficient, $F_{IC}$, was determined as an average over all loci within each colony, T1 – W12, with 95% confidence intervals generated by bootstrapping over all loci (Table 4.9).

Table 4.9 Mean intracolonial relatedness and inbreeding coefficients. Values in bold are significantly different from zero. Simple families are highlighted in green, extended in blue and mixed in yellow.

<table>
<thead>
<tr>
<th>Population</th>
<th>Mean Relatedness ± s.d.</th>
<th>$F_{IC}$ [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>0.832 ± 0.084</td>
<td>-0.47 [-0.73; -0.19]</td>
</tr>
<tr>
<td>T2</td>
<td>0.658 ± 0.138</td>
<td>0.05 [-0.33; 0.37]</td>
</tr>
<tr>
<td>T3</td>
<td>0.831 ± 0.114</td>
<td>-0.38 [-0.44; -0.24]</td>
</tr>
<tr>
<td>T4</td>
<td>0.822 ± 0.217</td>
<td>-0.14 [-0.37; 0.28]</td>
</tr>
<tr>
<td>M5</td>
<td>0.574 ± 0.190</td>
<td>0.12 [-0.15; 0.4]</td>
</tr>
<tr>
<td>M6</td>
<td>0.758 ± 0.130</td>
<td>-0.13 [-0.4; 0.21]</td>
</tr>
<tr>
<td>W7</td>
<td>0.659 ± 0.164</td>
<td>-0.11 [-0.48; 0.35]</td>
</tr>
<tr>
<td>D8</td>
<td>0.733 ± 0.207</td>
<td>-0.15 [-0.57; 0.36]</td>
</tr>
<tr>
<td>D9</td>
<td>0.866 ± 0.105</td>
<td>-0.06 [-0.59; 0.57]</td>
</tr>
<tr>
<td>D10</td>
<td>0.838 ± 0.173</td>
<td>-0.05 [-0.29; 0.1]</td>
</tr>
<tr>
<td>W11</td>
<td>0.859 ± 0.077</td>
<td>-0.62 [-1.0; -0.3]</td>
</tr>
<tr>
<td>W12</td>
<td>0.799 ± 0.130</td>
<td>-0.25 [-0.44; 0.19]</td>
</tr>
</tbody>
</table>

Only colonies T1, T3 and W11 showed a significant deviation from zero, tending towards active inbreeding avoidance. However, care must be taken when interpreting these results as colonies T1 and W11 are both simple families, while T3 is extended. By definition, both simple and extended families originate from a single pair of reproductives, implying that there are a very limited number of genotypes possible within the colony. This leads to a shortage of homozygotes and the significantly negative $F_{IC}$ values may therefore simply be a reflection the limited number of breeders.
That said, the negative $F_{IC}$ values also point to the possibility that the primary reproductives were likely outbred. If this is the case, it is probably due to the fact that *M. viator* colonies engage in synchronous release of alates (Coaton & Sheasby, 1974), and are thus provided with the opportunity to mate with unrelated individuals.

In conjunction with the lack of inbreeding, the generally high level of within-colony relatedness may be slightly surprising. However, it is important to bear in mind that relatedness is a relative measurement and, as such, the level of background relatedness must be taken into account. Furthermore, simply estimating intracolonial relatedness is something akin to a ‘snap-shot’ of a colony at a particular moment in time. Rather, looking at trends overtime or across colonies can provide us with a more detailed image of colony and population structure, from which we can better extrapolate functional strategies.

As $F$ statistics vary across populations, due to changes in age and local density of colonies (Thorne *et al*., 1999), we can utilise them to look for trends among colonies of the same family type. Thorne *et al*. (1999) and Bulmer *et al*., (2001) both conducted studies where they used computer simulations of several different reproductive strategies and scenarios, in order to determine the expected associated $F$ statistics and relatedness coefficients. Following Vargo (2003), Vargo *et al*., (2003), Husseneder *et al*., (2005) and Husseneder *et al*., (2012) the $F$ statistics and relatedness values for *M. viator* were compared to the simulations by Thorne *et al*., (1999) and Bulmer *et al*., (2001). When comparing the simulated coefficients for outbred and inbred simple families with the actual results from *M. viator* colonies (Table 4.10), it is clear that there is no significant difference between *M. viator* and the outbred simulation, even though the values for $F_{IT}$ and $F_{CT}$ are closer to the inbred values. These results are also in keeping with those from a study on *R. grassei*, where individual simple family colonies all had $F_{IC}$ values less than - 0.28 and, over all simple families, $F_{IC}$ was - 0.38 (Nobre *et al*., 2008). Average relatedness across *M. viator* simple family colonies is approximately 0.5, which is as expected from the offspring of outbred, monogamous reproductive pairs.
Table 4.10 F statistics and relatedness (r) coefficients for worker termites, under several different reproductive strategies. Under the simulation for simple families there is a single queen (Q) and king (K). Under extended families, there are scenarios for varying numbers of male (N_m) and female (N_f) neotenics and the number of generations (X) over which they are inbreeding. Under mixed families, the simulations refer to equal proportions (p = 0.5) of workers as the progeny from two distinctive sets of reproductives (amended from Bulmer et al., 2001 & Thorne et al., 1999).

<table>
<thead>
<tr>
<th>Reproductive Strategies and Scenarios</th>
<th>F_{IT}</th>
<th>F_{IT}</th>
<th>F_{CT}</th>
<th>r ± s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[95% CI]</td>
<td>[95% CI]</td>
<td>[95% CI]</td>
<td>± 0.2</td>
</tr>
<tr>
<td><strong>Simple Families</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. viator</em> (T1, D6, D9, W11)</td>
<td>0.2</td>
<td>0.43</td>
<td>-0.30</td>
<td>0.49</td>
</tr>
<tr>
<td>1. Outbred: Q = K = 1</td>
<td>0.00</td>
<td>0.25</td>
<td>-0.34</td>
<td>0.50</td>
</tr>
<tr>
<td>2. Inbred: Q = K = 1, X = 1</td>
<td>0.33</td>
<td>0.42</td>
<td>-0.14</td>
<td>0.62</td>
</tr>
<tr>
<td><strong>Extended Families</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. viator</em> (T3, M6)</td>
<td>0.2</td>
<td>0.39</td>
<td>-0.25</td>
<td>0.65</td>
</tr>
<tr>
<td>1. N_f = N_m = 1, X = 1</td>
<td>0.33</td>
<td>0.42</td>
<td>-0.14</td>
<td>0.62</td>
</tr>
<tr>
<td>2. N_f = N_m = 1, X = 3</td>
<td>0.57</td>
<td>0.65</td>
<td>-0.22</td>
<td>0.82</td>
</tr>
<tr>
<td>3. N_f = N_m = 10, X = 3</td>
<td>0.37</td>
<td>0.38</td>
<td>-0.02</td>
<td>0.56</td>
</tr>
<tr>
<td>4. N_f = 2, N_m = 1, X = 3</td>
<td>0.52</td>
<td>0.59</td>
<td>-0.17</td>
<td>0.78</td>
</tr>
<tr>
<td>5. N_f = 200, N_m = 100, X = 3</td>
<td>0.34</td>
<td>0.34</td>
<td>-0.00</td>
<td>0.51</td>
</tr>
<tr>
<td><strong>Mixed Families</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. viator</em> (T2, T4, M5, W7, D10, W12)</td>
<td>0.34</td>
<td>0.36</td>
<td>-0.04</td>
<td>0.43</td>
</tr>
<tr>
<td>1. Pleometrosis: Q = 2, K = 1</td>
<td>0.00</td>
<td>0.19</td>
<td>-0.23</td>
<td>0.38</td>
</tr>
<tr>
<td>2. Pleometrosis: Q = 2, K = 1, then N_f = N_m = 10, X = 3</td>
<td>0.27</td>
<td>0.29</td>
<td>-0.03</td>
<td>0.45</td>
</tr>
<tr>
<td>3. Mixed workers from unrelated nests: N_f = N_m = 1, X = 1, p = 0.5</td>
<td>0.33</td>
<td>0.20</td>
<td>0.17</td>
<td>0.29</td>
</tr>
<tr>
<td><strong>Budding</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. viator</em> (T1 &amp; T2)</td>
<td>0.06</td>
<td>0.21</td>
<td>-0.21</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>[0.41; 0.37]</td>
<td>[0.04; 0.33]</td>
<td>[-0.47; 0.08]</td>
<td>± 0.17</td>
</tr>
<tr>
<td><em>M. viator</em> (D9 &amp; D10)</td>
<td>0.24</td>
<td>0.31</td>
<td>-0.09</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>[-0.26; 0.68]</td>
<td>[0.13; 0.43]</td>
<td>[-0.58; 0.56]</td>
<td>± 0.18</td>
</tr>
<tr>
<td><em>M. viator</em> (W11 &amp; W12)</td>
<td>-0.25</td>
<td>0.14</td>
<td>-0.45</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>[-0.41; -0.01]</td>
<td>[0.11; 0.16]</td>
<td>[-0.64; -0.15]</td>
<td>± 0.14</td>
</tr>
<tr>
<td>1. X = 0, p = 0.5</td>
<td>0.33</td>
<td>0.37</td>
<td>-0.06</td>
<td>0.56</td>
</tr>
<tr>
<td>2. N_f = N_m = 1, X = 3, p = 0.5</td>
<td>0.66</td>
<td>0.56</td>
<td>0.22</td>
<td>0.68</td>
</tr>
<tr>
<td>3. N_f = N_m = 1, X = 3, p = 0.9</td>
<td>0.66</td>
<td>0.64</td>
<td>0.04</td>
<td>0.77</td>
</tr>
<tr>
<td>4. N_f = N_m = 100, X = 3, p = 0.9</td>
<td>0.43</td>
<td>0.41</td>
<td>0.03</td>
<td>0.58</td>
</tr>
</tbody>
</table>

In a study on *M. michaelseni*, Hacker et al. (2005) also found that sterile offspring from a monogamous pair showed relatedness close to 0.5, and the mean relatedness sampled between kings and queens of the species was zero. Likewise, the majority of mature R.
*flavipes* and *R. virginicus* colonies are founded by outbred primary reproductives, with only about 25% of colonies are headed by highly related reproductives (DeHeer & Vargo, 2006). Similarly, relatedness between monogamous reproductives in simple families of *Cubitermes* sp. *affinis subarquatus*, ranges from -0.55 to 0.6, with around 30% above 0.5 (Roy et al., 2010). Generally, evidence for inbreeding between colony founders is rare in termites and, when it occurs, is often a function of limited or inhibited dispersal ranges (Roy et al., 2010). While I was not able to sample primary reproductives from any of the *M. viator* colonies, based on *F* statistics and relatedness coefficients, it is likely that they are predominantly outbred.

