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Population genetic structuring in the common mole-rat, *Cryptomys hottentotus hottentotus*

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Declaration

This thesis reports the results of original research I conducted under the auspices of the Zoology Department, University of Cape Town, between 1998 and 2001. All the assistance that I received has been acknowledged. This work has not been submitted for a degree at any other university.

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Jacqueline Margaret Bishop

Abstract

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This thesis addresses the population genetic consequences of divergent social structure and dispersal regimes in two study populations of the common mole-rat *Cryptomys hottentotus hottentotus*. Aspects relating to the relationship between dispersal, gene flow and genetic variation, the roles of mutation and drift in promoting genetic structuring, and the role of genetic parentage in the species' mating system were investigated using a suite of *Cryptomys*-specific microsatellite markers.

C. h. hottentotus is a social species living in colonies of 2-14 individuals, permanently inhabiting a network of burrows. The species is a co-operative breeder whose colonies are composed of a breeding pair, their offspring and a number of potentially unrelated individuals. *C. h. hottentotus* is an obligate outbreeder whose offspring delay dispersal and the opportunities for independent reproduction until both social and ecological conditions are favourable. The species is widely distributed across South Africa from extreme arid regions where low rainfall restricts dispersal for much of the year, to highly mesic areas where dispersal is facilitated all year round. In response to varying ecological constraints on dispersal, the species displays differential degrees of social elaboration across its range. This appears to be in response to the high energetic costs of burrowing and the low probability of locating geophyte clumps in increasingly arid environments.

Population genetic analysis was supported by a detailed demographic study of the species, undertaken by Dr. Andrew Spinks (PhD thesis, Spinks 1998, Dept. of Zoology, University of Cape Town), across its ecological range. The two study populations, located in Somerset West, South Western Cape and Steinkopf, Northern Cape, South Africa represent an ecological range of increasing aridity. In addition to climate disparities, regional differences in geophyte food resources were also evident at the two sites. Geophytes occurred in clumps at both sites; however their density at Steinkopf was significantly lower than that at Somerset West. Resource density was further reflected in the density of colonies at the two sites, where colony density at Somerset West far exceeded that of Steinkopf. Microsatellite markers were used to specifically investigate the effects of differential social elaboration and dispersal patterns, in response to these disparities, on the population genetic structure of colonies at the two study sites. A species-specific microsatellite library, using a standard protocol, was constructed for *C. h. hottentotus*. Further microsatellite loci, isolated within the closely related Damaraland mole-rat *C. damarensis*, were also used in the population genetic analysis of the two study populations.

In order to assess the effects of divergent social stability and dispersal rates on genetic sub-structuring, variation and gene flow, colonies were analysed using *F*-statistics, interpreted within the framework of Chesser's breeding group models (Chesser 1991a, 1991b), Slatkin's R_{st}, (Slatkin 1995), and measures of allelic diversity and heterozygosity. The degree to which both populations were socially structured into colonies was found to be sufficient to promote significant levels of genetic structuring as measured by both F_{st} and R_{st}. Population genetic structuring at the two sites was found to be comparable when measured by F_{st}, but significantly different when measured by R_{st}. This appears to be reflective of the different roles random genetic drift and mutation have played in promoting sub-structuring, via contrasting dispersal and gene flow regimes, at the two sites. Measures of allelic diversity and heterozygosity were found to be unexpectedly higher within colonies at Steinkopf. Correlation analysis of colony H_{obs} and mean monthly rainfall indicated a significantly negative relationship such that colonies at Somerset West were characterised by an excess of homozygotes, relative to Hardy-Weinberg expectations, despite the greater opportunities for dispersal and gene flow. These unexpected results appear to be the consequence of highly localised dispersal patterns that may act to increase the chance of allelic identity by descent (IBD) among unrelated breeding pairs at Somerset West, thus resulting in both lower levels of heterozygosity and allelic variation.

Molecular analysis of the mating systems of an increasing number of species indicates that genetic systems of mating, as measured by relatedness and parentage, do not necessarily reflect social mating systems. Analysis of paternity was carried out in order to determine whether extra-colony paternity (ECP) characterises *C. h. hottentotus*. Within social species, the occurrence of extra-pair paternity has important consequences for the evolution of social and cooperative behaviours by kin selection and reciprocal altruism because of its influence on measures of relatedness and allelic IBD. In addition, paternity analysis was used to determine whether variation in social stability and dispersal regimes were reflected in differences in the genetic mating system of the species across its distributional range. Assignment of paternity to a within-colony male was found to occur with similar frequencies at the two study sites. However, assignment to the male originally designated as the breeder during the mark-recapture study, was greater within colonies at Steinkopf. Colonies at Somerset West were found to support a greater number of large unrelated males (>60g); as a result size criteria alone was found to be insufficient with which to determine the breeding male. In addition, genetic analysis of paternity appears to support Bennett's (1989) theory that colonies of *C. h. hottentotus* may support more than one resident breeding male. ECP was found to characterise colonies at both study sites and occurred with similar frequencies.

Furthermore ECP appears to have disparate genetic consequences within colonies of the two populations; whilst appearing to promote genetic variation within colonies at Steinkopf, ECP may act to increase the probability of allelic IBD within colonies at Somerset West, and thereby increasing the number of homozygote genotypes. Measures of relatedness were found to be comparable for a number of categories at both sites. However, relatedness between breeding pairs was found to be significantly higher at Somerset West, further supporting the reported effects of localised gene flow and increased allelic IBD on measures of heterozygosity.

This study assessed variation in genetic structure as a correlate of substantial differences in the ecology and social systems of two populations of *C. h. hottentotus.* Analysis of the species' population genetic structure and genetic mating system provides additional evidence for both the occurrence of extra-pair paternity in apparently monogamous mammals, and its potential consequences for the maintenance of genetic variation in wild populations. Furthermore, this study on *C. h. hottentotus* makes a significant and novel contribution to studies that aim to account for patterns of genetic variation within wild populations. Its main aim was to measure variation in genetic structuring as a correlate of differences in the ecology and social systems of two populations and this was achieved by assessing population genetic structure, genetic variation and gene flow of a single species, distributed along an ecological gradient, supported by detailed long-term demographic data.

Acknowledgments

I would like to express my sincere thanks to my supervisors, Professor Jenny Jarvis and Dr. Colleen O'Ryan, who provided continued guidance, motivation and support throughout the course of my PhD. I am particularly grateful to Professor Jenny Jarvis for providing me with the opportunity to expand my knowledge base into the exciting field of molecular ecology, and to Dr. Colleen O'Ryan for teaching me a vast range of molecular and analytical skills. In sharing their collective expert knowledge in two thought-provoking and stimulating fields, they have helped me bridge the gap between the ecological and genetic characteristics that distinguish this perplexing subterranean rodent species.

I am especially indebted to Prof. Nigel Bennett, University of Pretoria, for initiating the field project, and Dr. Andrew Spinks, University of Cape Town, who completed his PhD on the ecology of these two populations. Without their diligent mark-recapture fieldwork and resulting exhaustive data set, this thesis would not have been possible. Furthermore, I am very grateful for the initial guidance provided by Dr Andrew Spinks on the selection of colonies for genetic analysis that would best represent the demographic trends at the two study sites, and this was reflected in the intriguing and often perplexing genetic results. I am also extremely grateful to all those people who helped Nigel and Andrew collect all the toe-clip samples over the respective sampling periods at Steinkopf and Somerset West. Their backbreaking long days through hot days and cold nights are very much appreciated.

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I would like to thank and credit Dr. Tamsin Burland, Queen Mary and Westfield College, London for her all her technical advice and support during the construction of the microsatellite library, and in providing me with the unpublished *C. damarensis* primer sequences for my analysis. In addition, she provided invaluable advice in the use of the Relatedness v.5.08 software package and much-appreciated help in the interpretation of a number of confusing results.

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CHAPTER 1

General Introduction

THE BATHYERGIDAE

The African mole-rats, family Bathyergidae, are subterranean hystricognathous rodents endemic to sub-Saharan Africa. They are a unique mammalian group upon which to test hypotheses relating to the evolution and maintenance of sociality (Jarvis 1981, Jarvis and Bennett 1991), and its genetic consequences (O'Riain and Braude 2000, Faulkes and Bennett 2001). This is because the Bathyergidae display probably the widest range of sociality known for any mammalian family. This range extends from strictly solitary species e.g. the Cape dune mole-rat *Bathyergus suillis*, to the extreme eusocial organisation of the naked mole-rat *Heterocephalus glaber* (Jarvis 1981, Bennett 1989, Jarvis and Bennett 1991).

Several theories have been proposed as to the origin and evolution of sociality within the mole-rat family, of which the constraint based model of the Aridity-Food Distribution Hypothesis (AFDH)[1] currently represents the most convincing and comprehensive explanation (Jarvis *et al.* 1994, Lacey and Sherman 1997, Faulkes *et al.* 1997a, Spinks *et al.* 2000). The AFDH maintains that a number of ecological factors associated with increasing aridity have influenced the evolution of sociality present in the Bathyergidae. Lovegrove and Painting (1987) comment that this broad range of sociality represents an evolutionary stable response to the high energetic costs and low probability of locating food whilst excavating burrows by blind tunneling. Thus mole-rat sociality appears to have evolved in response to three main factors: i) the energetic costs of burrowing, ii) limited

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[1] Burda (1990) posed the life-history constraint hypothesis as an alternative to the AFDH. The theory argues that sociality within the Bathyergidae is the result of selective pressures acting to reduce body size, such that sociality has evolved independently of climatic conditions and related resource availability.

burrowing, and iii) the distribution of food resources in arid environments. Moreover, the impact of these factors is further exacerbated in arid areas where rainfall is both low and unpredictable. Therefore those species predisposed to sociality are predicted as being able to exploit arid areas, while solitary species are effectively excluded (Jarvis and Bennett 1991, Jarvis *et al.* 1994, Lacey and Sherman 1997, Bennett and Faulkes 2000). Indeed, using comparative analysis by independent contrast, Faulkes *et al.* (1997a) have shown that ecological variables, specifically rainfall and food distribution parameters, are significantly correlated with group size[2] in the 15 bathyergid species. This relationship is further reflected in the actual distribution of social and solitary species; solitary genera, with the possible exception of *Bathyergus janetta*, inhabit only mesic environments, in contrast to social species that have been able to exploit both arid and mesic areas (Bennett and Faulkes 2000).

The Common Mole-Rat *Cryptomys hottentotus hottentotus* (Lesson 1826)

Social structuring within the common mole-rat (Figure 1.1) is representative of an intermediate position within the social continuum that characterizes the Bathyergidae. The species is widely distributed throughout South Africa (Figure 1.2) where colonies of two to 14 individuals permanently inhabit a network of burrows, locating food as they 'blindly' extend foraging tunnels (Spinks 1998). The species feeds almost exclusively upon the underground storage organs of geophytes, which include rhizomes, corms, tubers and bulbs. From these, they are able to extract sufficient nutrients and water to meet all their physiological requirements, thereby reducing the need to surface where they are at risk of predation. *C. h. hottentotus* is a co-operative breeder whose colony structure is comprised of a linear social hierarchy (Bennett 1989), displaying the rare combination of group living and behavioural monogamy (McNutt 1996). Colonies consist of familial groups of parents and, on average, two litters composed of adolescents and infants (Bennett 1989, 1992). Reproduction within the colony is characterised by reproductive division of labour, comparable to that of the

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[2] Colony size used as an index of sociality (Faulkes *et al.* 1997)

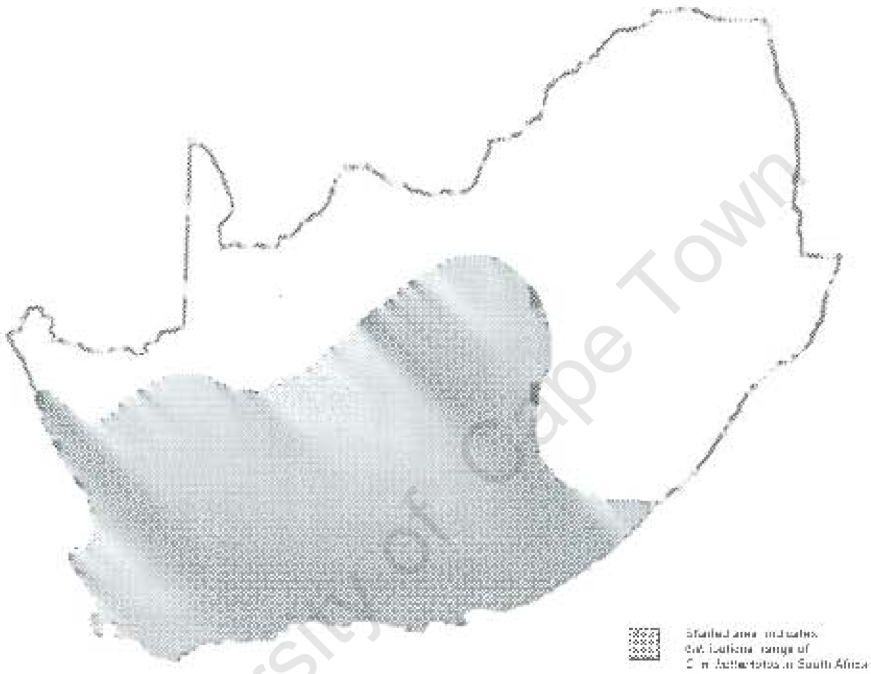
eusocial Damaraland mole-rat *Cryptomys damarensis* (Bennett 1989) and is generally restricted to the largest female and male of the colony. The remaining colony members are reproductively quiescent and show strict incest avoidance behaviour (Bennett 1989). The species is apparently unique amongst social bathyergids in displaying seasonal breeding (Bennett *et al.* 1991, Jarvis and Bennett 1991, Spinks *et al.* 1997), producing a maximum of two litters from November-January (Jarvis and Bennett 1991).

Figure 1.1 The common mole-rat *Cryptomys hottentotus hottentotus*

Photograph © Neville Eden

The common mole-rat appears to be an obligate outbreeder (Bennett 1989, Bennett and Faulkes 2000). Colony members are generally the offspring of the reproductive pair and do not reproduce until conditions, both ecological and social, favour dispersal and independent reproduction with non-kin (Jarvis *et al.* 1994). The combination of seasonal reproduction and litter sizes of only two to four pups result in *C. h. hottentotus* exhibiting the slowest rate of colony recruitment amongst social bathyergids and accounts for the species' comparatively small group size (Spinks *et al.* 2000 ; Spinks 1998).

Figure 1.2 Map of South Africa showing the estimated distributional range of *C. h. hottentotus* (De Graaf 1981 Bennett and Faulkes 2000)



SOCIALLY STRUCTURED SPECIES AND POPULATION GENETICS

Genetic structure occurs when sub-populations are partially isolated from one another (Wright 1965). This subdivision, usually geographical, may promote genetic differentiation between sub-populations as a result of mutation events and random genetic drift (Hartl and Clarke 1997). In turn, sub-populations of a species may evolve different life history traits in response to local environmental factors and stochastic events (Via and Lande 1985). Although the influence of geographical isolation on genetic structuring is well documented, it is less clear how social structure affects genetic sub-structuring within local populations (Dobson 1998, Sugg *et al.* 1996). A recent review of the literature by Storz (1999) suggests that social structuring does have a significant influence on the genetic properties of social species. Furthermore, a number of theoretical and empirical studies indicate that distinct mating and dispersal patterns lead to predictable values for the spatial heterogeneity and temporal dynamics of allele frequencies (Chesser 1991a, Dobson *et al.* 1997, 1998, Pope 1992). This is exemplified by one of the longest studies of a small mammal species, that of a population of black tailed prairie dogs *Cynomys ludovicianus*, at Wind Cave National Park, South Dakota, USA (Hoogland 1995). Dobson *et al.* (1998) report that within black tailed prairie dogs coteries, the relative magnitude of genetic structure brought about by social structuring and its associated behaviours is as great as that found amongst geographically isolated subpopulations. They conclude that social structure is therefore potentially as important to gene dynamics as geographic isolation.

Analysing population genetic structure using molecular markers

Over the past two decades advances in molecular biology have made it possible to reveal population structuring at the genetic level. However many of the commonly used markers e.g. allozymes and Restriction Fragment Length Polymorphisms (RFLP's) have limitations in their application as a result of their low levels of variability and the large amounts of starting material required. The advent of polymerase chain reaction (PCR)-based techniques has facilitated the development of a number of substantially more informative markers. Of these, microsatellite markers specifically are proving to be of great value as they have been shown to accurately determine the probability of identity at both the population (Paetkau *et al.* 1995) and individual level (Edwards *et al.* 1992) due to their highly polymorphic nature (Amos *et al.* 1993, Hughes and Queller 1993, Schlötterer *et al.* 1991). Furthermore, PCR-based markers require minute amounts of DNA from which to amplify informative loci (Saiki *et al.* 1988), making them ideal for the study of both endangered species and, particularly, small mammals by means of non-invasive sampling (Woodruff 1993).

Microsatellite markers

Microsatellite DNA is a relatively recently discovered class of genetic marker (Tautz and Renz 1984, Tautz 1989). Microsatellites originate in nuclear DNA, are inherited in a co-dominant fashion and display high levels of variability (Ashley and Dow 1994). Their high variability has been demonstrated by Hughes and Queller (1993) where 6 microsatellite loci examined in the social wasp *Polistes annularis* revealed a mean observed heterozygosity of 0.62 compared to that of 0.035 for 33 allozyme loci. Viard *et al.* (1996) also report on the extensive variation at microsatellite loci in the freshwater snail *Bulinus truncatus*, after initial allozyme data revealed almost no allozyme polymorphism.

Microsatellite DNA consists of short stretches of di- tri- or tetra-nucleotide repeat motifs e.g $(CAC)_n$ repeated a variable number of times at a given locus. They are highly abundant and interspersed throughout eukaryotic genomes where they rarely exceed approximately 70 repeat units (Schlötterer 1998). In mammals it is estimated that the most common motif, $(CA)_n$, occurs on average every 30kb (Schlötterer 1998). The potential variability at microsatellite loci has been reported by Schlötterer *et al.* (1991) where the presence of 58 alleles were identified at a single locus in a study on whales. This polymorphism is the result of high mutation rates thought to be the result of intra-allelic polymerase slippage during DNA replication (Schlötterer and Tautz 1993) and strand mismatch repair at meiosis (Strand *et al.* 1993). Within mammals mutation rates of 10^{-5} to 10^{-3} base pairs per locus per generation have been reported (Dallas 1992, Ellegren 1995, Weber and Wong 1993). These mutation rates are high enough to generate sufficient polymorphism for individual identification, but low enough to calculate statistically robust kinship coefficients within social groups, making them among the most powerful genetic markers currently available (Bruford *et al.* 1996, Goldstein and Pollock 1997, Jarne and Lagoda 1996, Sunnucks 2000).

GENERAL RESEARCH AIMS

In order to evaluate the validity of the AFDH Spinks (1998) carried out an intra-specific study detailing the distinct colony dynamics and social elaboration of two sub-populations of the common mole-rat along an environmental gradient of increasing aridity. Specifically, the study investigated aspects of local ecology, foraging behaviour, population demography and reproductive biology in order to assess how inter-habitat variation in ecological attributes influenced social structure and dispersal behaviour within the two sub-populations. Carried out over a period of three years, this work has produced a wealth of ecological and behavioural data, in addition to biological samples upon which to base a population genetic study. This wealth of information is of immense value as a detailed understanding of the pattern of population structure, both demographic and genetic, across heterogeneous environments is of fundamental importance to the evolutionary studies of species such as *C. h. hottentotus.* Indeed hypotheses and predictions pertaining to philopatry and dispersal behaviour, in addition to gene flow and genetic variation, are directly dependent upon such studies (Avise *et al.* 1992, Burland *et al.* 1999, Peacock and Ray 2000, Ross 2000). Furthermore, Slatkin (1987, 1993, 1994) emphasizes that the population structure of a species combines both the distinct, but inter-related, fields of population demography and the measurement of genetic parameters. However, many studies are often undertaken on species for which there is insufficient information on features such as dispersal and reproductive biology with which to explain observed genetic measures (Kalisz *et al.* 2001, Ross 2000).

Patterns of social evolution are influenced by changes in local dispersal and gene flow regimes (Wade and Breden 1987). This is because of the potential impact dispersal and gene flow regimes have on the distribution of genetic variance within and between social units (Chesser 1998, Dobson *et al.* 1998, Sugg *et al.* 1996, Wade and Breden 1987). This relationship between social structure and gene dynamics within populations can therefore have significant effects on behaviours such as social co-operation in the rearing of offspring, territory defense, dispersal and philopatry, foraging and the utilisation of limited resources (Dobson 1998, Chesser 1998). As a result, the combined approach of utilizing both demographic and genetic methodologies is of great value to the study of socially structured species.

Mammals range in social dynamics from solitary to highly social (Eisenberg and Kleiman 1983). It is clear, therefore, that studies of population genetic structure over a wide range of ecological conditions are becoming central to the continued understanding of aspects central to the evolution of social systems. Furthermore, Krebs and Davies (1993) note that comparative studies, of a single species distributed along an environmental gradient, will provide great insight into understanding the evolution of specific behavioural differences in response to differences in ecology. Indeed, Dobson (1998) comments that intermediate social systems, where kin structure occurs, specifically need investigating. The broad ecological range over which *C. h. hottentotus* occurs has made it a model species upon which to assess the impact of ecological constraints on social elaboration, co-operative breeding and dispersal behaviour (Spinks 1998). Indeed the influence of dispersal alone on species demography, spatial distribution, social elaboration and population genetic structure is well documented (Peacock and Ray 2000, Girman *et al.* 1997, Olivieri *et al.* 1995, Waser and Strobeck 1998). Accordingly, *C. h. hottentotus* is well suited to testing hypotheses and predictions relating to the population genetic causes and consequences of the flexibility inherent to its highly adaptable social system. The two study sites at Steinkopf and Somerset West, with their marked ecological differences, provide the perfect opportunity to assess the relative influences of ecological constraints on population genetic structuring and gene flow in the *C. h. hottentotus.* Indeed, this is further facilitated by it being one of the best studied of the social Cryptomids, supported by a wealth of ecological and behavioural data (Bennett 1989, Spinks *et al.* 1997, Spinks 1998). This thesis tests a number of hypotheses generated by the demographic data collected in Spinks' (1998) study and is based on microsatellite marker analysis of samples collected by Spinks.

The focal aims of this thesis are then to describe the population genetic outcome of social structure in the common mole-rat, specifically assessing the impact of divergent local ecological constraints on dispersal and gene flow and its consequences for the species' genetic mating system and maintenance of genetic variation.

