An investigation of the sociogenetic structure of the endemic fynbos ant, *Camponotus klugii*, via the use of microsatellites.

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Photograph courtesy of Hamish Robertson

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ABSTRACT

Eusocial insects, in particular ants, demonstrate great variability in their sociogenetic structure with regards to colony organization, queen number, queen mating frequency, levels of relatedness and worker reproduction. Within this study I perform an analysis on two groups of ant nests of the species *Camponotus klugii*, in order to investigate how the genetic structure may inform us of the sociogenetic structure of the species. Genetic differentiation between nests within each of the two groups (F_{ST} = 0.001 ± 0.004, p = 0.11; F_{ST} = 0.06 ± 0.074, p = 0.06) suggests that the groups of nests are each representative of an individual polydomous colony. Analysis of worker genotypes from each colony indicates that colonies are monogynous with an effective queen mating frequency of approximately k = 3. Within-nest genetic analysis revealed high relatedness values within each of the two colonies (r = 0.81; r = 0.66). These values are indicative of full-sister relationships between workers which conflicts with the established queen mating frequency. However, I argue that this discrepancy is due to low allelic variation and high allele frequencies. Finally, I argue against the theory of worker reproduction, proposed by Skaife (1961) and propose instead that the presence of brood within queenless nests of a queen-right polydomous colony are, in fact, fundamental to the structure of a polydomous colony and theoretically attributable to queen reproduction.

CHAPTER 1:
INTRODUCTION

Eusociality may be described as a cohabitation arrangement whereby adult members of a colony, who are derived from two or more overlapping generations, 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. Wilson and Holldobler (2005) suggest that eusociality may have arisen because of the selective advantage that organized cooperative groups are thought to exhibit over solitary individuals and pre-social groups. There are various examples of eusociality, the most notable of which fall into the Hymenoptera: namely ants, bees and wasps (Trivers & Hare, 1976; Schmid-Hempel & Crozier, 1999; Wilson & Holldobler, 2005). Of the Hymenoptera, ants undoubtedly exhibit the most striking example of eusociality.

Origins of Ants and the Evolution of Eusociality

Ants are thought to have originated during the Cretaceous period, with the oldest authenticated fossils being found in early Cretaceous amber (Wilson & Holldobler, 2005; Moreau *et al.*, 2006). From the Early Paleocene to the Late Cretaceous, during the rise of the angiosperms and angiosperm-gymnosperm mixed forests, was a time of great diversification for ants, hemipterans and beetles (Moreau *et al.*, 2006). Although ants initially remained rare, they are believed to have utilized the vegetation as an area for diversification, by proliferating in the ground layer as predators, granivores and collectors. It is also possible that the increased abundance of hemipterans during this period may have aided in the diversification of predatory ants both directly, as prey, and indirectly by providing honeydew as a food source (Moreau *et al.*, 2006). During the Eocene, due to increased diversification, ants were able to take advantage of new dietary niches, allowing them to expand into tropical forest canopies, temperate forests and xeric habitats. The result of this was that by the mid-Eocene ants were the numerically dominant insects on the planet (Wilson & Holldobler, 2005; Moreau *et al.*, 2006). They have remained numerically dominant to this day, as evidenced by the fact that ants and termites together are responsible for more than half of the insect biomass worldwide,

although they only account for approximately two percent of insect species (Wilson & Holldobler, 2005; Moreau *et al.*, 2006).

It appears that ant colonies are able to maintain this dominance via ecological control of nest sites in central foraging areas (Wilson & Holldobler, 2005). During the rise of eusociality this level of control played a central role in the establishment of dominance over both solitary individuals and pre-social cooperative groups. Furthermore there seems to have been a distinct shift in dietary behaviour, from hunters to gatherers, chiefly in association with hemipterans, which may have facilitated the evolution of particular social and group-directed behaviours. This, coupled with other colonial benefits such as increased genetic fitness within groups, appears to have cemented colonial existence (Wilson & Holldobler, 2005; Moreau *et al.*, 2006).

Haplodiploid Sex Determination

Haplodiploidy has evolved independently approximately 15 times in mite and insect species (Evans *et al.*, 2004). Approximately 15% of all animal species, including *C. klugii*, are haplodiploid. Generally this takes the form of diploid females (2n) and haploid males (n), although some exceptions to this rule do exist. For example in birds and snakes the sexes are reversed i.e. haploid females (n) and diploid males (2n) (Hedrick & Parker, 1997; Evans *et al.*, 2004). In a Haplodiploid system each sperm produced by a male inherits all of his genes, while each egg produced by a female inherits only half of her genes (Trivers & Hare, 1976). Therefore, all unfertilized eggs develop into males while fertilized eggs develop into females who inherit half of their mother's genes and all of their father's genes (Trivers & Hare, 1976; Hardy, 1994; Hedrick & Parker, 1997). The result of male-haploidy is thus the formation of relatedness asymmetries within a colony or group (Table 1).

Table 1. Relatedness within different relationships, in a haplodiploid sex-determination system, assuming complete outbreeding (after Trivers & Hare, 1976)

Relation	Relatedness (*r*)
Mother/Daughter	0.5
Mother/Son	0.5
Father/Daughter	1.0
Father/Son	0
Brother/Sister	0.25
Full Sisters	0.75
Half Sisters	0.25
Brother/Brother	0.5
Aunt/Nephew	0.375
Grandmother/Grandson	0.25

Colony Founding and Structure

Colony foundation may either be non-claustral, dependent upon worker assistance, or claustral, independent of worker assistance. Independent colony founding generally occurs after a virgin queen has left her natal nest and engaged in a nuptial flight. Alternatively dependent colony foundation, often referred to as 'budding', occurs when virgin queens engage in mating close to their natal nests, as the distance between the natal nest and the new colony site is limited by how far the workers are able to walk (Boomsma *et* al., 1995; Sanetra & Crozier, 2003; Viginier *et al.*, 2004; Zinck *et al.*, 2007).

Colony structure is primarily determined by the number of nests which comprise the colony. When a colony is housed within a single nest, it is referred to as being

monodomous. If a colony consists of several satellite nests centred on a queenright nest, it is referred to as being polydomous. Studies have found that in *Cataglyphis iberica*, a polydomous species, frequent mutual transport occurs between the satellite and queenright nests (Dahbi & Lenoir, 1998; Suarez *et al.*, 2002; Debout *et al.*, 2007).

A further feature of colony structure is that of the level of association between colonies within a population. Populations of colonies may either be multi or unicolonial. Multicoloniality is exemplified by well-defined and defended territories with a high level of inter-colonial aggression; independent of whether colonies are mono or polydomous (Suarez *et al.*, 2002; Debout *et al.*, 2007). Alternatively, when a population consists of a non-structured association of many colonies it is referred to as unicolonial (Suarez *et al.*, 2002). The number of colonies within an association may range anywhere from several hundred to thousands of colonies over a vast area, comprising a single population (Suarez *et al.*, 2002). Unicolonies tend to consist of non-hostile colonial and nest associations which lack distinct boundaries, and between which queens and workers may move freely (Heller, 2004, Giraud *et al.*, 2002). This has been well documented for the Argentine ant in its invasive range, for example just two super-colonies span across the entire area of Europe. It has been proposed that within this invasive range, due to a population bottleneck, a lack of sufficient genetic variation has not allowed for the development of distinct recognition cues. Therefore by focusing all their energy expenditure on foraging, rather than defence, they have become a dominant invasive species (Suarez *et al.*, 2002; Heller, 2004; Debout *et al.*, 2007).

Queen State

Colonies may either be queen-less or queenright; and if queenright then either monogynous or polygynous. A monogynous colony is one in which only a single queen is present, whereas a polygynous colony is one in which multiple, functional queens are present simultaneously (Sundstrom, 1997).

Genetic variation within a colony is increased by polygyny, resulting in lower levels of intracolonial genetic relatedness, or relatedness asymmetry, which in turn affects sex allocation, reproductive division and co-operation as well as possibly leading to conflict (Gertsch *et al.*, 1995; Liersch & Schmid-Hempel, 1998; Schmid-Hempel & Crozier, 1999; Bolton *et al.*, 2006). A study by DeHeer and Ross (1997) found that within polygynous colonies of *Solenopsis invicta* there are high levels of genetic variation due to the simultaneous presence of many functional, unrelated queens. Polygyny is thought to arise either due to pleometrosis, also known as joint foundress associations, or secondary polygyny, the subsequent addition of queens. An hypothesis regarding the evolution of pleometrosis is that it might have increased the probability of colony survival (Liersch & Schmid-Hempel, 1998, Holbrook *et al.*, 2007). It is also thought that the presence of multiple queens within a single nest may be attributed to spatial separation of queens within a nest, known as oligogyny or paragyny (Gertsch *et al.*, 1995).

Unless two queens are closely genetically related it is usually unlikely that they will share a nest due to competition (Fletcher & Blum, 1982), although this is not always the case. For example, a study by Ross (1993) of polygynous colonies of *Solenopsis invicta* showed an effective relatedness value, (r), between queens of $r = 0$. Nevertheless, workers are often responsible for removal of queens, to which they may even be related, thus some factor other than queen-queen competition may play a role in determining queen number within a colony. From their study on both monogynous and polygynous colonies of *Solenopsis invicta*, Fletcher & Blum (1982), suggest that queen cuticular hydrocarbon odour acts as a reliable signal of queen fecundity. Thus workers may assess the fecundity of a queen based upon the intensity of her odour. Furthermore they also propose that there may be an upper tolerance limit for queen odour within a colony such that, as less fecund queens produce less odour it is possible for a polygynous situation to arise. However, should the upper tolerance limit of the odour concentration within a nest be reached, either due to the addition of a new queen or the improvement in fecundity (via improved body condition) of a queen already present, the workers may respond by killing one or more of the least fecund queens present in the nest. Should a colony be orphaned, Fletcher and Blum found that workers of previously monogynous colonies

were far more discriminating of potential new foreign queens, than were workers of previously polygynous colonies. Similarly, orphaned monogynous colonies were less likely to accept a new queen from a polygynous colony.

In general, monogynous species tend to take part in nuptial flights and subsequently found nests independently of other queens and without the aid of workers (Liersch & Schmid-Hempel, 1998; Schmid-Hempel & Crozier, 1999; Macaranas *et al.*, 2001). This seems to produce a pattern of random mating and a lack of genetic structuring within a population of colonies (Zinck *et al.*, 2007). On the other hand, polygynous species exhibit decreased queen dispersal, with mating usually occurring near the natal nest, although this does not necessarily result in inbreeding (Sanetra & Crozier, 2003; Zinck *et al.*, 2007). Nests are founded via colony budding and tend to produce larger colonies despite a general decrease in the per-capita reproductive output, due to the presence of additional queens (Liersch & Schmid-Hempel, 1998; Schmid-Hempel & Crozier, 1999; Macaranas *et al.*, 2001). Generally this results in population viscosity: a level of genetic relatedness greater than expected by random colony foundation, between geographically closely situated colonies (Sanetra & Crozier, 2003). Within the genus *Camponotus* it is believed that colonies are almost exclusively monogynous, although rare examples of polygyny do exist (Gertsch *et al.*, 1995; Gadau *et al.*, 1996).

Queen Mating Frequency

Queens may mate either singly or multiply. A single mating is known as monandry; the alternative condition, known as polyandry, results in increased levels of genetic variation within a colony (Gadau *et al.*, 1996; Schmid-Hempel & Crozier, 1999). Mating occurs during early adulthood and, once mated, queens store the sperm in their spermatheca to be utilized as their life-time supply (Hardy, 1994; Holbrook *et al.*, 2007). Due to the haplodiploid sex-determination system, all unfertilized eggs develop into males (Hedrick & Parker, 1997), while fertilized eggs develop into females. This indicates that there is some level of female control over aspects associated with reproduction, such as the sex

ratio of offspring (Hardy, 1994). However, exactly how far this control extends is not yet fully understood. The fact that polyandry increases the level of genetic variation within the colony raises an interesting issue, as it implies that there is little or no sperm competition. The alternative to sperm competition would therefore be some level of female control (Liersch & Schmid-Hempel, 1998). While sperm competition is known to exist it may be strongly dependent upon the average colony population size, and is more likely to be a factor in very large colonies, where queens produce thousands of offspring (Robertson, *pers comm.*).

Thus, it is still not completely clear exactly where the benefit of polyandry lies for reproductive females (Pedersen & Boomsma, 1999). As previously mentioned, polyandry can result not only in increased worker conflict, but also the expenditure of time and energy as well as an increased risk of exposure to predators and pathogens for the queen (Schmid-Hempel & Crozier, 1999; Holbrook *et al.*, 2007). There have, on the other hand, been several theories proposed to explain the possible beneficial side effects of polyandry, including increasing the volume of sperm stored, deriving nutrients from sperm accessory substances, post-copulatory paternity biasing, increased genetic variation and alteration of the colony genetic structure (Trontti *et* al., 2006; Holbrook *et al.*, 2007). I will now discuss some of these theories in greater detail.

The sperm limitation hypothesis suggests that additional mates are required to ensure the queen has sufficient sperm stored for a lifetime of reproduction (Holbrook *et al.*, 2007). While this may be the case for very large and long-lived colonies, it is not always applicable. A survey conducted on virgin males from 12 species of the *Pogonomyrmex* genus found that all of the males sampled contained several times the volume of sperm as that of an inseminated queen (Holbrook *et al.*, 2007). This suggests that a single mating could provide more sperm that actually required by the queen and so does not fully explain the motivation behind polyandry.

While it is theoretically possible that queens are able to derive nutrients from the accessory substances accompanying sperm, in fact very little is known regarding this

within eusocial species. However a study which was conducted on *Apis mellifera* found peptides and lipids extracted from sperm had no discernable effect on female reproduction (Holbrook *et al.*, 2007).

Increased genetic variation may afford a colony a broader range of phenotypes, capable of withstanding a wider range of environmental conditions (Schmid-Hempel & Crozier, 1999). It has been proposed that another mitigating factor of increased genetic variation [illegible] in parasite load (Liersch & Schmid-Hempel, 1998; Schmid-Hempel &

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The above mentioned negative and positive aspects of polyandry apply not only to polyandrous but also to polygynous situations (Trontti *et* al., 2006; Holbrook *et al.*, 2007). Therefore whether polygyny and/or polyandry exists within a colony depends upon factors such as colony size and longevity, with respect to reproductive success, the number of queens in a colony, queen fecundity, the cost of additional matings, queen-queen competition and the prevailing parasite load (Liersch & Schmid-Hempel, 1998, Holbrook *et al.*, 2007). Furthermore it is thought that polyandry should be less prevalent in polygynous colonies, due to the increased genetic variation already accrued by the colony, as a result of multiple queens reproducing simultaneously (Schmid-Hempel & Crozier, 1999). This is supported by studies which have found that obligate polyandry exists only in monogynous species (Holbrook *et al.*, 2007).

Reproductive Skew and Sex Ratio

Within a colony it is important to consider reproductive division and, specifically, skew. Reproductive skew may be defined as the sharing of reproduction among breeding individuals within a group. When one individual dominates the reproductive output it is referred to as 'high reproductive skew' and vice versa (Bourke *et al.*, 1997, Bolton *et al.*, 2006). Skew theory provides a powerful means by which to investigate communal breeding. Facultative polygynous species, those which exhibit both polygyny and monogyny, present a unique situation in this regard. It is expected that in such colonies the skew should be low, so as not to negate the benefit of 'colony sharing' by queens. Since the 'sharing' is facultative, it must present some benefit to a queen, greater than the cost she would incur by founding a colony independently (Bourke *et al.*, 1997).