Among the extended families, where there is a greater range of possible scenarios, the results for *M. viator* seem to approximate the first simulated scenario (\(N_f = N_m = 1, X = 1\)) most closely (Table 4.10). In a study on *C. formosanus* extended family colonies, *F_{CT}* was large (0.31), confirming genetic differentiation between colonies, inbreeding was moderate (\(F_{IT} = 0.13\)) and *F_{IC}* was highly negative (-0.28), consistent with low numbers of reproductives, while relatedness was high (\(r = 0.55\)) (Husseneder et al., 2005). Similarly, in a study on *R. grassei*, all the extended family colonies also had negative *F_{IC}* values, indicating no significant inbreeding (Nobre et al., 2008). While results such as these are expected in simple family colonies, headed by an outbred primary reproductive pair, extended families are, by definition, inbred. However, while neotenic reproduction within a colony does increase homozygosity, it will not result in positive *F_{IC}* values (Thorne et al., 1999). Furthermore, the level of inbreeding within a colony is dependent on the number of neotenics and the number of generations over which they reproduce. Generally, multiple neotenics are required to ensure optimal colony growth, therefore they are produced in greater numbers than they are required and surplus neotenics are subsequently eliminated (Lenz, 1985). The *F_{IC}* values aid in estimating the number of neotenics reproducing as either few (\(< 6\)) or many (\(> 10\)), such that strongly negative values (\(< -0.2\)) indicate low numbers of neotenics (Husseneder et al., 2005; Vargo & Husseneder, 2011). Additionally, the fewer the number of neotenics and the more generations over which they inbred, the higher the inbreeding coefficient, \(F_{IT}\), and the greater the differentiation between colonies, *F_{CT}*, will be (Thorne et al., 1999; Vargo & Husseneder, 2011). It is also noteworthy that low *F_{IC}* values may arise in
extended family colonies with both primary reproductives and functional neotenics, thus resulting in colonies less inbred than those headed by neotenics alone (Husseneder et al., 2005). Based on this, it seems likely that the two extended family colonies, T3 and M6, are both quite young and have low numbers of neotenics, possibly reproducing in conjunction with a primary reproductive. The fact that the level of inbreeding is also not significantly greater in the extended family colonies, as compared to the simple family colonies, further corroborates that interpretation that the extended families are quite young (Husseneder et al., 2005). However, the sample size of extended families is very small, and so I extrapolate these results with caution.

When comparing the mixed family colonies of M. viator with the simulations, it is clear that results are best approximated by a pleometrotic colony, which has engaged in subsequent inbreeding (Table 4.10). It is also possible that rather than a pleometrotic association during colony foundation, an additional primary reproductive may have been adopted by the colony. The subsequent reproduction by neotenics suggests that these are fairly mature colonies, and lends further weight to the possibility that these mixed families may have arisen due to adoption rather than pleometrosis. Additionally, pleometrosis and adoption would both account for relatedness values below 0.5 (Husseneder et al., 2005; Nobre et al., 2008), and for the low $F_{IC}$ values (Husseneder et al., 2005). Had the mixed families arisen via colony fusion, as in the simulation of mixing workers from unrelated nests, we would have expected positive $F_{IC}$ values (Thorne et al., 1999; Husseneder et al., 2005). Although both T2 and M5 have slightly positive values of $F_{IC}$, neither was significantly different from zero.

Colony generation via budding is not common among termites (Husseneder et al., 2005; Brandl et al., 2005; Roy et al., 2006; Vargo & Husseneder, 2011), although it may occur more frequently in subterranean species (Vargo, 2003). While M. viator is technically a mound-building species, the bulk of their nest structure is subterranean and, as such, it is valuable to examine the data for any evidence of budding. My sampling strategy was, by necessity, opportunistic, and therefore there are only three pairs of neighbouring colonies (T1-T2, D9-D10 & W11-W12) within the sample. However, looking at the relationship between the colonies within each of these pairs
allows us to consider the possibility of budding as a strategy within *M. viator*. When considering each potentially budded pair, we can see that there is no significant difference between the coefficients of T1-T2 and D9-D10 (Table 4.10). However, although the coefficients of W11-W12 lie within the confidence intervals for the other two pairs, W11-W12 appears to have a narrower range of variability. If we then compare *M. viator* results with the simulations, we can see that the average relatedness between each pair is as high as would be expected in budded colonies. However, none of simulated scenarios is a good match for either T1-T2 or W11-W12. That said, the relationship between D9-D10 could be explained by either scenario 1 or 4 (Table 4.10). While the first scenario provides a fairly close approximation of the data, scenario 4 is based on the presence of 100 neotenics which, as already discussed, is unlikely in *M. viator*. Therefore, it may be possible that D10, a mixed family, gave rise to D9, a simple family. However, based on this limited sample size and unclear association, I would postulate that budding is unlikely, although not impossible, in *M. viator*. Furthermore, if budding were occurring, we would expect to see a high proportion of extended families, high population viscosity and high levels of inbreeding (Vargo, 2003; Brandl et al., 2005; Vargo & Husseneder, 2011). However, from the data there is a low proportion of extended families (± 16%) and when we look at intercolonial relatedness in conjunction with intercolonial distance (Fig. 4.9) across the whole population, we can see that there is no clear trend for decreasing relatedness with increasing colonial distance. Additionally, the inbreeding coefficients remain consistently low across the population. Finally, in termite species which have shown evidence of budding, such as *S. lamanianus* and *C. formosanus*, the nest areas tend to be very large – ranging from 50 to 100m (Hussender et al., 1998; Hussender et al., 2005), which can facilitate passive budding. However, *M. viator* colonies are generally only in the region of 20 to 30m, further undermining the likelihood of budding in *M. viator*. 
Conclusion

The primary aim of this investigation was to develop a general understanding of the colony and population genetics of *M. viator*, guided by predictions of outbred primary reproductives with moderate to strong differentiation between colonies and mild population viscosity with moderate to strong differentiation between geographical areas. Additionally, I anticipated a variable mix of family types within the population and a lack of evidence for budding as a mode of colony foundation.

On average, there are 5.8 alleles per locus, ranging from 2 to 14 alleles per locus over seven polymorphic loci. After a Bonferroni correction, all geographical populations were in Hardy-Weinberg equilibrium. As predicted, individual colonies show significant differentiation from one another, and likewise, if colonies are grouped geographically, each area is also distinct from each other area. These findings are supported by genetic distance analysis, which shows clear clustering of colonies within areas. Therefore, each colony can be considered to be a discrete entity, with low to moderate population viscosity.
Across all colonies, and within each area, a range of family types was expressed. The simple families appear to be headed by an outbred pair of reproductives, while the extended families generally support a low number of neotenics. These younger simple and extended family colonies tend to exhibit heterozygote excess. Within the population as a whole, there is a propensity towards mixed families, generated either via pleometrosis or adoption, likely with an additional female. The high frequency of mixed families may be in response to environmental stress. However, to confirm this hypothesis the frequency of mixed families would need to be determined across *M. viator*’s range and compared to rainfall pattern data. There is no compelling evidence of budding occurring as a mode of colony foundation, and thus the modest population viscosity is likely attributable to limited alate dispersal distances.

There is a fairly high level of background relatedness within the study population, and likewise elevated average relatedness within each geographical area. Although average relatedness within colonies is also high, the relatedness averages across family types are in keeping with those of other termite species and values postulated by computer simulations. There is no significant evidence of inbreeding within any individual colonies or across any family types. In fact, the only significant results generated for inbreeding were towards inbreeding avoidance, supporting the idea that colonies are founded by outbred reproductives. Given that alate release in *M. viator* is initiated by rainfall events (Coaton & Sheasby, 1974), it is like that there is synchronous release of alates between colonies within geographical areas, and that dispersal distances are likely in the region of several hundred meters – sufficiently far to generate outbred colonies, but limited enough to result in mild population viscosity.
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Chapter 5

Behavioural Interactions between Colonies of

*Microhodotermes viator*

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**Introduction**

The importance of recognition in social insects

Among eusocial insects, nestmate recognition - the ability to discriminate between colony members and non-members (Neoh *et al*., 2012) - is an important characteristic for two reasons. Firstly, there are normally only a few reproductives in a colony, as such the majority of the colony consists of sterile or non-reproductive workers (Dietemann *et al*., 2003). In order for non-reproductive individuals to maximize their genetic fitness, according to Hamilton’s theory of kin selection (Hamilton, 1964), it is essential that the benefit of their labour be directed towards kin (Greenberg, 1979; Carlin & Holldobler, 1983; Suarez *et al*., 2002; Yusuf *et al*., 2010). Hamilton’s theory of kin selection operates under the principal that the level of relatedness between an altruist and a beneficiary, must be sufficient that the benefit to the altruist, of helping the beneficiary, exceeds the cost incurred by the altruist. Although nestmates may not be full siblings, they are usually considered to be kin and treated as such, in order to maintain colony integrity (Buczkowski & Silverman, 2004). Secondly, the ability to discriminate is important for defence of resources from non-colony members – be they non-kin conspecifics or members of another species (Dahbi & Lenoir, 1998; Jmhasly & Leuthold, 1999; Hernandez *et al*., 2002; Buczkowski & Silverman, 2004; Jungnickel *et al*., 2004). This importance is exemplified by the numerous defensive mechanisms selectively employed by colonies, such as the construction of robust, and often cryptic, nests and the development of specialised defensive castes (Ishikawa & Miura, 2012), which protect the colony not only from predators but also from non-colony conspecifics. Ultimately, colony integrity must be maintained through a strong and efficient defence system, which minimises the costs associated with disease
transmission or reproduction with unrelated individuals, and maximises protection of the brood and colony resources (Dornhaus et al., 2004; Neoh et al., 2012). Initiating a defensive response however, is contingent on the ability to recognise or discriminate, and there are several means by which recognition may be achieved in social insects.