Within the framework of these aims I have chosen to include relevant background information about the study sites and a summary of Spinks' findings within this chapter. This information is positioned within the general introduction to act as a reference and it is hoped that it will assist in guiding the reader through the objectives and hypotheses that are developed and elaborated upon in the chapters that follow.

The ecological characteristics of the two study sites

Sampling of colonies was carried out at two localities representative of the broad ecological range over which *C. h. hottentotus* occurs (Figure 1.3):

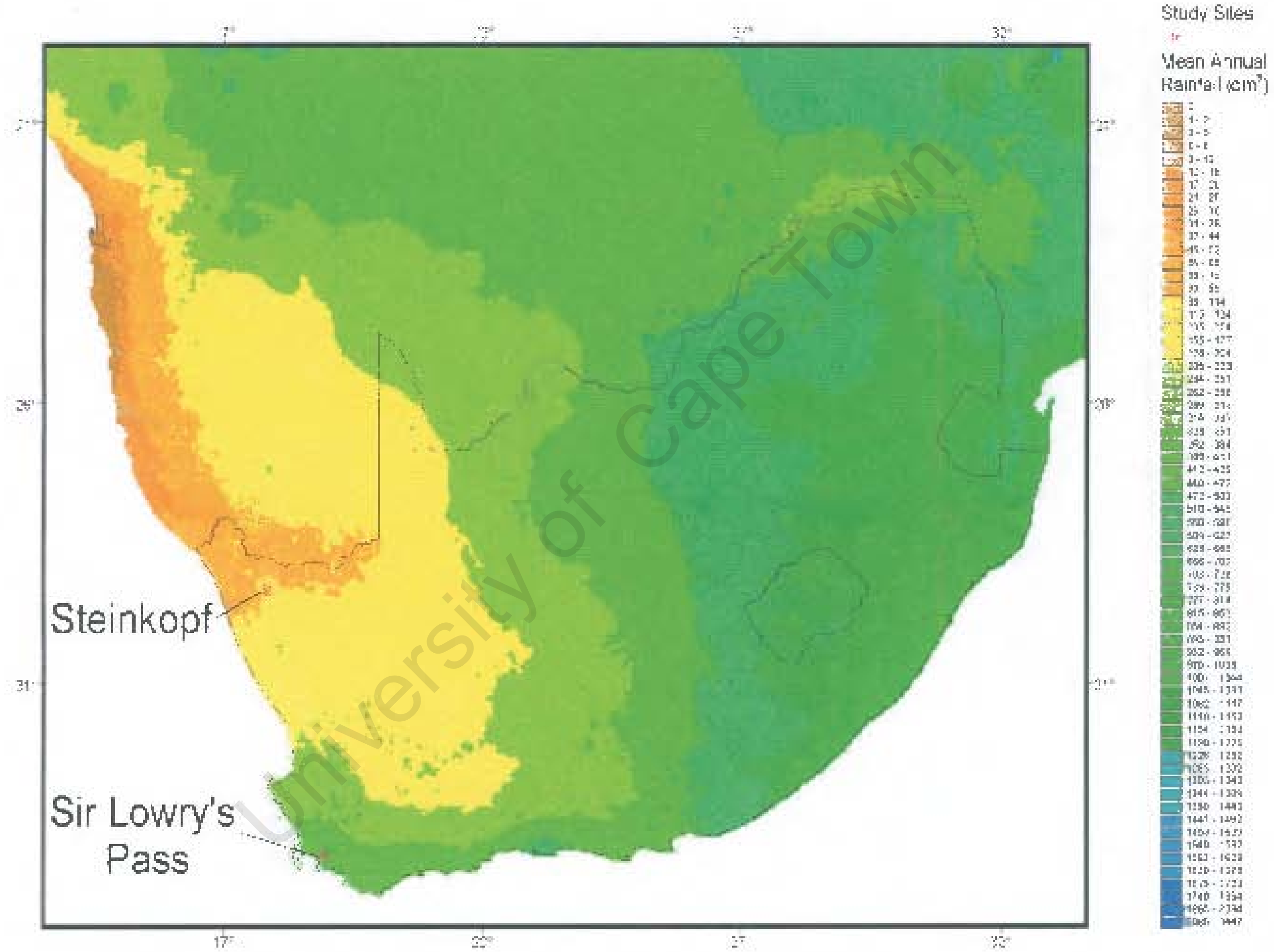
A. Sir Lowry's Pass, Somerset West (34°07' S; 18°55' E) in the Western Cape, South Africa

B. Steinkopf (29°20' S; 17°50' E) in the Northern Cape, South Africa

Lying at the distributional extremes for the species, these two sites characterised by marked ecological differences were specifically chosen on the basis of their inter-habitat divergence. Steinkopf is classified as an arid environment; defined by <150mm rainfall/annum with low predictability and high evaporation rates (McGinnes *et al.* 1968, Noy-Meir 1973). The area experiences low and sporadic levels of precipitation (145 ±9mm/annum) combined with a high co-efficient of variation in mean annual rainfall (63%) and high levels of evaporative water loss (3111mm mean annual evaporative loss). The area is characterised by the Bushmanland Nama Karoo biome (Low and Rebelo 1996), dominated by annuals and non-succulent shrubs, and poorly developed coarse sandy soils (Low and Rebelo 1996, Spinks 1998). This biome occurs in the most arid parts of South

Africa where the low unpredictable rainfall is the main factor determining the dynamics of the ecosystem (Low and Rebelo 1996, Cowling and Hilton 1999).

Figure 1.3 Map of South Africa showing mean annual rainfall (cm^{2}). The two study sites at Steinkopf and Sir Lowry's Pass. Somerset West are indicated. (Map created in ESRI ArcView using GPS spatially referenced data, rainfall data for Africa Hutchinson *et al.* 1995)



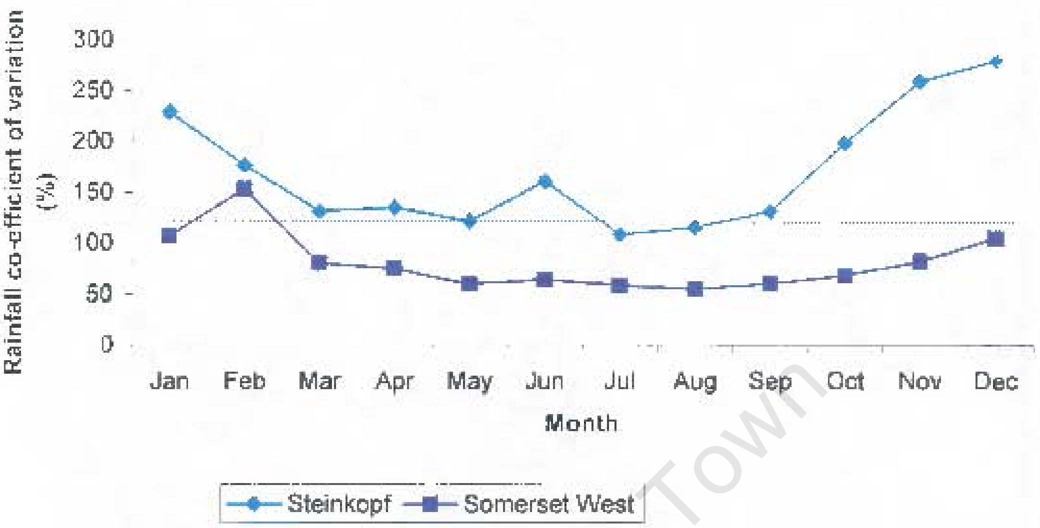
In contrast, the mesic Sir Lowry's Pass study site at Somerset West experiences high and predictable rainfall (652 ±17mm/annum) with a low co-efficient of variation in mean annual rainfall (27%), and a markedly lower level of evaporative water loss (1493 ±142mm). The vegetation associated with the site was, historically, West Coast Renosterveld on heavy clay and loamy soils (Low and Rebelo 1996). However, Low and Rebelo (1996) estimate that approximately 97% of this habitat has been transformed and the area currently exhibits highly disturbed vegetation dominated by invasive alien species.

Figures 1.4 and 1.5 illustrate the difference in both the amount of, and variability in, rainfall at the two study sites. Both mean annual rainfall and mean monthly rainfall at Somerset West (mesic) are significantly higher than at the arid site at Steinkopf. In addition, variability in mean monthly rainfall, expressed as the co-efficient of variation (*V*), was found to be significantly lower in the mesic area of Somerset West.

Figure 1.4 Mean monthly rainfall at Steinkopf (arid) and Somerset West (mesic) study sites. Error bars indicate SE's for each month at each study locality.

Note: dashed line represents the 20mm minimum rainfall cut-off line i.e. the minimum amount of rainfall per month required to stimulate a substantial increase in burrowing behaviour.

Figure 1.5 Monthly co-efficient of variation (*V*) in rainfall at Steinkopf (arid) and Somerset West (mesic) study areas. Note: dashed line represents *V* = 100%.



Rainfall is fundamental to the divergence in social structure that characterizes these two populations of *C. h. hottentotus*. This is because it appears to be the primary determinant of behaviours associated with burrow excavation, foraging and dispersal in mole-rats (Brett 1991, Lovegrove and Painting 1987, Jarvis 1978, Jarvis and Bennett 1991, Jarvis *et al.* 1994, Spinks 1998, Spinks *et al.* 2000). Jarvis *et al.* (1994) note that for soil to become sufficiently damp to work at the depths of the burrow systems of *C. damarensis*, at least 15-25mm of rain must fall within a short period of time (measured as 1mm of rain for every 1cm of soil penetrated to a burrow depth of 15-25cm). Spinks (1998) reports that burrow depths at both study sites were approximately 20cm, and concluded therefore, that at least 20mm of rain must fall to provide the animals with optimal digging conditions. Therefore if rainfall falls below 20mm the energetic costs involved in digging outweigh the potential for locating food, extending the burrow system or successful dispersal. As a result most digging activity then ceases. The low and sporadic levels of rainfall that characterise the Steinkopf site, ≤20mm/month for 8 months of the year, have been shown to have significant effects on levels of months of the year, have been shown to have significant effects on levels of foraging, burrow excavation and dispersal behaviour (Spinks 1998, 2000). This is in stark contrast to the mesic conditions at Somerset West where rainfall/month exceeds 20mm for all 12 months of the year, facilitating both burrow extension and potential dispersal by young adults.

General population parameters at the two study sites

The ecological disparities between the two sites have driven significant divergence in resource characteristics and individual densities at Steinkopf and Somerset West (Table 1.1). The substantial difference in area of the two study sites at Steinkopf and Somerset West, 43ha and 5ha respectively, reflects the considerable difference in *C. h. hottentotus* population densities in response to resource availability at the sites. Although geophyte densities differ significantly between the two areas, the extent to which geophytes are dispersed are comparable in that both sites support a significantly clumped arrangement of geophyte resources. However, the clumps are further apart at Steinkopf. Additionally, because geophytes in the arid regions are of a larger size, both sites support equivocal biomass of geophyte resources and total available energy (Spinks 1998).

Table 1.1 Colony and geophyte densities at Steinkopf and Somerset West (summarised from Spinks 1998)

Measure	*Steinkopf*	*Somerset West*
Study area	43ha	5ha
Mean individual density[1]	1.8 ±0.2	18.7 ±1.2
Mean geophyte density[2]	75.84 ±8.14	1424 ±232.13
Standardised Morisita Index of Dispersion (geophytes)[3]	+0.50	+0.51

[1] Measured as mean number of individuals per ha
[2] Measured as mean number of plants per m^{-2}
[3] Range: -1.0 perfectly uniform to +1.0 perfectly clumped (Krebs 1989)

The relationship that exists between colony and geophyte densities represents a key finding for support of the contention that it is the differential patterns of resource dispersion and density within mesic and arid areas that have aided in the promotion of bathyergid coloniality, and not the total available energy (Jarvis *et al.* 1994, Bennett 1988). Resource dispersion and density therefore have a direct impact on the probability of successful foraging by random burrow extension and on successful dispersal to new sites. Together with ecological constraints on dispersal and foraging, resource availability may therefore also influence the potential for differential gene flow at the two study sites.

A summary of the demographic trends reported for the two study populations is presented in Table 1.2.The two study populations were found to be comparable for a number of population measures including mean colony and litter size. Additionally, colonies at the two sites supported equivalent proportions of juveniles, non-reproductive adults and reproductive adults (Spinks 1998). However, marked differences in the nature of dispersal behaviour characterised the two study populations. Both colony attrition and recruitment rates were significantly lower at Steinkopf relative to Somerset West. Notably, the frequency of dispersal by young adults at Somerset West far exceeded that of Steinkopf, where colony membership and tenure by the resident reproductive pair displayed greater temporal stability. These dissimilarities reflect the divergence in ecological constraints upon dispersal at the two study sites and detailed discussions of their population genetic implications follow in Chapters 3 and 4.

Table 1.2 Summary of the demographic trends for *C. h. hottentotus* at Steinkopf and Somerset West (summarised from Spinks 1998 and Spinks *et al.* 2000)[3]

Demographic measure	***Steinkopf***	***Somerset West***	***Comment***[1]
Mean colony size	5.1 ±0.2	5.1 ±0.2	p=0.8
Mean litter size	2.6 ±0.2	2.3 ±0.2	p=0.2
Colony membership	88 ±3% (n=7)	71 ±2% (n=7)	No. marked animals recaptured in original colony between field trips (p=0.005)
Colony membership by reproductive ♀ and ♂	96 ±3% (n=7)	83 ±3% (n=7)	No. reproductives recaptured within original colony (p=0.01)
Colony membership by non-reproductive ♀ and ♂	83 ±3% (n=7)	64 ±3% (n=7)	No. non-reproductives recaptured within original colony (p=0.005)
Tenure by original reproductive pair	96 ±3% (n=7)	80 ±3% (n=7)	No. reproductive pairs recaptured within original colony for duration of study (p=0.0003)
No. of colonies with same reproductive pair for duration of study	64%	10%	p=0.02 (X^2)
Colony attrition	0.2 ±0.1 (n=7)	0.8 ±0.1 (n=7)	No. of animals lost / original group member / year (p=0.0003)
Colony recruitment	1.0 ±0.3 (n=7)	1.3 ±0.2 (n=7)	No. of animals gained / original group member / year (p=0.3)
% Colony recruitment via immigration	3%	26%	p=0.0003 (X^2 Goodness of Fit)

[1] All significance values calculated using Mann-Whitney U-test, unless indicated.

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[3] All reported results are based on observations carried out over a 3 year time period

ORGANISATION OF THIS THESIS

This thesis consists of three main chapters in addition to the General Introduction (Chapter 1) and General Synthesis and Conclusions (Chapter 5). Chapter 2, General Methodology, is divided into two sections: Part I details the sampling of individuals and selection of colonies for genetic analysis at the two study sites, whilst Part II details the isolation and characterization of *C. h. hottentotus* microsatellite markers. Chapters 3 and 4 then make use of the resolution of microsatellite markers at both the population and individual level. Chapter 3 is based on the assumption that differences in social cohesion and stability will be manifest in the genetic composition of a species. Thus Chapter 3 compares and contrasts the effects of ecological constraints on population genetic differentiation, variation and gene flow within and between colonies of *C. h. hottentotus* at Steinkopf and Somerset West. At the level of the individual, Chapter 4 describes the genetic mating system and levels of kinship within and between colonies of *C. h. hottentotus* that have come about as a result of the behavioural interactions that characterise both dispersing and philopatric individuals.

CHAPTER 2

General Methodology:

PART I: Colony sampling at Steinkopf and Somerset West
PART II: The characterization of microsatellite loci in the common mole-rat, *Cryptomys hottentotus hottentotus*

SUMMARY

Techniques used to describe both the demographic and population genetic consequences of social structure within colonies of the common mole-rat at Steinkopf and Somerset West are detailed in this chapter. A number of colonies at both study sites were sampled over three consecutive years. Individual mole-rats were captured by means of modified Hickman traps and permanently marked by toe-clipping. All captured individuals were weighed and sexed. Species-specific microsatellite loci were isolated using a standard non-enriched protocol and are reported in Burland *et al.* (2001). Toe-clips from sampled individuals were then used to generate individual allelic profiles at seven *Cryptomys*- specific microsatellite loci. Colonies selected for genetic analyses were those that best represented the demographic trends reported at the two sites. All loci were analysed for linkage and deviation from Hardy-Weinberg equilibrium. Furthermore, the cross-species amplification of loci isolated in *C. h. hottentotus* was investigated across all Bathyergid species.

PART I: Colony sampling at Steinkopf and Somerset West

Collection of field samples

C. h. hottentotus individuals were live-trapped at the two study sites, Steinkopf and Somerset West, over a period of three consecutive years (September 1992 - November 1995 at Somerset West, and September 1993 - November 1996 at Steinkopf). Sampling was timed to include trapping both within the breeding season (sampling undertaken during the period September to November) and out of the breeding season (sampling undertaken during the period February to June). More frequent trapping was avoided due to the potentially disruptive techniques employed. These required the excavation of small sections of the burrow systems, and the temporary housing of animals out of their burrow system until complete capture of the colonies were ensured (Spinks 1998). Animals were captured at both sites using modified Hickman live-traps (Hickman 1979a). Each individual was permanently marked by toe-clipping (Melchoir and Iwen 1965, American Society of Mammalogists 1987), sexed and weighed to the nearest 1g (Spinks 1998, Spinks *et al.* 2000). Colony members were then housed together until the entire colony had been captured i.e. until it included the reproductive pair, and if no further animals were found in the traps for three consecutive days after the last individual was caught (Spinks 1998). Following complete capture, the colony was then released at the original point of capture. Both the techniques employed, live-trapping and toe-clipping of animals, have been shown to have no adverse short-term effects on a broad range of rodent species (Korn 1987). In addition, the common mole-rat is a chisel-tooth digger, therefore minimal toe-clipping would not be expected to impede digging ability and hence opportunities for foraging and burrow extension.

Toe-clip samples were stored for genetic analysis in salt-saturated 20% DMSO at room temperature. Additional animals were removed from the field for laboratory studies. After sacrificing these individuals, liver, heart and kidney samples were stored at -70°C. The tissue was then used for the construction of a *C. h. hottentotus* microsatellite library (detailed in Part II).

Selection of colonies for genetic analysis

In order to assess the effect of inter-site differences in climate and resource availability on the genetic structure of *C. h. hottentotus*, colonies that best represented the demographic trends reported for each site were chosen for genetic analysis (Spinks, pers. comm.). Due to the disappearance of colonies between sampling periods, the misplacement of collected toe-clips and non-amplification of samples, analysis was limited to colonies that were as complete as possible. As a result of these difficulties a number of colonies that were included in the original field study, were excluded from the genetic analysis. Table 2.1 includes details as to colony identification, locality and colony size. In addition the total number of reproductive pairs per colony and the mean percentage of individuals retained within the colony between sampling periods is reported. Differences in these values at the two sites are reflective of the disparate colony dynamics reported by Spinks *et al.* (2000) and highlighted in Chapter 1.

Table 2.1 Details of colonies selected for genetic analysis.

Colony ID.	*Locality*	*No. of times colony sampled*	*Total no. individuals sampled*[1]	*Largest size colony attained over sampling period*[2]	*Total no. reproductive pairs / Mean % individuals retained in colony between sampling periods*
ST800	**Steinkopf, Northern Cape**	8	14 (4)	11	1 / 85.7%
ST1100		7	13	7	2 / 83.2%
ST2500		6	11 (1)	5	1 / 57.1%
ST600		7	17	10	1 / 73.8%
ST2000		8	12	9	1 / 85.7%
ST1000		8	15	11	1 / 84.3%
SW2100	**Somerset West, Western Cape**	5	18	8	3 / 43.9%
SW4400		8	12 (1)	5	2 / 75.7%
SW1800		6	17	8	2 / 46.3%
SW14000		3	7	7	2 / 54.7%
SW3000		8	22 (2)	11	3 / 67.3%
SW3400		3	8	6	1 / 58.3%
SW1200		8	25 (6)	14	5 / 57.6%

[1] Number of samples missing from genetic data set in parentheses.
[2] Expressed as largest number of individuals sampled at any one time.

A total of 6 colonies from Steinkopf and 7 colonies from Somerset West were selected. These represent 174 individuals collected at the two sites over their respective sampling periods. Figures 2.1 and 2.2 are schematic representations of the spatial arrangement of colonies at the Steinkopf and Somerset West study sites. These figures have not been drawn to a comparative scale. Solid-line circles indicate colonies included in the genetic study. The size of the solid-line circles is representative of the largest size the colony attained during the sampling period. Colonies that were included as part of the original population study by Spinks (1998), but not as part of the genetic study, are indicated by dashed-line circles.