Within the family Formicidae there are normally only one or a few reproductives in a colony and, as such the majority of the colony consists of sterile or non-reproductive workers (Peeters & Holldobler, 1995; Dietemann *et al.*, 2003). Such an existence may be considered to be altruistic in that the behaviour of the altruist, a worker, benefits another

individual, the queen, at a cost to the altruist, in this case the concession of life-time reproduction (Trivers & Hare, 1976; Wilson & Holldobler, 2005). Hamilton's theory of kin selection operates under the principal that the level of genetic relatedness between an altruist and a beneficiary must be sufficient that the benefit to the recipient exceeds the cost incurred by the altruist (Wilson & Holldobler, 2005). Therefore, in order for non-reproductive individuals to maximize their genetic fitness, according to Hamilton's theory (Hamilton, 1964), it is essential that the benefit of their labour be directed towards their kin (Trivers & Hare, 1976; Greenberg, 1979; Carlin & Holldobler, 1983; Suarez *et al.*, 2002; Bolton *et al.*, 2006). 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).

These patterns of relatedness between individuals within a colony are fundamental to understanding social behaviour and conflicts and, furthermore, predicting their outcomes (Zinck *et al.*, 2007). However due to the haplodiploid sex-determination system, combined with the social systems of monogyny or polygyny in conjunction with either monandry or polyandry, it is possible that the relationship between workers may lie anywhere between full sisters ($r = 0.75$) and totally unrelated ($r = 0$) (Trivers & Hare, 1976; Zinck *et al.*, 2007). Both polyandry and polygyny reduce the level of genetic relatedness between workers, thus resulting in a decrease in the indirect fitness benefits accrued by altruistic workers (Goodisman & Hahn, 2005 and Holbrook *et al.*, 2007). This can give rise to a situation such that it may be in the best interests of a worker, from a fitness point of view, to attempt reproduce parthenogenetically, as females are more closely related to their own sons ($r = 0.5$) than they are to their brothers ($r = 0.25$). However, females are also more closely related to their full sisters ($r = 0.75$) than they are to either their brothers or their own offspring (Trivers & Hare, 1976). Hamilton (1964) proposed that since the overall average relatedness of a worker to her full sister and brother combined ($r = 0.75 + 0.25 / 2 = 0.5$) was the same as to her own offspring, then, if given equal investment in each, haplodiploidy is not a driving force of altruistic behaviour or the evolution of eusociality. Trivers and Hare (1976) suggest that in order for the altruistic behaviour of a worker to be worth her while, some other factors, such as

female-biased sex ratio or some level of worker control over production of males, must be in effect.

Primarily, the sex ratio in effect within a colony appears to be a function of who controls reproduction: the queen or the workers? If the queen is in control then a male:female sex ratio of 1:1 should be in effect, as queens are equally related to sons and daughters (Nonacs & Carlin, 1990; Brown & Keller, 2000). If non-reproductive females are in control in a monogynous monandrous nest, following the relatedness values, the ratio should be 1:3. If, however, reproductive workers are in control then the favoured ratio should be 4:3, as females are more related to their own sons than they are to their nephews ($r = 0.375$).

The method by which queens may alter the sex ratio of eggs is via selective fertilisation, while workers may alter the ratio via culling of male eggs, laying male eggs or selective investment in eggs of a specific gender (Rosset & Chapuisat, 2006). This implies that in order for workers to bias the sex ratio they must be able to discriminate between male and female offspring, at some point, prior to completion of development. Generally it seems that the point at which discrimination between the sexes is possible, depends upon whether the colony in monogynous or polygynous.

In polygynous colonies, where relatedness asymmetries are less distinct, workers are able to identify the gender of eggs early on. This has been shown in *Monomorium pharaonis*, *Apis mellifera*, *Trigona postica*, *Bombus terrestris* and *Myrmica ruba* (Nonacs & Carlin, 1990). As mentioned above, workers are more closely related to their sisters than their nephews. Therefore, although the sex-ratio should theoretically approach the male bias of 4:3, worker policing keeps this to a minimum as workers would rather preferentially invest in their sisters than their nephews. The early identification of eggs is thus imperative to allow workers to effectively police one another (Nonacs & Carlin, 1990).

Within monogynous colonies queens are, for the most part, solely responsible for reproduction. Nevertheless this does not automatically negate the effects of male culling

and disparate investment in sisters by female workers. A study by Nonacs and Carlin (1990) of worker discrimination in the species *Camponotus floridans* found that workers were only able to distinguish between male and female brood after pupation. Once discrimination was possible, workers significantly favoured females with respect to care and energetic investment. The conundrum for workers is that once a male has pupated, and is readily identifiable, he has already received approximately one third of his energetic investment. This then leaves workers with a cost/benefit decision to make: cull the male, loose all investment to date and begin again by laying your own egg, or continue to raise the queen's son. Furthermore, as the colony sex-ratio approaches the female bias of 1:3 the relative value of a male increases and, in so doing, cumulatively reduces the female-bias. It is also important to mention that different colonies within a species may also specialise in the production of a particular sex (Trivers & Hare, 1976; Rosset & Chapuisat, 2006). For example, in a study of the facultatively polygynous species *Formica selysi*, Rosset and Chapuisat (2006) found that within monogynous colonies there was an extremely strong bias towards one particular sex. Similarly, several other studies have shown that in monogynous colonies sex-ratio investment tends to be female-biased (Brown & Keller, 2000). Queens that lay almost exclusively either haploid or diploid eggs essentially force workers to raise a particular sex, thereby significantly reducing the margin for worker manipulation of sex ratio. Finally it is very important to note that the above mentioned theoretical sex-ratios are usually never reached; and if they are reached they are generally not maintained for long periods of time. This is due to the constant power struggle between queens and workers which often results in some form of compromise being reached, and generally the sex-ratio in effect seems to fall into the region of a 1:2 female bias (Trivers & Hare, 1976, Boomsma, 1989).

Microsatellites

It has been found that genetic structuring may be observed at scales of anywhere from several meters to a few kilometres in ant species (Macaranas *et al.*, 2001). Within a population, structuring depends upon such features as the sex-determination system, the

number of reproductives and their mode of dispersal (Sanetra & Crozier, 2003). Furthermore, the amount of differentiation observed 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). We may therefore turn to genetic tools to aid us in elucidating these mechanisms.

Microsatellite markers are considered to be excellent at elucidating genetic structure and population history. They are easy to use, extremely prevalent in eukaryote genomes, 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). Microsatellites are tandem repeats of short nucleotide sequences, on average one to six base pairs long. They tend to have high heterozygosity and many alleles per locus (Queller *et al.*, 1993). The primary source of microsatellite polymorphism lies in the variation in the number of tandem repeats, along with variation in the length of the flanking sequences, both of these factors contribute to allelic variation (Queller *et al.*, 1993; Gertsch *et al.*, 1995).

Microsatellites are superior to other genetic techniques for several reasons. Firstly they are more variable than allozymes and are thus better suited to estimate relatedness in small groups of individuals. They are more comparable due to a simpler banding pattern across different gels than are DNA fingerprints, thereby reducing ambiguity when scoring. Finally they are also more cost effective as, with some careful planning, several different loci may be run on a single gel (Queller *et al.*, 1993).

Microsatellites can aid a study of eusocial species, via the use of neutral markers, by answering questions concerning nest inter-relationships such as kinship, inbreeding determination and paternity. For example, due to male-haploidy, if the queen and offspring's genotypes are known, paternity as well as mating frequency may be easily deduced (Crozier, 1977; Queller *et al.*, 1993; Gertsch *et al.*, 1995; Gadau *et al.*, 1996). 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).

A potential disadvantage of microsatellites is their mutation rate, with high mutation rates resulting in misleading genetic information. Male haploid individuals produce sperm in the absence of meiosis. Similarly it has been found that microsatellite mutation occurs in aphids in the absence of sexual reproduction, thus supporting the idea that meiosis is not required for microsatellite mutation. However, it is possible that the lack of meiosis does result in a decreased rate of mutation. Diploid males of other species 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. However in Hymenopterans it seems that a comparable number of germ cells are produced by both sexes, and typically mutation rates are less than 1×10^{-4}. Nevertheless it is important to bear in mind that longer alleles, those with a greater number of sequence repeats, will generally have a higher mutation rate that short alleles (Queller *et al.*, 1993; Crozier *et al.*, 1999).

Previous Research on Camponotus klugii

Approximately 11 800 species of ants, grouped into 60 genera, have been described to date (Moreau *et al.*, 2006). The genus *Camponotus*, the largest within the Family Formicidae (Picker *et al.*, 2002), is well represented in South Africa and is one of the most well represented world wide accounting for around 1000 species ants, of which *Camponotus klugii* is one (Gadau *et al.*, 1996; Brady *et al.*, 2000). However, very little research has been conducted on *C. klugii* and almost all of the various and variable facets of colony and sociogenetic structure I have described remain as yet undetermined.

An unpublished study by Eick investigated the genetic relatedness within and between individuals found in seven nests of *Camponotus klugii* on a single *Protea repens* bush. Three of the nests were each found to contain only a single foundress queen. Two of the nests were queenright with a single queen present in each and the two final nests

consisted only of workers. In one of the queen-right nests it was found that the queen's genotype was inconsistent with her being the mother of the workers found in that nest. In the other queen-right nest the queen's genotype was consistent with her being a sister, but did not exclude her as the mother, of the workers present in the nest. The queenless nests appeared to have been orphaned and the remaining queens on the bush were not the foundresses of these colonies. This suggests the possibility of queen turn-over within the colonies and requires further investigation. Genetic analysis of the *C. klugii* workers present in four of the seven nests indicated that their colonies were both monogynous and monandrous, however, this does not exclude the possibility that other *C. klugii* colonies or nests may in fact be polygynous and/or polyandrous, although polygyny is unlikely (Gertsch *et al.*, 1995; Gadau *et al.*, 1996).

The only other study performed on *C. klugii* was by Skaife (1961). He performed a census on 12 nests and reported small within-nest populations of no more than 100 individuals, which he proposed was due to the limitation of space within the nest. Many of the colonies he found were queenless, although brood was still present. He suggested that these queenless nests were extensions of queen right nests, again due to a limitation of space within the natal nest. However the author also purports that the presence of brood within queenless nests can be attributed to workers laying unfertilized, male eggs.

Rational and Objectives

The mating frequency of queens plays a central role in the determination of relatedness within the nest, which in turn affects sex ratio, reproductive regulation and division of labour (Gadau *et al.*, 1996). However, despite its importance comparatively little is known about the number of males with which queens of various ant species mate, and *C. klugii* is no exception. When researching polyandry in ant species it is virtually impossible to obtain the queen's mate/s because mating normally occurs away from the natal nest and males die shortly after copulation (Schrempf *et al.*, 2005). Since males are haploid their whole genotype is expressed in each of their daughters. Therefore, genetic

markers are a reliable means by which to determine paternity, and hence the level of polyandry within a nest. Of these genetic markers, microsatellite loci are very well suited to this endeavour, due to their highly variable repetitive DNA sequences (Gadau *et al.*, 1996).

While the *Camponotus* genus is thought to be almost exclusively monogynous, singular colonial exceptions have been found in various species (Gertsch *et al.*, 1995). It will therefore be invaluable to this study to determine the level of genetic relatedness between individuals within a nest as well as their maternity and possible shared paternity. It is also not known whether each nest represents a single colony, or whether the colony is polydomous. A study such as this would aid clarification of this matter and may allow us to formulate an idea of *C. klugii's* natural life histories, such as colony structure and number of queens.

Due to their preferred nesting sites on *Protea repens*, *C. klugii* may in fact also be able to play a role as an indicator species within fynbos. In order to best track the effects of disturbances upon a species the samples observed and/or obtained should be from within their endemic range, as it seems that the effects of disturbance are far more distinct when a species is within its optimal habitat (Hoffman & Andersen, 2003). Ants play important roles at many different trophic levels within an ecosystem (Underwood & Fisher, 2006). Therefore, the more we discover about this species, the more it may aid us in learning about other fynbos endemic species, as well as principals generally governing the extraordinary functioning of eusocial insect colonies.

Therefore, the aim of this study is to attempt to elucidate the sociogenetic colony structure of *C. klugii*, by focusing on the levels of relatedness within and between individuals and nests. Furthermore allele and genotype frequencies, Hardy-Weinberg equilibrium and linkage disequilibrium, various fixation indices, queen number and mating frequency will be investigated.

CHAPTER 2:
MATERIALS & METHODS

Sample collection and molecular analysis

(i) Sample collection

Eight nests, representing the total number of nests present, of *Camponotus klugii* were collected from a single *Protea repens* bush in the Cape of Good Hope Nature Reserve (34°16'S, 18°27'E), Cape Town, South Africa in September 2006 (Figure 2.1). A permit for collection was obtained from Table Mountain National Park. Each nest is located in the hollowed-out base of a dead *P. repens* inflorescence. These hollow cavities are the result of insect larval boring activities which occur while the inflorescence is still alive (Skaife, 1961). As the inflorescence desiccates, during senescence and subsequent to death, hardened hollow cavities are formed within the base of the inflorescence. No nests were found on neighbouring vegetation, within a three meter radius, around the focal bush. In addition to these eight 'focal' nests, two 'test' nests were obtained from a single *P. repens* bush several kilometers outside of the Cape of Good Hope Nature Reserve.

Figure 2.1 Management map detailing the Cape of Good Hope Nature Reserve (http://www.sanparks.org/parks/table_mountain/images/mngment_map.jpg 07/02/2008)

A second attempt to obtain further samples of *C. klugii* from the Cape of Good Hope Nature Reserve several months later was unsuccessful, despite extensive searching. Therefore the two test nests were included in this study, in order to increase the data set.

Whole nests were stored at -20°C and the eight focal nests were completely censused (Appendix A: i) prior to DNA extraction for microsatellite analysis. Of the ten nests recovered, only one of the focal nests contained a queen. However, all ten nests contained soldiers, minor workers and eggs, comparatively distinguished by eye, using a dissecting microscope. No males were identified in any of the nests. Within the focal group each individual nest population was quite small, on average 62, with numbers ranging from 29 to 105. The ratio of workers to soldiers was on average 3:1, but it ranged from as little as 1.1 to as much as 5.7, however there is some room for error here, because the morphological distinctions between soldiers and workers are not always clear, as there seem to be some intermediate individuals (Skaife, 1961).

Two workers from each nest collected, preserved in 96% ethanol, were deposited in the Iziko South African Museum, Cape Town, South Africa.

(ii) Nucleic acid extraction

In order to determine which DNA extraction protocol would be most well suited to my samples, I reviewed and compared several different methods (Appendix B: i & ii). Using common features from these methods, I then formulated two novel variations of the CTAB extraction procedure (Boyce *et al.*, 1989). The first has a digestion step using 1X CTAB and Proteinase K (Method 1) while the second uses only 2X CTAB for digestion (Method 2). I tested both methods each with three variations on the length of the digestion incubation step, namely one, two and three hours, using two samples for each variation. The DNA yield for each sample was measured twice, using a NanoDrop® ND-1000 Spectrophotometer and the associated software, NanoDrop Version 3.1.0. The average DNA yield for each variation of methods 1 and 2 was determined by averaging

over both samples. The variation which produced the highest average yield overall was Method 1 with a digestion incubation step of three hours. I therefore selected this procedure for all my DNA extractions and hence forth refer to it simply as a modified CTAB extraction method (Appendix B: iii).

Prior to extraction the head and gaster of each ant were removed using a sterile razor blade. The thorax was then homogenized, also using a razor blade. The blade, forceps and glass work surface were all sterilized with 96% ethanol between each ant dissection. Total genomic DNA was extracted from the legs and thorax of each individual ant using a modified CTAB extraction method. The reason for exclusion of the head in the DNA extraction procedure is that the head of *C. klugii* was found to contain pigments which inhibited Polymerase Chain Reaction, PCR, (Eick, unpublished data).

During my preliminary investigations, using 'test' samples, I found that my test sample DNA failed to amplify during PCR regardless of annealing temperature, magnesium chloride concentration or primer concentration. I therefore tested for the presence of inhibition by performing a PCR with samples containing only positive control DNA, only test sample DNA and a combination of both positive control and test sample DNA. While the positive control DNA was amplified, both the test sample DNA and the combination of positive control and test sample DNA failed to amplify, thus confirming the presence of inhibition. Therefore, due to the presence of persistent inhibition the extracted sample DNA was further purified prior to molecular analysis, using a Promega Wizard® SV Gel and PCR Clean-Up System.