**Means of recognition**

The underlying factor, upon which all recognition is based, is phenotypic variation (Husseneder et al., 1998). However, there are several different variable cues which may be utilised for recognition, and identifying which cues, or combinations of cues, are being used by a particular species, has been the focus of a large body of research. Several decades ago, most of the recognition research was conducted on ants. These studies have generally shown that ants have well-developed mechanisms for recognition that allow them to accept or reject individuals, based on the comparison of an encountered individual’s chemical cues to a template that the discriminating individual accepts as being representative of a colony member (Errard, 1994; Lahav et al., 1999; Buczkowski and Silverman, 2004; Yusuf et al., 2010). Although comparatively few studies on recognition have been conducted for termite species (Kirchner & Minkley, 2003), the ever-expanding body of research has brought to light the fact that recognition more likely functions as a complex hierarchical suite of cues (Bordereau & Pasteels, 2011). Two main categories of cues have been identified; chemical cues, having a genetic and/or environmental basis, and odour cues from symbiotic gut bacteria.

**Chemical recognition cues**

Hydrocarbon compounds are the major components of cuticular lipids, found on the epicuticle of all terrestrial insects (Haverty et al., 1988; Dietemann et al., 2003; Howard & Blomquist, 2005). Hydrocarbons are composed of an homologous series of long straight-chained, saturated alkanes, that can be modified though the addition of methyl groups and double bonds (Yusuf et al., 2010). These cuticular lipids are thought to play functional roles in sexual attraction, territory marking, recruitment, alarm pheromones (Howard et al., 1982), chemical mimicry, chemical communication and prevention of desiccation (Nowbahari et al., 1990; Hernandez et al., 2002; Lucas et al., 2004). By
encoding information about an individual’s species, caste, gender and age (Yusuf et al., 2010), cuticular hydrocarbons (CHC’s) also play a central role in recognition (Haverty et al., 1988; Bagneres & Morgan, 1990; Jungnickel et al., 2004; Howard & Blomquist, 2005). Behavioural studies confirm that termites exchange a continuous stream of chemical information via physical contact with one another, and both qualitative and quantitative differences in CHC profiles between conspecifics colonies have been demonstrated (Clement & Bagneres, 1998).

CHC’s may be genetically and/or environmentally derived (Errard, 1994). As such, a high degree of chemical specificity within, and variability between, species and colonies has been documented (Lahav et al., 1999; Jungnickel et al., 2004). Although CHC profiles alone cannot always fully explain intercolonial discrimination (Florane et al., 2004), their variability provides considerable potential for recognition. However, due to this extreme variability, there are also likely some further internal regulatory mechanisms, acting within colonies to standardise the colony odour (Clement & Bagneres, 1998).

(i) Genetically determined recognition cues

Endogenous, or genetically determined, hydrocarbons are internally synthesized and then transported to the insect cuticle (Howard & Blomquist, 2005). Due to that fact that nestmates tend to be highly related to one another (Buczkowski & Silverman, 2004), heritable genetic cues provide an important source of variation for recognition, and have been well demonstrated by many studies. Getty et al. (2000) found that behavioural trials between groups of Reticulitermes workers, which were from the same colony but collected on different occasions, always resulted in acceptance. Furthermore, Reticulitermes colonies can be maintained in the laboratory for an extended period of time, without losing their ability to discriminate between colony and non-colony members. Although these results are restricted to only a few Reticulitermes species, and are not considered typical for the genus, similar results were also noted using Microceroterms fuscotibialis (Olugbemi, 2013). Again, trials between groups of workers from the same colony, but collected at different times, showed no evidence of
significant aggression, regardless of the period of separation. This suggests that in particular species, the factors responsible for recognition remain functional over time, and may be predominantly genetic.

Support for genetically determined hydrocarbons can be found in chemotaxonomic studies, which use gas chromatography to identify species, by analysing the presence or absence and abundance of various components of the hydrocarbon signature (Sobti et al., 2009). Although this method has not been as widely used as DNA sequencing techniques to discriminate geographical variants, it has been used successfully to identify geographical populations of *Reticulitermes lucifugus* (Austin et al., 2002). A study comparing the hydrocarbons of *R. virginicus* and *R. flavipes*, which have sympatric ranges and comparable ecological niches, found that while they shared some similar components, their profiles were still distinctly different, which was supported by behavioural studies (Howard et al., 1982). Furthermore, using chemical analysis of *Zootermopsis angusticollis* colonies, Haverty et al. (1988) revealed that they had the exact same complement of hydrocarbons as found in a study by Blomquist et al. (1979), however the proportions were markedly different. Therefore, it is plausible to state that CHC’s tend to be species specific (Howard & Blomquist, 2005), and are often mediated by the environment.

Increased genetic variation within a colony, due to an increase in the number of reproductives, will extend the variation of the genetic component of chemical recognition cues within a colony, thus lowering the rejection threshold, by increasing the potential spectrum of similarity (Bagneres and Morgan, 1990; Errard, 1994; Sundstrom, 1997; Lahav et al., 1998). In ants, studies have found that workers from polygynous colonies are less discriminating between colony members and non-colony members, than workers from monogynous colonies (Sundstrom, 1997; Suarez et al., 2002). Therefore, hydrocarbon mediated interactions between colonies are, at least to some extent, dependent upon the number of reproductives within a colony (Lahav et al., 1998). Adams (1991), working on *Microcertotermes arboreus*, found that the probability of an aggressive encounter was associated with the level of relatedness between two colonies. As such, workers were far more aggressive towards unfamiliar,
unrelated workers, than they were towards unfamiliar kin. This result adds complexity to the understanding of genetic cues and indicates that they are functional, even in the absence of familiarity. However, these studies also suggest that recognition cues seldom appear to function in isolation.

Both Adams (1991) and Olugbemi (2013) proposed that more than genetic cues alone are used to facilitate recognition. In addition to finding that workers were capable of discriminating between unrelated workers and unfamiliar kin, Adams (1991) also found that workers are capable of discriminating between unfamiliar and familiar kin. He showed that unfamiliar kin were attacked significantly more often than familiar kin, despite the two groups having comparable levels of genetic relatedness. This highlights the fact that additional cues, such as environmental cues or odour cues from symbiotic gut bacteria, are also mediating recognition.

(ii) Environmentally determined recognition cues

Exogenously, or environmentally, derived hydrocarbons are dynamic and differ with respect to season, diet, habitat and nesting material (Beye et al., 1998; Buczkowski & Silverman, 2004). Florane et al. (2004), using solid phase microextraction (SPME), found that altering the diet of Coptotermes formosanus colonies resulted in a qualitative change in the chemical profile of the termites. In contrast, a study on R. virginicus found that all castes contained the same complement of hydrocarbons, but in different abundances (Howard et al., 1982). This is thought to be due to variations in the temperature and humidity to which individuals are exposed, based on where, and which tasks, they perform (Howard & Blomquist, 2005).

Dynamic environmental recognition cues are often capable of overriding more stable genetic cues (Beye et al., 1998; Buczkowski & Silverman, 2004). Cornelius & Osbrink (2009) showed a rapid and significant loss of intercolonial aggression between C. formosanus colonies, in relation to the length of time that they had been maintained in the laboratory. Obin (1986) reared multiple colonies of the ant Solenopsis invicta in the laboratory for 18 months under identical conditions. After this time, he investigated the level of aggression in trials between different laboratory-reared colonies and between
laboratory-reared colonies and field colonies. He found that laboratory colonies were significantly more aggressive towards workers from field colonies, than they were towards workers from other laboratory colonies. When genetically distinct laboratory colonies of *C. formosanus* were fed the same diet for three months, the levels of intercolonic aggressive behaviour was significantly reduced (Florane *et al.*, 2004). Essentially, by controlling for diet and nesting material, a level of variation between colonies is stripped away, with the result of a reduced expression of intercolonial aggression. Again showing the functionality of environmental variation, but in a converse manner, Nowbahari *et al.* (1990) found that intercolonial aggression was significantly correlated with intercolonial distance, a fact which the authors attribute to habitat variation across the intercolonial distance. Although levels of genetic relatedness between colonies were not investigated in these studies, the results still indicated that environmental cues mediate recognition. The effect of diet on recognition was particularly well demonstrated by Liang *et al.* (2001) who found that Argentine ant (*Linepithema humile*) workers were attacked by their nestmates post exposure to a prey item, the brown-banded cockroach (*Supella longipalpa*), due to the transfer of unrecognizable hydrocarbons from the cuticle of *S. longipalpa* to that of *L. humile*. However, in termites, which possess symbiotic gut bacteria, the effects of diet on recognition are more complex.

*Symbiotic gut bacteria odour recognition cues in termites*

Symbiotic gut bacteria have long been known to function in terms of cellulose degradation and nitrogen fixation in termites, however they are now also believed to play a role in recognition processes (Eutick *et al.*, 1978; Kirchner & Minkley, 2003). It has been found that there is a correlation between the distribution of bacterial genera across Isopteran families and the families themselves (Eutick *et al.*, 1978). In other words, there appear to be family-specific assemblages of gut bacteria. Supporting this, a study of gut bacterial assemblages in *Hodotermes mossambicus*, by Minkley *et al.* (2006), found that colonies possess similar, but yet distinctly different, gut bacteria. This is likely due to the fact that gut symbionts are heritable within colonies, insofar as they are transferred from one individual to another via proctodeal trophallaxis (Kirchner
& Minkley, 2003). This is an extremely important process, as termites lose the majority of their gut bacteria with each moult, which must then be replenished (Minkley et al., 2006). Trophallactic exchanges are considered to be a form of altruistic behaviour, and as such, should be preferentially directed towards kin (Kirchner & Minkley, 2003). There is thus a nice symmetry to the fact that the odours from the bacteria themselves should too play a role in nestmate recognition.