Figure 2.1 Schematic representation of colonies at the Steinkopf study site

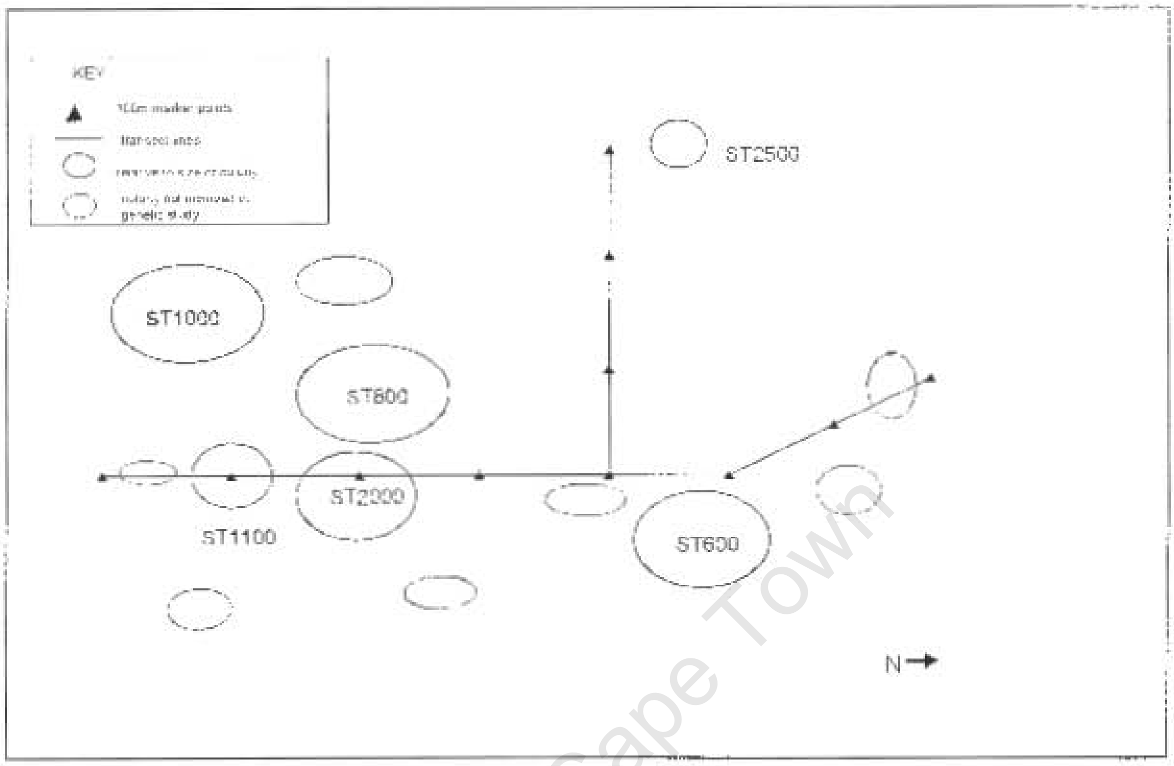
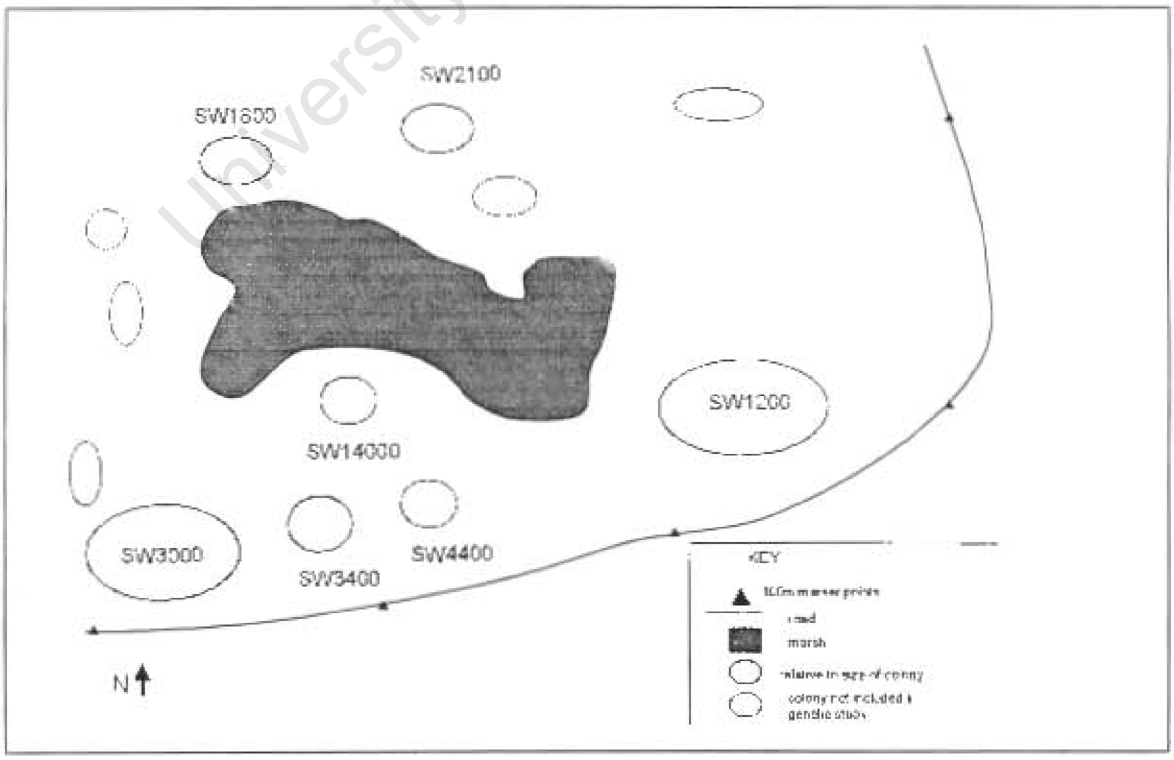


Figure 2.2 Schematic representation of colonies at the Somerset West study site



PART II: The characterization of microsatellite loci in the common mole-rat, *Cryptomys hottentotus hottentotus*

INTRODUCTION

While microsatellites are regarded as the marker of choice for population genetic studies (Sunnucks 2000, Bruford *et al.* 1996, Jarne and Lagoda 1996, Schlötterer and Pemberton 1994, Bruford and Wayne 1993), primers for the amplification of microsatellite loci are available for a limited number of species. This is, in part, due to the difficulties associated with developing species-specific microsatellite libraries (McDonald and Potts 1997, Primmer *et al.* 1996) in terms of financial, technical and time constraints. Surveys of primer conservation in a number of animal groups have demonstrated that loci developed in one species may be informative in both closely related and widely divergent taxa (Stallings 1995, Deka *et al.* 1994, Friedholm and Winterø 1995, Stallings *et al.* 1991, Moore *et al.* 1991). Indeed, Fitzsimmons *et al.* (1995) report the use of heterospecific primers developed in one family of marine turtles (Cheloniidae), and their successful amplification of loci in terrestrial tortoises, representing a divergence time of approximately 300 Myr. However, this is not always the case. The accumulation of mutations in both the microsatellite motif and its flanking regions may result in the loss of the microsatellite itself, or in non-specific binding of the primer sequence to template DNA during PCR, producing a non-informative fingerprint-like pattern (Moore *et al.* 1991, Primmer *et al.* 1996). The rate at which mutations accumulate within these non-coding regions of the genome varies both within and between species (Brooker *et al.* 1994, Rubinsztein *et al.* 1995a, Primmer *et al.* 1996). Thus the exceptional results reported by Fitzsimmons *et al.* (1995) may relate to the unusually slow rate of molecular evolution reported in turtles (Avise *et al.* 1992, Fitzsimmons *et al.* 1995). Although moderate levels of microsatellite conservation have been reported in mammalian groups, amplification of informative loci appears to be most successful in closely related species (Moore *et al.* 1991, Primmer *et al.* 1996, Schlötterer *et al.* 1991, Stallings *et al.* 1991). More distant taxa may have diverged sufficiently to lose the priming sites flanking the microsatellite, in turn, preventing the amplification of informative loci. As microsatellite primer sequences were unavailable for all species of Bathyergidae a partial genomic library was constructed in order to isolate microsatellite loci in the common mole-rat, *C. h. hottentotus*. Primers found to be polymorphic in the source species were then tested for cross-amplification in all species within the family Bathyergidae to determine their future use in population genetic studies. During this period additional primers were isolated in the Damaraland mole-rat, *C. damarensis*, by Dr. T.M. Burland (Queen Mary and Westfield College, London) and included in this study.

The isolation of microsatellite loci was carried out following a standard protocol outlined in Rassmann *et al.* (1991) and Schlötterer (1998). This method makes use of standard molecular biology principles in which fragments of genomic DNA are cloned into a plasmid vector, electro-transformed into a suitable *E. coli* strain and screened with radioactively end-labelled simple-sequence probes for the presence of microsatellite motifs. Positive colonies are then isolated and sequenced. The plasmid cloning vector pUC18 was chosen for its resistance to the antibiotic ampicillin and the presence of the *β*-galactosidase gene (Sambrook *et al.* 1989). Resistance to the antibiotic allows for the selection of plasmid containing bacterial colonies when plated on Luria broth-ampicillin agar media. Presence of the *β*-galactosidase gene allows for blue/white colony selection of transformed *E. coli* cells, where colonies containing recombinant vectors can be detected in the presence of IPTG, an enzyme inducer, and its substrate X-Gal (Sambrook *et al.* 1989).

Primers that were found to amplify polymorphic loci were then used to generate genotypic data for analysis of selected colonies from the two study sites.

METHODS[1]

DNA EXTRACTION

DNA extraction of tissue for microsatellite isolation

Genomic DNA was extracted from previously frozen kidney and heart muscle tissue collected during the field study (detailed in Part A) using a standard phenol:chloroform extraction protocol (Sambrook *et al.* 1989). Approximately 500mg of kidney and heart muscle tissue was finely sliced and placed in digestion buffer (100mM NaCl, 10mM Tris-HCl (pH 8.0), 25mM EDTA (pH 8.0), 0.5% SDS and 10mg/ml proteinase K) overnight at 56°C. Standard phenol:chloroform:isoamyl alcohol (25:24:1) precipitations were performed and the resulting DNA pellets were washed in 70% ethanol, air dried and re-suspended in 100μl Tris-EDTA (pH 8.0)

pH 8.

DNA extraction of toe clips for PCR amplification of individual genotypes

DNA was extracted from toe clips stored in salt-saturated DMSO. As samples comprised small sections of the animals' toes, and in many cases were limited to a single toe per individual, a High Pure PCR Template Preparation Kit (Roche Diagnostics, SA) was used to ensure as high a yield as possible. Samples were cleaned of residual DMSO with sterilised, distilled H_2O and DNA extracted as per manufacturer's instructions and stored at -20°C. For PCR amplification, DNA was diluted in sterilised distilled H_2O (pH8.0) to an approximate concentration of 25-50ng/μl and stored at 4°C to prevent shearing of the DNA by repeated freeze/thaw cycles.

ISOLATION AND SCREENING OF MICROSATELLITE LOCI

Microsatellite loci for *C. h. hottentotus* were isolated from a partial genomic library following a standard isolation protocol (Rassmann *et al.* 1991, Schlötterer 1998), with a number of minor modifications.

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[1] All concentrations reported are final concentrations

Size selection of digested DNA

Approximately 10μg of genomic DNA was digested to completion at 37°C in the presence of three four base-pair recognition restriction enzymes: *Alu*I (Promega, SA), *Hae*III (AEC Amersham, SA) and *Rsa*I (AEC Amersham, SA), all at a final concentration of 5U/μg DNA. The DNA digest was electrophoresed on a 1.5% (w/v) agarose gel, containing a final concentration of 0.5μg/ml ethidium bromide in 1x TBE, and sized using 600ng of *Dra*I digested λDNA size marker. DNA fragments in the 200-600bp range were excised from the gel using a scalpel and the gel slice purified using Genelute™ agarose spin columns (Sigma-Aldrich Co., USA).

Ligation of DNA into pUC18 plasmid vector

The plasmid cloning vector pUC18 (Roche Biochemicals, SA) was linearised with *Sma*I (Roche Biochemicals, SA) at a final concentration of 20U/μg DNA at 37°C for 2 hours. To increase the number of successful transformants linearised pUC18 was dephosphorylated using calf intestinal alkaline phosphatase (Roche Biochemicals, SA) added to a final concentration of 1U/2 pmoles 5'-terminal phosphate residues, recommended for blunt-ended reactions (Sambrook *et al.* 1989). The size-selected DNA fragments were ligated into pUC18 and incubated at 20°C for 15 to 18 hours. Ligations were performed in a final volume of 10μl containing 1.5U T4 DNA ligase (Promega, SA), 1x ligation buffer (final concentration: 30mM Tris-HCl (pH7.8), 10mM $MgCl_2$, 10mM DTT, 1mM ATP), 25ng *Sma*I-digested dephosphorylated pUC18 and 100ng size-selected DNA. The 1:5 plasmid to insert ratio was empirically determined to ensure optimal ligation reactions (Sambrook *et al.* 1989). DNA blank controls were included for comparison.

Transformation of plasmid vector into E. coli

Electro-competent *E. coli* JM109 cells were prepared following a standard protocol (Sambrook *et al.* 1989) and stored in 10% glycerol at -70°C. An additional washing step was included in the protocol to remove any remaining NaCl residue in the culture as an excess of NaCl interferes with the electro-transformation of cells causing 'arcing' and cell death.

Cell transformation efficiencies using native pUC18 were calculated as 1×10^7 CFU (colony forming units) per µg of DNA, which is within the 1×10^7 to 1×10^9 recommended range for electrocompetent cells (Sambrook *et al.* 1989). Transformation of the cells was then carried out using 1µl of the ligation reaction added to 40µl electrocompetent *E. coli* JM109 glycerol cell stock. The sample was then placed in a chilled 0.2cm gap electroporation cuvette (Biorad, USA) and transformed using a Biorad Gene Pulser® and Pulse Controller Unit. The settings used were as follows: 2.5kV, 200Ω resistance and 25µF. RCF Time constant values ranging from 4.5 to 4.9 were observed (within manufacturer's recommended range). Immediately after electroporation, 1ml of chilled SOC[2] buffer was added to the cuvette. The transformed cells were then transferred to a sterile 10ml recovery tube and incubated at 37°C for 1 hour with shaking. Following incubation, 50µl of the cell suspension was plated onto Luria broth agar selection media in small petri dishes, containing 50µg/ml ampicillin antibiotic, 4µl IPTG (10mg/ml) and 40µl X-GAL (10mg/ml). The plates were then incubated at 37°C overnight to allow the transformed colonies to develop. Recombinant plasmids containing insert DNA were then identified using insertional inactivation of the β-galactosidase gene, where blue colonies indicate the absence of insert DNA and white colonies indicate the presence of insert DNA. Individual colonies positive for insert DNA were then picked and grown up in 96-well microtitre dishes containing 120µl Luria broth media and ampicillin (50µg/ml) per well, and incubated at 37°C for 2 hours.

Colony lifts

In order to screen the colonies for microsatellite repeat motifs the colonies were then transferred from the microtitre dishes onto Hybond-XL nylon membrane (AEC Amersham, SA) placed on luria broth agar medium (containing 50µg/ml ampicillin) in 245mm x 245mm Nunclon™ BioAssay dishes (Nunc, Denmark), following the procedure of Buluwela *et al.* (1989). The orientations of the membranes were marked for later reference and

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[2] Standard buffers in Appendix I

alignment, and the plates returned to 37°C to incubate overnight. Following incubation, the membranes were lifted and the cells lysed by placing the membranes onto Whatmanns No.1 filter paper pre-soaked in 10% SDS for 3 minutes. The membranes were then transferred to filter paper pre-soaked in denaturation buffer (0.5M NaOH, 1.5M NaCl) for 5 mins and then a final 5 mins on filter paper pre-soaked in neutralising solution (1.5M NaCl, 0.5M Tris-HCl (pH 7.5)). In order to remove proteinaceous debris, the membranes were briefly washed by agitation in 2 x SSC. The DNA was then fixed to the nylon membranes by microwaving at 800W for 1-2 mins until the membranes were completely dry (as per manufacturer's instructions).

Colony screening

The bacterial colonies were screened for the presence of microsatellite repeat motifs using γ^{32}P-dATP radioactively end-labelled oligonucleotide probes (Sambrook *et al.* 1989). The two probes, $d[GA]_{20}$ and $d[CA]_{20}$, used to screen the library were end-labelled using 20U T4 Polynucleotide kinase (AEC Amersham, SA) per 250 pmoles probe in a final volume of 100µl. The membranes were separated by mesh and pre-hybridised for 1 hour in 200ml Church and Gilbert Buffer (0.5M Phosphate buffer, 7% SDS, 10mM EDTA (pH8.0), 1% BSA) at 65°C in a rotisserie Hybridisation Oven (AEC Amersham RPN 2510/2511, SA). After pre-hybridising for 1 hour, 100µl of both probes were then added simultaneously to the hybridisation buffer and the membranes returned to the hybridisation oven and incubated at 65°C for a further 12-16 hours. Once hybridisation was complete, the membranes were washed, removing excess hybridisation buffer and any non-specifically bound probe, in 200ml low stringency wash buffer (2 x SSC, 0.1% SDS) for 15 minutes at the hybridisation temperature. This was repeated until the wash buffer no longer produced a radioactive signal, as measured by a Geiger-Mueller counter. Whilst still wet, the membranes were then wrapped in cling-film, placed in autoradiograph cassettes and exposed to Cronex® Medical X-Ray film (Du Pont, USA) at -70°C. Length of exposure was dependent upon the strength of the radioactive signal as measured by a Geiger-Mueller counter, and ranged from 1-4 days. On development of the autoradiographic film and alignment with the original microtitre plate, colonies producing a positive signal to the $d[GA]_{20}$ and $d[CA]_{20}$ probes were identified and grown up in 5ml Luria Broth - Ampicillin cultures (as before) overnight with shaking at 37°C.

Purification of plasmid DNA from positive clones

DNA was purified from the bacterial cultures using a QIAPrep Spin Miniprep Kit (Qiagen, Germany) as per the manufacturer's instructions. The resulting DNA yield was then determined using a Shimadzu UV-1601 spectrophotometer. To check for the presence of an insert in the purified plasmid samples, prior to sequencing, a 10µl aliquot of each mini-preparation was restricted with 10U *Hind*III (Roche Biochemicals, SA) restriction enzyme at 37°C overnight. 5µl of the digested sample was then electrophoresed on a 1.5% agarose gel and run against 500ng of dephosphorylated *Sma*I linearised pUC18. Samples that displayed a linear fragment larger than that of the *Sma*I linearised pUC18 were then sequenced for the presence of a microsatellite repeat motif.

Sequencing of plasmid DNA

Using the chain termination protocol of Sanger et al. (1977), positive clones were cycle sequenced using a GeneAmp PCR System 9700 (Perkin Elmer Applied Biosystems, USA) with the Cy5™ Thermo Sequenase Dye Terminator Kit (APBiotech, USA) on an ALFexpress DNA Automated Sequencer (AEC Amersham, SA). The sequencing reaction was electrophoresed on a 5% Long Ranger Gel for 13 hours at 55°C according to manufacturer's specifications. The captured sequence data was processed and edited by ALFwin 2.1 software (AEC Amersham, SA). All positive clones were initially sequenced using the pUC18 forward -40 primer. Reactions that failed to resolve sequence toward the end of the clone were then reverse sequenced using the -40 reverse primer. Colonies that produced positive sequence results for the presence of microsatellite repeat motifs were then stored as permanent stab cultures as per Ausubel *et al.* (1989).

PRIMER DESIGN

Polymerase chain reaction (PCR) primer pairs were designed using DNAMan 4.13 software (Lynnon Biosoft Inc.). Primer pairs were then selected from those suggested by the software using standard selection criteria (Innis *et al.* 1999). Where the software failed to yield suitable primer pairs, the sequence was inspected manually and potential primer pairs then checked for complementarity, T_m (melting temperature) and %GC through DNAMan 4.13. Selected primers were then synthesized on a Beckman Oligo 1000 DNA Synthesizer (Dept. of Molecular and Cell Biology, University of Cape Town).

PRIMER TESTING, OPTIMISATION AND GENOTYPING

Primer testing

Primer pairs were tested for amplification of polymorphic product using 8-16 *C. h. hottentotus* individuals that best represented the geographical sampling range of colonies, and in so doing, increasing the probability of identifying the full allelic range of the species.

Primers were tested over a range of conditions, manipulating the annealing temperature, primer and $MgCl_2$ concentrations until a repeatable microsatellite motif for the test samples was produced. Prior to PCR amplification, 0.5 to 1.0pmoles of the forward primer was end-labelled with $\gamma^{32}P$ dATP (3000µCi mmol^{-1} stock diluted to 20µCi/µl, Amersham, SA) using 5U T4 Polynucleotide Kinase (AEC Amersham, SA) as per Sambrook *et al.* (1989). PCR amplification was then carried out in 10µl reaction volumes using 0.5U BIOTAQ™ *Taq* polymerase (Bioline UK Ltd.), 1 x *Taq* Buffer (final concentration: 16mM $[NH_4]_2SO_4$; 67mM Tris-HCl (pH 8.8); 0.01% Tween-20), 0.2mM dNTP, 1 to 4mM $MgCl_2$, 0.5 to 1.0pmoles unlabelled reverse primer and 50 to100ng DNA. PCR was performed under mineral oil (Sigma-Aldrich, USA) on an MJ Research, Inc. PTC-100™ Thermal Cycler using 0.2ml thin-walled PCR tubes. Primer optimisation cycling conditions were as follows: initial denaturation at 94°C for 3 minutes, 30-35 cycles at the locus-specific test annealing temperature and 60s at 72°C, followed by a final extension step of 10 minutes at 72°C. On completion, 4µl Stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to each reaction. The reactions were then electrophoresed on standard 6% polyacrylamide denaturing (8M urea) sequencing gels (Sambrook *et al.* 1989) in 1x TBE buffer at 1500V for approximately 2-5 hours, depending on the size of the PCR product. M13 control ladder was included on the gel to act as an absolute size marker. The M13 ladder was sequenced with -40F primer end-labelled with $\gamma^{32}P$ dATP (as above) using the T7 Sequenase Kit (AEC Amersham, SA). Prior to loading, the DNA was denatured by heating to 94°C for 5 minutes and then snap-cooled on ice. On completion, the gel was lifted onto Whatmanns No.1 filter paper, dried under vacuum and exposed to Cronex® Medical X-Ray film (Du Pont) at room temperature for 3-48 hours, depending on the strength of the radioactive signal as measured by a Geiger-Mueller counter. Loci producing multiple-banding stutter patterns were then re-tested using a touchdown PCR protocol of decreasing annealing temperatures in order to prevent spurious binding of the *Taq* polymerase (Don *et al.* 1991) and thereby reduce the number of stutter bands visualized on the gel.

Additional primers tested

Primers isolated in the closely related Damaraland mole-rat, *C. damarensis* (courtesy of Dr. T. M. Burland, Queen Mary and Westfield College, University of London) were also tested in *C. h. hottentotus.* In addition to these, a universal primer reported for cross-mammalian order amplification of a polymorphic microsatellite locus within the 3' untranslated end of the mammalian neural cell adhesion molecule, NCAM (Moore *et al.* 1998), was also tested in *C. h. hottentotus.*

Genotyping of C. h. hottentotus samples

Individuals were genotyped at each microsatellite locus using optimal annealing temperatures and $MgCl_2$ concentrations determined during testing (detailed in Tables 2.3 and 2.4). Reaction volumes and reagents were as previously described. Individual alleles were sized at each microsatellite locus using the M13 size marker. Previously analysed samples of known allele sizes and a DNA blank were included in each PCR to ensure reproducibility and check for contamination respectively. PCR products were separated on polyacrylamide gels and visualised by auto-radiography.

Cross-species amplification of C. h. hottentotus microsatellite loci within the family Bathyergidae

Cross-species amplification of the *C. h. hottentotus* microsatellite loci was investigated in 10 bathyergid species, representative of the five genera found in the family (detailed in Table 2.6). DNA was extracted from frozen tissue samples using a salt/chloroform method (Müllenbach *et al.* 1991) and resuspended, after air-drying, in 100µl Tris-EDTA (pH 8.0). PCR test reactions were carried out in a final volume of 10µl as previously described. Cycling conditions were as previously described, however to allow for mutations in the forward and reverse priming regions, the optimal annealing temperatures at each locus was lowered, reducing the stringency of the PCR.