(iii) Microsatellite amplification

Approximately 37 workers from each of the eight nests were screened for microsatellite amplification at seven microsatellite loci: Camp 4, Camp 8, Ccon 12, Ccon 20, Ccon 42, Ccon 70 and Ccon 79 (Table 2.1).

Table 2.1 Characteristics of microsatellites amplified in *Camponotus klugii*

Locus	Source Species	Reference	No. of alleles amplified in source species	Repeat Type
Camp 4	*Camponotus lingiperdus*	Gertsch *et al.*, *1995*	1	$(AT)_4A(AT)_3G(TA)_3(CA)_4$
Camp 8	*C. lingiperdus*	Gertsch *et al.*, *1995*	4	$(GT)_7(TG)_4TC(TG)_2A(GT)_2$
Ccon 12	*Camponotus consobrinus*	Crozier *et al.*, *1999*	8	$(GA)_3GG(GA)_4AA(GA)_9$
Ccon 20	*C. consobrinus*	Crozier *et al.*, *1999*	11	$(TC)_9$
Ccon 42	*C. consobrinus*	Crozier *et al.*, *1999*	13	$(GA)_{11}$
Ccon 70	*C. consobrinus*	Crozier *et al.*, *1999*	38	$(GA)_2AA(GA)_{27}$
Ccon 79	*C. consobrinus*	Crozier *et al.*, *1999*	28	$(GA)_5(AG)_2GGGAA(GA)_{12}$

A range of annealing temperatures as well as primer and magnesium chloride concentrations were tested at each locus until a repeatable microsatellite motif for the test samples was produced. The forward primer of each locus was fluorescently labeled with either HEX or FAM. DNA amplification reactions were performed in 0.2ml PCR tubes in 20µl reaction volumes containing the following reagents: 0.25 units/µl GoTaq® DNA Polymerase, 1X Colourless GoTaq® Flexi Buffer and Magnesium Chloride Solution (Table 2.2) (GoTaq® Flexi DNA Polymerase, Promega), 0.2mM/7µl dNTP's (Bioline), fluorescently labeled forward primer & reverse primer (Table 2.2 & 2.3), Millipore water and 2µl of template DNA. For each PCR performed the master mix cocktail was assembled and aliquoted out into individual sample reaction tubes in a DNA-free environment, prior to addition of template DNA to each reaction volume in the laboratory. Two blank amplifications 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 my 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 3 minutes at 94°C followed by 35 cycles composed of 94°C for 30 seconds, Ta for 45 seconds (Table 2.2) and extension at 72°C for 45 seconds, followed by a final extension step at 72°C for 10 minutes. Each PCR reaction was stopped with a rapid cool-down to 4°C. Samples were maintained at 4°C prior to electrophoresis.

Table 2.2 Characteristics of microsatellite loci amplified in *Camponotus klugii*. Numbers of alleles and allelic size range are based on the genotypes of 314 individuals

Locus	T_a (°C)	Primer Concentration (µM)	$MgCl_2$ (mM)	No. of alleles amplified	Size Range (bp)
Camp 4	51	0.3	1.5	4	208 – 219
Camp 8	55	0.5	1.5	1	127
Ccon 12	53	0.3	1.5	3	167 – 173
Ccon 20	55	0.3	1.0	5	286 – 294
Ccon 42	53	0.4	1.5	1	258
Ccon 70	60	0.5	2.5	5	162 – 172
Ccon 79	57	0.3	2.0	3	356 - 368

Table 2.3 DNA sequences of primers used for amplification of microsatellite loci in *Camponotus klugii*

Locus	Forward Primer Sequence 5′ - 3′	Reverse Primer Sequence 5′ - 3′
Camp 4	GAT GAT GTT GGC ACA GGA AT	ACG TCT TCT CGC CTC AAG AG
Camp 8	ACA CGA TAG ACC TAT TGG CT	TGG TTT CAG AGT AAG GCA AG
Ccon 12	CGG ACCAGA GTC GCG TAA GT	CGG TGC GTT TTA CCG GAA TG
Ccon 20	GGT GCG ATG CAA AAG CAT TG	ACA TGC GAG CGG ACG TTC
Ccon 42	CGA TGG AAT GCC TTC ATG CGA	TCC GAA GAA TGG TAT ACT C
Ccon 70	GCA TTA AAG TCG GGA CGG AC	CAG ATG CGA AGA GCT CGC
Ccon 79	GAA CCT GCC CAT AAA TCG AG	TGA CGC CTC TTT ACT CGT GA

(iv) Agarose gel electrophoresis

All samples were visualized on a 1.5 % or 2 % agarose gel (Appendix B: iv) 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). Each gel contained the following reagents; agarose powder, 1X TBE (Appendix B: ix) and ethidium bromide (Appendix B: x) to a final concentration in the gel of 0.5µg/ml.

(v) Polyacrylamide gel electrophoresis and scoring

The appropriate dilution for each sample was determined from the intensities of the DNA visualized on the agarose gel (Table 2.4). One microlitre of loading dye (ABI), 2.5 µl of formamide, 0.3 µl of GeneScan™ 350 or 500 ROX™ Size Standard (ABI) and either three or 4µl of the sample, or dilution thereof, were loaded into each lane of the gel after heating at 95°C for three minutes followed by snap cooling on ice. Samples were electrophoresed according to the manufacturer's specifications on a 6% acrylamide-urea gel (Appendix B: xii) in 1 X TBE buffer for four hours on an ABI 373 automated DNA Sequencer. Allele sizes were scored with the GENESCAN corresponding software. A

positive control, of known size, specific to each locus was run on every gel in order to control for size variation due to minor differences between gels.

Table 2.4 PCR sample dilutions prior to genotyping

Relative Visibility of Band	Dilution	Ratio of Sample to Distilled Water (µl)	Amount of Sample/Dilution Loaded into Gel (µl)
Barely visible	-	-	4
Visible	1/5	1:5	3
Bright	1/10	1:10	3
Very Bright	1/20	1:20	3

(vi) Genotyping of *Camponotus klugii* individuals

In total 293 workers and one queen, from the eight 'focal' nests and 20 workers from the two 'test' nests were genotyped across seven loci.

Genetic Data Analysis

Estimating Genetic Parameters within a Haplodiploid Organism

Haplodiploid organisms require special consideration during statistical analysis. Primarily this is due to the fact that males of such species are haploid (n) while females are diploid (2n), however they usually exhibit other factors such as a female-biased sex ratio and low heterozygosity which must also be taken into consideration when analysing data (Liu & Smith, 2000). If both males and females are jointly analysed then many of the statistical tests commonly employed need to be modified appropriately for these special circumstances (Hedrick & Parker, 1997). However, as males are often difficult to obtain for sampling, it is often the case, and indeed in this study, that only females are sampled and analysed. As such it is no longer necessary to modify the statistical tests employed; nevertheless care must still be taken to interpreting the results in a biologically meaningful way, given the haplodiploid condition.

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 or False Positives, erroneous rejection of the null hypothesis. The two most accepted methods by which to correct errors arising from multiplicity are the Bonferroni correction procedure and the False Discovery Rate (FDR). The FDR is less restrictive than the classical Bonferroni correction and aims to control the expected proportion of errors within the suite of rejected hypotheses, by functioning as a '*post hoc* maximizing procedure'(Benjamini & Hochberg, 1995; Garcia, 2004). However, in certain instances a very strict correction, such as the Bonferroni correction, may be appropriate. Within this study I have chosen to use the Bonferroni correction (for the formula see: Appendix C: i).

(i) Allele and Genotype Frequencies

(a) Allele frequencies

Allele frequencies were calculated for each locus in the program RELATEDNESS 4.2 (Queller & Goodnight, 1989). Although observed frequencies can be used as estimates of true allele frequencies (Weir, 1996), because all individuals within my focal nests and all individuals within my test nests are related, the problem of bias was one which needed to be addressed. If background population allele frequencies are calculated from a group of related individuals, the resulting frequencies are biased and thus lead to an underestimation of relatedness within the focal groups, as each individual contributes too much to the population mean (Queller & Goodnight, 1989). Although my sample size is large and the issue of bias can be ignored if the whole population is sampled, and does diminish with increasing number of groups, alleviating the bias insofar as possible is still preferable to disregarding it. Therefore all 313 workers from the ten nests were collectively used to calculate the population background allele frequencies, with group 1, focal nests A – H, and group 2, test nests Ta and Tb, weighted equally. By weighting the groups equally the large variation in group size, 293 to 20, can be accounted for.

(b) Genotype Frequencies

Genotype frequencies were calculated in both the focal and test nest groups using the web implementation of GENEPOP (version 3.4) (Raymond and Rousset, 1995).

(ii) Hardy-Weinberg Equilibrium and Gametic Disequilibrium

(a) Hardy-Weinberg Equilibrium

Population-level departures of allele frequencies, from those expected under Hardy-Weinberg equilibrium, were tested for in the web implementation of the GENEPOP (version 3.4) (Raymond and Rousset, 1995). Both the focal and test nests were individually tested, in each instance using all the worker genotypes across the seven loci, 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 (for the formula see: Appendix C: ii).

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; 500 batches and 1000 iterations per batch.

(b) Gametic Disequilibrium

Gametic disequilibrium, also known as linkage disequilibrium, was tested for with version 3.1 of the program Arlequin (Schneider *et al.*, 2000) (for the formula see: Appendix C: iii).

Nests were tested both as individual populations and then combined as a single focal or test population. The test performed by the program is based on a likelihood ratio test. The null hypothesis, that there is no association between loci, is compared to the likelihood of the sample, when association is allowed. The significance of the observed likelihood ratio is thus found by computing the null distribution of this ratio, under the null hypothesis of linkage disequilibrium, using a permutation procedure. The number of permutations performed was 16 000 and the number of initial conditions for the Expectation-

Maximization (EM) algorithm (utilized for multi-locus genotype data where the gametic phase is unknown) was 10.

(iii) Genetic Variation

(a) Observed and Expected Heterozygosity

Observed and expected heterozygosity (H_O and H_e respectively) were determined using the web implementation of GENEPOP (version 3.4) (Raymond and Rousset, 1995). For both the focal and test nests H_O and H_e were determined, within each of the two groups, as an average at each locus with all nests combined and at each locus within each separate nest. H_e was calculated based on observed allelic counts, under Hardy-Weinberg equilibrium. The associated *p* values and standard deviations were calculated in version 3.1 of the program Arlequin (Schneider *et al.*, 2000) using 1000 000 steps in the Markov Chain Method and 100 000 permutations. The overall H_O and H_e values were calculated in version 2.9.3.2 of the program FSTAT (Goudet, 2001).

(b) Genetic Diversity

Genetic diversity was calculated using version 2.9.3.2 of the program FSTAT (Goudet, 2001) (for the formula see: Appendix C: iv). For both the focal and test nests gene diversity was determined, within each of the two groups; as an average over all loci and all nests, as an average at each locus with all nests combined and at each locus within each separate nest.

(iv) Inbreeding Coefficient

The inbreeding coefficient, F_{IS}, was determined for all polymorphic loci, within both the focal and test nest groups, as an average over all loci with all nests combined and over all loci within each separate nest. Groups of varying sample sizes were weighted equally.

The analysis was performed in version 2.9.3 of the program FSTAT (Goudet, 2001) (for the formula see: Appendix C: v).

(v) Genetic Differentiation

Genetic differentiation, F_{ST}, is a correlation coefficient which quantifies the level of differentiation between subdivisions within a population and is based on allele frequency (Weir, 1996; Hedrick, 2000), such that:

$$F_{ST} = F_{IT} - F_{IS} \; / \; 1 - F_{IS}$$

Where:

F_{IS} = the deviation from Hardy-Weinberg equilibrium within subpopulations

F_{IT} = the deviation from Hardy-Weinberg equilibrium within the total population.

Genetic differentiation is usually a positive value, with a high value indicative of a high level of differentiation. The analysis was performed in version 2.9.3 of the program FSTAT (Goudet, 2001) (for the formula see: Appendix C: iv.a).

F_{ST} was determined within each of the focal and test nest groups, for each locus over all the nests within each group and as an overall value for each group. Associated p values were determined using the web implementation of GENEPOP (version 3.4) (Raymond and Rousset, 1995), under the null hypothesis that genotypes are drawn from the same distribution in all populations.

An analogous measure of genetic differentiation is that of R_{ST}, which calculates variance between populations or groups thereof, across all loci, based on the size of the repeat unit and the length (number of base pairs) of the flanking sequence of each locus. The analysis was performed in the program RST CALC (Goodman, 1997), with 1000 permutations and 1000 bootstrap tests (for the formula see: Appendix C: iv.b).

The associated *p* values generated relate to the null hypothesis that the value returned for R_{ST} is not significantly different from zero. The assumptions of this formula are that all samples are of equal size and that all loci have equivalent variances. In order to overcome these assumptions the data must be globally standardized prior to calculation of R_{ST}.

Firstly, to account for the variance in allele size, alleles must be converted to standard deviations from a global mean using the formula:

$$Ys = (Y\text{-}GM) / \text{standard deviation}$$

Where:
Ys = the standardized value of allele 'Y' at locus 'L'
GM = the mean allele size, in repeat units, for locus 'L' over the whole data set
Standard deviation = the deviation of the standard deviation of allele size in terms of repeat units for locus 'L' over the whole data set.

Secondly, in order to mitigate the effects of varying sample size variance components are calculated with the formula:

$$Rho = Sb / (Sb + SW)$$

Where:
Sb = the component of variance between populations.
SW = twice the estimated variance in allele size *within* each population.

Pairwise R_{ST} values were calculated between nests within each of the focal and test nest groups, as a global value for each of the two groups and between the focal and test nest groups, with each group considered as a single population.

(vi) Genetic Variance

An Analysis of Molecular Variance (AMOVA) was performed in version 3.1 of the program Arlequin (Schneider *et al.*, 2000) with 1000 permutations (for the formula see: Appendix C: vii). 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 both the focal and test nest groups as for a group of populations, where each nest is considered as a single population, with within-individual level analysis.

(vii) Queen Genotypes and Mating Frequency

Using version 1.0 of the program MATESOFT (Moilanen *et al.*, 2004) genotypes of both unknown queens and potential mates were generated from the female offspring genotype data, coupled with the background population allele frequencies derived in the program RELATEDNESS (see: (i) Allele and Genotype Frequencies). Queen genotypes were generated for both the focal and test nest groups under both the 'narrow' and 'broad' deductions. The narrow deduction always assumes monandry insofar as possible to explain all offspring genotypes. However, if it becomes clear during the analysis that the queen has in fact been multiply mated, then alternative queen genotypes will be suggested. On the other hand, the broad deduction assumes that monandry and polyandry are both equally likely and will thus generate all possible queen genotypes. The broad deduction therefore has a greater level of accuracy in determining queen genotypes, but it may also generate too many potential mate genotypes and thus care must be taken when analysing this portion of the data.

Queen mating-frequency was determined for each of the narrow and broad deductions, within each of the focal and test nest groups, by using the FQM, (**F**emale offspring, **Q**ueen and potential **M**ate), genotype data generated by MATESOFT during the previous

analysis step. It is important to note that if the number of potential mates generated in a particular analysis exceeds two, then when estimating queen mating-frequency there are no means available of calculating the dispersal measures. Furthermore, jackknifing over groups and/or bootstrapping by groups are not reliable estimators when there are less than five groups available.

When determining queen and consequently mate genotypes it is important to quantify, insofar as possible, the amount of error which may occur, either due to insufficient sampling or non-detection. To this end the probability of having all queen alleles represented within your sample or the power, β, of correctly deducing queen genotypes is determined for each analysis (for the formula see: Appendix C: viii).