The functionality of gut symbionts was demonstrated in an interesting experiment by Kirchner & Minkley (2003), who examined trophallaxis in *H. mossambicus*. Colonies were reared in the laboratory from pairs of sexuals, where very few aggressive interactions were observed between non-nestmates. The recognition experiment took place in two stages. Firstly, as a control, a group of individuals were starved for 24 hours, known as recipients, and a second group, known as donors, were provided food *ad libitum*. Donors and recipients were then paired in nestmate and non-nestmate couples and the number of trophic interactions observed in an arena over a three minute period. In the second part of the experiment, individuals were either left untreated or they received a tetracycline treatment via their drinking water. As with the controls, half the individuals in each treatment were then starved to generate recipients and donors. The results showed that two tetracycline-treated non-nestmates behaved as nestmates, while two nestmates, one tetracycline treated and the other not, behaved more like non-nestmates. The caveat to the above experiment is that significant results were only obtained for larval pairings, and workers showed no significant tendency to feed nestmates more than non-nestmates. However, this may be explained by caste-specific roles, as Nel et al. (1969) found that *H. mossambicus* workers were almost incapable of feeding themselves and, in fact, relied on large larvae to provision them with partially digested food. Nevertheless, similar results have also been shown in *Reticulitermes seperatus, Macrotermes subhyalinus* and *Macrotermes bellicosus* (Kirchner & Minkley, 2003).

It is not yet clear whether the gut symbionts are introduced to the colony by the king and queen, and then subsequently disseminated by workers, or if they originate in the colony food source. However, studies seem to indicate that it is likely a combination of
both avenues of introduction (Minkley et al., 2006). Furthermore, genetic variation could also possibly affect the colonisation by microbes, through its impact on cuticular hydrocarbon profiles within a colony (Calleri et al., 2006).

Modes and means of recognition

During the process of recognition, individuals ‘read’ one another’s labels and then compare them to some form of a ‘template’. This process requires that each individual acquire and express their own label, and learn a template against which to compare labels (Kaib et al., 2004; Yusuf et al., 2010). There are several different explanations for how labels may be acquired.

The individualistic model proposes that labels are genetically derived and non-transferrable, while the environmental model purports that labels are environmentally derived, and are thus by nature transferrable. Alternatively, it may be that labels are originally disseminated by the king and queen (Kaib et al., 2004). However, regardless of the origin/s of the labels, many studies have shown that colonies express a distinct and generally uniform colony profile (Howard et al., 1982; Clement & Bagneres, 1998; Austin et al., 2002; Sobti et al., 2009). The concept of a colony profile is well explained by the gestalt hypothesis.

The gestalt hypothesis proposes that a uniform colony odour is created by the mixing of individual hydrocarbons, through behavioural contact such as trophallaxis and allogrooming (Lucas et al., 2004; Lahav et al., 1999; DeHeer & Ross, 1997). As such, the specific odour of each individual in a nest is representative of the entire colony (Dahbi & Lenoir, 1998). This may also explain how inquilines achieve colony integration. Studies have shown that beetle termitophiles (Staphylinidae) have achieved complete social integration into R. flavipes colonies by exactly mimicking their host’s cuticular hydrocarbon profiles (Howard et al., 1982). Subterranean termites generally live in closed systems of interconnecting galleries, with essentially static air movement, thus the primary means of communication will be tactile and chemosensory (Howard et al., 1982; Clement & Bagneres, 1998; Yusuf et al., 2010). As hydrocarbons are non-
volatile, physical contact between two individuals is required for full behavioural expression of acceptance or rejection (Lahav et al., 1999; Jungnickel et al., 2004). A cooperative colony requires a level of social regulation (Bordereau, 1985), and the gestalt odour is likely beneficial to colonies by increasing the overall productivity of the nest, and by reducing inter-worker aggression (Greenberg, 1979). This may be particularly important in colonies where all individuals are not equally related, such as those headed by multiple neotenics.

**Agonism**

The primary purpose of recognition, is to maintain an efficient defence strategy (Buczkowski & Silverman, 2004; Jungnickel et al., 2004), in order to maximise genetic fitness of the colony (Suarez et al., 2002; Yusuf et al., 2010). Thus recognition facilitates agonistic, or competitive, interactions between colonies. Agonism can be defined as the combination of offensive and defensive responses between competing individuals (Polizzi & Forschler, 1999). Agonistic behaviour generally occurs in the presence of non-nestmates, such as when termites attempt to acquire or defend resources (Fuller et al., 2004; Yusuf et al., 2010; Olugbemi, 2013), and manifests as fighting, fleeing or submission (Haverty & Thorne, 1989; Olugbemi, 2013). As such, termites which exhibit aggression towards one another are generally considered to be from different colonies (Getty et al., 2000). However, while agonism between colonies is considered to be the norm, passive encounters between conspecific colonies are often recorded (Getty et al., 2000) and warrant further consideration.

Levels and expressions of agonism are highly variable, both between and within termite species (Clement & Bagneres, 1998; Polizzi & Forschler, 1999; Fuller et al., 2004; Cornelius & Osbrink, 2009; Neoh et al., 2012). For example, both Cornelius & Osbrink (2009) and Florane et al. (2004) noted that *C. formosanus* colonies display variable levels of intercolonial agonism, with some colony pairs being highly aggressive while others interact amicably. Similarly, workers from different colonies of *Macrotermes carbonarius* can often be found peaceably foraging together (Neoh et al., 2012). Given the relative importance of recognition, it may seem surprising that some colonies appear not to recognise one another as foreign. However, it is important to bear in mind that
aggression is the most dramatic form of discriminatory behaviour (Jmhasly & Leuthold, 1999), and that a lack of aggression does not equal a lack of recognition. Colonies of Microtermes species have been observed isolating themselves from one another through the construction of soil barriers, rather than engaging in aggressive behaviour (Olugbemi, 2013). Therefore, it would seem that there are additional factors mediating the expression of agonistic behaviour.

Agonism between colonies varies with colony circumstance and environmental influences (Sundstrom, 1997; Dahbi and Lenoir, 1998; Lepage & Darlington, 2000). Colony circumstance includes genetic composition, age, health and reproductive status of colonies. For example, raising nymphs to the alate stage is energetically expensive and colonies may choose to defend themselves more aggressively during this phase (Neoh et al., 2012). Family type may also affect aggression. For example, in a study of simple (closed) family colonies of Reticulitermes grassei, it was found that there was a high level of genetic differentiation between colonies and a very high level of intercolonial aggression (Clement & Bagneres, 1998). Environmental influences include season and geographical distribution of the population (Clement & Bagneres, 1998; Fuller et al., 2004; Korb & Foster, 2010; Yusuf et al., 2010; Neoh et al., 2012). For example, colonies of R. lucifugus and R. grassei will interact amicably with conspecifics during summer, but aggressively during winter (Clement & Bagneres, 1998). As such, both the ecology and the environment mediate the relative costs and benefits of species interactions (Korb & Foster, 2010), and at times engaging in territorial fighting may simply be too costly for a colony (Neoh et al., 2012).

**Recognition in Microhodotermes viator**

Most ‘higher’ termites, from the family Termitidae, are able to discriminate between nestmates and non-nestmates (Fuller et al., 2004), however this has not been as well demonstrated in the ‘lower’ termite families. M. viator is a ‘lower’ termite from the family Hodotermitidae (Picker et al., 2002; Inward et al., 2007), which constructs earth-mound nests, the bulk of which is subterranean (Picker et al., 2007). M. viator engages in ‘separate’ type foraging, such that the nest and foraging sites are independent of one
another (Legendre et al., 2008). In winter, *M. viator* forages during the day out in the open with thousands of workers emerging simultaneously to gather food and return it to the nest (personal observation). Foraging *en masse* may be beneficial by improving the efficiency of exploiting aggregated resources, and reducing the amount of time spent outside the safety of the nest (Hassell & Southwood, 1978). However, foraging behaviour has a high level of associated risk - not only from predators and the natural elements, but also from both hetero- and conspecifics (Hassell & Southwood, 1978). Therefore, an additional benefit of group foraging may be that colonies are able to harness the effects of cooperative defence, which can play an effective role if a significant number of the group would survive an attack (Hassell & Southwood, 1978).

The nest system of *M. viator* comprise very large (up to 20 m diameter) ‘heuweltjies’ (earth mounds) – sand layers which cover the largely subterranean nest itself and its central hive. The age of the mounds has been estimated at thousands of years (Moore & Picker 1991; Midgley et al. 2002), suggesting that may be a site of sequential colonisation by generations of *M. viator*.

Given *M. viator*’s foraging pattern⁶, and very long-lived nest system, it is hypothesised that this species should employ a high level of innate colony defence. Furthermore, if both environmental and genetic factors play a role in recognition cues, then the greater the geographical distance between two colonies, the more likely they should be to recognise one another as non-nestmates, and respond with appropriately scaled agonistic behaviour (Nowbahari et al., 1990).

Levels of intraspecific aggression are informative of relationships between different colonies (Getty et al., 2000). Thus, the aim of this investigation is to gain a better understanding of the behavioural interaction between colonies of *M. viator*, across

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⁶ Across their range, *heuweltjies* are non-randomly distributed, with consistent inter-mound distances of 45 to 50m (Coaton & Sheasby, 1974; Lovegrove & Siegfried, 1989; Midgley & Musil, 1990; Lovegrove, 1991; Moore & Picker, 1991; Laurie, 2002; Cramer et al., 2012). This underdispersed spatial pattern is reflected in an R value (Clark & Evans, 1954) of 1.7 (Lovegrove & Siegfried, 1989).
variable geographic distances, by conducting laboratory-based behavioural interaction bioassays.

**Materials and Methods**

*Termite collection*

Sampling was conducted in the Western Cape Province of South Africa, from May to July 2011. The Western Cape experiences Mediterranean-like winters which are cold and wet, with average day-time temperatures of 18°C and rainfall of about 80mm per month (The Weather Channel). In order to obtain sufficient samples from each colony, colonies engaging in *en masse* foraging activities were targeted for collection, where thousands of workers emerge simultaneously. Foraging *en masse* was only observed on warm, sunny, windless days after recent rain. As emergence of *M. viator* is known to be unpredictable (Dean, 1993), foraging colonies were opportunistically identified by walking through renosterveld vegetation rich areas on days when the climatic conditions were suitable for foraging. Due to the necessitated sampling strategy, only two colonies (W7 and W12) were sampled off identifiable *heuweltjies*. In total, twelve discrete colonies were sampled from across four distinct geographical areas - Tygerberg, Malmesbury, Darling and Worcester (Table 5.1, Fig. 5.1).