STATISTICAL ANALYSIS

Expected heterozygosities and departures from Hardy-Weinberg and linkage equilibrium at all loci were calculated using GenePop v3.3 (Raymond and Rousset 1995). Estimation of exact probabilities was carried out using a Markov chain method with the following default parameters: dememorisation 1000, batches 100, iterations per batch 1000. Significance values for deviations from Hardy-Weinberg expectations were obtained following Fisher's method of combining exact test probabilities. Linkage disequilibrium i.e. the non-random association of allele frequencies between loci, was considered significant when $\alpha<0.05$ after a Bonferroni correction for multiple applications of the same test (Rice 1988, Weir 1996). Departures from Hardy-Weinberg Equilibrium are calculated by X^2 Goodness of Fit from allele frequency data by expansion of the binomial distribution $p^2+2pq+q^2=1$. Linkage equilibrium is tested because alleles at different loci may have frequencies that show statistical association that is independent of whether the loci are physically mapped to the same chromosome (Rice 1988). This is because non-random associations among alleles at unlinked loci may come about as an artefact of population sub-structuring, founder effects and selection favouring particular multilocus allelic combinations (Avise 1994, Hartl and Clark 1997, Ohta 1982).

RESULTS

Isolation of microsatellite clones

A breakdown of the positive clones is provided in Table 2.2. Of the total number of colonies screened (N=2208) with the d[CA]$_{20}$ and d[GA]$_{20}$ probes, 27 (1.2%) were found to be positive for the presence of a microsatellite repeat motif. However, after sequencing, it was found that only 12 of the 27 clones were suitable for primer design and testing. Of the remaining sequences, 5 were found to be large d[CA]$_n$ or d[GA]$_n$ repeats that lacked sufficient flanking region for primer design as a result of asymmetric cloning. In addition, a further two clones that supported sufficient sequence length for primer design lacked the suitable base composition, supporting sections of poly [A]'s and poly [T]'s (Innis *et al.* 1999). A further five clones were found to be false positive, most probably the result of insufficiently stringent washing conditions. As a result of this, the non-specifically bound probe was not removed from the nylon membrane, producing a false-positive result. One clone was found to have unreadable sequence, i.e. the sequencer was unable to discern the individual bases. This was probably as a result of it being a double clone, where two different colonies were picked as one and grown together in the microtitre well.

Table 2.2 Breakdown of microsatellite clone isolation

Number of clones screened	***2208***
Positive clones identified	**27 (1.2%)**
Insufficient flanking region	5
Unable to design primers to sequence	2
Unreadable sequence	1
Identical insert	2
False positives	5
Sequences for which primers designed	**12**

Primer testing and optimisation

In addition to the 12 *C. h. hottentotus* loci, primers isolated in the closely related Damaraland mole-rat *C. damarensis* (T.M. Burland, Queen Mary and Westfield College, London) and a universal mammalian primer, NCAM (Moore *et al*. 1998), were also tested for amplification of polymorphic (allele number > 2) product in *C. h. hottentotus*. These results are detailed in Table 2.3 and show that of the 12 loci isolated in *C. h. hottentotus*, only four amplified polymorphic product. The remainder either failed to amplify visible PCR product, with or without radioactive end-labelling (2/12), or produced a monomorphic (allele number ≤2) pattern (6/12). Three of the five loci isolated in *C. damarensis* produced polymorphic results. Of the remaining two loci, DMR5 produced a polymorphic result but appeared fixed for two alleles, and DMR1 produced multiple 'stutter' bands that were not possible to score consistently. These 'stutter' or 'shadow' bands, due to strand slippage during PCR, are a common feature when amplifying microsatellite loci, and in particular when using cross-species primers (Don *et al.* 1991, Primmer *et al.* 1996). This may result in the incorrect assignment of genotypes, and the locus was therefore excluded from further analysis. The NCAM locus, reported for cross-mammalian order amplification of a polymorphic microsatellite, did produce a polymorphic result in the *C. h. hottentotus*. However, despite a number of attempts at optimising the locus, the alleles were often difficult to resolve from the 'smudgy' PCR product and the locus was excluded from further analysis.

Table 2.3 PCR testing of microsatellite loci isolated in *C. h. hottentotus*, *C. damarensis* (courtesy of T.M. Burland, Queen Mary and Westfield College, London) and the mammalian neural cell adhesion molecule NCAM (Moore *et al.* 1998). Note: Clone ID refers to the microtitre plate number and well position of the *C. hottentotus* clones.

Clone I.D.	***Microsatellite repeat motif***	***PCR Product***	***Result***
AD10	$(CA)_{13}$	Yes	Monomorphic
CG11	$(GA)_{35}$	Yes	Monomorphic
DD4	$(CA)_{17}$	Yes	Polymorphic
EF12	$(GT)_{12}GA(GT)_{6}$	Yes	Polymorphic
HG2	$(CA)_{17}$	Yes	Monomorphic
HG3	$(CA)_{18}$	No	-
PA2	$(GT)_{16}$	Yes	Polymorphic
SD5	$(CA)_{4}(CG)_{2}(CA)_{2}CG(CA)_{10}$	Yes	Polymorphic
VB11	$(GT)_{12}$	Yes	Monomorphic
XA6	$(CA)_{11}$	Yes	Monomorphic
XE2	$(CA)_{16}$	Yes	Monomorphic
WD2	$(GT)_{15}$	No	-
DMR1[1]	$(CT)_{18}$	Yes	Polymorphic but unable to resolve alleles from multiple stutter bands
DMR3	$(CT)_{17}$	Yes	Polymorphic
DMR4	$(CT)_{17}$	Yes	Polymorphic
DMR5[1]	$(CT)_{21}$	Yes	Polytypic but fixed for two alleles in *C. h. hottentotus* populations
DMR7	$(TG)_{16}$	Yes	Polymorphic
NCAM (Moore *et al.* 1998)	$(CA)_{29}$ (reported in Rodents)	Yes	Polymorphic but unable to resolve alleles in 'smudgy' PCR product

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[1] See Burland *et al.* (2001) for sequences

Characterization of microsatellite loci

A total of seven polymorphic loci were selected for PCR analysis of the samples and the characterization of these loci is detailed in Tables 2.4 and 2.5. A total of 174 *C. h. hottentotus* individuals from 12 colonies were genotyped at the seven loci, identifying a total of 62 alleles[3]. At all seven loci, observed heterozygosity (H_{obs}) values were lower than expected (H_{exp}) under Hardy-Weinberg equilibrium. All loci isolated in *C. h. hottentotus* were found to be highly variable, with a mean value of 10.8 alleles per locus. Loci isolated in *C. damarensis* and subsequently used in the analysis of *C. h. hottentotus* samples were found to be less variable, supporting a mean value of 6.3 alleles per locus.

Table 2.4 Characterisation of polymorphic microsatellite loci isolated in *C. h. hottentotus*.

Locus GenBank Accession No.	***Primer sequence*** *(5' - 3')* *Forward primer sequence first*	***Microsatellite repeat motif***	*T_a (°C)* *$MgCl_2$*	***Allele no.***	***Allele size range (bp)***	*H_{obs}/H_{exp}*
CH1 AF380172	AGTGGCTTGCTTGCAGAC GGAAGAGAAGAGGCATTTCA	$(GT)_{12}GA(GT)_{10}$	**60** 1.0mM	13	103-137	0.64 / 0.72
CH2 AF380173	GGCTTTGGCTAGATCTGATG GGATGCTACACATGCCAGAG	$(CA)_{17}$	**60** 1.0mM	13	110-134	0.74 / 0.78
CH3 AF380174	CCATTGTATTGGGAGAACAG GCGGTTGATTTGCCAGGAG	$(CA)_4(CG)_2(CA)_2CG(CA)_{10}$	**59-57**[1] 1.5mM	8	120-136	0.65 / 0.79
CH4 AF380175	CCATAGAACCCAACTCTGGG GAACACCTATGAGAAGCTAG	$(GT)_{16}$	**58-55**[1] 2.0mM	9	82-110	0.63 / 0.79

[1] For the first five cycles of the PCR reaction the higher annealing temperature was used, followed by the lower temperature for the remaining cycles.

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[3] Allele frequency histograms of all study colonies at all loci analysed presented in Appendix II

Table 2.5 Characterization of polymorphic *C. damarensis* microsatellite loci in *C. h. hottentotus*

Locus GenBank Accession No.	**Primer sequence** ***(5' - 3')*** *Forward primer sequence first*	***Microsatellite repeat motif***	***T_a (°C)*** *$MgCl_2$*	***Allele no.***	***Allele size range (bp)***	***H_{obs}/H_{exp}***
DMR3 AF380167	GCCTAGAAGAGCTTATTTTCGC GGCTGACTCCAATGCAGGATG	$(CT)_{17}$	**56** 2.5mM	5	137- 141	0.47 / 0.65
DMR4 AF380168	GGGCGTGGGGAGAACTAC CGTGGGAGCTAACATCGG	$(CT)_{17}$	**58** 1.5mM	6[1]	198- 208	0.28 / 0.61
DMR7 AF380171	CATAGCACTTTAGCTTCACGTC CAATGGGACCCGCCTAC	$(TG)_{16}$	**62** 2.0mM	8[1]	124- 138	0.49 / 0.69

[1] Some *C. h. hottentotus* alleles for loci DMR4 and DMR7 differed by 1 base pair

Note: For all loci annealing temperature (T_a), $MgCl_2$ concentration, number of alleles, allele size range and observed/expected heterozygosities (H_{obs}/H_{exp}) are reported.

Hardy-Weinberg and linkage equilibrium

The test for linkage disequilibrium, calculated for each pair of loci across all colonies, showed disequilibrium for only one of the 21 pairwise comparisons, that of locus CH1 and locus DMR4 ($p<0.05$ after Bonferroni correction for multiple tests). When analysed within colonies, disequilibrium was found for only two out of 273 pair-wise comparisons; colony SW1800 at loci CH1 and DMR4 and colony SW3000 at loci CH2 and DMR3 ($p <0.05$). As consistent linkage of loci across colonies was not found, normal segregation of alleles was assumed (Hartl and Clark 1997).

Estimates of Hardy-Weinberg and linkage equilibrium were calculated for each colony/locus combination and overall for each locus and colony. The results are detailed in Table 2.6. When tested at particular loci, a number of colonies were found to show significant departure from Hardy-Weinberg equilibrium. Further analysis of allelic frequencies indicated these to be as a result of both heterozygote and homozygote excess, depending on the colony of interest. However when analysed across all loci only two colonies maintained a significant departure ($p<0.05$ after Bonferroni correction for multiple tests), those of ST2000 and SW1200, both as a result of an excess of homozygotes. When analysed across all colonies, only two of the seven loci, locus CH3 and locus DMR4, showed a significant departure from Hardy-

Weinberg equilibrium. When analysed across all loci/across all colonies, departure from Hardy-Weinberg was found to be highly significant ($p<0.0001$).

Table 2.6 Hardy-Weinberg equilibrium calculated for each colony/locus combination, combined across loci for each colony and combined across colonies for each locus.

	LOCUS							
COLONY NUMBER	***CH1***	***CH2***	***CH3***	***CH4***	***DMR3***	***DMR4***	***DMR7***	**ACROSS ALL LOCI**[1]
ST800	NS	NS	NS	NS	NS	NS	NS	**NS**
ST1100	NS	NS	NS	*	NS	NS	NS	**NS**
ST2500	NS	NS	NS	*	NS	NS	NS	**NS**
ST600	NS	NS	NS	*	NS	*	NS	*
ST2000	NS	NS	NS	NS	* * * *	* *	NS	* * * *
ST1000	NS	NS	* *	NS	-	NS	NS	**NS**
SW2100	NS	NS	NS	NS	NS	NS	-	**NS**
SW4400	NS	NS	NS	NS	NS	NS	-	**NS**
SW1800	NS	NS	NS	NS	NS	NS	NS	**NS**
SW14000	NS	NS	NS	NS	NS	-	-	**NS**
SW3000	NS	NS	NS	NS	NS	NS	NS	**NS**
SW3400	-	NS	NS	NS	*	-	NS	**NS**
SW1200	NS	* *	NS	NS	NS	-	NS	*
ACROSS ALL COLONIES[2]	**NS**	**NS**	*	**NS**	* * * *	**NS**	**NS**	* * * *

NS = p>0.05, * = p<0.05, * * = p<0.01, * * * = p<0.001, * * * * = p<0.0001
'-' = Analysis not possible due to presence of insufficient alleles.
[1] Sequential Bonferroni applied to correct for multiple application of tests (p<0.05).

Cross-species amplification of C. h. hottentotus microsatellite loci within the family Bathyergidae

The results of cross-species amplification within the family Bathyergidae are detailed in Table 2.7 (see also Burland *et al.* 2001). Amplification was extensive across all species of *Cryptomys*. However, allelic diversity at locus CH4 was poor in all species when compared to other loci tested. Amplification within the solitary genera of *Bathyergus* and *Georychus* was widespread.

Table 2.7 Cross-species amplification of *C. h. hottentotus* microsatellite loci within the Bathyergidae. For each species-locus combination, the number of alleles, size range in base pairs and number of individuals tested (in parentheses) are given[1].

Locus	***CH1***	***CH2***	***CH3***	***CH4***
T_a (°C) in source species[2]	*60*	*60*	*59-57*	*58-55*
Cryptomys damarensis	6 115-131bp (92)	9 121-139bp (92)	7 116-134bp (41)	1 104bp (13)
C. darlingi	3 118-124bp (5)	5 105-125bp (5)	4 131-137bp (5)	1 100bp (5)
C. mechowi	3 116-122bp (7)	6 108-128bp (7)	3 115-121bp (7)	2 100-102bp (7)
C. bocagei	3 102-114bp (4)	3 106-124bp (4)	2 119-123bp (4)	2 100-102bp (4)
C. h. 'pretoriae'	6 121-139bp (5)	6 114-126bp (5)	4 119-127bp (5)	1 104bp (5)
Georychus capensis	2 98-100bp (4)	5 100-108bp (4)	4 119-133bp (4)	2 80-82bp (4)
Bathyergus janetta	6 110-126bp (5)	2 90-92bp (5)	6 97-111bp (5)	3 80-88bp (5)
B. suillis	6 109-125bp (6)	2 91-93bp (6)	3 101-105bp (6)	-
Heliophobus argentocinereus	7 118-130bp (8)	5 120-128bp (8)	1 102bp (8)	2 137-141bp (8)
Heterocephalus glaber	-	1 104bp (6)	-	-

[1] $MgCl_2$ concentrations are as in Table 2.8.3.
[2] Test annealing temperatures were 2-5°C lower than that reported in the source species.
'-' Denotes no PCR product visible on the gel image.
Note: Species names as in Bennett and Faulkes (2000).

Furthermore, of the four loci tested in the naked mole-rat, *Heterocephalus glabur*, only one locus amplified product, and this was found to be monomorphic. It is worth nothing that cross-species amplification of the loci was assessed from a limited number of samples of each species and this could account for the low numbers of alleles observed at some loci.

DISCUSSION

The isolation and characterisation of microsatellite loci

Several methods detailing enriched approaches for the isolation of microsatellite loci from genomic libraries are available in the literature. All these methods act to increase the initial yield of microsatellites and include the use of triplex affinity capture (Nishikawa *et al.* 1995), biotinylated probes (Hammond *et al.* 1998), hybridisation selection (Armour *et al.* 1994) and RAPD-PCR enrichment (Ender *et al.* 1996). Despite their efficiency, these methods are costly if not already optimised within a laboratory. A standard, non-enriched approach, with minor modifications, was therefore used in this study.

Initial screening for microsatellite repeats directly followed the protocol detailed by Sambrook *et al.* (1998). This involved screening small transformed *E. coli* colonies that were lifted onto nylon membrane from large bioassay dishes containing Luria Broth agar. However, after hybridisation, colonies repeatedly failed to produce a signal. It was then decided to grow individual colonies singly within microtitre wells and screen these larger colonies for the presence of microsatellite repeats. As a result of the increased copy-number of insert DNA within the bacterial colonies strong signals to the probe were produced. The larger concentration of DNA most likely facilitates the binding and retention of the probe after removal of non-specific binding by washing. Of the 2208 colonies screened, 1.2% (27) indicated the presence of a microsatellite repeat motif. However, after sequencing it was found that only 12 of these clones contained sequence suitable for the design of PCR primers. Of these 12 loci, only four amplified polymorphic informative product. The remainder either failed to amplify any product (2/12) or the amplified product was found to be monomorphic (allele number ≤2, 6/12) and therefore uninformative.

The relatively high number of negative clones (5/27) was thought to be as a result of two factors: the presence of d(GA)- and d(CA)-rich sequence in the genome of *C. h. hottentotus* facilitating the binding of the probes, and insufficiently stringent washing conditions. On close inspection of these sequences it was found that two of the five clones supported areas of repeat-like motifs. This would account for the probe binding and producing a 'positive' result. False-positives can be prevented by increasing the stringency of the probe removal i.e. increasing the stringency of the wash buffer or adjusting both the temperature and the duration of the membrane washing steps.

Asymmetric clones accounted for five of the 27 positive clones. These occur when the DNA fragment is cloned both close to or directly within the microsatellite sequence, thereby producing insufficient flanking region for the design of PCR primers, and are a difficult problem to overcome. Although initial size-selection of larger fragments of genomic DNA may increase the potential length of flanking sequence, the size of the insert itself is constrained by the efficiency of the ligation step i.e. smaller fragments clone more successfully than do larger ones (Sambrook *et al.* 1989). On inspection of the sequences it was found that of the sequences supporting the presence of a microsatellite repeat motif, only fragments in the 200 to 300bp size range had been successfully ligated into the vector. This could be improved by initial selection of DNA in the 300-600bp size range, selecting for the incorporation of longer fragments, and thereby reducing the number of sequences that fail to support sufficient flanking region for primer design. The use of a combination of four base-pair recognition restriction enzymes should limit restriction of the DNA within a microsatellite repeat. However, the restriction enzyme *Alu*I recognises the sequence 'AG/CT', cutting between the G and the C, and is associated with the isolation of minisatellite repeat sequences (Armour *et al.* 1990, Longmire *et al.* 1998). This could account for the 'GA' repeat asymmetric clone isolated in this library if 'TC' preceded the repeat motif. Likewise, the recognition sequence of the enzyme *Rsa*I, that of 'GT/AC', could account for the 'GT' repeat asymmetric clone isolated. Clearly, despite methodology to reduce their occurrence, there is always a small chance of these fragments being cloned and sequenced.

Genotype frequencies, used to calculate Hardy-Weinberg equilibrium, are determined in part by the pattern of mating observed within a species. Deviations from Hardy-Weinberg equilibrium, caused by associations amongst alleles, can act as indicators of the evolutionary forces acting upon a population. These deviations can then be characterised by further analysis of allele frequency data and the generation of fixation indices (Wright 1965). A combination of both heterozygote excess and heterozygote deficiency within the sampled colonies explained the observed departures from Hardy-Weinberg equilibrium. This is expected when the assumptions of the Hardy-Weinberg model are not met as a result of genetic sub-structuring within the population (Hartl and Clark 1997, Paxton *et al.* 1996, Weir 1996). A combination of factors interact to bring about sub-structuring and, in this case, may include non-random mating, overlapping generations, rapid mutation rates at microsatellite loci and the effects of random genetic drift on small populations (Avise 1994, Hartl and Clark 1997, Weir 1996). The lower than expected heterozygosity values at all loci, calculated under Hardy-Weinberg equilibrium, are most likely the result of an unintentional Wahlund effect. This may come about when data from highly structured sub-populations are pooled for analysis (Hartl and Clarke 1997). *C. h. hottentotus* is a colonial species and, as such, populations of this species are highly sub-structured (Bennett and Faulkes 2000). A detailed discussion of the effects of sub-structuring on population genetic parameters in this species follows in Chapter 3.

Cross-species amplification of loci within the Bathyergidae

Cross-species amplification of *C. h. hottentotus* loci was found to be extensive in species representative of all five Bathyergid genera. All four loci amplified polymorphic product within species of the social genus *Cryptomys*. Although amplification was found to be widespread among the solitary genera *Heliophobus*, *Georychus* and *Bathyergus*, allelic diversity appeared reduced in *Heliophobus* at loci CH3 and CH4. However, low allelic diversity at CH4 was found among all species tested. Of interest was the lack of amplification of polymorphic product in the eusocial naked mole-rat *Heterocephalus glabur*, where three of the four loci failed to amplify any product. Phylogenetically, the naked mole-rat is basal to the other four genera (Faulkes *et al.* 1997) and increasing evolutionary distance from the source species often results in a reduction in the proportion of loci amplified (Moore *et al.* 1991, Lagerkrantz *et al.* 1993, Primmer *et al.* 1996). Mechanisms of microsatellite evolution have been invoked from the many reported trends associated with increased use of cross-species primers (Valdes *et al.* 1993, Estoup *et al.* 1995, Garza *et al.* 1995, Ellegren 1995). These trends include reductions in both levels of allelic polymorphism and allele size with increasing genetic distance from the source species (Fitzsimmons *et al.* 1995, Ellegren *et al.* 1995, Primmer *et al.* 1996, Ellegren *et al.* 1997). Within this study the number of alleles observed at each locus in related species may not reflect the true level of polymorphism as a result of the small sample sizes. In addition, although there does appear to be a trend of reduced allele size in non-source species, this could be accounted for by an ascertainment bias in the selection of loci i.e. a result of the procedure used to isolate the microsatellite motifs. Techniques used select for the longest repeats in the source species and, as a result, are therefore more likely to be longer than their homologues in related lineages (Ellegren *et al.* 1995, Primmer *et al.* 1996, Crawford *et al.* 1998). This may account for the apparent difference in the mean number of alleles per locus observed at loci isolated in *C. h. hottentotus* and *C. damarensis*, with values of 10.8 and 6.3 respectively.

Conclusions pertaining to the evolution of these loci within the Bathyergidae therefore require further detailed sequencing and analysis of the microsatellite motifs, their flanking regions and associated allele frequencies.