A particular feature of the program MATESOFT is that a patriline is determined for each individual offspring genotype in combination with each individual queen genotype. Before presenting the final results the program removes non-compulsory patrilines and merges identical patrilines, which is where the error of non-detection usually arises. However, as part of the results package, the program also reports the number of identical patrilines which were present prior to the merge. This allows the amount of non-detection error to be clearly quantified and furthermore enables the user to determine an upper and lower limit on the number of patrilines present.

(viii) Relatedness

Genetic relatedness was calculated in the program RELATEDNESS 4.2 (Queller & Goodnight, 1989) (for the formula see: Appendix C: ix). Relatedness was calculated as an average, between all individuals within a data set, for each individual nest within each of the focal and test nest groups and as a symmetrical average between the focal and test nest groups. Pairwise relatedness estimates were determined, between all individuals, within each of the focal and test nest groups, with individuals weighted equally. Pairwise relatedness estimates were also determined between the single queen, found within the

sample, and each other individual within the focal nests group. Furthermore an unpaired student t-test was performed to compared the pairwise relatedness estimates between the queen and her potential offspring to the theoretical value of R = 0.5. Standard errors were obtained by jack-knifing over loci.

CHAPTER 3:

RESULTS

(i) Allele and Genotype Frequencies

Within the focal nest group there appears to be low allelic variation: Figure 1 summarises the allele frequencies for the seven loci examined in the focal nests. Camp 8 and Ccon 42 are both monomorphic while the five remaining loci have only two alleles each. Table 1 summarises the genotype frequencies corresponding to the allele frequencies in Figure 1 and indicates that there are fewer genotypes than would be expected based on the allele frequencies. Figure 2 summarises the allele frequencies for the same seven loci also examined within the test nests. There are four monomorphic loci, Camp 8, Ccon 12, Ccon 42 and Ccon79, and three polymorphic loci with a total of 12 alleles. Table 2 indicates the genotypes associated with the allele frequencies found in Figure 2. There are fewer genotypes at Camp 4 than would be expected, based on the allele frequencies. Figure 3 indicates the background population allele frequencies determined for the total combined focal and test nest data set. There are five alleles at the most polymorphic loci, Ccon 20 and Ccon 70, and the two monomorphic loci appear to be fixed for the whole population. The associated genotype frequencies can be found in Appendix A: ii.

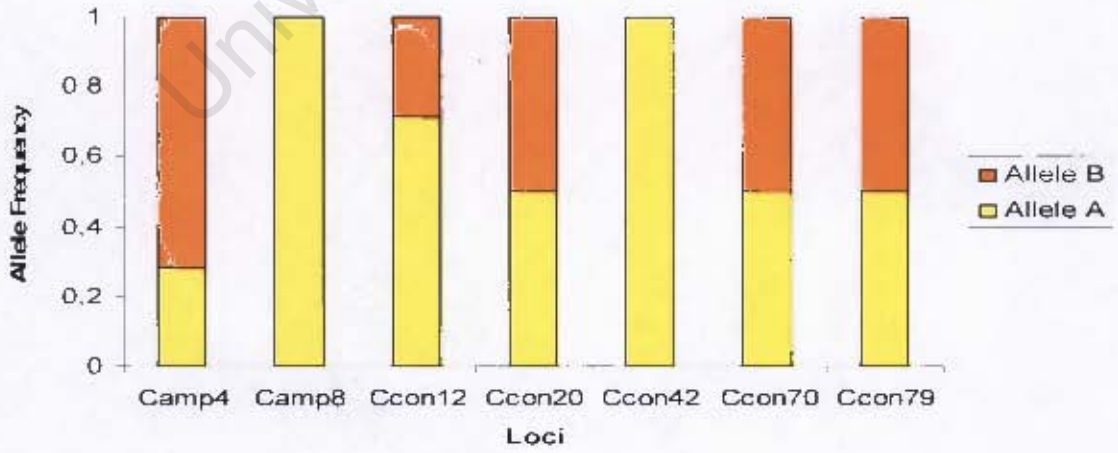

Figure 1. Focal nest allele frequencies in *Camponotus klugii*

Table 1. Focal nest genotype frequencies in *Camponotus klugii*.

Locus	Genotype	Frequency
Camp4	208/208	0.02
	208/210 *	0.52
	210/210	0.46
Camp8	**127/127** *	1.00
Ccon12	167/167	0.43
	167/173 *	0.56
	173/173	0.01
Ccon20	**286/290** *	1.00
Ccon42	**258/258** *	1.00
Ccon70	**162/170** *	1.00
Ccon79	**356/366** *	1.00

*values in bold indicate the genotype expressed by the queen

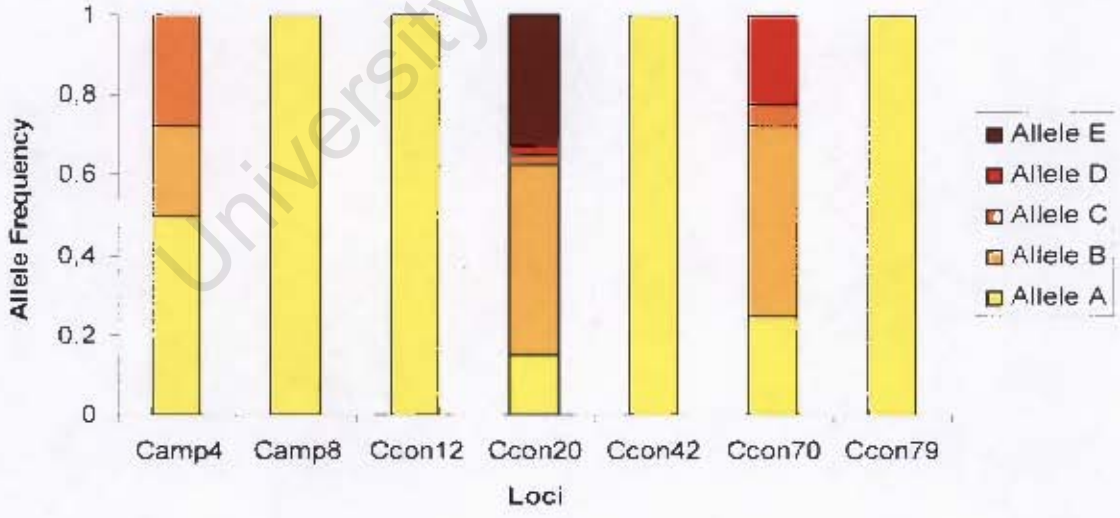

Figure 2. Test nest allele frequencies in *Camponotus klugii*

Table 2. Test nest genotype frequencies in *Camponotus klugii*.

Locus	Genotype	Frequency
	208/214	0.45
Camp4	208/220	0.55
Camp8	127/127	1.00
Ccon12	169/169	1.00
	286/286	0.05
	286/288	0.10
	288/288	0.10
Ccon20	288/290	0.05
	288/292	0.05
	286/294	0.10
	288/294	0.55
Ccon42	258/258	1.00
	166/166	0.05
	166/168	0.15
Ccon70	168/168	0.25
	170/168	0.10
	166/172	0.30
	168/172	0.15
Ccon79	358/368	1.00

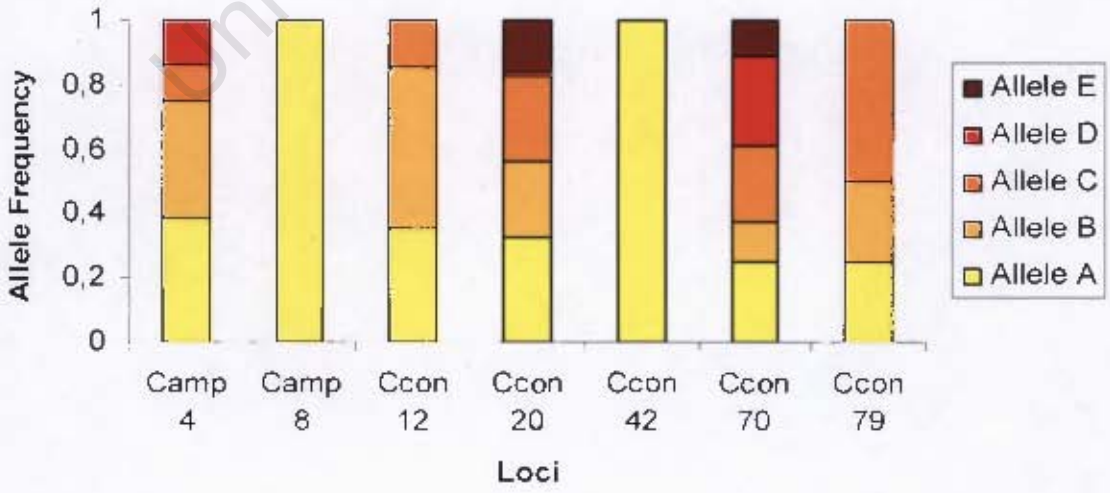

Figure 3. Background population allele frequencies in *Camponotus klugii* obtained using 293 individuals from the group of focal nests and 20 individuals from the group of test nests.

(ii) Hardy-Weinberg Equilibrium and Gametic Disequilibrium

(a) Hardy-Weinberg Equilibrium

(i) Focal Nests

Focal nests were treated both as a global population and as each nest representative of a single population. When each nest was considered as a separate population, some individual loci within particular nests were found to be in Hardy-Weinberg equilibrium (Nest A - Camp4: $p = 0.08$, Ccon12: $p = 0.23$; Nest B – Camp4: $p = 0.64$, Ccon12: $p = 0.06$; Nest D – Camp4: $p = 0.65$; Nest F – Camp4: $p = 0.44$, Ccon12: $p = 0.57$; Nest H – Camp4: $p = 0.08$, Ccon12: $p = 0.08$). However when all loci in each nest were considered overall, none were found to be in equilibrium ($p < 0.001$). Similarly when equilibrium was considered as each locus with all nests combined (p = highly significant, $\chi^2 = 1199.34$, d.f. = 80), and overall loci with all nests combined (p = highly significant, $\chi^2 = \infty$, d.f. = 4), none were found to be at Hardy-Weinberg equilibrium.

(ii) Test Nest

Test nests were treated both as a global population and as each nest representative of a single population. When each nest was considered as a separate population, some individual loci within particular nests were found to be in Hardy-Weinberg equilibrium (Nest Ta – Ccon20: $p = 0.05$, Ccon70: $p = 0.79$; Nest Tb – Ccon20: $p = 0.49$, Ccon70: $p = 0.38$). When each locus was considered with both nests combined the same loci were still found to be in equilibrium (Ccon20: $p = 0.06$, Ccon70: $p = 0.17$). However when each nest was considered over all loci, only test nest Tb was found to be in equilibrium ($p = 0.07$, $\chi^2 = 11.57$, d.f. = 6). Nevertheless after a strict Bonferroni correction was applied, the amended significance level for the test became $p = 0.37$, and thus nest Tb can not be considered to be at Hardy-Weinberg equilibrium either. Furthermore when both nests were jointly considered, over all loci, there was a significant departure from Hardy-Weinberg equilibrium ($p < 0.001$, $\chi^2 = 29.42$, d.f. = 6).

(b) Gametic Disequilibrium

When gametic or linkage disequilibrium was tested for between all pairwise combinations of the seven loci in the focal nests, treated as a single population, it was found that all pairs of loci, save one, were significantly linked at the 0.05 level. However, when the nests were analysed individually, only three significant linkage associations (Ccon20:Ccon70, Ccon20:Ccon79 & Ccon70:Ccon79) were found to commonly occur in all eight nests. The tests nests were similarly treated and, when considered as a single population, no significant linkage was found. Therefore when the same three pairs of loci, significantly linked in the focal population, were examined in the test population they were found to be unlinked. Thus the non-random linkage associations that are present are not representative of true chromosomal linkage and I have therefore considered all pairs of loci to be unlinked.

(iii) Genetic Variation

Observed and expected heterozygosity (H_O & H_e) were determined, within each of the Test and Focal colony groups, as an average at each locus with all nests combined and at each locus within each separate nest (Table 3). Similarly gene diversity was determined within each of the two groups as an average over all loci and all nests, as an average at each locus with all nests combined and at each locus within each separate nest. In all instances H_O was found to exceed H_e, with genetic diversity being a very close approximation of H_e. The associated *p* values indicated that the observed and expected heterozygosity values are significantly different.

Table 3. Observed and expected heterozygosity, H_O and H_e respectively, with standard deviation (s.d.) across all polymorphic loci, in each of the Test and Focal nest groups.

	Focal Nests		Test Nests	
Loci	**H_O ± s.d.**	**H_e**	**H_O ± s.d.**	**H_e**
Camp 4	0.52 ± 0.0	0.4	1.0 ± 0.0	0.64
Ccon 12	0.56 ± 0.0	0.41	**	**
Ccon 20	1.0 ± 0.0	0.5	***0.85** ± 0.0002	0.66
Ccon 70	1.0 ± 0.0	0.5	***0.7** ± 0.0004	0.69
Ccon 79	1.0 ± 0.0	0.5	**	**
Overall	0.58	0.33	0.36	0.27

* Bold values indicate associated $p > 0.05$; ** Indicates missing data due to a non-polymorphic locus within that particular group

(iv) Inbreeding Coefficient

The inbreeding coefficient, F_{IS}, was determined for all polymorphic loci within each of the focal and test nest groups, as an average over all loci with all nests combined and over all loci within each separate nest. For each individual nest, within both groups, F_{IS} was always found to fall between 0.0 and -1. The average F_{IS} within each of the focal and test groups, over all nests and all loci was F_{IS} = -0.77 (range 0.0 to -1.0) and F_{IS} = -0.34 (range 0.0 to -0.57) respectively.

(v) Genetic differentiation

Genetic differentiation, F_{ST}, was determined within each of the focal and test nest groups, for each locus over all the nests within each group and as an overall value for each group (Table 4). Within the focal nests group the F_{ST} values for each locus, overall nests, ranged from 0.0 to 0.008 and within the test nests group the values for each locus, again over all nests, ranged from 0.0 to 0.18.

Table 4. Overall F_{ST} values between and within Focal and Test colony groups.

Genetic Differentiation	F_{ST} ± SE	*p*
Between focal nests	0.001 ± 0.004	0.11
Between test nests	0.06 ± 0.074	0.06
Between focal & test nests	0.53 ± 0.06	<0.001

Global R_{ST} values were determined for each of the focal (Table 5) and test nest groups as well as between the two groups (Table 6). R_{ST} values for each polymorphic locus across all nests ranged from -0.004 to 0.003 within the focal nests groups and -0.04 to 0.001 for the test nests group.

Table 5. Pairwise R_{ST} values for Focal nests

	Nest A	Nest B	Nest C	Nest D	Nest E	Nest F	Nest G
Nest B	-0,02						
Nest C	-0,01	-0,01					
Nest D	-0,01	-0,02	-0,01				
Nest E	-0,01	-0,01	-0,01	-0,01			
Nest F	-0,01	-0,01	-0,01	**0,001 ***	**0,01 ***		
Nest G	-0,01	-0,01	-0,01	-0,01	-0,01	-0,01	
Nest H	-0,01	-0,02	-0,01	-0,01	-0,01	-0,01	-0,01

* Bold values indicate associated $p < 0.05$

Table 6. Global R_{ST} values between and within Focal and Test nest groups.

Genetic Differentiation	R_{ST}	p
Between focal nests	-0.008	0.12
Between test nests	-0.03	0.36
Between focal & test nests	0.39	<0.001

(vi) Genetic Variance

The AMOVA was performed on both the focal (Table 7) and test (Table 8) nest groups as for a group of populations, where each nest is considered as a single population, with within-individual level analysis. Within the focal group the AMOVA indicates that only 0.12% of the genetic variance was due to genetic differentiation between nests and that differentiation between individuals essentially accounted for 99.88% of the variance. Similarly within the test group only 5.99% of the variance was due to differentiation between nests and essentially the differentiation between individuals accounted for 94.01% of the variance.