The distances between the four areas ranged from 34.37 – 102.63km, and the intercolonial distances within areas ranged from 0.69 – 3.04km. Two to three hundred individuals from each of the 12 colonies were returned to the laboratory and maintain in nest boxes prior to behavioural analysis.
Table 5.1 Location of 12 *M. viator* colonies, sampled for genetic and behavioural analysis from four discrete geographical areas.

<table>
<thead>
<tr>
<th>Area</th>
<th>Colony Name</th>
<th>GPS coordinates (decimal degrees &amp; minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tygerberg</td>
<td>T1</td>
<td>33.87471° S 18.5960° E</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>33.87827° S 18.59659° E</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>33.85188° S 18.60552° E</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>33.85479° S 18.59888° E</td>
</tr>
<tr>
<td>Malmesbury</td>
<td>M5</td>
<td>33.45976° S 18.74372° E</td>
</tr>
<tr>
<td></td>
<td>M6</td>
<td>33.46712° S 18.74000° E</td>
</tr>
<tr>
<td>Darling</td>
<td>D8</td>
<td>33.37391° S 18.37657° E</td>
</tr>
<tr>
<td></td>
<td>D9</td>
<td>33.38763° S 18.38231° E</td>
</tr>
<tr>
<td></td>
<td>D10</td>
<td>33.38926° S 18.38229° E</td>
</tr>
<tr>
<td>Worcester</td>
<td>W7</td>
<td>33.60986° S 19.44442° E</td>
</tr>
<tr>
<td></td>
<td>W11</td>
<td>33.62693° S 19.43371° E</td>
</tr>
<tr>
<td></td>
<td>W12</td>
<td>33.62689° S 19.43435° E</td>
</tr>
</tbody>
</table>

Fig. 5.1 Satellite image of the Western Cape of South Africa showing the location of 12 colonies of *M. viator* (Image source: Google™ Earth). Elevation approximately 172 km.
Colony maintenance within the laboratory

Each colony was maintained in a separate nest box in a constant temperature room, set at 22°C and 50% humidity on a 12:12 light:dark cycle. Although 22°C was slightly higher than the average day-time temperature during the collection period, the termite colonies did not fare well in the laboratory at lower temperatures.

Each nest box (Fig. 5.2) was comprised of two compartments, a nest area and a foraging arena, joined together by a 10cm piece of clear tubing with a 1cm diameter, referred to as the foraging portal. The nest area consisted of an opaque box (15 x 21 x 9cm), with a window in the lid to allow unobtrusive observation of the termites. Additional surface area within the nest was provided by large sheets of filter paper with a corrugated fold. The foraging arena was a transparent box (15 x 22 x 6cm), and it was in this area that food and water were provided.

![Fig. 5.2 Termite colony nest boxes, showing the opaque nest area, with a window in the lid, the transparent foraging arena and the foraging portal connecting the two.](image)

Food, in the form of a mixture of cereal crumbs (Weet-Bix, Bokomo brands) and oat bran, was provided *ad libitum*. Water was also provided *ad libitum*, in 0.2µl thin-walled PCR tubes which were simply filled up and laid on their sides. As the diameter of the PCR tubes was so small, the surface tension of the water was maintained, allowing the termites’ easy access to the water without the risk of drowning. All surface areas within the nest box, including the inside of the foraging portal and PCR tubes used for water, were lightly abraded with a fine-grain sandpaper to allow surface traction for the termites.
Although soldiers are the specialised defensive caste in many termite species, workers form the bulk of the colony workforce. In *H. mossambicus* soldiers are only occasionally found outside the nest and often do not move more than a few centimetres from the foraging portal (Kok & Hewitt, 1990), and it is likely that soldiers behave similarly in *M. viator*. Workers, on the other hand, engage in *en masse* foraging, brood-care, nest building and maintenance and even defence, as they have been shown to be able to actively respond to changes in their environment, irrespective of age or morphology (Polizzi & Forschler, 1999; Ishikawa & Miura, 2012). Furthermore, workers are well suited to defence, due to their strong mandibles (Haverty & Thorne, 1989). This is particularly true for major workers among the harvesters, which are primarily responsible for cutting vegetation, while minor workers are responsible for transporting vegetation (Duncan & Hewitt, 1989). As such, ideally all behavioural assays would be conducted with major workers, however minor workers outnumber major workers by approximately 4:1 (Watson, 1973), making obtaining a sufficient number of major worker samples challenging. Additionally, although there is a significant difference in head capsule width between the two worker classes within any given colony, the absolute size of the two worker classes varies widely from one colony to the next (Watson, 1973). Therefore, all behavioural interactions were conducted between a variable mix of both major and minor workers.

One-on-one experimental and control trials were performed in the same manner in a neutral arena, comprised of a 6.5cm Petri dish lined with a disc of Whatmans No.1 filter paper, moistened with 200µl of water. After each trial, the disc of filter paper was discarded and the Petri dishes were wiped out with 96% ethanol to remove any hydrocarbons which may have been deposited during the previous trial.

Prior to use in a trial, individuals were marked with a very small dot of either white or pink Tipp-Ex on their thorax. The mark was made by dipping the tip of a toothpick in the Tipp-Ex and then lightly dotting it on the thorax, while the individual was still in its nest box. Paint and ink markings are commonly used to identify individuals for
behavioural research (Hagler & Jackson, 2001). This marking method was chosen as it allowed for many individuals to be marked in quick succession and minimized handling of the termites, as well as being cost-effective, quick-dying, lightweight and highly visible. After marking, individuals were allowed to remain in their nest boxes for at least 20 minutes, prior to use in a trial.

Prior to commencing a trial, the two target individuals were removed from their nest boxes, using soft (larval) forceps, and placed in separate sterile 1.5ml Eppendorf vials for five minutes. The two individuals were then simultaneously introduced into the Petri dish by opening and inverting the Eppendorfs.

Each trial was conducted for three minutes, commencing from first contact between the two individuals. During the trial, a range of behaviours and their time of occurrence were recorded, following a modified version of aggression scores used by Adams (1991) (Table 5.2). If at the end of the three minute trial period both individuals were still alive, then they were left in the Petri dish for at least 12 hours. If, after 12 hours, either individual was dead or moribund, with clear evidence of aggression having occurred (e.g. amputated limbs), the outcome of the trial was recorded as rejection. After completion of each trial, surviving individuals were frozen at -20°C; no individual was used more than once.

<table>
<thead>
<tr>
<th>Behaviour Code</th>
<th>Description of Behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>Neutral response: Contact without examination or attack</td>
</tr>
<tr>
<td>A</td>
<td>Antennation</td>
</tr>
<tr>
<td>MF</td>
<td>Mandible Flaring</td>
</tr>
<tr>
<td>Def</td>
<td>Defecation: deposition of anal fluid on, or near, opponent</td>
</tr>
<tr>
<td>P/N</td>
<td>Pulling and/or ripping - without visible damage</td>
</tr>
<tr>
<td>B</td>
<td>Severe biting – with visible damage</td>
</tr>
<tr>
<td>D</td>
<td>Death (or moribund) of one or both individuals</td>
</tr>
</tbody>
</table>

Table 5.2 Range of behaviours recorded during termite aggression trials.
In total, 376 one-on-one behavioural interactions were conducted (Table 5.3); 12 control trials, with five or six replicates of each trial (average = 5.9), and 66 experimental trials, with between one and six replicates of each trial (average = 4.6).

<table>
<thead>
<tr>
<th></th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>M5</th>
<th>M6</th>
<th>W7</th>
<th>D8</th>
<th>D9</th>
<th>D10</th>
<th>W11</th>
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<td>5</td>
<td>1</td>
<td>6</td>
<td>6</td>
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</tr>
</tbody>
</table>

Data analysis

Statistical significance was set to $\alpha = 0.05$ for all statistical tests. Basic descriptive statistics were performed in Microsoft Office Excel 2007.

Fishers exact probability test, between the occurrence of biting and trial outcome, was performed in Statistica 11 (StatSoft, Inc., 2012).

A generalised linear mixed model (GLMM) was constructed to examine the data for significant predictors of trial outcome. A GLMM combines the statistical approaches of linear mixed models, which can accommodate random effects, and generalised linear models, which can accommodate non-normal data (Bolker et al., 2008). As such, GLMMs quantify the magnitude of variation among a number of measured variables
against a response, while taking random effects into account. The modelling was done using Stata/MP 11 (StataCorp, 2009).

In the model, the random effect was considered to be the pairing of different colonies in each experiment (Table 5.4). By taking the variation within and between different colony pairings into account, it allows the model to better identify significant fixed effects. Death, as a proxy for rejection, was used as the binary response variable and, as such, a logistic mixed model was used. The fixed effects variables considered for inclusion in the model were; ‘neutral response’, ‘antennation’, ‘mandible flaring’, ‘pulling & nipping’, ‘distance’ and ‘relatedness’. Distance was factorised to two biologically meaningful levels – ‘close’ (0.06 – 3.04 km) and ‘far’ (34.37 – 102.63 km). Attempts to further factorise distance, to the same fine scale used above, were not successful. Mean intercolonial pairwise relatedness (calculated, as per Ch.4, using the program IDENTIX 1.1.5, Belkhir et al., 2002) was factorised to four levels, according to the data. Biting and defecation, although fixed effects, were not used for this analysis, as both perfectly predict death (autocorrelated), therefore the model is unable to compute an odds ratio for either behaviour.