CHAPTER 3

Factors influencing population genetic structure and gene flow in *Cryptomys hottentotus hottentotus*

SUMMARY

Microsatellite markers were used to investigate the effects of divergent dispersal regimes and social structure on population genetic structuring in two populations of *C. h. hottentotus*. Colonies within the two study areas of Steinkopf and Somerset West were characterised by significantly different dispersal regimes and colony dynamics in response to ecological constraints associated with increasing aridity. The degree to which populations were socially structured into colonies composed of breeding pairs and their offspring was found to be sufficient to promote significant levels of genetic structuring. As a result, population genetic structuring was found to be comparable at the two sites when measured by F_{st}, but significantly different when measured by R_{st}. This is reflective of the different roles random genetic drift and mutation play in promoting sub-structuring at the two sites. Long-term ecological constraints on dispersal at Steinkopf were reflected in the accumulation of new mutations and the greater degree of structuring measured by R_{st}. In addition, differences in the nature of recruitment within colonies at the two sites were reflected in the degrees to which the two populations were genetically sub-structured. Measures of allelic diversity and heterozygosity were found to be unexpectedly higher within colonies at Steinkopf. Correlation analysis of colony H_{obs} and mean monthly rainfall indicated a significantly negative relationship such that colonies at Somerset West were characterised by an excess of homozygotes, despite the greater opportunities for dispersal and gene flow. These unexpected results appear to be the result of highly localised dispersal patterns increasing the chance of allelic identity by descent among unrelated breeding pairs, resulting in both lower levels of heterozygosity and allelic variation.

INTRODUCTION

Two major factors are thought to contribute to the extent to which populations of plants and animals show patterns of spatial population genetic structure. These are the patterns and distances that characterise dispersal of individuals and their gametes (Ennos 1994, Rousset 2001, Bohonak 1999, Ross 2001) and the extent to which local selection then acts upon the ensuing genetic structure (Hedrick 1986, Slatkin 1985a). At the population level, the predicted effects of varying patterns of dispersal on genetic variation and structure are well understood (Slatkin 1985), as are the theoretical consequences of local selection (Felsenstein 1976, Slatkin 1985a). These factors then govern the development and maintenance of spatial genetic structure within populations as a result of local selection, mutation, random genetic drift and the relationship between dispersal and gene flow (Slatkin 1985a, Barton and Clark 1990).

Factors that influence the degree to which populations show genetic structuring include geographic isolation, isolation by distance and behavioural isolation (Rank 1992, Wright 1943a). Behavioural isolation comprises factors intrinsic to a species, including dispersal patterns, mating systems and social structure (Chesser 1983).

Measures of dispersal, both direct from mark-recapture data and inferred from genotypic data, are of particular importance in population genetics as they respectively act as measures of potential and realised gene flow (Rousset 2001). Dispersal is responsible for continuously adjusting the effects of selection and genetic drift on population structure by changing the effective population size, N_e, and thus the availability of genetic variation upon which selection can act (Slatkin 1985a). Indeed Rousset (2001) notes that the probability as to whether local adaptation will occur or not, depends specifically on parameters associated with dispersal, N_e and population densities. These in turn depend on species-specific life history traits which act together to directly determine N_e. Differences in any of these traits among populations may then lead to differences in levels of genetic variation and the respective roles of drift, mutation and selection in determining population structuring (Matocq *et al.* 2000).

The AFDH asserts that differences between mole-rat populations in arid and mesic areas, resulting from ecological constraints characteristic to each environment, should be reflected in differential social structuring of bathyergid species occurring there (Jarvis *et al.* 1994). Indeed this has been found to be the case in *C. h. hottentotus*. Spinks (1998) and Spinks *et al.* (2000) report that differential social elaboration at the two study sites of Steinkopf and Somerset West exists as a consequence of ecological constraints on dispersal behaviour. Spinks' demographic study revealed significant differences in both the qualitative and quantitative nature of the dispersal of individuals in the two populations. Most notably dispersal occurred less frequently at the arid site, where colonies demonstrated greater temporal stability with more predictable group membership over time (refer to Table 1.2 in Chapter 1 for a summary of the demographic findings for *C. h. hottentotus* at the two study sites).

All social mole-rats exhibit reproductive skew (Bennett and Faulkes 2000). Theory predicts that reproductive skew will increase concomitantly with increased ecological constraints on independent reproduction (Vehrenkamp 1983b, Keller and Reeve 1994). This increase in reproductive skew will then have important consequences for N_e and its relationship with genetic variation (Chesser 1991a, 1991b). Any differences therefore in the degree to which reproductive skew manifests itself in the two study populations will in turn be reflected in population genetic measures dependent upon it. A greater level of reproductive tenure by breeding pairs was observed within colonies at Steinkopf. In addition, reproductive individuals at Somerset West disappeared from colonies more frequently, resulting in faster replacement and turnover of breeders than at Steinkopf (Spinks 1998, Spinks *et al.* 2000). These differences, within colonies at Steinkopf and Somerset West, therefore appear to reflect an adaptive variation in social behaviour between the two regions and suggest that individual survival rate in arid areas benefits to a greater degree from delayed dispersal and co-operative behaviour than that of individuals in mesic areas. Variation in the number of individuals contributing to the gene pool at any one time will therefore have important implications on measures of population genetic structuring at the two study sites.

Classical models of population structure emphasize the respective roles played by population differentiation and rates of inbreeding in the maintenance of genetic variation (Wright 1951, Hartl and Clark 1997). Social structure is often inferred from the resulting data based on a sampling system that largely ignores it (Sugg *et al.* 1996). Structuring that results from a sampling regime that takes into account the social units of a population provides an additional means with which to explain the distribution of genetic variation. In so doing, it provides further information as to the relative influence of said social structure, and potential mechanisms that maintain genetic variation within genetically sub-structured populations.

Fixation indices, developed by Wright (1951, 1965), demonstrate the way in which genetic variation, in terms of heterozygosity, is partitioned into hierarchical population level components that then reflect its structure and allow inference of various population parameters (Jarne and Lagoda 1996). These *F*-statistics, that simply estimate the correlations of alleles within and between individuals, describe the proportion of variation within individuals relative to that expected in the sub-population (F_{is}) and the total population (F_{it}), and the proportion of variation in subpopulations relative to the total variance in the population sampled (F_{st}). However, the observation that populations of many species are further subdivided into behaviourally isolated breeding groups maintained by a system of philopatry, dispersal and territorial exclusion of foreign individuals (Chepko-Sade and Shields 1987, Clutton-Brock 1989a, Greenwood 1980, Pusey 1987) has led to the development of models that take into account this level of population structure.

Chesser (1991a, 1991b) and Sugg and Chesser (1994) have developed a theoretical framework for the interpretation of *F*-statistics in socially structured populations (reviewed by Sugg *et al.* 1996) i.e. Chesser's (1991a, 1991b) breeding group models. Such that F_{st} provides a measure of the genetic variance apportioned among social groups relative to the subpopulation sampled, F_{is} a measure of allelic correlations within individuals relative to the social group, and F_{it} a measure of allelic correlations within individuals relative to the entire collection of social groups comprising the subpopulation. One of the most important consequences of these models is that coancestry, as a measure of allelic correlations and kinship, can develop more rapidly within social groups than inbreeding (Sugg *et al.* 1996). In this context the term coancestry is used to represent genetic similarity that has arisen through the spatial arrangement of social lineages and not as a measure of true inbreeding between close relatives. As a result, individuals within social groups may have an increased chance of sharing alleles by descent, or identity by descent (IBD), whilst still exhibiting behavioural mechanisms that avoid true inbreeding. These models can then account for the influence that coancestry may have on genetic variation, its apportionment within populations and therefore on measures of structure. In so doing, an hierarchical approach of this sort can then identify the spatial scales at which differentiation is occurring and provide insight into the significance of each differentiating mechanism (Perault *et al.* 1997, Routman 1993, Trexler 1988).

Within *C. h. hottentotus* populations, social colonies constitute an identifiable and distinct hierarchical level of population structure as a consequence of demographic constraints restricting dispersal and thereby promoting natal philopatry within the confines of a co-operatively breeding unit. Therefore in this chapter, partitioning of genetic variance within and among social colonies of *C. h. hottentotus* was interpreted in terms of Wright's (1951, 1965) hierarchical *F*-statistics as applied to the socially defined levels of population structure of Chesser (1991a, 1991b).

Direct measures of dispersal are often difficult to generate from mark-recapture techniques, as the data do not always represent the full distribution of individual movements (Peacock and Ray 2001). This is particularly problematic when dispersal events are very rare or highly episodic, thereby making it difficult to generate direct measures of a species' demography (Rowe *et al.* 2000, Wilson *et al.* 2000). Genetic analyses that allow dispersal and gene flow rates to be inferred, rather than directly observed, offer a potentially valuable tool with which to provide further insight into the population dynamics of a species (Peacock and Ray 2001, Wilson *et al.* 2000).

The partitioning of genetic variation, in terms of the frequency and distribution of genotypes measured at microsatellite loci, has particular bearing on the evolution of altruistic behaviours that characterise social species (Hamilton 1964, Bourke 1997). Therefore, the assessment of factors that ultimately promote the evolution of altruism is of fundamental importance to the study of social species. Of these factors, those most pertinent to the establishment of a species' population level genetic structure include: i) the pattern of social dispersion that characterises a species; ii) the actual physical patterns of individual dispersal events and their collective effect on gene flow; and iii) the genetic outcomes of a species' mating system. [Note: Aspects relating to the genetic outcomes of *C. h. hottentotus'* mating system are reported and fully discussed in Chapter 4]

RESEARCH AIMS

The main aims of this chapter are to describe the genetic consequences of differential social elaboration, dispersal and philopatry in two populations of *C. h. hottentotus,* specifically to test the following hypotheses:

1. The degree to which *C. h. hottentotus* is socially structured into colonies composed of familial units will be sufficient enough to impact upon the partitioning of genetic variation into localised lineages, resulting in significant levels of sub-structuring at the colony level.
2. Coancestry will be greater within colonies of *C. h. hottentotus* in arid environments as a result of increased social elaboration in response to greater constraints on individual dispersal and independent reproduction.
3. A predictable relationship will exist between the amount of genetic variation found within colonies and the extent to which ecological constraints are manifested in dispersal patterns within the two study populations. Colonies at Somerset West will be characterised by greater levels of genetic variation in response to increased opportunities for dispersal and hence gene flow.

Within this framework we are provided with an ideal opportunity to then further assess the validity of the AFDH as an explanation for the evolution of sociality within the Bathyergidae and thereby examine a number of its predictions at the genetic level.

METHODS

Molecular Analyses

A total of 176 *C. h. hottentotus* individuals were analysed at seven *Cryptomys*-specific microsatellite loci (detailed in Chapter 3, Burland *et al.* 2001). Allele frequency data used to generate all analyses is included in Appendix II as allele frequency histograms of all study colonies at all loci analysed.

Genetic Data Analyses

Allelic variation at each locus was scored manually by comparison with aligned M13 standard size fragments and positive control individuals on all gels. Although an individuals genotype was represented only once in the data set, a large proportion of the animals sampled were present in the focal colonies for more than one sampling period at the two study sites. In addition, measures of population sub-structure did not show significant differences when analysed over all colonies per sampling period, suggesting that genotypic data could be pooled to yield single, larger data sets per colony that would be representative of their structure and typical patterns of gene dynamics within the study areas at any one sampling point.

Intrapopulation genetic variation

Within-population genetic diversity was quantified as the average number of alleles per locus (A), observed heterozygosity (H_{obs}) and Hardy-Weinberg expected heterozygosities (H_{exp}) (Nei 1973). These were calculated for each colony and averaged over all loci using FSTAT v2.9.3 (Goudet 1995). Deviations from Hardy-Weinberg expectations at each site were investigated using exact tests based on Markov Chain iterations as implemented in GENEPOP v3.3 (Raymond and Rousset 1995b). Critical significance levels for multiple applications of the same test were corrected using a sequential Bonferroni procedure (Rice 1989). The extent to which both loci and colonies deviated from Hardy-Weinberg expectations was quantified using Weir and Cockerham's (1984) inbreeding co-efficient F_{is} in FSTAT v2.9.3 (Goudet 1995). Significant deviations from zero were calculated for colonies at both sites using permutation tests. F_{is} measures changes in heterozygosity within individuals relative to the sub-population, providing a measure of allelic correlations within individuals relative to the social group; in this case the colony (following Chesser's breeding group models where F_{is} represents the variation within individuals relative to the social lineage). As the main effect of inbreeding is to reduce the number of heterozygote individuals relative to that expected with random mating in the same sub-population, F_{is} values indicate whether deviations from Hardy-Weinberg expectations are due to an excess or deficit of heterozygote individuals (Hartl and Clarke 1997). In addition, the measure F_{IT} was estimated at each study site in FSTAT v2.9.3 (Goudet 1995), assessing the degree to which allelic correlations within individuals relative to the total sample indicate differences in coancestry and sub-structuring. Significant deviation from a zero value was calculated by bootstrapping loci 1000 times.

Due to the combination of limitations associated with mark-recapture techniques (Peacock and Ray 2001) and the highly episodic nature of dispersal in *C. h. hottentotus*, it was not possible to generate direct measures of dispersal frequency and distance per sampling period for the study colonies at Steinkopf and Somerset West (Spinks 1998). However, rainfall has been shown to be one of the most important determinants of dispersal behaviour within *C. h. hottentotus* (Spinks 1998, Spinks *et al.* 2000). As a result, rainfall should therefore directly impact upon the potential for gene flow among colonies at the two sites and can be used as an indirect measure of potential dispersal behaviour (Spinks 1998). Therefore in order to investigate a potential association between rainfall, as a result of its influence on dispersal opportunities, and gene flow between colonies of *C. h. hottentotus*, the statistical relationship between rainfall and H_{obs} was assessed using correlation analysis. Both mean monthly rainfall and the co-efficient of variation in mean monthly rainfall were used to determine the possible impact rainfall might have on levels of $H_{obs.}$ Because H_{obs} may not to be normally distributed, all correlations were non-parametrically determined using Spearman's r_s in SPSS v9.0 (SPSS Inc. 1998). Mean individual H_{obs} per sampling period was calculated from the raw allele data and correlated with the associated rainfall measure for that sampling period.

Hierarchical population structure

The null hypothesis of homogeneity in allele frequencies at both sites was tested using an approximation of Fisher's exact test (Raymond and Rousset 1995a) as implemented in GENEPOP v3.3 (Raymond and Rousset 1995b). Estimation of exact probabilities was carried out using a Markov chain method.

The extent of population sub-structuring at each study site was assessed using both the infinite allele model (IAM, Ohta and Kimura 1973) based F_{st}, and the stepwise mutation model (SMM, Kimura and Crow 1964) based analogue of F_{st}, Slatkin's R_{st} (1995). This is because *F*-statistics do not take into account the proposed stepwise mutation mechanism and high mutation rate involved in microsatellite evolution (Slatkin 1995). [Note: F_{is} and F_{it} are not dependent upon models of mutation as they describe deviations from expectations from Hardy-Weinberg equilibrium and therefore do not require analogues when analysing microsatellite data (Jarne and Lagoda 1996)]. Pair-wise colony and overall F_{st} (IAM) values were computed using Weir and Cockerham's (1984) θ in FSTAT v2.9.3 (Goudet 1995). In order to account for differences in sample size and potential sample variances, unbiased pair-wise and overall estimates of Slatkin's R_{st} (SMM) (Slatkin 1995) were calculated using RstCalc (Goodman 1997). Significant deviations from a zero R_{st} value were determined using permutation tests (x 2000). Because the measures of F_{st} and R_{st} behave differently with regard to drift and mutation, their comparison can provide insight into the factors influencing population differentiation (Rousset 1996, Goodman 1998, Castric *et al.* 2001). In order to assess the degree to which these two measures were concordant at each site, Mantel tests were carried out on colony pairwise F_{st} and R_{st} matrices. A randomisation test was used to determine the significance of the correlation between matrices (Mantel 1967).

Genetic assignment tests

A genetic assignment test was carried out on all individuals at both study sites using the programme GeneClass v1.0.02 (Cornuet *et al.* 1999). The programme employs a likelihood-based method using allele frequency data, where individuals are assigned to the 'population' in which the likelihood of their genotype is highest. In the case of *C. h. hottentotus* the assignment of individuals is to the colony. A Bayesian-based approach (Rannala and Mountain 1997) implemented within the programme was selected for calculating the assignment values. Recent evaluation of the performance of various assignment tests by computer simulation has shown that allele frequency-based methods, using a Bayesian approach, generally out-perform other available methods (Cornuet *et al.* 1999, Primmer *et al.* 2001). Assignment of individuals to reference 'populations' or colonies was calculated using the 'leave one out' option. In so doing, the reference allele frequencies are calculated using all individuals in the data set except for the individual that is being assigned. This is to prevent a bias that would be introduced by counting the current individual in its population when estimating reference allele frequencies.

A measure of confidence for each assignment is realised by simulating multilocus genotypes from each reference 'population' and then determining the probability of an individual's assignment value falling within the distribution of said reference 'population' (Cornuet *et al.* 1999). Consequently, this allows not only the assignment of individuals, but also the exclusion of individuals, with a specified degree of confidence.

When there is a sufficient degree of population sub-structuring, so as to generate significantly different allele frequency distributions as measured by F_{st} (Cornuet *et al.* 1999), the assignment test can provide an indication of 'interpopulation' dispersal rates (Waser and Strobeck 1998, Davies *et al.* 1999, Paetkau *et al.* 1995). In addition, the test is particularly sensitive to contemporary dispersal behaviour, measured within the last one to two generations (P. Waser pers. comm.). The degree to which individual mole-rats are correctly assigned to their source 'population', or colony unit, can therefore act as a genetic measure of the stability of social cohorts at the two study sites. Concomitantly, the degree to which individuals are mis-assigned i.e. an individual who's genotype is assigned to a colony other than the colony within which it was sampled, can provide an indication of the extent to which colonies are linked by dispersal and gene flow (Paetkau *et al.* 1995, Paetkau *et al.* 1998, Waser and Strobeck 1998, Mossman and Waser 1999).

Principal component analysis

Principal component analysis (PCA) was performed on allele frequency data at both the inter- and intrapopulation level using PCA-GEN v1.2 (Goudet 1999b). PCA is a multivariate statistical method that uses as few factors or components as possible to explain the majority of the total variation in a data set. It does this by reducing the dimensionality of the data, whilst still maintaining as much of its original structure, and then expressing it in the form of a number of principal components (Kleinbaum *et al.* 1988). If the data set is highly structured i.e. that variation in the data is specifically partitioned, the first two principal components should account for a large majority of the variation in the data (> 50%) (Everitt and Dunn 1991). Component scores are obtained for each sample within the data set and reflect the relationship between each variable and each principle component. Additionally, these scores act as coordinates for the plotting of each sample on the axes of a dispersion matrix. If there is a high degree of genetic sub-structuring within a species, a relationship, in the form of a correlation, should exist between the locus-specific allele frequencies of individuals within colonies. It is this correlation then, that will be identified by PCA and allow for a graphical representation of the relationship when plotted on a dispersion matrix.

PCA-GEN v1.2 (Goudet 1999b) specifically links this form of multivariate analysis to a known measure of population differentiation, that of Wright's F_{st} (1965). It achieves this by the linear combination of alleles expressing most of the variance in allele frequency within the data set; thereby accounting for the degree to which a population is sub-structured. Through a series of simulations, based on a number of population genetic models of gene flow (Island model, One D stepping stone model, Hierarchical island model), the technique has been shown to demonstrate a consistent relationship between F_{st} and the graphical representation of the PCA. F_{st} and the dispersion matrix measure the total inertia present within a data set and the % inertia allocated to each axis respectively. In so doing, the technique reveals a direct association between the apportionment of genetic variation within principal component space and the actual physical apportionment of genetic variation within population units.

Genetic distance measures

Using a number of genetic distance measures the evolutionary relationships of all colonies from both study sites were determined and are presented in the form of radial phenograms. Goldstein *et al.*'s delta μ^2 (1995b), Cavalli-Sforza and Edward's chord distance D_{CE} (1967) and Nei *et al.*'s D_A (1983) were chosen to determine the associations between colonies at the two study sites. Delta $\mu 2$ is a distance measure that was specifically developed for application to microsatellite evolution assuming the SMM (Kimura and Crow 1964), and makes use of the difference in size between alleles, which is expected to carry useful information under the SMM model. In addition it takes into account the high variance inherent to microsatellite data and is very robust to differences in sample size (Goldstein *et al.* 1995b, Goldstein and Pollock 1997), lending itself well to the analysis of social cohorts that are unlikely to be of equal size. Cavalli-Sforza and Edward's (1967) D_{CE} and Nei *et al.*'s (1983) D_A do not assume a model of mutation. Instead they simply make use of the sum of the products of allele frequencies shared between samples and have been shown to perform very well in the construction of trees from microsatellite data when operational taxonomic units (OUT's) are closely related (Goldstein and Pollock 1997, Takezaki and Nei 1996). Indeed Takezaki and Nei (1996) note that for very recently separated populations, distances that make use of the product of allele frequencies shared between populations are the most accurate measures for establishing their current relationships. This is particularly so when both allelic variance and therefore mutation rate is high; both of which characterise microsatellite DNA markers (Takazaki and Nei 1996, Goldstein *et al.* 1995b, Goldstein and Pollock 1997). In addition, Takezaki and Nei (1996) also note that of the three measures, D_{CE} has a higher probability of depicting the correct tree topology because it makes no assumptions regarding equal mutation rates over loci or constant population size.

Genetic distances were calculated from microsatellite allele frequency data using the program POPULATIONS v1.2.21 (Langella 2001), which calculates both distance matrices and representative trees. Radial phenograms were constructed using UPGMA (Sneath and Sokal 1973) as this method of cluster analysis is well suited to the task of generating trees that simply reflect similarities between OTU's. UPGMA relies on the rate of evolution among different lineages being approximately equal; in the case of microsatellite evolution within a species, this is most likely to be the case (Goldstein and Pollock 1997, Goldstein *et al.* 1997, Schlötterer 1998). UPGMA, however, is not considered a good algorithm for construction of phylogenetic trees and it is important, therefore, not to draw phylogenetic inferences from the clustering pattern seen with this method. It does, however, prove extremely useful in identifying similar isolates from distance measures based on recent changes within populations (Swofford *et al.* 1996). Confidence in tree topologies were assessed by bootstrapping loci 1000 times. Trees were edited using TREEVIEW v1.6.6 (Page 1996).

In addition to assessing the degree of concordance among the distance measures used, Mantel tests were also used to assess the degree to which distance-based matrices corresponded to the traditional estimates of population differentiation, R_{st} and F_{st}, which measure variance in allele size and changes in levels of heterozygosity respectively. A randomisation test was used to determine the significance of the correlation between the matrices (Mantel 1967).