Table 7. AMOVA results for the focal nest group

Level of Variation	df*	% Variation	Fixation index	p
Among Nests	7	0.12	$F = 0.001$	0.2
Among individuals within nests	285	-90.47	$F = -0.9$	1.0
Within individuals	293	190.35	$F = -0.9$	1.0

* Degrees of freedom

Table 8. AMOVA results for the test nest group

Level of Variation	df	% Variation	Fixation index	p
Among Nests	1	5.99	$F = 0.06$	0.04
Among individuals within nests	18	-31.61	$F = -0.34$	1.0
Within individuals	20	125.62	$F = -0.26$	1.0

(viii) Queen Genotypes and Mating Frequency

Genotypes of unknown queens and potential mates were generated from the female offspring genotype data, coupled with the background population allele frequencies. Queen genotypes were generated for both the focal and test nest groups under both the 'narrow' and 'broad' deductions.

(a) Focal Nests

Using the genotype of the single queen found within one of the eight focal nests, both the narrow and broad deductions were in complete agreement with regards to the genotypes of potential mates. Three patrilines were suggested with eight possible genotypes for each patriline all with the identical probability of 0.125 (Appendix A: iii, Table A.3). Similarly the same average, weighted queen mating-frequency (k = 3) was generated through both the narrow and broad deductions. No identical patrilines were merged during either analysis.

When the sampled queen was disregarded, both the narrow and broad deductions were in complete agreement with regards to the genotypes of potential queens and mates, generated by using the genotypes of all the sampled offspring found within my eight focal nests (Appendix A: iii, Table A.4). Both deductions generated eight queen genotypes all with an equal probability. For each queen three patrilines were generated. Of the three patrilines one of them always had two possible alternative genotypes with unequal probabilities. The average, weighted queen mating-frequency, (k = 3), was also in agreement. Once again no identical patrilines were merged, in either analysis, for any of the eight queen genotypes proposed.

(b) Test Nests

When the narrow deduction was performed on the test nests one matriline with a single queen genotype was determined (Appendix A: iii, Table A.5). In order to account for all the genotypes of the workers analysed from the two nests nine different patrilines were suggested. Three identical patrilines were merged during the analysis, therefore, in order to account for the sampled offspring's genotypes, given the queen's genotype, between nine and eleven mates were required and according to the queen mating-frequency analysis the average weighted number of mates was $k = 9$.

When the broad deduction was performed on the test nests two matrilines, with a single queen genotype each, were put forward (Appendix A: iii, Table A.6). In conjunction with the first queen nine patrilines, with a single genotype each, were suggested. Three identical patrilines were merged during the analysis of potential mates of the first queen therefore in order to account for the sampled offspring's genotypes, given the queen's genotype, between nine and eleven mates were required. In the case of the second queen 12 patrilines were suggested. Eleven of the 12 patrilines had a single genotype and one patriline had four possible genotypes with unequal probabilities. Only one patriline merger occurred during the analysis, therefore the second queen had either 12 or 13 mates. The queen mating-frequency analysis found that the average weighted number of mates was $k = 10.46$.

(vii) Relatedness

Genetic relatedness was calculated as an average, between all individuals within a data set, for each individual nest within each of the focal and test nest groups and as a symmetrical average between the focal and test nest groups. Within each of the focal nests (Table 9) there is a high level of average relatedness corresponding to that of full-sisters or higher. Within the test nest group (Table 10) the average within-nest relatedness values are similar to those expected for full-sisters, if a little low. Between the focal and

test nest groups (Table 11) there is essentially zero level of relatedness indicating that these two groups are totally distinct.

Table 9 Average within-nest and overall relatedness values for the focal nests, weighted by group

Nests	Relatedness (R)	Standard Error	95% Confidence Interval
A	0.80	0.10	0.24
B	0.81	0.10	0.25
C	0.84	0.09	0.21
D	0.82	0.10	0.26
E	0.85	0.09	0.23
F	0.74	0.13	0.32
G	0.84	0.09	0.21
H	0.81	0.09	0.22
Overall	0.81	0.09	0.24

Table 10 Average within-nest and overall relatedness values for the test nests, weighted by group

Nests	Relatedness (R)	Standard Error	95% Confidence Interval
Ta	0.68	0.19	0.46
Tb	0.64	0.20	0.48
Overall	0.66	0.19	0.46

Table 11 Average relatedness for the combined focal nests versus the combined test nests, weighted by group

Colonies	Relatedness (R)	Standard Error	95% Confidence Interval
Focal vs. Test	- 0.05	0.1	0.24

Pairwise relatedness estimates were determined, between all individuals, within each of the focal (Figure 4) and test (Figure 5) nest groups. Within the focal nest group the pairwise estimates are indicative of a very high level of relatedness between all individuals, with only about five percent of the estimates falling below 0.6. Within the test nest group the pairwise estimates are far more normally distributed, although the class of relatedness within which the most estimates fall corresponds to that of full-sisters.

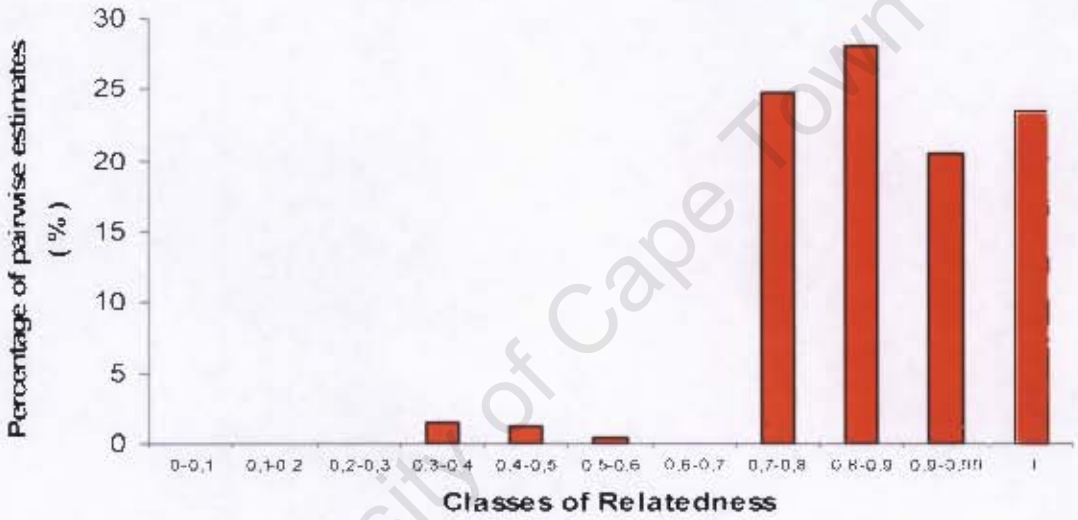

Figure 4 Pairwise relatedness estimates, based on the background population allele frequencies and genotypes of all sampled individuals present in all eight focal nests, with individuals weighted equally.

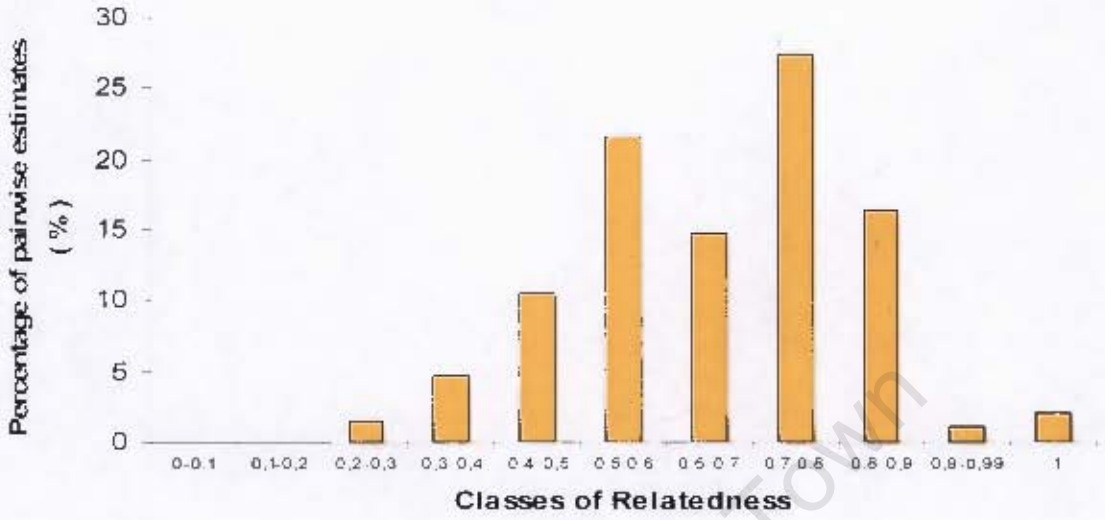

Figure 5 Pairwise relatedness estimates, based on the background population allele frequencies and genotypes of all sampled individuals present in both the test nests, with individuals weighted equally.

Pairwise relatedness estimates were determined between the single queen, found within the sample, and each other individual within the focal nests group (Figure 6). An unpaired Student t-test, performed to compare the pairwise relatedness estimates between the queen and her potential offspring to the theoretical value of R = 0.5, indicated that the mean of the pairwise estimates (0.832 ± 0.12) was significantly different than R = 0.5 ($p < 0.0001$).

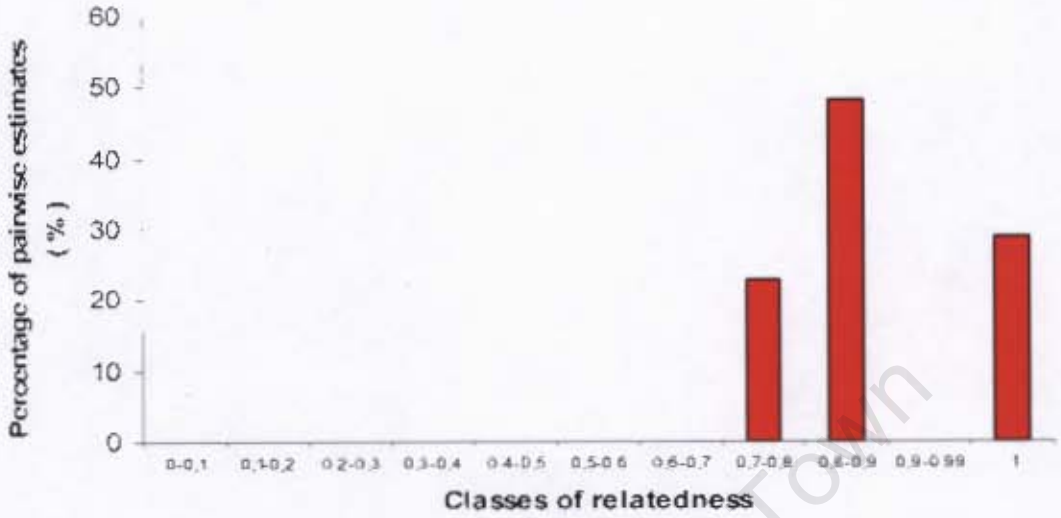

Figure 6 Pairwise relatedness estimates, based on background population allele frequencies and genotypes, of the queen versus all other sampled individuals present in the eight focal nests, with individuals weighted equally.

CHAPTER 4:
DISCUSSION

Population Definition

A recent review questioned the validity of genetic studies examining population structure, that did not first clearly define what they meant by a population (Waples and Gaggiotti, 2006). Furthermore, that these studies' results were not necessarily translatable within a broader context and did not contribute to the greater understanding of that particular species. There are three general paradigms within which a plethora of population definitions can be classified. Firstly, the Statistical Paradigm encompasses aggregates of things which may or may not represent individuals. Secondly, the Biological/Ecological Paradigm generally refers to a group of individuals that share some biological attribute and occupy the same space at the same time. Finally the Evolutionary Paradigm refers to groups of individuals from a sexually reproducing species within which random mating may occur.

Within *C. klugii* groups of individuals are readily found together in single nests, given the biological/ecological paradigm, these groups could theoretically be considered as individual populations. However, frequent mutual transport between nests is known (Dahbi & Lenoir, 1998), thus *groups of nests* need to be considered. Furthermore, even though *Camponotus* is a sexually reproducing species, given the life history of the species it is quite unlikely that random mating would occur within a group of nests which may, quite possibly, constitute a colony. Therefore, it seems that neither the biological nor the evolutionary paradigms are individually sufficient. The cohesive forces within the biological paradigm are demographic, while in the evolutionary paradigm they are genetic. Since shared relatedness and group cohabitation are both fundamental features of eusocial existence I have chosen to subscribe to both the Biological/Ecological and Evolutionary Paradigms within this study as both are applicable in their own right. I have, therefore, considered a group of nests to be representative of a single population, as such, all eight of the focal nests are considered to be a population and henceforth I will interchangeably refer to them as either the focal nest group or the focal population. Similarly, while the two test nests are almost certainly only two of a larger group of nests

I will also interchangeably refer to them as either the test nest group or the test population. Finally, within both groups, if required, and where appropriate, nests may be referred to as sub-populations.

However, the above definitions are applicable only to this study and I have no doubt that given further field sampling it may become evident that populations are in fact made up of multiple groups of nests or colonies. Nevertheless, while the above definitions are appropriate within the frame-work of this study, care should be taken when extrapolating the results. It is also important to bear in mind that at this point the ability to interchange the word 'population' with the word 'colony' has yet to be determined. Therefore, until the point at which that has been determined the two terms are *not* considered to be interchangeable, and each is deliberately used where most appropriate.

Colony Founding and Structure

Only one of the nests contained a queen, from the census performed on the eight focal nests. All eight nests contained soldiers, minor workers and brood. Skaife (1961) found a similar demographic distribution: he investigated 12 nests of *C. klugii*, only four of which contained queens, however, all of which contained brood at some level of development. Similarly a study by Pfeiffer and Linsenmair (1998) of the monogynous polydomous species *Camponotus gigas* found that in addition to the queen-right nest, which contained the most brood, some of the peripheral nests also contained brood. This study also found that each individual nest population was quite small, on average 62 individuals, and the ratio of workers to soldiers was on average 3:1. Again Skaife (1961) found similar results of an average of 100 individuals per nest and an average worker to soldier ratio of 2.7:1.

The fact that no winged males or females were identified in any of the nests is probably due to the timing of the sampling, which occurred at the beginning of spring. Winged forms are produced around the middle of summer, the timing of which is controlled by both temperature and photoperiod. The reproductives remain in the natal nest until spring

when they are triggered to emerge and engage in a nuptial flight, usually by heavy rainfall (Skaife, 1961; Pfeiffer & Linsenmair, 1997).

The aggregation of nests on a single bush raises the possibility that the group of nests may form a polydomous colony. A particular benefit of polydomy is the reduction in energetic costs by reducing the overlap of foraging paths, furthermore by spreading the foragers out they are more efficiently able to take advantage of randomly distributed food sources (Pfeiffer & Linsenmair, 1998). In a recent review by Debout *et al.* (2007) a working definition of polydomy was proposed as an ant colony composed of at least two nests, spatially separated by a distance greater than that between two separate chambers within a nest. Furthermore, in order for a structure to be considered a nest it should house both workers and brood, but may contain any number of queens, if any at all. According to the above definition, the nest distribution of my focal colony can best be described as polydomous. The distribution of my focal colony is in accordance with that previously described by Skaife (1961) for *C. klugii* and as found in other *Camponotus* species, (Pfeiffer & Linsenmair, 1997; Debout *et al.*, 2007).

The idea that all eight of the focal nests comprise a single colony is corroborated by the significant lack of genetic differentiation between the nests. The average between-nest differentiation, based on allele frequencies, was very low (F_{ST} = 0.001 ± 0.004; p = 0.11). Similarly, the pairwise comparisons of between-nest differentiation, based on the size of the repeat unit and the length of the flanking sequence of each locus (R_{ST}), generated negative values in all but two of the comparisons. F_{ST} is considered to be a more reliable estimator than R_{ST} when the number of loci is less than ten or the population size is less than 50 (Sanetra & Crozier, 2003). However, within this study both estimators produced similar results. Furthermore, in populations with high microsatellite variability, F_{ST} is thought to underestimate the amount of genetic differentiation (Sanetra & Crozier, 2003). Therefore, given these two lines of evidence I believe that both F_{ST} and R_{ST} can be considered reliable estimators within this study and deserve further consideration.