**Table 5.4 Terms and effects used to build the GLMM.**

<table>
<thead>
<tr>
<th>Model Terms</th>
<th>Effect</th>
<th>Form of the Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony-Pair</td>
<td>Random</td>
<td>Text</td>
</tr>
<tr>
<td>Death</td>
<td>Response</td>
<td>Binary (0/1)</td>
</tr>
<tr>
<td>Neutral response</td>
<td>Fixed Effect</td>
<td>Binary (0/1)</td>
</tr>
<tr>
<td>Antennation</td>
<td>Fixed Effect</td>
<td>Binary (0/1)</td>
</tr>
<tr>
<td>Mandible Flaring</td>
<td>Fixed Effect</td>
<td>Binary (0/1)</td>
</tr>
<tr>
<td>Pulling &amp; Nipping</td>
<td>Fixed Effect</td>
<td>Binary (0/1)</td>
</tr>
<tr>
<td>Distance</td>
<td>Fixed Effect</td>
<td>Factorised (Close or Far)</td>
</tr>
<tr>
<td>Relatedness</td>
<td>Fixed Effect</td>
<td>Factorised (0.4-0.5; 0.5-0.6; 0.6-0.7; 0.7-0.8)</td>
</tr>
</tbody>
</table>
Results and Discussion

Control trials

Participants in control trials only exhibited three behaviours; neutral responses, antennation or pulling & nipping (Fig. 5.3). The first behaviour exhibited during a trial was almost always a neutral response, with accompanying antennation. Although, for one replicate in colony M6, pulling and nipping was the initial response. Pulling & nipping only occurred in two other colonies, M5 and D9, although not as a first response. All of the control trial replicates had an ultimate outcome of acceptance.

The lack of aggression during the control trials is as expected, although the low frequency of mild aggression (pulling & nipping) is slightly unusual. However, in a study by Haverty & Thorne (1989) on Zootermopsis, similar results were noted. They also conducted behavioural assays in Petri dish arenas, and found that when termites were introduced to the arena, some were on ‘alert’ and engaged in transient and mild aggressive behaviour, which was not sustained unless the interaction was truly aggressive. Therefore, the occurrence of pulling & nipping in the three control trial replicates, is likely due to the bioassay design rather than any true agonism, particularly since all three interactions ended in acceptance.

![Fig. 5.3 Behaviours which were exhibited during control trials and the number of control trial replicates in which each behaviour was observed.](image-url)
**Experimental trials**

During the experimental trials, the full range of behaviours was exhibited. The most common initial response was antennation, followed by the neutral response. However, pulling & nipping and biting, often accompanied by defecations, was observed in all trials which ended in rejection (Fig. 5.4).

Of the 305 experimental trial replicates, only 70 (23%) ended in rejection, which is a much smaller percentage than expected. A possible explanation may be seasonal variations in aggression, known to be operational in termites. In this study, colonies were sampled during the rainy season, when food sources are abundant. Thus the resource-rich environment may provide little impetus for aggressive defence and the potential costly loss of workers during this foraging-intensive period.

In order to examine the relationship between biting (the most severe of all agonistic interactions) and interaction outcome (acceptance/rejection), Fisher’s exact probability test was performed against the null hypothesis that biting and outcome are independent of one another. The results of the test indicate a strong significant association ($p < 0.0001$) between biting and outcome, with a markedly higher proportion of trial replicates ending in rejection when biting occurs.

![Fig. 5.4](image)

*Fig. 5.4 Behaviours which were exhibited during experimental trials and the total number of experimental trial replicates in which each behaviour was observed.*
When the level of aggression exhibited by each colony was compared, using the percentage of trial replicates ending in rejection, there was a fair amount of variability (Fig. 5.5; Table 5.4). Naturally, the outcome of an interaction would be dependent on several different factors, such as intercolonial distance or colony state. However, \textit{ceteris paribus}, some colonies appear to be more aggressive than others. Generally, 20 to 30% of trial replicates appear to end in rejection, with T3 exhibiting the highest score of 50% and W11 and W12 the lowest scores of 2 and 6% respectively.

![Graph showing percentage of total trial replicates for each colony which ended in rejection.](image)

**Fig. 5.5** Percentage of total trial replicates for each colony which ended in rejection.

There are several reasons as to why colonies might display variable levels of aggression. Firstly it may be a function of colony state (Neoh \textit{et al.}, 2012), which unfortunately was not something that this study was able to ascertain. However, given that colonies within geographical areas appear to be on the same seasonal developmental cycle, this is an unlikely explanation. A second reason may be due to family type. However, T3 is an extended family, while W11 is simple and W12 is mixed (see Chapter 4). Therefore, it is unlikely that family type alone can account for this variation. A final explanation may lie with colony size. The size of a particular nest is often proportional to the colony size (Emerson, 1938), and may mediate the level of expressed aggression. Although no significant relationship between mound size and level of aggression was found in \textit{M. subhyalinus}, \textit{M. bellicosus} (Jmhasly & Leuthold, 1999) and the ant \textit{Rhytidoponera confusa} (Crosland, 1990), this relationship has never been explicitly tested in \textit{M. viator}.
and therefore remains a plausible factor in explaining variation in aggression between colonies.

An interesting outcome of this study was that many of the colony pairings produced inconsistent results between trial replicates. Of the 66 colony pairings, 35 showed complete acceptance for all trial replicates, while only six pairings showed complete rejection for all trial replicates (Table 5.5). The latter included colony pairing from both within and between geographical areas. However, it is important to note that a portion of these trials were based on only a single replicate. The other 25 colony pairings exhibited final outcomes of both acceptance and rejection among the trial replicates. The occurrence of inconsistent replicate outcomes between a single colony pairing may be due to several factors. It is possible that the behavioural assay was too artificial to elicit a natural response, however all the control trials exhibited consistent outcomes. Alternatively, colonies may be using a complex suite of cues upon which recognition is based (Kaib et al., 2004). Additionally, there is likely a division of labour among workers (Watson, 1973) such that major workers may be better suited, and therefore more inclined, to engage in aggressive behaviour (Polizzi & Forschler, 1999).

Table 5.5 Matrix of experimental colony pairings. Pairings highlighted in orange indicate that all trial replicates ended in rejection, while those highlighted in blue indicate that all trial replicates ended in acceptance.
In behavioural studies, bioassay design and management has certainly been shown to affect levels of aggression between colonies (Cornelius & Osbrink, 2009). Laboratory-based agonism assays tend to be executed in highly artificial environments, which may mediate normal social behaviour (Polizzi & Forschler, 1998). The commonly utilised Petri dish arena may not adequately simulate natural conditions, and therefore result in reduced agonistic behaviour, especially since behavioural and ecological contextual data are absent or biased (Cornelius & Osbrink, 2009). When Cornelius & Osbrink (2009) compared trial outcome between Petri dish arenas and Y-tube arenas, they did find that less aggression was displayed in the Petri dishes. However, many studies using Petri dish arenas have still demonstrated high levels of intercolonial aggression (Jmhasly & Leuthold, 1999; Getty et al., 2000; Delphia et al., 2003; Florane et al., 2004; Olugbemi, 2013). Furthermore, as M. viator routinely forages in the open, the open test arena may not have been particularly disruptive. An alternative consideration of bioassay design relates to the number of individuals used in each trial replicate, and the number of replicates conducted for each colony pairing.

The number of individuals used in each replicate can affect the outcome, and generally using up to 10 individuals from each colony, per a replicate, produces more reliable results (Cornelius & Osbrink, 2009). However, Delphia et al. (2003) found no obvious difference in results when comparing one-on-one interaction outcomes with 10-on-10 interaction outcomes. Additionally, an insufficient number of trial replicates may also lead to false conclusions. Polizzi & Forschler (1998) recommend at least 30 replicates for each assay, however typically fewer number of replicates are used, and generally researchers appear to use between 4 and 10 replicates (Delphia et al., 2003). Due to the logistics of collection and laboratory maintenance, as well as resource constraints, using large numbers of individuals in each interaction and performing large numbers of trial replicates, is often not realistically possible, especially if freshly collected termites from the same collection are to be used in all cross trials. Field trials might have yielded different results but, given the large intercolonial distances and erratic emergence of foragers, this was not a feasible approach for this study. Despite these caveats of laboratory-based bioassays, studies that have performed trials in the laboratory and then
repeated them again in the field, found that the outcome of laboratory trials are generally a good predictor for the outcome of field trials (Cornelius & Osbrink, 2009).

Animals modulate their behaviour in response to rapid changes in the environment, a plasticity that is highly evident in social insects (Ishikawa & Miura, 2012). Thus, in addition to bioassay design, the laboratory environment likely mediates the outcome of behavioural trials. Shelton & Grace (1997) found no agonism between conspecific laboratory colonies of *C. formosanus*, while pairings of field and laboratory groups displayed reduced levels of aggression. Given that recognition is based on a combination of factors such as genetic, environmental and odour cues, which work in conjunction with one another, it may be that for aggression to be elicited, a particular combination and intensity of cues need to be displayed (Polizzi & Forschler, 1999). In laboratory colonies, environmental variation in odour is usually reduced, due to standardisation of nesting material and food type (Adams, 1991; Polizzi & Forschler, 1999). This reduced variation in recognition cues, manifests in the commonly observed phenomenon that levels of aggression decrease in accordance with length of time in the laboratory (Polizzi & Forschler, 1999; Cornelius & Osbrink, 2009), although this was not observed in *Zootermopsis* colonies (Haverty & Thorne, 1989). The *M. viator* colonies were not maintained in the laboratory for an extended period of time, as they were collected and assayed within a 3 month period. Furthermore, on occasion the laboratory colonies were replenished with fresh field collections, thus 3 months is an outside estimate, and likely not long enough to fully explain the low levels of intercolonial aggression observed. It is also noteworthy, that when the fresh field collections of termites were introduced to the existing laboratory colonies, no aggressive behaviour was ever observed.

Low temperatures have also been reported to significantly decrease aggression (Getty *et al.*, 2000). However, over a two year period, no seasonal fluctuation in aggressive behaviour was noted in field colonies of *Reticulitermes* (Polizzi & Forschler, 1998). All the behavioural trials conducted in this study were performed within a single season, and the constant laboratory temperature was set 4°C higher than the average ambient temperature. So, while seasonal fluctuation in aggression may occur in *M. viator*, it
cannot explain the variability in these results, although it may account in part for the generally low levels of aggression observed.