RESULTS

Intrapopulation genetic variation

A total of 67 alleles over seven loci were identified from individuals sampled at the two study sites. At both the population and colony level, *C. h. hottentotus* at Steinkopf had greater levels of allelic variation than at Somerset West (Table 3.1). Colonies at Steinkopf were found to have 58 of the total number of alleles, compared to only 41 alleles at Somerset West. The mean number of alleles per locus was found to be significantly greater at Steinkopf (8.3 alleles, Wilcoxon Signed Rank Test; p=0.05) than at Somerset West (5.8 alleles). However, though the mean number of alleles per colony at Steinkopf and Somerset West did differ, with 4.4 and 3.1 alleles per colony respectively, this difference was not found to be significant. Both sites were found to support similar proportions of rare alleles (frequency ≤ 0.05), when expressed as a percentage of the total number of alleles present in the population (approximately 44%). However, the two study sites differed in the total number of private alleles (Slatkin 1985) present in their respective populations. Of the total number of alleles present within colonies at Steinkopf, 44.8% of these were found to be private i.e. they were only found within colonies at the Steinkopf study site. This is in comparison to only 17.1% of alleles at Somerset West that were found to be private to colonies at this site.

Table 3.1 Summary of allelic variation at Steinkopf and Somerset West

	Steinkopf	*Somerset West*
Total no. of alleles	58	41
Mean no. alleles/locus	8.3	5.8*
Mean no. alleles/colony	4.4	3.1
Total no. rare alleles (*f* <0.05)	26/58 = 44.8%	18/41 = 44%
Total no. private alleles	26/58 = 44.8%	7/41 = 17.1%

*p<0.05 Wilcoxon Signed Rank Test

In addition, the degree to which colonies were fixed for the presence of a particular allele was greater at Somerset West, where 10% of sample points (calculated from 7 colonies at 7 loci) indicated the fixation of a particular allele within a colony. This is contrast to Steinkopf, where only 4.7% of sampling points indicated a colony to be fixed for a specific allele. [For details see allele frequency histograms for all colonies in Appendix II]

Observed heterozygosity (H_{obs}) values at the two sites ranged from a minimum of 0.35 for SW1200 to a maximum of 0.77 for ST2500 (Table 3.2).

Table 3.2 Measures of genetic variation within colonies of *C. h hottentotus* at Steinkopf and Somerset West.

Colony	*A*[1]	H_{obs}[2]	H_{exp}
Steinkopf			
ST800	21	0.40	0.55
ST1100	28	0.76	0.63
ST2500	28	0.77	0.65
ST600	31	0.68*	0.61
ST2000	26	0.67***	0.57
ST1000	28	0.63	0.51
Colony mean	**27** **(SD ±3.3)**	**0.66*** **	**0.58**
Somerset West			
SW2100	23	0.48	0.45
SW4400	23	0.53	0.49
SW1800	25	0.65	0.56
SW14000	20	0.51	0.42
SW3000	24	0.34	0.40
SW3400	18	0.44	0.36
SW1200	25	0.35*	0.39
Colony mean	**22.6** **(SD ±2.6)**	**0.48** **	**0.44**

[1] A - total number of alleles per colony

[2] Values found to deviate significantly from Hardy-Weinberg expectation using χ^2 exact tests: *p < 0.05, **p < 0.01, ***p < 0.001 (Goudet 1995)

In addition, mean H_{obs} over all colonies for all loci was found to be higher at Steinkopf, 0.66, than at Somerset West, 0.48. However, at both sites average observed heterozygosity was found to deviate significantly from Hardy-Weinberg expectations (χ^2 exact test; $p<0.01$).

When tested at particular loci, a number of colonies were found to show significant departure from Hardy-Weinberg expectations at the 95% confidence interval using chi-squared analysis (See Table 2.6, Chapter 2). Associated F_{is} values (Table 3.3) indicate these deviations to be due to a significant excess of heterozygote individuals in ST2500 and ST600 at locus CH4, ST1000 at locus CH3 and SW3400 at locus DMR3. A significant excess of homozygote individuals characterised deviations from Hardy-Weinberg expectations in ST600 at locus DMR4 and SW1200 at locus CH2. Departure from Hardy-Weinberg expectations over all loci was found in only two colonies, both from Steinkopf; ST600 supporting a significant excess of heterozygotes and ST2000 supporting an excess of homozygotes. When analysed across all loci/across all colonies, departure from Hardy-Weinberg at both study sites was found to be highly significant ($p<0.0001$). F_{is} values indicated overall departure from Hardy-Weinberg expectations at Steinkopf to be as a result of an excess of heterozygote individuals (F_{is} -0.12, $p=0.05$) and as a result of an excess of homozygote individuals at Somerset West (F_{is} +0.04, $p=0.05$). It is however worth noting that heterozygosity within groups in excess of Hardy-Weinberg expectations characterised colonies at both Somerset West and Steinkopf. This is reflected in the fact that both sites displayed overall H_{obs} in excess of Hardy-Weinberg expectations. Observed deviations from expectation may be attributed to a number of causes, particularly when assumptions of the Hardy-Weinberg model are not met (Hartl and Clark 1997, Paxton *et al.* 1996, Weir 1996) and may include non-random mating, overlapping generations, rapid mutation rates at microsatellite loci and the effects of random genetic drift on small populations.

Table 3.3 F_{is} values (Weir and Cockerham 1984) for each locus per colony, and totalled over all loci per colony at Steinkopf and Somerset West.

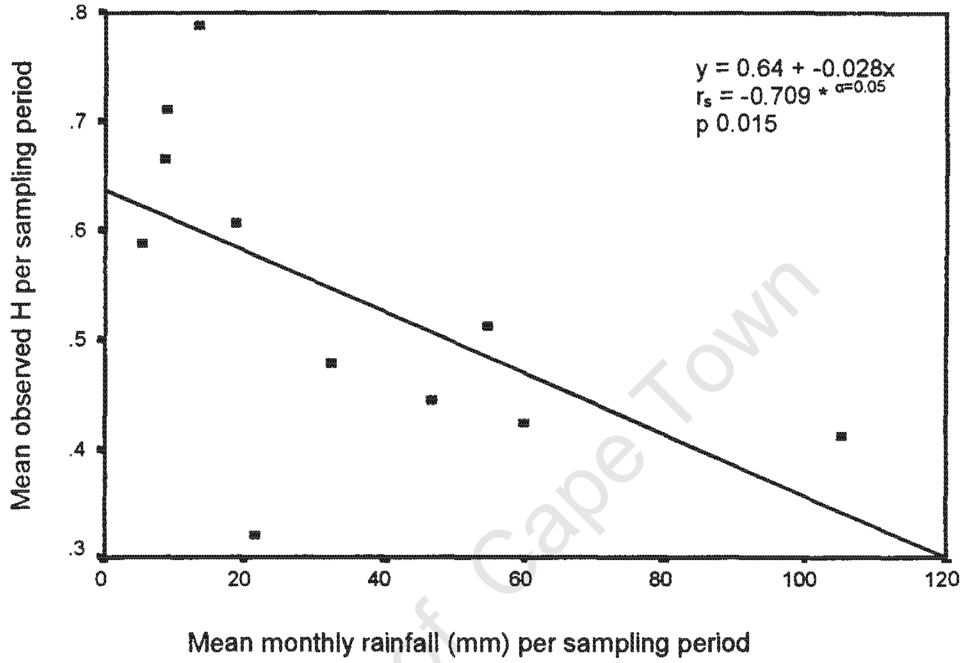
Locus *Colony*	*CH1*	*CH2*	*CH3*	*CH4*	*DMR3*	*DMR4*	*DMR7*	***Total F_{is} over all loci***
Steinkopf								**-0.12***
ST800	-0.41	-0.40	-0.57	0.25	-0.20	0.21	-0.37	**-0.20***
ST1100	-0.14	-0.13	-0.33	0.30	-0.24	-0.20	-0.32	**-0.13***
ST2500	-0.26	-0.19	-0.48	-0.07	-0.21	0.37	-0.30	**-0.13***
ST600	0.08	-0.20	0.06	-0.15	-0.21	0.23	-0.27	**-0.1**
ST2000	-0.33	-.025	-0.22	-0.21	1	1	-0.22	**+0.05**
ST1000	-0.13	-0.22	-0.61	-0.17	NA	0.13	-0.20	**-0.21****
Somerset West								**+0.04***
SW2100	-0.14	-0.18	-0.10	0.02	0.27	-0.15	NA	**-0.03**
SW4400	-0.13	-0.21	0.21	-0.11	0.14	-0.18	NA	**-0.02**
SW1800	0.02	-0.35	0.02	-0.27	-0.10	-0.15	0.10	**-0.13***
SW14000	-0.36	-0.18	0.14	-0.50	0.05	NA	0.00	**-0.15***
SW3000	0.39	-0.06	0.15	-0.12	0.30	0.40	0.21	**+0.17***
SW3400	NA	-0.10	0.28	-0.31	-0.42	0.00	-0.29	**-0.17**
SW1200	0.27	0.16	0.01	0.29	0.26	NA	0.15	**+0.18****

Significant deviation from zero determined by randomisation tests (FSTAT v 2.9.3, Goudet 1995); $^*p < 0.05$, $^{**}p < 0.01$, NA - analysis not possible from alleles present in data set

Measures of F_{it}, measuring inbreeding within individuals relative to the sub-population, at Steinkopf and Somerset West, were both found to be significantly greater than zero at p=0.05. However, this homozygote excess within individuals relative to Hardy-Weinberg expectations of the total population was accordingly greater at Somerset West (F_{is} = 0.12, Steinkopf F_{is} = 0.04).

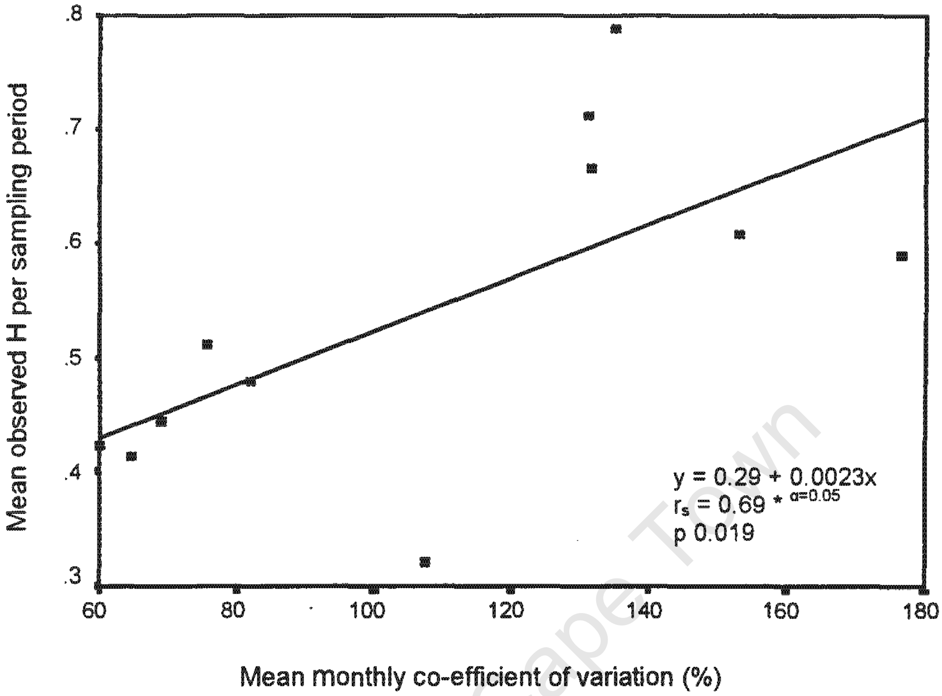
Analysis of the relationship between measures of rainfall and observed heterozygosity (H_{obs}) in *C. h. hottentotus* revealed significant and unexpected results. Spearman's rank correlation analysis indicated a significantly negative relationship between H_{obs} and mean monthly rainfall (r_s -0.709, $p<0.05$) in *C. h. hottentotus* (Figure 3.1). Regression line equation is shown on figure.

Figure 3.1 The relationship between mean monthly rainfall (mm) and observed heterozygosity (H_{obs}) levels in *C. h. hottentotus.*



Concomitantly, correlation analysis between H_{obs} and the mean monthly coefficient of variation in rainfall indicated a significantly positive relationship (r_s 0.69, $p<0.05$) (Figure 3.2). This reflects the unpredictable nature of the rainfall patterns that characterise the Steinkopf study site, where low monthly rainfall appears to promote the accumulation of genetic variation within colonies of *C. h. hottentotus*. As high rates of dispersal behaviour are generally associated with an increase in the number of heterozygote genotypes (Hartl and Clark 1997), these relationships were not as expected.

Figure 3.2 The relationship between the mean monthly co-efficient of variation in rainfall (%) and observed heterozygosity (H_{obs}) levels in *C. h. hottentotus.*



Hierarchical population structure

Highly significant levels of heterogeneity in allele frequencies were found at all loci tested at both the Steinkopf and Somerset West study sites (Fisher's exact test; $p<0.0001$). In addition, 82.7% of colony pair-wise comparisons at Steinkopf supported $p<0.05$, in contrast to only 57.1% colony pair-wise comparisons at Somerset West. *C. h. hottentotus* sub-populations at both sites therefore appear to be composed of colonies that demonstrate potentially high levels of genetic differentiation.

The multilocus values of F_{st} and R_{st} for combined data of all colonies at each site (Table 3.4) indicated a highly significant degree of genetic differentiation between the two *C. h. hottentotus* sub-populations (F_{st} p=0.01, R_{st} p< 0.0001). Overall F_{st} and R_{st} were comparable, measuring 0.3 and 0.31 respectively. Furthermore, measures of differentiation among colonies at each site were comparable when measured as variation in allele frequency by F_{st}; both Steinkopf and Somerset West displayed 'moderate' genetic differentiation

(Wright 1978) of F_{st}=0.13 and F_{st}=0.12 respectively, significant at p=0.01 However, this was not the case when using the measure of R_{st}, based on variance in allele length. Overall R_{st} amongst colonies at Steinkopf was more than four times greater than that calculated amongst colonies at Somerset West, measuring R_{st}=0.26 and R_{st}= 0.06 respectively. At both sites, however, overall R_{st} was found to be significantly greater than zero by permutation tests and bootstrapping over loci.

Table 3.4 Overall measures of population genetic differentiation at Steinkopf and Somerset West

	Total F_{st}[1]	*Total R_{st}*[2]
Steinkopf *v* Somerset West	0.3**	0.31****
Steinkopf	0.13**	0.26****
Steinkopf (without ST2500 + ST600)	0.11**	0.15****
Somerset West	0.12**	0.06 ***

[1] *p=0.05, ** p=0.01 (FSTAT, Goudet 1995)
[2] *** p< 0.0001, **** p< 0.00001, (RstCalc, Goodman 1997)

In order to assess whether the difference in overall sub-structuring at the two sites, as revealed by R_{st}, was influenced by the highly dispersed spatial arrangement of colonies at Steinkopf, analysis of F_{st} and R_{st} was then repeated excluding colonies ST2500 and ST600. These two colonies were relatively isolated, both from one another and the main complex of colonies comprising ST800, ST1000, ST1100 and ST2000. After removal from the data set, both F_{st} and R_{st} were found to be marginally lower. However the overall trend remained the same; F_{st} values were still comparable, now measuring 0.11 at Steinkopf, and although R_{st} was calculated as lower, at 0.15, it was still significantly greater than zero (p< 0.00001) and two and a half times greater than R_{st} measured at Somerset West. Therefore, despite the larger area and greater distances between colonies at the Steinkopf study site, measures of differentiation based on all six colonies are representative of dynamics within this arid-adapted sub-population.

Pair-wise colony F_{st} values ranged from 0.05 to 0.21 at Steinkopf, and from 0.04 to 0.22 at Somerset West. Pair-wise colony R_{st} values ranged from 0.003 to 0.25 at Steinkopf and 0.008 to 0.14 at Somerset West (detailed in Tables 3.5 and 3.6).

Table 3.5 Pairwise estimates of genetic differentiation for all colonies sampled at Steinkopf (ST); R_{st} (Goodman 1997)[1] above diagonal, F_{st} (Goudet 1995)[1] values below diagonal.

Colonies	*ST800*	*ST1100*	*ST2500*	*ST600*	*ST2000*	*ST1000*
ST800	–	*0.20*	*0.20*	*0.15*	*0.06*	*0.002*^{NS}
ST1100	0.12	–	*0.15*	*0.14*	*0.24*	*0.21*
ST2500	0.17	0.18	–	*0.20*	*0.20*	*0.25*
ST600	0.13	0.16	0.16	–	*0.25*	*0.18*
ST2000	0.11	0.09^{NS}	0.18	0.18	–	*0.09*
ST1000	0.05^{NS}	0.14	0.21	0.15	0.14	–

[1]All R_{st} values significant at $p \leq 0.05$ except where indicated ^{NS}, all F_{st} values significant at p=0.05 except where indicated ^{NS}. Significant deviation from zero determined by permutation tests and bootstrapping x 1000 over loci (RstCalc, Goodman 1997).

Table 3.6 Pairwise estimates of genetic differentiation for all colonies sampled at Somerset West (SW); R_{st} (Goodman 1997) above diagonal, F_{st} (Goudet 1995) values below diagonal.

	SW2100	*SW4400*	*SW1800*	*SW14000*	*SW3000*	*SW3400*	*SW1200*
SW2100	–	*0.05*	*0.12*	*0.03*^{NS}	*0.10*	*0.08*	*0.08*
SW4400	0.08^{NS}	–	*0.05*	*0.05*	*0.01*^{NS}	*0.04*^{NS}	*0.03*^{NS}
SW1800	0.09	0.11	–	*0.03*^{NS}	*0.07*	*0.11*	*0.03*^{NS}
SW14000	0.14	0.07^{NS}	0.14	–	*0.09*	*0.01*^{NS}	*0.03*^{NS}
SW3000	0.12	0.06^{NS}	0.16	0.15	–	*0.14*	*0.04*
SW3400	0.18	0.17	0.22	0.13	0.23	–	*0.09*
SW1200	0.04^{NS}	0.08^{NS}	0.12	0.08^{NS}	0.05^{NS}	0.13	–

[1]All R_{st} values significant at $p \leq 0.05$ except where indicated ^{NS}, all F_{st} values significant at p=0.05 except where indicated ^{NS}. Significant deviation from zero determined by permutation tests and bootstrapping x 1000 over loci (RstCalc, Goodman 1997).

Mean pair-wise colony R_{st} and F_{st} values were both found to be significantly larger amongst colonies at Steinkopf (Mann-Whitney *U*-test; one tailed, F_{st} $p=0.01$, R_{st} $p<0.001$) reflecting the greater overall degree of population sub-structuring evident within the arid site.

Mantel correlation tests of the degree of concordance between pair-wise colony F_{st} and R_{st} matrices (Table 3.7) at Steinkopf indicated a highly significant relationship ($p<0.01$). However, at Somerset West no relationship between the two measures of pair-wise colony differentiation existed. This reflects the differential effects of mutation and genetic drift on changes in allele frequency and allele length, and the relative influence each mechanism has on the establishment of colony-level sub-structuring evident at the two sites. As a result, colonies at Somerset West were found to be divergent with respect to allele frequencies where genetic drift alone was responsible for differentiation between colonies. This was in contrast to Steinkopf where colonies were found to be divergent with respect to both allele frequencies and allele length, reflecting the combined role of mutation and genetic drift in promoting population differentiation.

Table 3.7 Mantel correlation coefficients between R_{st} and F_{st} at Steinkopf and Somerset West

Study site	*Correlation coefficient*	*p-value (α = 0.05)*[1]
Steinkopf	0.82	0.005
Somerset West	0.13	0.13

[1] Significance levels computed from 1000 matrix permutations.

Genetic assignment tests

The overall degree to which individuals were correctly assigned to their source colony, expressed as a percentage of the total number sampled, was significantly greater at Steinkopf (Mann-Whitney *U*-test; one tailed $p=0.05$). Of the total number of individuals sampled at Steinkopf, 90.9% were correctly assigned to the colony within which they were sampled. This is in contrast to 57.6% of individuals correctly assigned at Somerset West (Table 3.8).

Table 3.8 Assignment statistics for *C. h. hottentotus* individuals sampled at Steinkopf and Somerset West

Study area	*% individuals correctly assigned to source colony*	*% of individuals assigned with 100% confidence*[1]	*% individuals mis-assigned*	*% of individuals assigned to colonies not included in study*
Steinkopf	90.9	64.3	11.7	6.5
Somerset West	57.6	24.6	41	7.1

[1] All other sampled colonies excluded as possible sources (p=0.01)

Of the individuals correctly assigned to their source colony at Steinkopf, 64.3% were assigned with 100% confidence i.e. all other colonies were excluded as possible sources at p=0.01. This value was found to be significantly greater than assignment at Somerset West where only 24.6% of the total number sampled were assigned with 100% confidence (Mann-Whitney *U*-test; one tailed p<0.05). The degree to which individuals were mis-assigned i.e. individuals who's genotypes were assigned to a colony other than that within which it was sampled, was found to be significantly greater at Somerset West (Mann-Whitney *U*-test; one tailed p=0.01). Of the total number of mole-rats sampled 41% were mis-assigned in contrast to only 11.7% at Steinkopf. The extent to which individuals were assigned to an unsampled colony did not differ significantly at the two sites, regardless of the differences in study area and colony densities. A total of 6.5% and 7.1% of individuals at Steinkopf and Somerset West respectively could not be assigned with any confidence to one of the colonies included in this analysis. These comparatively low values suggest the sampled colonies to be a representative selection from which to assess colony dynamics at the two sites.

Table 3.9 Summary of assignment statistics at Steinkopf

Colony	*% individuals correctly assigned to source colony*	*% individuals assigned with 100% confidence*	*Colony mis-assigned individuals most likely to come from*
ST800	100	20	-
ST1100	92.3	83.3	ST2000
ST2500	90	100	Unsampled colony
ST600	82.4	85.7	ST1100, unsampled colony x 2
ST2000	83.3	80	Unsampled colony x 2
ST1000	86.7	30.8	ST1100, ST2000

All colonies at Steinkopf included a component of assignment with 100% confidence of individuals to their source, in contrast to only two of the seven colonies at Somerset West (Tables 3.9 and 3.10 respectively). Adult individuals (>44g Spinks 1998) mis-assigned within colonies at Steinkopf were most likely to come from colonies ST2000, ST1100 and additional colonies not included within this sample. These mis-assignments of individuals, however, do not represent effective dispersal between colonies that were consistently nearest-neighbours. Furthermore at Somerset West, although there did not appear to be a clear pattern as to where mis-assigned individuals were most likely to come from, 42% of these individuals were assigned to nearest-neighbour colonies. In addition, individuals were more likely to be mis-assigned to a colony not included in the sample at Steinkopf (55.6% of mis-assigned individuals) than at Somerset West (20% of mis-assigned individuals). In all probability this would appear to be as a result of the difference in study area size, such that colonies beyond the sampling range at Steinkopf may have been source colonies for dispersers. Additionally, the physical limits on dispersal distance achievable by a single mole-rat would result in the tendency, therefore, for most successful dispersal to be between nearest-neighbour colonies.