There are two situations under which negative values of genetic differentiation may arise. Firstly, as the estimator is slightly biased, if the true value is very small and thus close to zero, the estimator may in fact generate a slightly negative value. The alternative, which I believe to be very interesting in this particular case, is intraclass correlation (Weir, 1996). Essentially this means that there may be less differentiation *between* nests, than within them. This refutes the possibility of genetic structuring between the nests and clearly supports the theory that all eight nests, in fact, form a single polydomous colony. Following on from this, as individuals become more related *within* groups, the level of genetic differentiation *between* groups should increase (Weir, 1996). However, if individuals are not becoming more related within groups, but rather show a high level of relatedness overall, then the level of genetic differentiation between groups should decrease. Within the focal population there is a high level of pairwise relatedness between all individuals. Approximately 95% of the estimates indicated a minimum level of relatedness of full-sisters. In effect this means that nests, within the focal population, are arbitrary subdivisions of a colony, rather than discrete genetic entities.

Similarly, for the two test nests, genetic differentiation between the nests, based on allele frequency was very low (F_{ST} = 0.06 ± 0.074; p = 0.06), and differentiation based on the size of the repeat unit and the length of the flanking sequence of each locus was also slightly negative. Relatedness estimates, on the other hand, were more widely distributed than in the focal nests. However, within each individual nest, the average estimate fell between half and full-sisters. Following on from this, in the pairwise relatedness estimates, the greatest percentage of estimates fell within the two categories of half-sisters and full-sisters. Therefore, although these two nests almost certainly only represent a subset of a larger group they clearly indicate that the trends described in the focal nest group are maintained.

When calculating relatedness it is important that the allele frequencies utilized are independent of the individuals between which the level of relatedness is being quantified, as observed allele frequencies within a sample do not accurately reflect background population allele frequencies unless the sample size is sufficiently large (Hedrick, 2000).

Unfortunately, as discussed in a previous section, I was unable to determine independent allele frequencies and although I attempted to alleviate this bias insofar as possible, some still remains. The result of the bias is an underestimation of the level of relatedness between the individuals of interest (Queller & Goodnight, 1989). In other words, the already high levels of relatedness between individuals within my focal and test populations should be even higher. However, due to the haplodiploid sex-determination system it is clear that the level of relatedness between two workers should be no higher than the theoretical value of $r = 0.75$ within an out-breeding colony. Why then are the pairwise relatedness estimates within the focal and, to a lesser extent, test populations higher than expected? There are two factors which need to be considered: allele frequency and inbreeding.

Within both the focal and test nest groups the overall inbreeding coefficients were found to be strongly negative (Focal: $F_{IS} = -0.77$; Test: $F_{IS} = -0.34$). This indicates a departure from random mating within the population. Any departure from random mating will either be towards or away from inbreeding. Active inbreeding-avoidance will cause F_{IS} to be negative (Weir, 1996). Furthermore, inbreeding tends to result in the production of excess homozygotes (Hartl & Clark, 1989). Given that the most basic measures of genetic diversity within a population are allele and genotype frequencies, much information can be derived from genotype frequencies and particularly heterozygote frequencies, as each heterozygote carries two different alleles and thus represents variation within a population (Weir, 1996). As can be seen from the focal population results, there are a far greater proportion of observed heterozygotes than were expected. Although the distinction between observed and expected heterozygotes was less pronounced in the test population, the trend holds as there were still a far greater percentage of heterozygotes than homozygotes. Within both colonies the average expected proportion of heterozygotes was low (Focal: $H_e = 0.33$; Test: $H_e = 0.27$). For example a study of *C. consobrinus* by Crozier *et al.*, (1999) found that the average expected heterozygosity of 6216 individuals from 207 colonies across five loci was $H_e = 0.82$. Similar values have been reported in other genera as well, such as *Nothomyrmecia*, *Atta* and *Myrmica* (Sanetra & Crozier, 2003). Although breeding within the colony as a

whole is naturally not random, given that there are normally only one or a few reproductives in a colony and as such the majority of the colony consists of sterile or non-reproductive workers (Peeters & Holldobler, 1995; Dietemann *et al.*, 2003), there is clear evidence that the reproductives within the colony, which are responsible for offspring production, are actively avoiding inbreeding. However, low genetic variation, as evidenced by the reduction in expected heterozygosity, may in fact lead to inbreeding depression (Sanetra & Crozier, 2003). Nevertheless, if inbreeding is not the cause of the over-inflated relatedness estimates, then allele frequencies must be considered.

Within the focal nest group the average number of alleles per locus was 1.7, with no more than two alleles at any particular locus and therefore no more than three possible genotypes at any particular locus. Furthermore, there were two totally monomorphic loci: Camp 8 and Ccon 42. Similarly, within the test nest group, the average number of alleles per locus was 2.3, ranging from one to five. However, although there was a greater allelic richness, there were in fact four monomorphic loci: Camp 8, Ccon 12, Ccon 42 and Ccon 79. The greatest difference between the focal and test populations appears to be with regards to the average number of alleles per *polymorphic* locus. Genetic polymorphism may be defined as "...the occurrence in the same population of two or more alleles at one locus, each with appreciable frequency." (Hedrick, 2000). Most commonly an appreciable frequency is designated as the arbitrary value of 0.99, which the frequency of the most common allele, at a particular locus, must not exceed (Hedrick, 2000). Within the focal population there is an average of two alleles per polymorphic locus while in the test population this value is four, even though there are five polymorphic loci in the focal population as opposed to three in the test population. In general, for both groups, there was a significant population-level departure from Hardy-Weinberg Equilibrium, indicative of the non-random union of gametes. The appearance of the non-random union of gametes may be as a consequence of non-random mating or inbreeding, gametic disequilibrium, overlapping generations, selection, mutation, migration; or a combination thereof (Weir, 1996; Liu & Smith, 2000). The issue of non-random mating and inbreeding has already been discussed and Hardy-Weinberg equilibrium is not particularly sensitive to selection, mutation and migration, (Hartl & Clark, 1989),

however, gametic disequilibrium and overlapping generations warrant some further consideration.

In a randomly mating population, one in Hardy-Weinberg equilibrium, alleles at different loci asymptotically approach random association. This random association is, however, reduced by linkage (Hedrick, 2000). Linkage disequilibrium is therefore the investigation of the non-random associations between alleles, with regards to their frequencies, at different loci. Alleles from different loci that have frequencies which indicate non-random associations are generally referred to as being in linkage disequilibrium, although this may in fact have nothing to do with linkage, as the loci may reside on two different chromosomes (Weir, 1996 & Hedrick, 2000). It is for this reason that a new term, gametic disequilibrium, is increasingly being used rather than the term 'linkage disequilibrium'. Gametic disequilibrium is more encompassing as it considers the non-random association of alleles, which may occur in gametes, and can theoretically attribute said associations, not only to linkage but also, to other factors such as low genetic diversity and high allele frequencies (Hedrick, 2000). While evidence of linkage was found within each of the focal and test nest groups individually, no common linkage was found to occur within the two groups. Therefore it seems that while there appears to be linkage, in fact, it is simply an artefact of high allele frequencies.

A particular feature of eusociality is that it is a cohabitation arrangement whereby adult members of a colony are derived from two or more overlapping generations (Wilson & Holldobler, 2005). This immediately violates the assumption of Hardy-Weinberg that there is no overlap of generations. In a population which does not violate the prerequisite assumptions, Hardy-Weinberg frequencies are expected to be reached after one generation. However, in a population with overlapping generations, Hardy–Weinberg frequencies are only expected to be reached after several generations (Hartl & Clark, 1989). It is therefore possible that over time both the test and focal nest group allele frequencies may have developed into something more closely resembling those predicted by Hardy-Weinberg equilibrium. However, as we do not know the age of either group, it is impossible to predict this with any kind of certainty.

Therefore, to come back to the original question of colony structure: the low levels of genetic differentiation between and within nests of either group, the high levels of within-population relatedness as opposed to between populations, inbreeding avoidance, high allele frequencies and pseudo-linkage all seem to clearly support the idea that *C. klugii* exhibit a polydomous colony structure. Interestingly, polydomy is also thought to be an ecological response to the possibility of nest destruction (Debout *et al.*, 2007). Thus *C. klugii's* preferred nesting site within the fynbos, a habitat inextricably linked to fire, may partly explain the functionality of polydomy within this species.

Queen State, Turn-Over and Mating Frequency

A single extant queen was recovered within the focal colony while sampling. During the analysis it was found that if she was the colony queen she would have required a minimum of three mates. The genotypes of her putative mates could not, however, be unequivocally determined; for each of the three patrilines eight possibilities exist, all with an equal probability. A caveat of the above scenario is the level of relatedness between the queen and workers. Pairwise relatedness estimates, determined between the queen and each individual within the colony, fell between $r = 0.7$ and $r = 1.0$. This level of relatedness is indicative of her being a sister to the workers, rather than a mother which would have resulted in relatedness estimates of around $r = 0.5$. Bearing in mind the discussion in the previous paragraph regarding high allele frequencies and an over-inflated estimate of relatedness, some alternative possibilities should still be considered.

Although the genotypes of workers and the 'queen' are not inconsistent, the irregularities in relatedness could be indicative of a high queen-turnover. High queen-turnover may be as a result of either a short queen life-span or queens leaving nests to begin new nests by colony budding (Bourke *et al.*, 1997). Skaife (1961) found that queenright colonies of *C. klugii*, which he reared in the laboratory, survived for many years suggesting that this species has very long-lived queens. A study by Schrempf *et al.*, (2005) found virgin

queens mated with fertile males had increased longevity and the authors suggest that multiple matings may further enhance this effect. Therefore, within *C. klugii*, a polyandrous species, we might expect a similar trend. High queen turn-over is also quite closely related to low levels of relatedness between age cohorts within the colony (Brown & Keller, 2000). Since both the focal and test colonies had brood within their nests, and both colonies had expanded to the point of requiring more than a single nest, it is safe to assume that there were multiple age cohorts within the samples. Therefore the high level of relatedness between individuals within each of the two colonies refutes the possibility of high queen turn-over. Supporting this theory is a study by Bargum *et al.*, (2007) which found absolutely no queen turn-over in 16 monogynous colonies of *Formica fusca* over a four year period.

The high level of relatedness between the sampled queen and the rest of the focal colony is consistent with her being a sister. Therefore, in order for her to be mated to propagate the colony *and still* have such a high level of relatedness to the workers within the colony, (given the possibility of high queen turn-over), she would have had to have left the colony, engaged in mating and returned to her natal colony for her to be the 'new' queen. Naturally this scenario is highly unlikely within a monogynous species and *Camponotus* colonies are believed to be almost exclusively monogynous (Gertsch *et al.*, 1995; Gadau *et al.*, 1996). In general, monogynous species tend to take part in nuptial flights and subsequently found nests independently, which strongly refutes the possibility of colony budding, normally a feature of polygynous colonies (Liersch & Schmid-Hempel, 1998; Schmid-Hempel & Crozier, 1999; Macaranas *et al.*, 2001).

However, if the scenario of queen-turnover suggested above *is* accurate, then the possibility that *C. klugii* is polygynous should be considered. Firstly, if the queen is in fact an inseminated 'daughter queen', then given a polygynous situation, one really would expect to find more than a single queen present. Interestingly enough, when the focal colony was analysed *sans* the queen to investigate the possibility of alternative queen genotypes, eight were proposed. However, each queen genotype had three associate patrilines, two of which were always definitive. It is believed that polyandry is

far less prevalent in polygynous colonies, due to the increased genetic variation already accrued by the colony, as a result of multiple queens reproducing simultaneously (Schmid-Hempel & Crozier, 1999). This is supported by studies which have found that obligate polyandry exists only in monogynous species (Holbrook *et al.*, 2007). Finally, the eight queen genotypes proposed during the analysis all had equal probabilities, which implies that each queen individually is equally likely.

Within the test population no queens were recovered during sampling, however, two possibilities arose during analysis. Either the individuals analysed could be attributed to a single, multiply inseminated queen or to two multiply inseminated queens. As mentioned above multiple inseminations within polygynous colonies are quite unlikely (Schmid-Hempel & Crozier, 1999).

Thus essentially the two possible scenarios are either a monogynous polyandrous colony, (with over-inflated estimates of relatedness in the focal population), or a polygynous polyandrous colony, (with only a single queen present in the focal population). In a situation such as this, where multiple possibilities exist, it is often prudent to employ Occam's Razor, until further evidence for or against either hypothesis is uncovered. Therefore, given the discussion above, I would support the theory that *C. klugii* colonies are monogynous and polyandrous.

Within the focal colony the over-inflated estimates of relatedness are as a result of high allele frequencies and a maximum of only two alleles at any particular locus. This suggests that the queen and her mate must share at least one allele at every locus, which may be the consequence either of inbreeding or of low allelic variation within the source population of the reproductive individuals. As we have already established that inbreeding is not occurring, low genetic variation within the source population of the colony reproductives seems to be the most plausible explanation at this point.

Worker Reproduction

Skaife (1961) proposed that the presence of brood in nests, other than the queen-right nest, was indicative of worker reproduction. However, he also asserted that the colonies of *C. klugii* were polydomous. While the presence of brood in queenless nests could possibly be attributed to worker production, it is very important to understand that it is also fundamental to the nature of a nest and furthermore a polydomous colony. Brood within a nest, any nest, require a certain level of care and provisioning and as such facilitate the expression of group-directed behaviours, like nest defence (Debout *et al.*, 2007). Furthermore studies have found that in polydomous species frequent mutual transport, of workers and brood alike, occurs between the satellite and queenright nests (Dahbi & Lenoir, 1998; Suarez *et al.*, 2002; Debout *et al.*, 2007).

An important indicator feature is perhaps that although brood may be spread across several satellite nests, the queenright nest tends to contain the most brood. This was found to be the case for *Camponotus gigas*, (Pfeiffer & Linsenmair, 1997), and similarly within my study the queenright nest contained the second highest number of brood. When examining Skaife's (1961) nest census there was a greater than average number of brood present in three out of four queenright nests. However, he makes no distinction between nests which may belong to the same colony, thus it is possible that if the queenright nests were compared to their associated satellite nests, the trend would hold fast.

A factor affecting worker reproduction is that of queen mating-frequency and, by association, the level of relatedness between workers. Polyandry reduces the level of genetic relatedness between workers, thus resulting in a decrease in the indirect fitness benefits accrued by altruistic workers (Goodisman & Hahn, 2005; Holbrook *et al.*, 2007). This can give rise to a situation such that it may be in the best interests of a worker, from a fitness point of view, to attempt to reproduce parthenogenetically. Within the focal colony the effective queen mating-frequency of three, suggests that the ratio of half-sisters to full-sisters should be about 2:1, in other words there should be a far greater percentage of half-sisters within the pairwise relatedness estimates than there are. As

previously discussed the high allele frequencies and low variation within the focal population are almost certainly the cause of the skewed relatedness estimates. A study by Brown and Keller (2000) has strongly cautioned against using indirect measures, such as relatedness asymmetry, to extrapolate results of colony sex-ratios. Therefore, in order to address the issue of worker reproduction, in this situation, it best to take a slightly theoretical approach.