Another recognition cue which may likely mediate trial outcome, but which is not affected by the laboratory environment, is genetic relatedness. Kaib et al. (2004) found that intercolonial aggression in the mound-building species *M. subhyalinus*, was strongly correlated with differences in chemical profiles, such that genetically similar colonies had similar chemical profiles. Both high (Florane et al., 2004) and low (Neoh et al., 2012) levels of relatedness may result in reduced aggression. When relatedness is high between colonies, a layer of variability is reduced, while in colonies headed by multiple unrelated reproductives, the spectrum of similarity is increased. The average relatedness between *M. viator* colony pairs ranged from $r = 0.456$ to $r = 0.78$ (See Chapter 4), which is relatively high, given that the average between parents and offspring or full siblings is normally $r = 0.5$. Therefore, the high average relatedness between colonies may underlie reduced variability of recognition cues, and could explain the low levels of observed aggression.

A final consideration for the low levels of observed aggression is that of division of labour among workers. It has been proposed that due to division of labour, only a particular portion of workers within a colony may respond aggressively to hetero- and conspecifics. Both Polizzi & Forschler (1999) and Delphia et al., (2003) found that workers identified as aggressive during a behavioural trial remained aggressive in subsequent trials. Furthermore, if this is in fact the case, then using limited numbers of workers in behavioural trials further decreases the possibility that there will be a “fighter” among the group (Polizzi & Forschler, 1998; Delphia et al., 2003). In order to avoid possible habituation, each worker was used only once in this study.

*Generalised linear mixed model analysis*

In order to better examine the effects of different variables, such as intercolonial distance and relatedness, on trial outcome, a GLMM was created. Only three variables emerged as significant in the minimum model (Table 5.6) in explaining trial outcome. If
antennation occurred during a trial replicate, the interaction was 98.4 % less likely to end in rejection. When interactions occurred between individuals from colonies which are ‘far’ apart, the occurrence of a neutral response resulted in a 93 – 95% decrease in the likelihood of rejection. In other words, rejection/death was most likely to occur when colonies were close together and in the absence of antennation or a neutral response. Neither intercolonial distance or intercolonial relatedness emerged as significant predictors of trial outcome, although it is possible that in the case of relatedness, the variability in outcome of replicates within a given trial, may be masking effects which are in fact present. However, these results are not unprecedented and Adams et al., (2007) similarly found that neither of these factors was significantly associated with trial outcome in their study of 60 independent pairs of *Nasutitermes corniger* colonies.

Table 5.6 Significance of fixed effects on the outcome of behavioural aggression trials.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Odds Ratio</th>
<th>P value</th>
<th>Std. Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antennation</td>
<td>0.0162</td>
<td>&lt; 0.001</td>
<td>0.0109</td>
</tr>
<tr>
<td>Neutral, when Distance = close</td>
<td>1.006</td>
<td>0.994</td>
<td>0.8047</td>
</tr>
<tr>
<td>Neutral, when Distance = far</td>
<td>0.0534</td>
<td>&lt; 0.001</td>
<td>0.0314</td>
</tr>
<tr>
<td>Distance, when N = Neutral</td>
<td>0.076</td>
<td>0.004</td>
<td>0.0679</td>
</tr>
<tr>
<td>Distance, when N = no Neutral</td>
<td>1.44</td>
<td>0.64</td>
<td>1.128</td>
</tr>
</tbody>
</table>
Conclusion

The aim of this investigation was to better understand the behavioural interactions between colonies of *M. viator*, across variable geographic distances, as these underpin the underdispersed spatial patterning of *heuweltjies* in the landscape, especially at the local scale. This investigation was based on the hypotheses, that there should be a high level of innate colony defence related to defence of a foraging territory in the close vicinity of the nests/heuweltjie (Picker *et al.* 2007), and that increasing the intercolonial distance should increase the level of intercolonial aggression, as both genetic and environmentally derived recognition cues are likely to diversify with increasing intercolonial distance.

However, while *M. viator* is clearly capable of discriminating between nestmates and non-nestmates, the levels of intercolonial aggression were highly variable, and unpredictable. Furthermore, neither intercolonial distance nor relatedness emerged as a significant predictor of trial outcome. Given that intercolonial distance can be used as a proxy for environmental variation across the study area, it is likely that environmental factors are not the main source of recognition cues for *M. viator*. Alternatively, this result may be due to the loss of environmental recognition cues within the laboratory and/or the artificial nature of the behavioural bioassays. As such, a non-aggressive result from a laboratory interaction may not be sufficient to predict behaviour under natural conditions (Neoh *et al.*, 2012). However, if environmental factors are ruled out as a key contributor to recognition cues, then genetic recognition cues need to be considered - yet these too do not appear to satisfactorily explain the observed results.

Therefore, it is still not clear as to which recognition cues *M. viator* is utilising for discriminatory purposes, although it is quite likely that recognition in this species functions as a complex hierarchical suite of cues (Bordereau & Pasteels, 2011). As such, it will be beneficial for future research to investigate both CHC profiles and odours from symbiotic gut bacteria.
References


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Chapter 6

Overview of Population Genetic Structure and Behavioural Interactions in *Microhodotermes viator*

In order to develop a complete picture of the population structure of any species, one has to understand its reproductive organisation, modes of dispersal, interaction between individuals and external environmental factors that impact on the life history traits of the species at the population level. Among eusocial insect species, populations are organised such that individuals form colonies, and colonies form populations. Therefore colonies often function as discrete entities within a population.

The genetic composition of colonies examined in this thesis suggests that *M. viator* follows a standard mode of termite colony foundation, given the lack of any evidence for budding. Alate release is likely synchronous within geographical areas, given that it is initiated by rainfall events (Coaton & Sheasby, 1974), with dispersal distances probably up to 500m (Vargo et al., 2003; DeHeer & Vargo, 2006; Vargo & Husseneder, 2011), to several kilometres, possibly via passive drift on prevailing winds, as has been shown in *Macrotermes michaelseni* (Brandl et al., 2005). This range of dispersal distance is supported by the distinctive clustering of particular alleles at similar frequencies within geographic areas, leading to mild population viscosity, as evidenced by the higher level of average genetic relatedness within geographic areas (range: $r = 0.59 - 0.67$), compared to the BGP ($r = 0.42$). The combined effect of synchronous alate release and moderate dispersal distances is that alates are afforded the opportunity to mate with unrelated individuals. This is reflected by the lack of significant inbreeding ($F_{IC}$ values range from 0.12 to -0.62), and moderate to strong levels of genetic differentiation between colonies within geographic areas ($F_{ST}$ values range from 0.1 – 0.29). Although the genetic structure of individual *M. viator* colonies suggests that colonies are generally founded by a monogamous pair of outbred reproductives, which
gives rise to simple families, other family types were also observed, with most colonies exhibiting mixed families.

Simple families were present in the study population at around 33%, in keeping with observations in other termite species (Vargo & Husseneder, 2011). When compared to computer simulations of different reproductive strategies, the average intercolonial level of genetic relatedness between simple family colonies was approximately $r = 0.5$, as would be expected from an outbred pair of reproductives. Over time, one or both of the primary reproductives will die and be replaced by neotenics from within the colony, giving rise to an extended family.

Extended families, which are common among the ‘lower’ termites (Hacker et al., 2005; Vargo & Husseneder, 2011), are present in the study population at around 17%, which is also in keeping with observations in other termite species (Vargo & Husseneder, 2011). The extended family colonies had a higher level of average genetic relatedness ($r = 0.65$), as expected due to neotenic reproduction, but appear to support fairly low numbers of neotenics (likely < 6), as evidenced by the strongly negative $F_{IC}$ values ($-0.25$) (see also Husseneder et al., 2005; Vargo & Husseneder, 2011).

A third family type, mixed families, was also present in the global study population at a surprisingly high proportion (50%). In M. viator, mixed families appear to arise either due to pleometrosis or later adoption of an additional reproductive. Average relatedness within mixed family colonies is reduced ($r = 0.43$), compared to the other family types, as would be expected by the functional presence of an additional unrelated reproductive. It is possible that the high proportion of mixed families may be as a response to environmental stress (Brandl et al., 2004), and a comparison of the distribution and density of mixed family colonies to rainfall gradients across M. viator’s range, is an interesting avenue for future research. Additionally, the mixed families may also be indicative of an older population. Given the extreme longevity of heuweltjies (Moore & Picker 1991; Midgley et al., 2002; Potts et al., 2009), the adoption of unrelated reproductives may facilitate increased longevity of colonies. As such, colonies may be utilising a system of sequential addition of reproductives over time. This is evidenced
by the high number of alleles at specific loci within the mixed family colonies. For example, at Mvit 17, in colony T4, there are nine alleles, necessitating at least five unrelated reproductives. Further research into this hypothesis may help to reconcile the potential longevity of *heuweltjies* with the potential longevity of the colonies within them and which are responsible for their development and maintenance.

Within mixed family colonies, the presence of an additional unrelated functional reproductive results in decreased average relatedness and a reduction in accrued fitness benefits by workers, through weaker kin selection (Hamilton, 1964). The development of mixed families generally necessitates a reduced rejection threshold, to allow the infiltration of an unrelated reproductive into what is normally a closed group (Dahbi & Lenoir, 1998; Jmhasly & Leuthold, 1999; Hernandez *et al.*, 2002; Buczkowski & Silverman, 2004; Jungnickel *et al.*, 2004). In *M. viator*, this reduced rejection threshold was evident as there were low levels of aggression expressed during the experimental behavioural trials between colonies.

*M. viator* was capable of discriminating between ‘nestmates’ and ‘non-nestmates’, however levels of aggression exhibited between colonies varied considerably. Generally, *M. viator* was only mildly aggressive or had a reduced rejection threshold, possible due to insufficient variation in recognition cues required for discrimination. Additionally, variable behavioural outcomes were noted, not only between colonies but also between replicates within a given trial. This observation leads to two inferences. Firstly, variability in trial replicate outcomes suggests that some individuals may be more aggressive than others (Polizzi & Forschler, 1998; Delphia *et al.*, 2003). Among the harvester termites there is a division of labour among major and minor workers, such that majors may be better adapted to nest defensive behaviour (Duncan & Hewitt, 1989). Ideally all behavioural assays would have been conducted with major workers, however as minor workers out-number major workers by approximately 4:1, and the absolute size of the two worker classes varies widely from one colony to the next (Watson, 1973), the use of both major and minor workers was necessitated. Secondly, variability in overall aggressiveness between colonies, may suggest that colony circumstance (genetic composition, age, health and reproductive status of colonies) too
plays a role in mitigating defensive behaviour. However, the aggression which was observed could not be fully attributed to levels of genetic relatedness, environmental variation or family types as seen from the generalised linear mixed model analysis. Therefore, within _M. viator_, recognition probably functions via a complex suit of cues, as has been observed in other termite species (Bordereau & Pasteels, 2011). Furthermore, odours attributable to bacterial symbionts may also play a role in discrimination, as has been shown in colonies of _Hodotermes mossambicus_ (Kirchner & Minkley, 2003; Minkley et al., 2006), providing an important avenue for future research. However, the effective spacing of _M. viator_ colonies must be mediated through some form of aggressive behaviour, likely occurring during foraging bouts, resulting in the observed under dispersion of the nests (= _heuweltjies_).