Table 3.10 Summary of assignment statistics at Somerset West

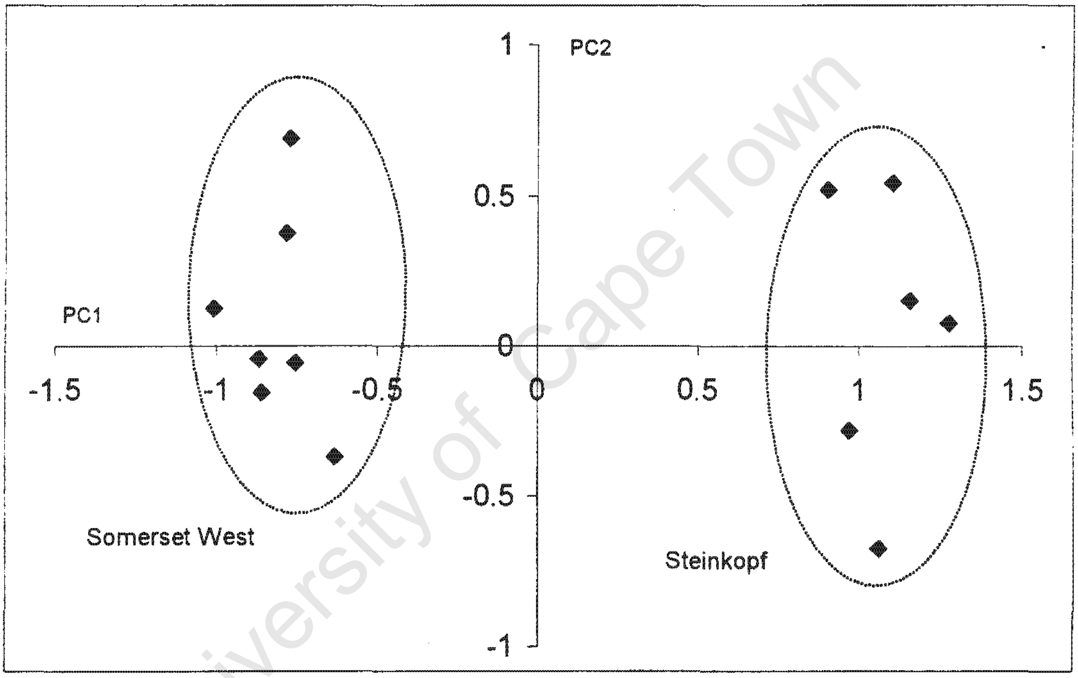
Colony	*% individuals correctly assigned to source colony*	*% individuals assigned with 100% confidence*	*Colony mis-assigned individuals most likely to come from*
SW2100	66.7	16.6	SW1800, SW4400, SW1200
SW4400	81.8	0	SW1200
SW1800	88.2	70.5	SW4400, SW2100
SW14000	100	0	-
SW3000	46.7	0	SW3400, SW4400, SW2100, unsampled colony
SW3400	75	0	SW4400, unsampled colony
SW1200	11	0	SW4400, SW14000, SW1200, SW3000, SW2100, SW1800, unsampled colony x 2

Principal components analysis (PCA)

Principal component analysis of population-level structuring of the *C. h. hottentotus* at Steinkopf and Somerset West (Figure 3.3) noticeably separated colonies within the dispersion matrix according to their geographic location without any overlap. This was largely due to the fact that all colonies from Somerset West displayed negative values along PC1, whilst all colonies at Steinkopf displayed positive values. Together PC1 and PC2 accounted for 70.7% of the variation in allele frequencies at the two study sites, reflecting the structured nature of the allele frequency data.

Figure 3.4 details the relationships between colonies at Steinkopf within principal component space, where PC1 and PC2 accounted for 51.8% of the variation in the data. The factor map clearly represents the dispersed spatial arrangement of colonies ST2500 and ST600, and the clumped arrangement of colonies ST1000, ST800, ST1100 and ST2000 (represented by encircled colonies). Similarly, PC analysis of allele frequency variation among colonies at Somerset West was also able to detect the overall spatial arrangement of colonies (Figure 3.5). Principal components 1 and 2 were found to account for 60.2% of the variation within the data, separating colonies on either side the marsh area where dispersal of individual mole-rats would involve burrowing around the area.

Figure 3.3 Factor map of the two main axes of the Principal Component Analysis (PCA) on all colony genotypes at Steinkopf and Somerset West. The *x*-axis represents 61.6% inertia (F_{st} = 0.18), while the *y*-axis represents 9.1% inertia (F_{st} = 0.03). Note: Ellipses not statistically generated.



The efficiency with which this technique was able to detect spatial arrangements at both study sites was as a result of similar F_{st} values amongst colonies at Steinkopf (F_{st} = 0.13) and Somerset West (F_{st} = 0.12). The study sites therefore exhibit sufficient structuring at both the colony and individual level for correlations in allele frequencies to exist. These correlations then provide an accurate indication of colony spatial relationships inherent in the data. [See Figures 2.1 and 2.2 in Chapter 2 for detailed layout of colonies at the two study sites]

Figure 3.4 Factor maps of the two main axes of the Principal Component Analysis (PCA) of colonies at Steinkopf. The *x*-axis represents 24.6% inertia (F_{st} = 0.05), while the *y*-axis represents 27.2% inertia (F_{st} = 0.04). Note: Ellipse not statistically generated.

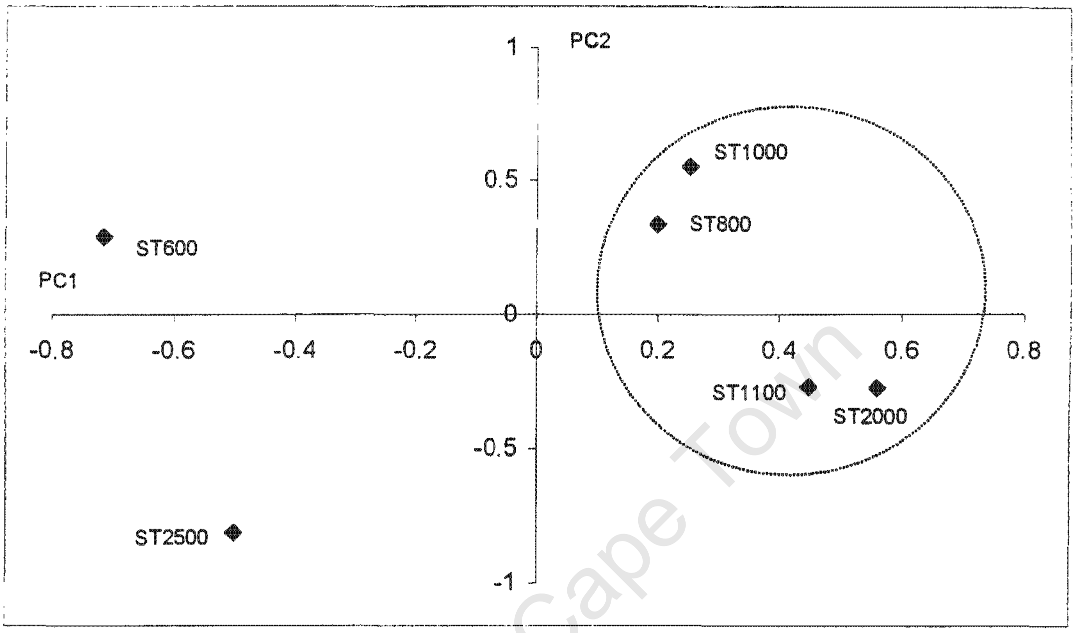
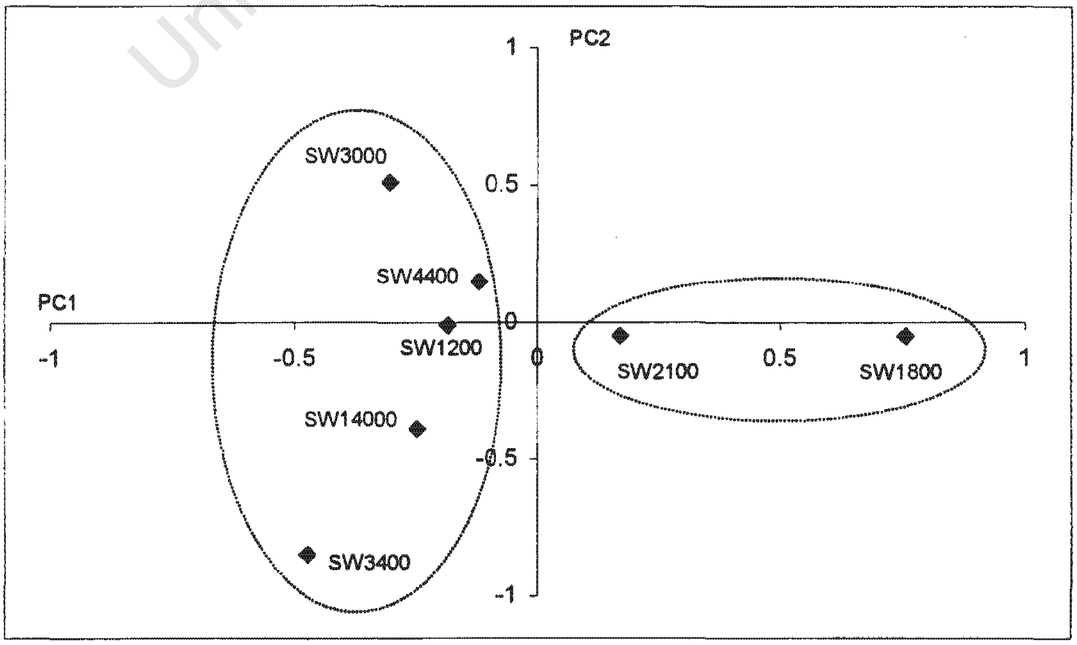


Figure 3.5 Factor map of the two main axes of the Principal Component Analysis (PCA) of colonies at Somerset West. The *x*-axis represents 33.4% inertia (F_{st} = 0.04), while the *y*-axis represents 26.8% inertia (F_{st} = 0.03). Note: Ellipses not statistically generated.



Genetic distance measures

Pair-wise comparisons of colonies at Steinkopf exhibited significantly greater genetic distances than colonies at Somerset West at all three measures used (Table 3.11). The mean pair-wise estimate of Nei's D_A among colonies at Steinkopf, 0.32 (SE 0.02) was double that of 0.16 (SE 0.01) at Somerset West (Mann-Whitney *U*-test; $p<0.01$). Cavalli-Sforza and Edward's D_{CE} measured 0.49 (SE 0.02) and 0.33 (SE 0.01) at Steinkopf and Somerset West respectively (Mann-Whitney *U*-test; $p<0.01$). The microsatellite-specific measure of Goldstein's Delta μ^2 displayed the greatest degree of difference in mean pair-wise colony distances at the two sites, measuring 3.26 (SE 0.55) between colonies at Steinkopf and only 0.28 (0.03) at Somerset West (Mann-Whitney *U*-test; $p<0.001$).

Table 3.11 Measures of genetic distance among colonies at Steinkopf and Somerset West

Distance	**Steinkopf**		**Somerset West**	
	Mean[1]	*Range*	*Mean*[1]	*Range*
D_A	0.32 (0.02)	0.15 - 0.48 (0.33)	0.16 (0.01)	0.07 - 0.30 (0.23)
Delta μ^2	3.26 (0.55)	0.26 - 7.52 (7.26)	0.28 (0.03)	0.07 - 0.52 (0.45)
D_{CE}	0.49 (0.02)	0.30 - 0.61 (0.31)	0.33 (0.01)	0.24 - 0.48 (0.24)

[1] Pairwise mean, standard error in parentheses

In addition, the ranges of all three genetic distance measures were found to be larger as measured among colonies at Steinkopf. This was as expected due to the larger size of the study area at Steinkopf and the greater physical distance between colonies.

Mantel tests found highly significant correlations amongst all measures of pair-wise colony distance (Table 3.12). Matrix correspondence analysis of all distance measures used was significant at $p<0.001$. In addition, matrices of all three measures were found to be concordant with measures of pair-wise F_{st} and R_{st} at $p<0.001$.

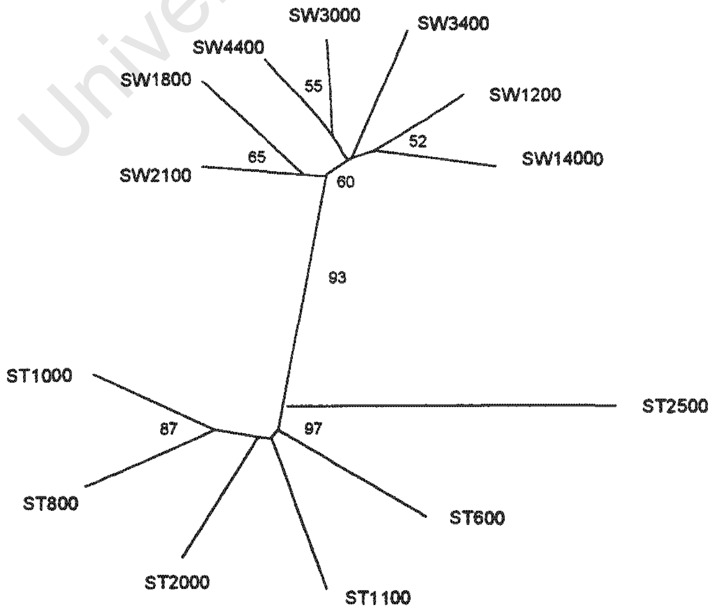
Table 3.12 Mantel correlation coefficients between distance matrices of all colonies at Steinkopf and Somerset West. Note: All correlations significant at $p<0.001$[1].

	Delta μ^2	D_{CE}	D_A	F_{st}
D_{CE}	0.87			
D_A	0.84	0.97		
F_{st}	0.81	0.93	0.96	
R_{st}	0.87	0.89	0.92	0.93

[1] Significance levels computed from 1000 matrix permutations.

This degree of concordance amongst all three measures of genetic distance is reflected in their similar tree topologies. UPGMA trees based on D_A, D_{CE} and Delta μ^2 of all colonies at both sites consistently grouped same-site colonies together (Figures 3.6, 3.7 and 3.8 respectively). Percentage support for nodes that received more than 50% bootstrap support in 1000 replicate trees appears adjacent to the node. Bootstrap confidence values on tree nodes were highest when using Nei's D_A, however the topology of the tree based on Delta μ^2 provides the best representation of the degree to which the two sites are structured as a function of differential social elaboration.

Figure 3.6 UPGMA phenogram of *C. h. hottentotus* colonies at Steinkopf and Somerset West using Cavalli-Sforza and Edward's chord distance D_{CE} (Cavalli-Sforza and Edward 1967).



Steinkopf colony branch lengths were generally longer than those for Somerset West colonies for all three genetic distance measures. This is particularly apparent in the Delta μ^2 tree topology where the large range in pair-wise distances measured amongst colonies at Steinkopf is reflected in the considerably longer branch lengths. Regardless of the measure used, genetic differences between the two study sites were generally greater than differences between colonies within each study site. The exception to this trend was colony ST2500. On all three trees the longest branch length was associated with colony ST2500, reflecting its divergence from colonies at both Steinkopf and Somerset West. Both Nei's D_A and Cavalli-Sforza and Edward's D_{CE} placed ST2500 within the Steinkopf grouping, where it was first to branch off. However Goldstein's Delta μ^2 placed it within its own clade i.e. it was found to be as dissimilar from the Steinkopf grouping as it was from Somerset West.

Figure 3.7 UPGMA phenogram of *C. h. hottentotus* colonies at Steinkopf and Somerset West using Nei's D_a (Nei *et al.* 1983).

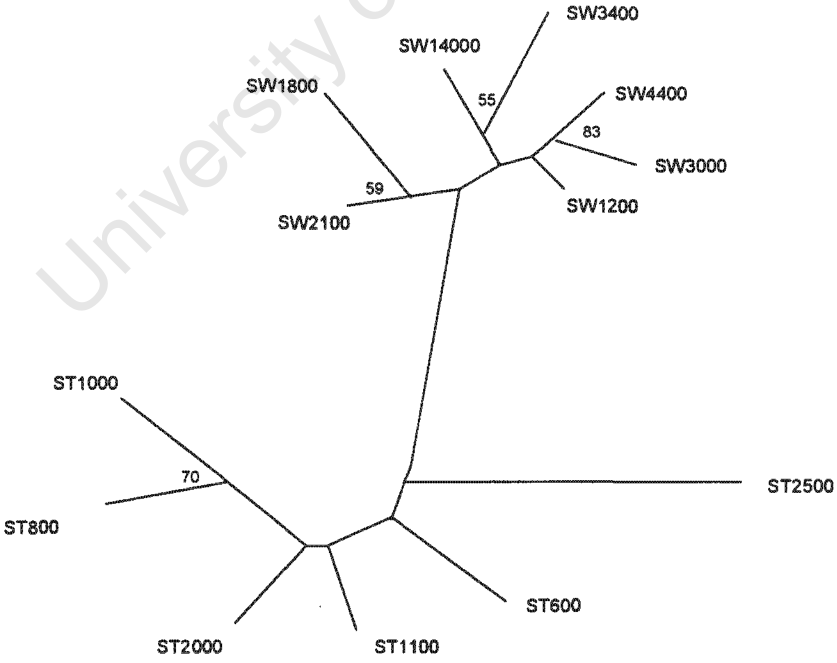
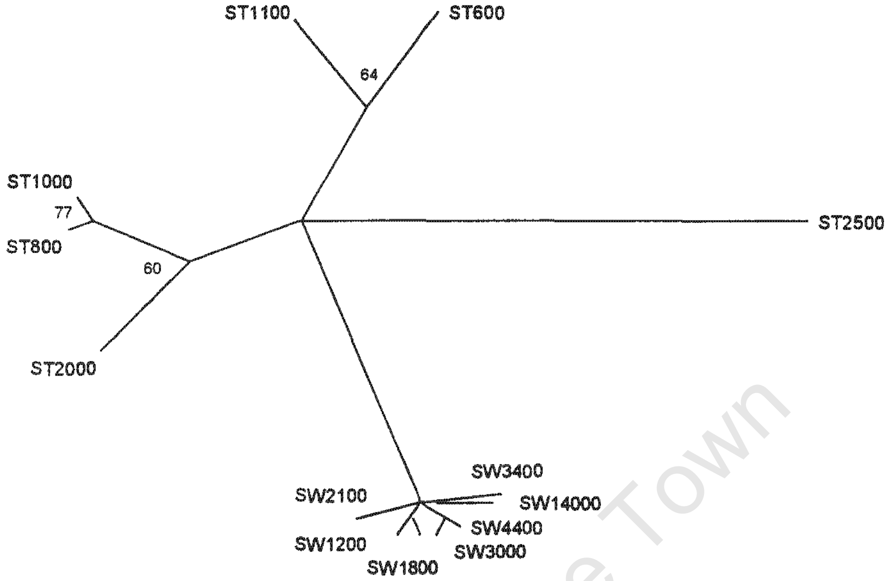


Figure 3.8 UPGMA phenogram of *C. h. hottentotus* colonies at Steinkopf and Somerset West using Goldstein's delta μ^2 (Goldstein *et al.* 1995a).



Although tree topology accurately reflected the degree to which colonies were sub-structured at the two sites they were unable to detect a consistent relationship between branching pattern and the spatial arrangement of said colonies.

DISCUSSION

Estimates of population genetic differentiation

Common mole-rat colonies within the two study sites are characterised by divergent dispersal patterns and group dynamics. Spinks *et al.* (2000) summarised these as a combination of two distinguishing features: i) markedly lower rates of dispersal at arid Steinkopf relative to mesic Somerset West, and ii) a greater degree of social cohesion among mole-rats occurring in arid areas, reflecting adaptive variation in cooperative behaviour in response to enhanced ecological constraints on dispersal behaviour in arid areas.

If dispersal away from the natal group is constrained, the degree to which individuals within groups display coancestry will increase over time. This trend will be intensified by the reproductive tenure of the resident breeding pair, such that each colony will represent a progressively smaller proportion of the genetic variability in the total population. Genetic differentiation among colonies, as measured by R_{st} and F_{st}, should therefore increase as a function of this accumulation of spatially localised allelic similarity and impose a social lineage effect on population sub-structuring. Both F_{st} and R_{st} indicate that social structuring within the *C. h. hottentotus* is sufficient to promote the accumulation of lineage-specific genetic similarity such that gene flow between colonies is too low to counteract differentiation by a combination of drift and mutation. F_{st} values among colonies at the two sites ranged from 0.05 to 0.25, indicative of 'moderate to great' levels of genetic differentiation according to Wright's (1978) qualitative guidelines. This is in line with maximal F_{st} values of 0.16 reported for black-tailed prairie dog coteries, *Cynomys ludovicianus*, within wards (Dobson *et al.* 1997, 1998), and red howler monkey troops, *Alouatta seniculus*, within a number of localities where F_{st} values ranged from 0.09 to 0.22 dependent upon locality (Pope 1992, 1998).

Overall F_{st} values were comparable at the two study sites and were reflected in the proficiency with which both principal component analysis and genetic distance measures were able to resolve both geographical and site-specific spatial relationships among colonies at Steinkopf and Somerset West. Radial unrooted trees, based on a number of genetic distance measures, were consistently able to resolve the structured nature of allele frequency data at both sites. In addition, all three measures of genetic distance were found to be representative of the divergent spatial scales over which colonies were distributed at the two sites. The microsatellite-specific measure Delta μ^2 was found to be particularly sensitive to the structured nature of the data at the two sites and best represented the genetic dissimilarities and spatial arrangement of colonies.