The average level of pairwise relatedness between workers, within each of the focal nests, was $r = 0.81$ and appears to indicate that they are full-sisters. As workers are more closely related to their sons ($r = 0.5$) than their brothers ($r = 0.25$) and furthermore, as full-sisters, more closely related to their nephews ($r = 0.375$) than to their brothers it should be in their best interests, from a fitness point of view, to engage in reproduction (Trivers & Hare, 1976). However, although contrary to the relatedness estimates, we have already established the presence of at least three patrilines, which must be taken into consideration. As half-sisters are only related by $r = 0.25$, any worker is only related to her half-sister's son by $r = 0.125$. It is clearly not within said workers best fitness interests to allow her half-sister to lay eggs. All things being equal, the "break-even" point for worker reproduction should be reached when there are equal numbers of offspring from two patrilines, in other words the full-sister to half-sister ratio should be 1:1 (Villesen & Boomsma, 2003). As the proportion of half-sisters increases workers should refrain from reproduction (Suni *et al.*, 2007). Therefore, given the full-sister to half-sister ratio, in the focal colony, of 1:2, based on the queen effective mating frequency, theoretically worker reproduction should not be occurring.

Within the test colony the effective queen mating frequency is nine. This results in a full-sister to half-sister ratio of 1:8. We should therefore find a far greater percentage of pairwise relatedness estimates falling into the category of half-sisters rather than full-sisters. Within the test colony, the average within-nest relatedness estimate was $r = 0.66$. The percentage of pairwise relatedness estimates, falling within the half-sister category, were higher in the test colony as opposed to the focal colony, however, the percentage is

still not nearly what is expected with a queen effective mating frequency of nine. Once again this may be due high allele frequencies and low genetic diversity.

Having established the fact that worker reproduction *should* be inhibited, based on the queen effective mating frequency, it is important to consider how this inhibition may be in effect. In polygynous colonies, where relatedness asymmetries are less distinct, workers are able to identify the gender of eggs early on (Nonacs & Carlin, 1990). This situation allows for worker policing, as workers would rather preferentially invest in their sisters than their nephews. The early identification of eggs is imperative as it allows policing to occur prior to expensive energetic investment in male eggs (Nonacs & Carlin, 1990). Within monogynous colonies queens are, for the most part, solely responsible for reproduction. Nevertheless this does not automatically negate the effects of male culling and disparate investment in sisters by female workers. A study by Nonacs and Carlin (1990) of worker discrimination in the species *Camponotus floridans* found that workers were only able to distinguish between male and female eggs after pupation.

Therefore, either workers of *C. klugii* are capable of early discrimination between male and female eggs or they are not. If discrimination is possible it may be due to the fact that in colonies where there are multiple patrilines the level of relatedness between workers is reduced. Thus, even though the colony is monogynous, this may simulate a situation similar to that found within polygynous colonies. If this is the case, then worker policing will almost certainly be in effect. If, on the other hand, regardless of multiple patrilines discrimination is not possible, then one can predict a strongly biased colony sex-ratio and possibly disparate investment in male and female brood. If the level of relatedness between workers is low one can expect the colony to invest predominantly in males. If, as is the case within the focal colony, the level of relatedness between workers is high, then one can expect the colony to specialize in producing reproductive females (Boomsma & Grafen, 1990).

Without further investigation it is impossible to favour either of these theories, however, neither theory favours the possibility of worker reproduction. Interestingly enough

though, it has been found that the larger the work-force, the less control the queen retains (Nonacs, 1986), for example, colonies of the monogynous, polyandrous species *Pogonomyrmex barbatus*, can have around 10 000 workers (Suni *et al.*, 2007). Thus, given the relatively small colony size of *C. klugii* the queen may have a significant level of control over the workers, further reducing the possibility of worker reproduction. It is important to note that while my data do not support Skaife's (1961) theory, I cannot negate the possibility that worker reproduction may very well occur in the event that the colonies were no longer queenright. Examples of this behaviour have been found in the *Apterostigma, Tachymyrmex* and *Sericomyrmex* genera (Villesen & Boomsma, 2003).

Shortcomings of the Current Study

In retrospect certain areas within this study could have been executed differently and improved. Firstly the size of my data set could have been increased. The area that was sampled was about two square kilometres, however clearly very few nests were found within this area. Had I been able to source more nests, by sampling more widely, unbiased background-population allele frequencies could have been determined. This would have allowed for a more accurate estimate of relatedness within the colonies. Similarly, more nests would have allowed me to increase my data set. Consequently a larger data set would have allowed the emerging trends to be more easily identifiable and confirmed these trends as sociogenetic characteristics of the species. However, on subsequent sampling no additional *C. klugii* nests were found and time constraints limited further sampling.

Secondly, the number of polymorphic loci in the data set could have been increased. Rather than focusing on one colony and one nest at a time, completing all it's genotyping before moving on to the next nest, a small subset from each nest and colony should have been genotyped first. This would have allowed for earlier detection of the two totally monomorphic loci, Camp 8 and Ccon 42. This would have saved both time and money and allowed the detection of additional polymorphic loci.

Thirdly, the sperm stored within the queen's spermatheca could have been genotyped. In order for this to be a viable option it is best that the queen's gaster is never frozen and that the spermatheca is dissected out while still fresh. At the time of collection I was unfortunately not aware of these constraints and by the time an attempt was made to dissect out the spermatheca, the queen had been frozen and thawed three times. The result of this was that the tissue within her gaster was macerated making it impossible to identify, let alone remove, her spermatheca.

Future Research

A particularly interesting avenue for further research would be to examine colony sex ratio. The sex ratio within a colony appears to be a function of who controls reproduction: the queen or the workers? If the queen is in control then a male:female sex ratio of 1:1 should be in effect, as queens are equally related to sons and daughters (Nonacs & Carlin, 1990). If the workers are in control and there is a high relatedness asymmetry within the colony one would expect production of predominantly female reproductives, and if the relatedness asymmetry is low, male reproductives (Brown & Keller, 2000). Sampling for such a study would have to be carefully timed as winged forms are produced around the middle of summer and only remain in the natal nest until spring (Skaife, 1961; Pfeiffer & Linsenmair, 1997). To unequivocally address the question of worker reproduction, one could also look at mitochondrial DNA as it is maternally inherited (Hedrick, 2000).

One could also investigate polyethism: the role genetics plays in determining to which caste an ant will belong, worker or soldier. Genetically influenced polyethism has been found in the ant *C. consobrinus* (Fraser *et al.*, 2000). A feature of this is the presence of intermediate phenotypes, which may in fact be in response to a range of available tasks (Fraser *et al.*, 2000). Similarly, Skaife (1961) found intermediate individuals when performing a census of *C. klugii* nests, and I encountered "intermediates" during the census of my nests as well.

Another avenue for research is the role *C. klugii* may play as an indicator species within the fynbos. It has been well documented that insects are extremely useful for both long and short term ecosystem monitoring as they are sensitive to environmental change and respond rapidly to such changes (Underwood & Fisher, 2006). In order to best track the effects of disturbances upon a species, the samples observed and obtained should be from within their endemic range, because it seems that the effects of disturbance are far more distinct when a species is within its optimal habitat (Hoffman & Andersen, 2003). This may be particularly so for *C. klugii* due to the low genetic variance it seems to exhibit as a species, as it has been shown that low variance is strongly correlated with a lack of resilience to detrimental environmental change (Oldroyd & Fewell, 2007). If field colonies were obtained and maintained within the laboratory, it would be very informative to simulate the climate changes predicted due to global warming. Over time one could experimentally alter environmental conditions, such as temperature and humidity, and document the effects these changes may have upon the population, for example to egg number or colony sex ratio, with regards to genetic variation. A laboratory based study in conjunction with repeated field sampling over time could result in producing genuinely informative information for conservation management decisions.

APPENDIX A

Raw Data

i. Nest Census

Table A.1 A complete census of all eight nests comprising the focal colony.

Nest	Minor Workers	Major Workers	Queens	Total	Number Genotyped	Brood
1	58	18	0	76	39	35
2	15	14	0	29	27	4
3	76	29	0	105	39	57
4	57	20	0	77	38	75
5	40	7	0	47	38	57
6	27	11	0	38	36	8
7	26	15	0	41	38	1
8	68	15	1	84	39	72
Total	367	129	1	497	294	309

ii. Background Population Allele Frequencies

Table A.2 Background population allele frequencies in *Camponotus klugii* based on the genotypes of 314 individuals.

Locus	Allele	Allele Frequency
Camp4	208	0.38993
	210	0.36007
	214	0.11250
	220	0.13750
Camp8	127	1.0
Ccon12	167	0.35751
	169	0.5
	173	0.14249
Ccon20	286	0.32500
	288	0.23750
	290	0.26250
	292	0.01250
	294	0.16250
Ccon42	258	1.0
Ccon70	162	0.25000
	166	0.12500
	168	0.23750
	170	0.27500
	172	0.11250
Ccon79	356	0.25000
	366	0.25000
	368	0.5

iii. Queen Genotypes and Mating Frequency

Each matriline is numbered Q**1**, Q**2**, Q**3** etc... The patriline associated with a particular queen is numbered accordingly M**1**, M**2**, M**3** etc...Additional patrilines associated with a particular queen are numbered M1/**1**, M1/**2**, M1,**3** etc...In the event that there are multiple possible genotypes for a particular patriline, they will all be identically numbered but each will have a specific probability associated with it.

Table A.3 Genotypes of potential mates generated, with both a narrow and broad deduction, from the combined data of all eight focal nests.

Q/M	P*	Camp4	Camp8	Ccon12	Ccon20	Ccon42	Ccon70	Ccon79
Q 1	**1.0**	**208/210**	**127/127**	**167/173**	**286/290**	**258/258**	**162/170**	**356/366**
M 1/1	0.125	210	127	167	286	258	162	356
M 1/1	0.125	210	127	167	286	258	162	366
M 1/1	0.125	210	127	167	286	258	170	356
M 1/1	0.125	210	127	167	286	258	170	366
M 1/1	0.125	210	127	167	290	258	162	356
M 1/1	0.125	210	127	167	290	258	162	366
M 1/1	0.125	210	127	167	290	258	170	356
M 1/1	0.125	210	127	167	290	258	170	366
M 1/2	0.125	210	127	173	286	258	162	356
M 1/2	0.125	210	127	173	286	258	162	366
M 1/2	0.125	210	127	173	286	258	170	356
M 1/2	0.125	210	127	173	286	258	170	366
M 1/2	0.125	210	127	173	290	258	162	356
M 1/2	0.125	210	127	173	290	258	162	366
M 1/2	0.125	210	127	173	290	258	170	356
M 1/2	0.125	210	127	173	290	258	170	366
M 1/3	0.125	208	127	167	286	258	162	356
M 1/3	0.125	208	127	167	286	258	162	366

M 1/3	0.125	208	127	167	286	258	170	356
M 1/3	0.125	208	127	167	286	258	170	366
M 1/3	0.125	208	127	167	290	258	162	356
M 1/3	0.125	208	127	167	290	258	162	366
M 1/3	0.125	208	127	167	290	258	170	356
M 1/3	0.125	208	127	167	290	258	170	366

*P = Probability

Table A.4 Queen and potential mate genotypes generated with both a narrow and broad deduction, the power of which was $\beta = 1.0$, from the combined data of all eight focal nests.

Q/M	P*	Camp4	Camp8	Ccon12	Ccon20	Ccon42	Ccon70	Ccon79
Q 1	**0.125**	**208/210**	**127/127**	**167/173**	**286/286**	**258/258**	**162/162**	**356/356**
M 1/1	1.0	210	127	167	290	258	170	366
M 1/2	1.0	208	127	167	290	258	170	366
M 1/3	0.28	208	127	173	290	258	170	366
M 1/3	0.72	210	127	173	290	258	170	366
Q 2	**0.125**	**208/210**	**127/127**	**167/173**	**286/286**	**258/258**	**162/162**	**366/366**
M 2/1	1.0	210	127	167	290	258	170	356
M 2/2	1.0	208	127	167	290	258	170	356
M 2/3	0.28	208	127	173	290	258	170	356
M 2/3	0.72	210	127	173	290	258	170	356
Q 3	**0.125**	**208/210**	**127/127**	**167/173**	**286/286**	**258/258**	**170/170**	**356/356**
M 3/1	1.0	210	127	167	290	258	162	366
M 3/2	1.0	208	127	167	290	258	162	366
M 3/3	0.28	208	127	173	290	258	162	366
M 3/3	0.72	210	127	173	290	258	162	366
Q 4	**0.125**	**208/210**	**127/127**	**167/173**	**286/286**	**258/258**	**170/170**	**366/366**
M 4/1	1.0	210	127	167	290	258	162	356

M 4/2	1.0	208	127	167	290	258	162	356
M 4/3	0.28	208	127	173	290	258	162	356
M 4/3	0.72	210	127	173	290	258	162	356
Q 5	**0.125**	**208/210**	**127/127**	**167/173**	**290/290**	**258/258**	**162/162**	**356/356**
M 5/1	1.0	210	127	167	286	258	170	366
M 5/2	1.0	208	127	167	286	258	170	366
M 5/3	0.28	208	127	173	286	258	170	366
M 5/3	0.72	210	127	173	286	258	170	366
Q 6	**0.125**	**208/210**	**127/127**	**167/173**	**290/290**	**258/258**	**162/162**	**366/366**
M 6/1	1.0	210	127	167	286	258	170	356
M 6/2	1.0	208	127	167	286	258	170	356
M 6/3	0.28	208	127	173	286	258	170	356
M 6/3	0.72	210	127	173	286	258	170	356
Q 7	**0.125**	**208/210**	**127/127**	**167/173**	**290/290**	**258/258**	**170/170**	**356/356**
M 7/1	1.0	210	127	167	286	258	162	366
M 7/2	1.0	208	127	167	286	258	162	366
M 7/3	0.28	208	127	173	286	258	162	366
M 7/3	0.72	210	127	173	286	258	162	366
Q 8	**0.125**	**208/210**	**127/127**	**167/173**	**290/290**	**258/258**	**170/170**	**366/366**
M 8/1	1.0	210	127	167	286	258	162	356
M 8/2	1.0	208	127	167	286	258	162	356
M 8/3	0.28	208	127	173	286	258	162	356
M 8/3	0.72	210	127	173	286	258	162	356

*P = Probability

Table A.5 Queen and potential mate genotypes generated, with a narrow deduction power of $\beta = 0.99$, from the combined data of both test nests.

Q/M	Camp4	Camp8	Ccon12	Ccon20	Ccon42	Ccon70	Ccon79
Q	**214/220**	**127/127**	**169/169**	**286/288**	**258/258**	**166/168**	**368/368**
M 1	208	127	169	294	258	172	368
M 2	208	127	169	294	258	168	368
M 3	208	127	169	288	258	168	368
M 4	208	127	169	294	258	166	368
M 5	208	127	169	294	258	170	368
M 6	208	127	169	292	258	170	368
M 7	208	127	169	290	258	168	368
M 8	208	127	169	288	258	172	368
M 9	208	127	169	286	258	172	368

Table A.6 Queen and potential mate genotypes generated, with a broad deduction power of $\beta = 0.99$, from the combined data of both test nests.

Q/M	P*	Camp4	Camp8	Ccon12	Ccon20	Ccon42	Ccon70	Ccon79
Q 1	**0.51**	**214/220**	**127/127**	**169/169**	**286/288**	**258/258**	**166/168**	**368/368**
M 1/1	1.0	208	127	169	294	258	172	368
M 1/2	1.0	208	127	169	294	258	168	368
M 1/3	1.0	208	127	169	288	258	168	368
M 1/4	1.0	208	127	169	294	258	166	368
M 1/5	1.0	208	127	169	294	258	170	368
M 1/6	1.0	208	127	169	292	258	170	368
M 1/7	1.0	208	127	169	290	258	168	368
M 1/8	1.0	208	127	169	288	258	172	368
M 1/9	1.0	208	127	169	286	258	172	368
Q 2	**0.49**	**208/208**	**127/127**	**169/169**	**286/288**	**258/258**	**166/168**	**368/368**
M 2/1	1.0	220	127	169	294	258	172	368
M 2/2	1.0	214	127	169	294	258	168	368
M 2/3	1.0	214	127	169	294	258	172	368
M 2/4	0.19	220	127	169	286	258	166	368
M 2/4	0.38	220	127	169	286	258	168	368
M 2/4	0.15	220	127	169	288	258	166	368
M 2/4	0.28	220	127	169	288	258	168	368
M 2/5	1.0	220	127	169	294	258	168	368
M 2/6	1.0	214	127	169	294	258	170	368
M 2/7	1.0	214	127	169	292	258	170	368
M 2/8	1.0	220	127	169	290	258	168	368
M 2/9	1.0	214	127	169	288	258	168	368
M 2/10	1.0	220	127	169	294	258	166	368
M 2/11	1.0	220	127	169	288	258	172	368
M 2/12	1.0	214	127	169	286	258	172	368

*P = Probability

APPENDIX B

Protocols and Reagents

i. DNA extraction method review

Table B.1 A brief review of different DNA extraction method protocols with respect to a variety of samples.