In terms of _M. viator_ population structure, it is clear from the $F_{CT}$ values that each colony forms a discrete entity, with mild population viscosity observable over a scale of several kilometres within geographic areas, and strong differentiation between areas. These results are attributable to a combination of synchronous alate release within geographical areas, moderate alate dispersal distances and method of colony foundation. In terms of alate dispersal, habitat fragmentation might limit gene flow currently, as there is some habitat fragmentation at each of the sites. The Tygerberg, Malmesbury and Darling sites were located in and around residential areas, while the Worcester sites were located near residential areas. Furthermore, the intervening areas between the study sites are comprised predominantly of urban areas and agricultural land, as well as the Hottentots Holland mountain range between Worcester and the other three study sites, presenting both man-made and natural barriers to gene flow. The vegetation of the area is a mosaic of fynbos (from which _M. viator_ is excluded) and renosterveld (Mucina & Rutherford, 2006), and this reinforces the isolation between the Worcester population and the others. Additionally, the distance between the Worcester colonies and other sites ranges from 66 to 102km. Thus the Worcester population is geographically and ecologically isolated from the other three populations. With regards to colony foundation strategy, budding is clearly not utilised as a mode of dispersal, as evidenced by the strong differentiation between geographic areas and lack of strong population
viscosity or any clear relationship between genetic relatedness and intercolonial distance.

The overall level of relatedness within the population \((r = 0.42)\) is quite high, given the lack of inbreeding or colony budding and the strong differentiation between colonies, which may be evidence of an historical bottleneck, however, due to the small sample size this could not be tested for. With regards to inbreeding, the life history of many termite species is characterised by having cycles of outbreeding and inbreeding ( Calleri et al., 2006). However, recent evidence suggests that inbreeding may not be as extensively utilised by termite as previously thought, and in fact inbreeding depression may be functioning ( Vargo & Husseneder, 2011). The results presented here indicate a lack of significant inbreeding. Generally, colony breeding structure is thought to be sensitive to environmental conditions, with ecological factors, such as environmental stress, selecting against inbreeding ( Vargo & Husseneder, 2011; Brandl et al., 2004), as may well be the case for M. viator.

In spite of M. viator’s saturated population density (covering 14 to 25% of the land surface area (Lovegrove & Siegfried, 1989; Picker et al., 2007) in many winter rainfall landscapes in the Western Cape Province of South Africa and south-western parts of Namibia, and its ecological engineering impacts on the landscape (Knight et al., 1989; Midgley & Musil, 1990; Lovegrove, 1991; Corinna et al., 2005), the relationship between the heuweltjies and this species of termite is still debated. This thesis provides support for an M. viator origin of heuweltjies, and hypothesises that the high frequency of mixed family colonies may be indicative of a system to increase colony longevity by periodic acceptance of unrelated reproductives into the colony, which is likely relevant to the extreme longevity of heuweltjies.

M. viator is believed to have spread to the Western Cape Province of South Africa during the Holocene, when climatic changes at the end of the LGM facilitated a shift from grassland to shrublands (Duncan & Hewitt, 1989). It is currently restricted to the winter rainfall biomes of South Africa, with the closely-related H. mossambicus taking over where there is a switch to summer rainfall, and a grass-dominated landscape.
(Picker et al., 2002). In the eastern part of *M. viator*’s range, smaller subterranean nests are formed which may be capped over due to hardening of the clay soils (Coaton & Sheasby, 1974). Similar clay-capped nests occur rarely in other parts of *M. viator*’s range (e.g. at VanRhynsdorp), in areas where soils have a high clay and gypsum content (Mucina & Rutherford, 2006). However, in the western and central parts of its range, *M. viator* is nearly always associated with *heuweltjies* which have the same nest structure, but include a capping of sand (Moore & Picker, 1991). This change in nest structure across the range is possibly due to changes in soil structure, such as a higher clay content. Additionally, in *heuweltjies* there is the formation of a calcrete base over time (Midgley & Hoffman, 1991; Francis et al., 2012). *M. viator* foragers collect and return organic matter to their nests where much is converted into biomass and then excreted as frass. In addition, unused foraged vegetation in storage chambers would degrade and accumulate. The result is an altered nutrient status and an increase in soil pH due to an accumulation of basic cations, such as calcium and magnesium (Francis et al., 2012). Over time, these alkaline *heuweltjie* soils, in conjunction with rainfall, ground water and evaporation, facilitate calcrete precipitation (Midgley & Hoffman, 1991; Francis et al., 2012). The ongoing debate in the literature stems in part from the failure of Coaton & Sheasby (1974) to mention or describe *heuweltjies* in their survey of the termites of South Africa. While some studies have dated the calcrete below mature *heuweltjies* (30m in diameter) using C14 dating and returned ages in the region of 20 – 30 kya BP (Midgley et al., 2002; Potts et al., 2009), it is likely that this dating method is inappropriate for calcrete, and as such these results may be too old. On the other hand, the results obtained by Moore & Picker (1991), in the region of 5000 yrs BP, are likely too young given what is known about the rate of calcrete formation (Candy et al., 2005). Therefore, more research is required to resolve the debate regarding the age range of mature *heuweltjies*, preferably using a more appropriate method for dating calcrete such as U-series isochron dating.
References


Appendix A

Micro-Checker

The genotype scoring was checked for the presence of stochastic effects, such as stuttering, large-allele dropout and, at a population level, null alleles, using the program MICRO-CHECKER (version 2.2.3) (van Oosterhout et al., 2004). While there was no evidence of large allele dropout or null alleles, some loci exhibited stuttering (Table A.1). Mvit 4 and 21, which have only two alleles each, showed evidence of stuttering in five and three of the colonies respectively.

Table A.1 Results indicating which colonies and loci have potential challenges associated with the scoring of genotypes.

<table>
<thead>
<tr>
<th>Colony</th>
<th>Locus</th>
<th>Stuttering</th>
</tr>
</thead>
<tbody>
<tr>
<td>BGP</td>
<td>Mvit 4</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>Mvit 21</td>
<td>yes</td>
</tr>
<tr>
<td>T4</td>
<td>Mvit 4</td>
<td>yes</td>
</tr>
<tr>
<td>M5</td>
<td>Mvit 4</td>
<td>yes</td>
</tr>
<tr>
<td>D10</td>
<td>Mvit 4</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>Mvit 21</td>
<td>yes</td>
</tr>
<tr>
<td>W12</td>
<td>Mvit 4</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>Mvit 21</td>
<td>yes</td>
</tr>
</tbody>
</table>

Stuttering, which is common in dinucleotides, is caused by slippage during PCR amplification and tends to be the result of the combined effects of low amounts of template DNA and a high number of PCR cycles (Butler & Hill, 2010; van Oosterhout et al., 2004). Stuttering results in the production of additional peaks that differ from the correct peak/s by multiples of the repeat unit length (van Oosterhout et al., 2004). Due to the extraction reagents used during the ‘dilution protocol’ (using the Phire® Animal Tissue Direct PCR Kit), the NanoDrop® ND-1000 Spectrophotometer was not able to get a clear ‘read’ to quantify the DNA yield in each extraction. However, given the
large amount of macerated tissue used (one whole leg from each individual), it seems unlikely that the amount of template DNA was very low. There were, however, a high number of PCR cycles used (40), as per the manufacturer’s instructions. In Mvit 4 (Fig. A.1) the stutter peaks were consistent across samples but generally too small to size, except in Fig. A.1.c. Similarly, the banding pattern was also consistent across samples.

![Fig. A.1](image)

Fig. A.1 Allele peak profiles at Mvit 4, indicating the consistent presence of stutter peaks, approximately 1bp smaller than the true allele peaks, for both homozygotes (a, b, c & d) and heterozygotes (e & f). The banding patterns are consistent across different samples. Peak labels indicate Height, Area and Size of each peak.

Similarly, in Mvit 21 (Fig. A.2) the stutter peak was consistently present at 117bp. Therefore, for both loci, I was able to accommodate for the stutter and not erroneously identify it as a true allele peak. Furthermore, the banding patterns of the true allele peaks were replicable across different PCR’s and consistent across samples, therefore they can be considered to be reliable (Butler & Hill, 2010).
Fig. A.2 Allele peak profiles at Mvit 21, indicating the consistent presence of a stutter peak at 117bp, as well as the reproducibility of the homozygote banding pattern across different PCR's for the same sample (a & b) and the consistency of the heterozygote banding pattern across different samples (c & d). Peak labels indicate Height, Area and Size of each peak.

References


Appendix B

Allele & Genotype Frequency Histograms

Fig. B.1 Genotype frequencies at locus Mvit 4, across all populations

Fig. B.2 Genotype frequencies at locus Mvit 14, across all populations
Fig. B.3 *Genotype* frequencies at locus Mvit 21, across all populations

Fig. B.4 *Allele* frequencies at locus Mvit 23, across all populations

Fig. B.5 *Genotype* frequencies at locus Mvit 23, across all populations
Fig. B.6 Genotype frequencies at locus Mit 17, across all populations
Fig. B.7 Allele frequencies at locus Mvit 18, across all populations
**Fig. B.8** Genotype frequencies at locus Mit 18, across all populations

<table>
<thead>
<tr>
<th></th>
<th>BGP</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>M5</th>
<th>M6</th>
<th>D8</th>
<th>D9</th>
<th>D10</th>
<th>W7</th>
<th>W11</th>
<th>W12</th>
</tr>
</thead>
</table>

*Note: The image shows the distribution of genotypes across different populations, with each population represented by a different color.*