SPECIES	INDIVIDUAL	EXTACTION METHOD	EXTRACTION REFERENCE	JOURNAL REFERENCE
Formica exsecta	workers & pupae	proteinase K/SDS & phenol chloroform purification	Sambrook *et al.*,1989	Brown & Keller, 2000.
Formica paralugubris	workers	proteinase K/SDS & phenol chloroform purification	Sambrook *et al.*,1989	Chapuisat *et al.*, 1997.
Leptothorax acervorum	workers	CTAB buffer, proteinase K & phenol chloroform purification	Hamaguchi *et al.*, 1993	Bourke *et al.*, 1997.
Dendromyrmex, Camponotus, Formica & Oecophylla sp.	Ethanol preserved workers	CTAB buffer, proteinase K & phenol chloroform purification & DNA extraction kit, ID Pure	Hunt & Page, 1994	Brady *et al.*, 2000.
Camponotus floridanus	workers	proteinase K/SDS & phenol chloroform purification	Schenkel *et al.* 1985; Heinze *et al.* 1994	Gadau *et al.*, 1996.
Camponotus ephippium complex	workers	Rapidgene Genomic DNA purification kit	Amresco, USA	Macaranas *et al.*, 2001.
Rhytidponera sp.	workers	modified CTAB method	Boyce *et al.* 1989	Tek Tay *et* al., 1997.
Pissodes nemorensis (bark weevils)	adults	modified CTAB method	Doyle & Doyle, 1987	Boyce *et al.*, 1989.
Calliphora erythrocephala (fly)	Embryos, adults & fat bodies	proteinase K/SDS	novel	Schenkel *et* al., 1985.
Human bone	fresh, 3 & 9 months	proteinase K/SDS & sodium acetate	novel	Cattaneo *et al.*, 1995.

ii. DNA extraction method comparison

Table B.2 A comparison of five different DNA extraction method protocols.

Modified CTAB (Palle Villesen, unpublished)	CTAB (Boyce *et al.*, 1989)	Sodium acetate (Cattaneo *et al.*, 1995)	Phenol:chloroform (Sambrook *et al.*, 1989)
homogenise	homogenise	homogenise	
place sample & 350µl 1xCTAB buffer in 1.5ml eppendorf	place sample & 500µl 2xCTAB buffer in 1.5ml eppendorf	1.5g sample & 3ml white cell lysis buffer (Tris-HCL; NaCl; EDTA)	add = volumes DNA solution & phenol/chloroform (25:24)
add 2µl Proteinase K		add 25µl Proteinase K & 50µl SDS	
mix by tapping		mix by tapping	
incubate @ 60°C for 60min	incubate @ 65°C for 45 - 60min	incubate @ 42°C overnight (waterbath)	
	centrifuge for 15sec @ 13000 rpm		
	transfer supernatant to new eppendorf		
	dissolve pellet in 300µl CTAB		
	incubate @ 65°C for 10min		
	centrifuge for 10min @ 13000 rpm		
	transfer supernatant to eppendorf containing first supernatant		
add 350µl chloroform/isoamylalcohol (24:1)	add = volumes DNA solution. & chloroform/isoamylalcohol (24:1)	add 1ml saturated sodium acetate	
mix by vortexing		shake for 30sec	mix by vortexing
centrifuge for 4min @ 13000 rpm		centrifuge for 10min @ 4000 g	centrifuge for 15sec @ 12000g
transfer supernatant to new eppendorf		transfer supernatant to new eppendorf	transfer supernatant to new eppendorf
			add = volumes DNA solution. & chloroform
			mix by vortexing
			centrifuge for 15sec @ 12000g
			transfer supernatant to new eppendorf
precipitate with 875µl (2.5 vol.) freezer-cold 100% ethanol	precipitate with freezer-cold isopropanol	precipitate with 4ml 100% isopropanol	precipitate with 2 - 2.5 vol freezer cold 100% ethanol
incubate @ -70°C over night		mix for 10min	
centrifuge for 25min @ 13000 rpm		centrifuge for 10min @ 4000 g	
discard supernatant, wash with 100µl 96% freezer-cold ethanol	wash with 70% ethanol	discard supernatant, wash with 250µl 70% ethanol	
centrifuge for 5min @ 13000 rpm		centrifuge for 10min @ 13 000 g	
discard supernatant, dry tube on tissue	discard supernatant, dry tube on tissue	discard supernatant, dry tube on tissue	
dissolve pellet in 30/50/100µl TE buffer or water	dissolve pellet in 30/50/100µl TE buffer	dissolve pellet in 250µl sterile water	
incubate @ 37°C for 30min			

Table B.2 continued.

Proteinase K/SDS (Schenkel *et al.*, 1985)	**Method 1**	**Method 2**
homogenise	homogenise	homogenise
combine sample & 250µl buffer A (Tris-HCL; NaCl; EDTA; spermidine;spermine & sucrose)	place sample & 350µl 1xCTAB buffer in 1.5ml eppendorf	place sample & 500µl 2xCTAB buffer in 1.5ml eppendorf
add 250µl buffer B (Tris-HCL; NaCl; EDTA; SDS & Proteinase K)	add 2µl Proteinase K	
mix by tapping	mix by tapping	
incubate @ 37°C for 45 - 90min	incubate @ 60°C for 60min/120min/**180min**	incubate @ 65°C for 60/120/180min
		centrifuge for 15sec @ 13000 rpm
		transfer supernatant to new eppendorf
		dissolve pellet in 300µl CTAB
		incubate @ 65°C for 10min
		centrifuge for 10min @ 13000 rpm
		transfer supernatant to eppendorf containing first supernatant
add = volumes DNA solution. & phenol (pH 8)		
add = volumes DNA solution. & chloroform/isoamylalcohol (24:1)	add 1/5 volume saturated sodium acetate	add 1/5 volume saturated sodium acetate
	shake for 30sec	shake for 30sec
	centrifuge for 10min @ 4000 g	centrifuge for 10min @ 4000 g
	transfer supernatant to new eppendorf	transfer supernatant to new eppendorf
adjust to 1M NaCl & 1% SDS		
add = volumes DNA solution. & chloroform		
incubate on ice for 10 min		
precipitate with ethanol	precipitate with 875µl (2.5 vol) freezer-cold 100% ethanol	precipitate with freezer-cold isopropanol
	incubate @ -70°C over night	
	centrifuge for 25min @ 13000 rpm	
	discard supernatant, wash with 100µl 96% freezer-cold ethanol	wash with 70% ethanol
	centrifuge for 5min @ 13000 rpm	
	discard supernatant, dry tube on tissue	discard supernatant, dry tube on tissue
dissolve pellet in TE	dissolve pellet in 30/50/100µl TE buffer or water	dissolve pellet in 30/50/100µl TE buffer
	incubate @ 37°C for 30min	

iii. Modified CTAB DNA extraction method

1. Homogenise the sample
2. Place the sample & 350µl of 1X CTAB buffer in a 1.5ml eppendorf
3. Add 2µl of Proteinase K (Promega)
4. Mix by tapping
5. Incubate at 60°C for 3hrs
6. Add a 1/5 volume of saturated sodium acetate
7. Shake for 30 seconds
8. Centrifuge for 10 minutes at 13 200 rpm
9. Transfer supernatant (350µl) to a new eppendorf
10. Precipitate with 2.5 volumes freezer-cold 100% isopropanol
11. Incubate at -70°C for 12hrs
12. Centrifuge at 13 200 rpm for 25 minutes
13. Discard the supernatant
14. Wash with 100µl of freezer-cold 96% ethanol
15. Centrifuge for 5 minutes at 13 200 rpm
16. Discard the supernatant
17. Air dry the tube to allow any latent ethanol to evaporate
18. Dissolve the pellet in 50µl of TE buffer
19. Incubate at 37°C for 30 minutes

iv. Agarose Gel Electrophoresis Protocol (Sambrook *et* al., 1989)

1. Combine three microlitres of each sample with two microlitres of 1X tracking dye
2. Load the sample into the gel, with a single sample in each lane.
3. Load five microlitres of the restriction enzyme marker Dra I (Promega) into a single lane of the gel.
4. Submerge the gel in 1X TBE buffer in a tank and electrophorese at between 65 and 130 volts.
5. Run the gel for 30 to 40 minutes
6. Removed the gel from the tank and exposed to UV light in a dark room and photograph.

Table B.3 Characteristics of agarose gels used to visualize amplified samples

Concentration of Gel	Agarose (g)	1X TBE (ml)	Ethidium Bromide (µl)	Size Range (bp)
1.5%	1.5	100	5	200 – 300
2%	2	100	5	50 - 200

Table B.4 Characteristics of restriction enzyme Dra I

Restriction Enzyme	Recognition Sequence	No. of Recognition Sites	Generated Fragments (relevant fragments in bold)
Dra I	TTT/AAA	13	**92, 174, 228, 533**, 695, 1071, 2152, 2303, 3599, 6038, 6816, 7834, 8370, 8596

Table B.5 Voltage range used for agarose gel electrophoresis with respect to the distance between electrodes

Voltage to Distance Ratio	Distance between Electrodes (cm)	Voltage range (V)
1 – 5V:cm	13	13 – 65
1 – 5V:cm	26	26 - 130

v. Decontamination of ethidium bromide solutions (Sambrook *et al.*, 1989)

1. Combine 100mg of activated charcoal powder with every 100ml of solution
2. Allow to stand, with occasional agitation, for at least one hour
3. Filter the solution through Whatman No.1 filter paper and discard filtrate
4. Dispose of the charcoal with hazardous waste

vi. 1X CTAB Buffer (Ausubel *et al.*, 2002)

1. Dissolve 1g HAB (hexadecyltrimethyl ammonium bromide) in 75ml of a 1M NaCl solution
2. Add 5ml of a 1M Tris solution
3. Add 2ml of a 0.5M EDTA solution

4. Adjust to a final volume of 100ml

vii. TE Buffer (Ausubel *et al.*, 2002)

1. Combine 6.055g of Tris with 1.86g of EDTA
2. Add 500ml of water

viii. 0.5M EDTA (Sambrook *et al.*, 1989)

1. Dissolve 93.05g of EDTA in 400ml of water
2. While stirring vigorously add 10g of NaOH pellets
3. Adjust to a final volume of 500ml

ix. 10X TBE Buffer (Sambrook *et al.*, 1989)

1. 108g Tris
2. 55g Boric acid
3. 7.4g EDTA
4. 1 litre distilled water
5. Combine the reagents while stirring and heating gently
6. For 1X TBE: dilute 10X TBE to a 1:10 ratio with distilled water

x. 10mg/ml Ethidium Bromide (Sambrook *et al.*, 1989)

1. 0.1g Ethidium Bromide
2. 10ml Distilled water
3. Combine the reagents and stir until fully dissolved

xi. 6X Tracking Dye (Sambrook *et al.*, 1989)

1. 62.5mg bromophenol blue
2. 10g sucrose
3. Dissolve both in 15ml distilled water
4. Add 1ml EDTA
5. Make up to final volume of 25ml with distilled water
6. For 1X Tracking Dye: dilute 6X tracking dye 1:6 with distilled water

xii. 6% Acrylamide-urea Gel (GENESCAN 672 Software – User's Manual)

1. 18g Urea
2. 40ml Millipore water
3. 5ml 10X TBE
4. 5ml 40% Acrylamide solution
5. Combine all the reagents and de-gas the solution through a Millipore durapore ™ with a 0.22µm filter.
6. Quickly add 250µl of 10% Ammonium persulfate (AMPS) and 35µl of TEMED to the solution
7. Carefully dispense between the gel plates and allow to set for 1hr before use

APPENDIX C

Formula

i. Bonferroni correction (Weir, 1996)

$$\alpha = 1 - (1 - \acute{\alpha})^{1/L}$$

Where:

$\acute{\alpha}$ = The original significance level $p = 0.05$

α = The corrected significance level

L = The number of tests performed

ii. Exact Hardy-Weinberg Test (Weir, 1996)

$$\Pr(n_{AA}, n_{Aa}, n_{aa} \mid n_A, n_a) = \frac{n! n_A! n_a! 2^{n_{Aa}}}{n_{AA}! n_{Aa}! n_{aa}! (2n)!}$$

Where A and a are two alleles found at a particular locus and n is the number of individuals in the sample, such that:

n_A = the number of A alleles in the sample

n_a = the number of a alleles in the sample

n_{AA} = the number of homozygote AA genotypes in the sample

n_{aa} = the number of homozygote aa genotypes in the sample

n_{Aa} = the number of heterozygote Aa genotypes in the sample

iii. Gametic Disequilibrium (Schneider *et al.*, 2000)

$$S = -2 \log(L_{H*}/L_H)$$

Where:

S = the likelihood-ratio statistic

L_{H*} = the likelihood of the data assuming linkage equilibrium

L_H = the likelihood of the data *not* assuming linkage equilibrium

iv. Genetic Diversity (Goudet, 2001)

$$H_{sk} = (n_k / n_k - 1)(1 - \Sigma p_{ik}^2 - H_{ok} / 2n_k)$$

Where:

n_k = the number of individuals in sample k

p_{ik} = the frequency of allele A_i in sample k

H_{ok} = the proportion of observed heterozygotes within sample k.

v. Inbreeding Coefficient (Goudet, 2001)

$$F_{IS}k = 1 - H_O k / H_S k$$

Where, in a population of k individuals:

H_O = the observed heterozygosity

H_S = the average expected heterozygosity or within-sample gene diversity

vi. Genetic Differentiation

a. G_{ST}' (Goudet, 2001)

$$G_{ST}' = H_t' - H_S / H_t'$$

Where G_{ST}' is the equivalent estimator of F_{ST}, independent of the number of samples:

H_t' = the overall gene diversity, also independent of the number of samples

H_S = the within sample gene diversity.

b. R_{ST} (Goodman, 1997)

$$R_{ST} = (S|-SW)/ S|$$

Where:

S| = twice the estimated variance in allele size across populations

SW = twice the estimated variance in allele size *within* each population.

vii. Genetic Variance (Schneider *et al.*, 2000)

$$F_{ST} = \sigma^2a / \sigma^2_T \qquad F_{IT} = \sigma^2a + \sigma^2b / \sigma^2_T \qquad F_{IS} = \sigma^2b / \sigma^2b + \sigma^2c$$

Where:

σ^2a = associated covariance component of the group

σ^2b = associated covariance component of the population

σ^2c = associated covariance component of haplotypes within a population within a group

σ^2_T = total sum of expected mean squares

viii. Queen Genotype Deduction (Moilanen *et al.*, 2004)

$$\beta = \prod^{N}_{i=1} \prod^{l}_{k=1} \left(1 - 2\,(1/2)^{n_{ik}} \right)$$

Where:

n = the number of offspring at a particular locus

N = the number of groups scored

l = the locus

ix. Genetic Relatedness (Queller & Goodnight, 1989)

$$R = \Sigma_x \Sigma_k \Sigma_l (P_y - P^*) / \Sigma_x \Sigma_k \Sigma_l (P_x - P^*)$$

Where, when all individuals are equally weighted:

x = individuals within the data set

k = loci within the data set

l = allelic position

P_x = the frequency of the allele at position 'l' in locus 'k' of the individual 'x'

P_y = the frequency of the same allele in the set of individuals to which you are measuring x's relatedness

P* = the frequency of the same allele within the whole population, excluding all putative relatives of 'x'

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