In contrast to F_{st}, both overall and pair-wise R_{st} values were found to differ significantly at the two study sites. This trend held even after the removal of spatially isolated colonies at Steinkopf, accounting for differences in scale at the two study sites that may have caused a bias in the data. These results suggest that factors such as genetic drift and mutation, which influence the distribution of allele frequencies and sizes respectively, affect genetic structure at the two sites to differing degrees. Alleles that differ in frequency characterise recently diverged lineages as a function of dispersal and genetic drift (Slatkin 1995). As a result, F_{st} is an appropriate measure with which to determine the effect of current population dynamics on population genetic sub-structuring. Accordingly, it may therefore be an inappropriate measure with which to determine the long-term effects of differential social elaboration and dispersal patterns on population genetic sub-structuring within sub-populations of *C. h. hottentotus* (Slatkin 1995). Because R_{st} compares variances in allele size, which come about as a result of mutational events, it should be more sensitive than F_{st} to changes over a longer time frame when using microsatellite data (Slatkin 1995). Indeed R_{st} calculated both overall and between colonies at Steinkopf and Somerset West, does indicate differential structuring as a function of mutation. Consistently higher R_{st} values, both overall and pair-wise, at Steinkopf suggest that the combination of long term reduced dispersal and resultant colony stability in response to arid conditions has had a significant effect on the partitioning of variation within colonies. Additionally, the number of private alleles within a population is reflective of the relative effects of gene flow and mutation on differentiation. Steinkopf was characterised by a larger proportion of private alleles, thereby invoking the significant effect mutation and low levels of gene flow have had on promoting genetic differentiation at the site. Ecological constraints at Steinkopf appear then to promote both the accumulation of mutations, preventing their loss by random genetic drift, and their maintenance at low frequencies within colonies. This appears to be the result of long-term reproductive isolation of colonies and low rates of effective dispersal that characterise the species' life history response to aridity.

Furthermore, the greater reliability with which individuals were assigned to their source colony at Steinkopf attests to the overall increased stability of social cohorts occurring there. Socially mediated barriers to gene flow may therefore be an important determinant of population genetic structuring within the common mole-rat, and this may be especially relevant to colonies at Steinkopf where philopatry and kin-selection through co-operative breeding reflect an adaptive response to the physical constraints on successful dispersal and gene flow.

Genetic estimates of dispersal rates and gene flow

Movement between groups of small mammals are most likely to be characterised by a stepping-stone type of model (Shields 1987, Patton *et al.* 1996) based on the likely physical limit on dispersal distances that small rodents are capable of achieving. As a result, gene flow is then the result of effective dispersal between nearest-neighbour 'population' pairs. This is in contrast to Wright's island model (Wright 1931) where dispersal and gene flow have the potential to occur between any possible pair. Dispersal between colonies of *C. h. hottentotus* is further restricted by the subterranean habitat, as the species often appears to disperse by burrowing and not by movement over ground (J.U.M. Jarvis pers. comm.). This may act to increase the likelihood of detecting successful dispersal and gene flow between those colonies separated by shorter rather than longer distances. The use of an assignment test to detect the likely source of an individual, and thereby infer gene flow and dispersal rates, represents a potentially informative method with which to estimate a parameter that both theoretical methods and mark-recapture techniques often fail at (Rousset 2001). This is because the detection of dispersal, both directly and indirectly, is particularly problematic when its occurrence is rare or when it hasn't taken place in the last few generations (Peacock and Ray 2001, Rousset 2001). This approach has the potential to revolutionize estimates of contemporary dispersal between colonies and populations of subterranean species. This approach is possible due to the high levels of polymorphism inherent to microsatellites, and has facilitated their use in a number of studies of individual identification (e.g. Paetkau *et al.* 1995, Favre *et al.* 1997, Haig *et al.* 1997, Estoup and Engers 1998, Waser and Strobeck 1998, Cornuet *et al.* 1999, Primmer *et al.* 2000). Despite the assignment of 42% of individuals to nearest-neighbour colonies at Somerset West, assignment tests of individuals at both sites were not able to clearly identify a prevailing pattern of nearest-neighbour effective dispersal in

C. h. hottentotus. The high degree of stability within colonies at Steinkopf, where 90.9% of individuals were correctly assigned to their source colony, prevents an accurate attempt at inferring patterns of dispersal at the site. This is further compounded by the assignment of individuals to unsampled colonies. Indeed Spinks (2000) notes that all immigrant animals at Steinkopf were unmarked, suggesting that both the temporal and spatial scale over which the field study took place at Steinkopf may have impacted upon the degree to which detailed dynamics of effective dispersal by assignment tests could be determined. Pair-wise F_{st} estimates among colonies at Steinkopf, however, did indicate a degree of increasing differentiation with distance. Within the colony complex of ST800/ST2000/ST1100/ST1000 both F_{st} and assignment tests were unsuccessful at revealing a stepping-stone pattern of gene flow. Nonetheless, when compared to colonies ST2500 and ST600, representing a larger spatial scale, F_{st} values did then indicate greater differentiation with distance. Pair-wise estimates of F_{st} at Somerset West were not able to detect a similar pattern. This is most likely due to a combination of the small spatial scale over which individuals were sampled, comparable to that over which the ST800 colony complex occurred at Steinkopf, the significantly greater density of colonies and higher colony turnover at the mesic site. Such that continual dispersal by individuals at any one time may involve movements to both nearest and more distant neighbours simultaneously, resulting in irregular spatial differentiation as measured by F_{st}. As a result of this dynamic, effective gene flow may be expected to homogenize the degree to which to which colonies are differentiated with respect to distance at Somerset West.

Distribution of genetic variation

Chesser's breeding groups models (1991a, 1991b) account for population differentiation at the colony level as a function of sex biased dispersal, variance in reproductive success by at least one sex, and a variety of mating strategies. This combination of factors then appears to consistently promote socially structured systems characterised by significant F_{st} among colonies, and an excess of heterozygotes, as measured by F_{is}, within colonies (Storz 1999). However, in many socially organised species, including *C. h.*

hottentotus (Spinks 1998, Spinks *et al.* 2000), dispersal is undertaken, to a lesser or greater degree, by both sexes (Greenwood 1980). Additionally, a number of socially structured mammal species characterized by a lack of sex-biased dispersal do indeed display significant F_{st} along with negative F_{is} values (Dobson *et al.* 1997, 1998, Mathews and Porter 1993, Pope 1992, 1998). Chesser (1991b) thus notes that lineage effects alone may be sufficient to generate differentiation among colonies that are characterised by an excess of heterozygote individuals. As a result, the degree to which populations of *C. h. hottentotus* are socially structured into colonies may alone be sufficient for the accumulation of genetic differentiation at the spatial scale by which colonies are distributed; thereby invoking the influence of social behaviour on mechanisms of population genetic structuring at the colony level (Chesser 1998, Dobson *et al.* 1997).

Greater levels of overall genetic variation characterised colonies and individuals of *C. h. hottentotus* at Steinkopf relative to Somerset West. Allelic variation exceeded that of Somerset West in terms of the total number of alleles, mean number of alleles per locus and mean number of alleles per colony. In addition, the relationship between rainfall and heterozygosity was contrary to that expected from the disparate dispersal regimes at the two sites. Rainfall at Somerset West exceeds the minimum level required to promote burrowing activity for much of the year. This is in complete contrast to Steinkopf where aridity is the prevailing condition. The reduced environmental constraints on dispersal at Somerset West were expected to facilitate both gene flow and the maintenance of higher levels of genetic variation, as measured by heterozygosity. Furthermore, though both sites were characterised by comparable levels of sub-structuring as revealed by F_{st}, Somerset West displayed significantly positive F_{is} values indicating an excess of homozygotes whilst Steinkopf was characterised by a significant excess of heterozygote individuals. The scenario reported for Steinkopf is therefore in accordance with Chesser's models, where genetic subdivision among breeding groups is accompanied by strongly negative F_{is} values (Chesser 1991a, 1991b, Storz 1999, Storz *et al.* 2001). Nonetheless positive F_{is} values have been reported in black-tailed prairie dogs *Cynomys ludovicianus*

(Chesser 1983) and long-tailed macaques *Macaca fasciculatis* (de Ruiter 1994) where they appear to be as a result of features specific to local dispersal and mating systems. Therefore, while both sites demonstrate the socially mediated accumulation of coancestry within colonies, local differences in dispersal patterns appear to have had significantly divergent effects on the actual variation being apportioned. Furthermore, recruitment via reproduction dominated increases in colony size at Steinkopf, whereas recruitment mainly via immigration of foreign individuals characterised colonies at Somerset West. These patterns of recruitment may then be expected to influence genetic variation within colonies at the two sites; promoting the incidence of genotype variability within colonies at Steinkopf, and increasing the localised concentration of allelic identity by descent within colonies at Somerset West.

The high density of food resources at mesic Somerset West (1424 geophytes/m^{-2}) successfully supports a similarly high density of colonies (18.7 individuals per ha) within a relatively small area (5ha). This is in complete contrast to the arrangement of geophytes (75.8 geophytes/m^{-2}) and mole-rat colonies (1.8 individuals per ha) at the arid 43ha Steinkopf site. Dispersing mole-rats at Somerset West therefore have an increased chance of i) successfully dispersing into a nearby colony with the potential to become a breeder, and ii) successful access to nearby food resources. Together, these features reduce the energetic costs of burrowing and negate the need for long distance dispersal. However, as a direct result of this highly localised movement and exchange, individuals inadvertently appear to increase the incidence of breeding with foreign conspecifics that share allelic identity by descent, by chance alone. In turn this results in fewer possible allelic combinations and the detected decrease in H_{obs} relative to Steinkopf. In contrast to Somerset West, environmental constraints on potentially effective dispersal at Steinkopf appear to actively promote high levels of genetic variation between lineages. Inter-colony differences in both allele frequency and size that is a result of reduced dispersal then facilitates the exposure of a greater proportion of the species' variation at microsatellite loci to selection. In turn, the accumulation of new mutations is promoted, their loss by genetic drift is limited, and a dispersal regime involving relatively long distances by individual mole-rats results in matings between foreign conspecifics with a reduced chance of sharing alleles by descent; and H_{obs} at Steinkopf is then inadvertently promoted.

CONCLUDING REMARKS

Within these sub-populations of the common mole-rat it is clear then that genetically differentiated lineages are able to both preserve and actively promote genetic polymorphism in a manner similar to genetic differentiation at the levels associated with classical models of population structure. Local variation in breeding group structure and stability is able to impose a prevailing affect on both the accumulation of population level genetic variation, and its apportionment. Consequently divergent population genetic structuring with an important spatial element characterises these two study sites. These differences appear to be as a result of both local dispersal and mating systems, and the way in which local selection operates both within and among colonies. Together, these processes demonstrate their potential to either reinforce or abate the spatial aggregation of individuals sharing coancestry, irrespective of the obligate outbreeding nature of the species.

CHAPTER 4

Genetic consequences of the mating system of *Cryptomys hottentotus hottentotus*

SUMMARY

Molecular analysis of parentage in an increasing number of species indicates that genetic systems of mating do not necessarily reflect social mating systems. Within social species, the occurrence of extra-pair paternity has important consequences for the evolution of social and cooperative behaviours by kin selection and reciprocal altruism because of its influence on measures of relatedness and allelic identity by descent (IBD). Microsatellite markers were used to assess genetic parentage and measures of relatedness within and between colonies of *C. h. hottentotus* at Steinkopf and Somerset West. Assignment of paternity to a within-colony male was found to occur with similar frequencies at the two study sites. However, assignment to the originally identified breeding male was greater within colonies at Steinkopf. Colonies at Somerset West were found to support a number of large unrelated males; as a result size criteria alone were insufficient with which to determine the breeder. In addition, genetic analysis of paternity appears to support Bennett's (1989) theory that colonies of *C. h. hottentotus* may support more than one resident breeding male. Extra-colony paternity (ECP) characterised colonies at both study sites and was found to occur with comparable frequencies of 21.5% and 25% at Steinkopf and Somerset West respectively. The occurrence of ECP appears to have divergent genetic consequences within the two populations; whilst potentially promoting genetic variation within colonies at Steinkopf, it may act to increase the chance of allelic IBD within colonies at Somerset West, thereby increasing the number of homozygote genotypes. Relatedness values were found to be comparable for a number of categories at both sites. However, relatedness between breeding pairs was found to be significantly higher at Somerset West, reflecting the effects of localised gene flow and increased chances of allelic IBD on measures of heterozygosity. Analysis of the genetic mating system of *C. h. hottentotus* provides further evidence for the occurrence of extra-pair paternity in apparently monogamous mammals, and suggests that behavioural dynamics within wild populations may be more complex than previously perceived.

INTRODUCTION

A detailed understanding of a species' mating system, both behavioural and genetic, are essential to explaining the evolutionary causes and effects underpinning socially structured species. The use of molecular methods for parentage analysis and estimates of genetic relatedness between interacting individuals provide further opportunity to promote our understanding of mating systems, and their genetic and evolutionary consequences. Indeed, our understanding of the ecology and evolution of mating systems has been revolutionized by molecular techniques (Burke 1994) and it is becoming increasingly apparent that the genetic outcome of many species' mating systems may differ notably from their apparent social mating system (Avise 1994, Møller and Ninni 1997, Davis *et al.* 2001, Gagneux *et al.* 1999, Vigilant *et al.* 2001). This trend is most prevalent in the birds and many fundamental assumptions about mating systems have been modified following molecular analysis (Birkhead and Møller 1992, 1995, Birkhead *et al.* 1987, Westneat and Sherman 1997). For example, although social monogamy characterises more than 92% of bird species (Lack 1968, Møller 1986), 75% of over 100 socially monogamous species, in which molecular techniques have been used to investigate paternity, have indicated extra-pair fertilizations (Birkhead and Møller 1992, 1995, Møller and Ninni 1997).

Less than 3% of mammal species are characterised by an apparently monogamous mating system, largely restricted to the Primates, Rodents, Ungulates and Canids (Kleiman 1977). In these too, field and genetic studies are increasingly reporting extra-pair copulations, resulting in extra-pair paternity of offspring (Goossens *et al.* 1998, Reichard 1995, Roemar *et al.* 2001, Fietz *et al.* 2000). These resulting offspring, sired by males other than the attendant male, affect the outcome of estimates of male and female reproductive fitness, measures of reproductive success and skew, estimates of N_e and the processes by which genetic variation is maintained.

Much research now focuses on the genetic and social factors that may promote or hinder extra-pair mating, most notably in social species (Davies 1991, Møller and Ninni 1998, Petrie *et al.* 1998, Petrie and Kempenaars 1998). Indeed, social organisation and demography have important consequences for the genetic outcomes and evolution of a species' mating system (Davies 1991). Furthermore, the genetic structure of a species has significant consequences for the evolution and maintenance of social and cooperative behaviours by kin selection (Hamilton 1964a, 1964b) and reciprocal altruism (Trivers 1971). Both theories require higher degrees of relatedness within social groups relative to that in the population as a whole. Therefore the ability to estimate relatedness between any two interacting individuals is of great value to the study of the evolution of sociality (Queller and Goodnight 1989, Pamilo 1989, Morin *et al.* 1994a).

Assessment of relatedness itself is evolutionarily significant as genetic similarity can be brought about by factors other than direct relatedness as measured by pedigrees. Apparent inbreeding, or the chance sharing of alleles by descent, may be exacerbated by highly localised natal dispersal (discussed in Chapter 3), particularly in species with low vagility such as *C. h. hottentotus*. A species' mating system may then facilitate the accumulation of allelic identity by descent (IBD) as a result of contingent coancestry, despite behavioural outbreeding, which in turn, may become apparent in estimates of genetic relatedness (Chesser 1991b).

Estimates of extra-pair mating and relatedness are often difficult to measure from field observations alone (Hughes 1998). For example, field observations of chimpanzees, *Pan troglodytes,* have led to estimates of extra-group copulations accounting for only 1 to 13% of offspring, whilst molecular analysis has indicated this value to be closer to 50% (Gagneux *et al.* 1997, 1999)[1]. Indeed behaviours such as mating, parental care and dispersal are near impossible to observe in subterranean species such as *C. h. hottentotus*. Therefore, genetic analysis of parentage and relatedness provides us with invaluable insight into the genetic dynamics of individual mole-rats both within and between colonies. Moreover, studies on all subterranean taxa will clearly benefit from this combined approach.

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[1] It is worth noting that this is currently refuted with analysis based on strict exclusion criteria (Vigilant *et al.* 2001).

As discussed in Chapter 1, social organisation within *C. h. hottentotus* represents the rare combination of group living and behavioural monogamy (Bennett 1989, Bennett and Faulkes 2000, Spinks 1998). Colonies are characterised by a single breeding female and one to three potentially breeding males[2] that rear a maximum of two litters during the summer period from late November to January (Bennett 1989, Skinner and Smithers 1990, Jarvis and Bennett 1991, Spinks 1998, Spinks *et al.* 2000). Adolescent mole-rats from previous litters forego independent reproduction and remain within the natal colony where they assist in the rearing of young until such time as conditions favour their dispersal and opportunities to breed (Bennett 1989). Molecular analysis of population genetic structuring at Steinkopf and Somerset West (Chapter 3) revealed the disparate effects of local dispersal patterns on gene flow. Regional variations in a species' life history responses are clearly fundamental to its success and may, in turn, be reflected by flexibility in the mating system. This association has been reported in the common brushtail possum, *Trichosurus vulpecula* (Taylor *et al.* 2000), and the European rabbit, *Oryctolagus cuniculus* (Cowan and Garson 1985, Fuller *et al.* 1997), where the ability to adapt their mating system to local conditions has facilitated both species' rapid expansion into a variety of habitats. Therefore the different constraints on dispersal that are apparent in measures of gene flow and genetic structure at Steinkopf and Somerset West may be further evident within the corresponding mating systems of the two populations.

The social organisation and demography of the common mole-rat has been described in considerable detail by Bennett (1988, 1989) and Spinks (1998, Spinks *et al.* 2000). However, reproductive behavioural interactions between wild-living animals have not been described due to the obvious constraints to observations of the subterranean environment. Analysis of parentage and estimates of relatedness were therefore carried out to investigate specific

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[2] Observations of a captive wild-caught colony (Bennett 1989) noted the potential presence of more than one breeding male/colony, based on dominant mounting behaviour by subordinate males. However ejaculation did not always follow mounting behaviour by these less dominant males. Therefore in the absence of genetic data the largest dominant male was presumed to be the most likely breeder.

hypotheses pertaining to the species' mating system and the disparate findings of population genetic structure at the two study sites.

RESEARCH AIMS

The broad aim of this chapter is thus to describe some of the genetic consequences of the mating system of *C. h. hottentotus* at Steinkopf and Somerset West in order to examine the following hypotheses:

1. Extra-colony paternity (ECP)[3] is more likely to occur within colonies at Somerset West, as a result of relaxed constraints on dispersal and the reduced levels of aggression displayed to foreign immigrants.

Reproductive function in non-breeder males is not under physiological control (Bennett 1989, Spinks *et al.* 1997). Dispersing males therefore have as much chance of successfully siring young as established breeding males within colonies. In addition, significantly higher turnover of colony members and immigration of foreign individuals into colonies characterized population dynamics at Somerset West. As a result, the occurrence of behavioural interactions between breeding females and mature foreign males would be greater than at Steinkopf. Paternity of litters was therefore analysed to examine the prediction that colonies at Somerset West were more likely to exhibit ECP than those at Steinkopf, where constraints on dispersal itself limit year round movement of young adult dispersers.

2. A negative relationship between pairwise relatedness estimates and colony H_{obs} is predicted because the increased occurrence of allelic IBD at Somerset West directly impacts upon the possible number of local genotypes.

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[3] Within the context of this thesis the term 'colony' is used to represent all individuals sampled from a single burrow system, and therefore considered as belonging to a single group or colony.

Highly localised patterns of dispersal and hence gene flow among colonies at Somerset West have resulted in lower levels of H_{obs}. In addition, a significantly negative relationship exists between colony H_{obs} and mean monthly rainfall across the distributional range of *C. h. hottentotus*. Correlation analysis was undertaken to test the prediction that lower levels of H_{obs} reported within colonies at Somerset West are the consequence of localised dispersal patterns. These patterns act to increase the chance of allelic IBD and thereby, genetic relatedness.

3. Differential recruitment within colonies at Steinkopf and Somerset West will be reflected in the relationship between relatedness estimates and colony size.

Mean relatedness within colonies at both sites is predicted to increase with colony size as a function of the accumulation of lineage-based relatedness. Furthermore, as sex biased dispersal has not been demonstrated in *C. h. hottentotus* (Spinks *et al.* 2000), the degree to which male and female colony members are genetically related, both within and between colonies, should not differ.

METHODS

Molecular Analyses

A total of 176 *C. h. hottentotus* individuals were analysed at seven *Cryptomys*-specific microsatellite loci (detailed in Burland *et al.* 2001). As described in Chapter 2, allelic variation at each locus was scored manually by comparison with aligned M13 standard size fragments and positive control individuals on all gels. Allele frequency data was then used to generate parentage and relatedness analyses and is included in Appendix II as allele frequency histograms of all study colonies at all loci analysed.

Genetic Data Analyses

Assessment of Parentage

In determining parentage, either paternal or maternal, individuals are assessed by the proportion of loci at which they share alleles with the offspring in question (Marshall *et al.* 1998). Parentage is ordinarily then assigned to the candidate matching the offspring at all loci tested. In doing so, this approach based on exclusion criteria rejects all candidate parents where allelic mismatches occur. However, parentage assignment by exclusion may be ambiguous, despite the use of a number of polymorphic microsatellite loci, as mismatch at a single locus results in all likely parents being excluded. This may become problematic because multiple males/females may be genetically compatible with the offspring of interest as a result of typing errors (Marshall *et al.* 1998), null alleles (Pemberton *et al.* 1995) and the high mutation rates associated with microsatellite loci (Queller *et al.* 1993). All these factors act to increase the likelihood of parent-offspring mismatch, resulting in Type II errors and the underestimation of possible extra-group paternity. In addition, caution in the interpretation of results is of particular importance when potentially related males may compete for matings (Double *et al.* 1997) and the accumulation of localised coancestry both act to increase the chance sharing of allelic identity by descent. The program CERVUS v2.0 (Marshall *et al.* 1998) overcomes these problems by using a likelihood approach to identify parent-offspring pairs. Through simulation, the program generates criteria for parentage to be assigned at a given level of confidence. Allele frequency data is used to generate a likelihood ratio to each candidate parent presented to the program, which is expressed as the natural logarithm of the ratio, termed the LOD score. A Monte-Carlo simulation, based on observed allele frequencies at all loci and taking into account the number of candidate parents, is then used to determine the required critical difference in LOD scores between the first and second most likely parents for assignment of parentage with ≥95% confidence. The critical difference, termed *Delta*, is simply the difference between the first and second most likely parent's LOD scores that is required for assignment with statistical confidence. It is important to note however that the use of the *Delta* criteria results in parentage remaining unassigned when 2 or more most-likely parents have similar LOD scores. CERVUS v2.0 (Marshall *et al.* 1998) was thus used to explore specific questions pertaining to parentage within the colonies sampled at Steinkopf and Somerset West.

Paternity analysis

The morphological determination of breeding males within colonies of *C. h. hottentotus* is generally less clear than identification of the most likely breeding female (Spinks 1998), who is distinguished by size and the presence of prominent teats (Jarvis and Bennett 1993). This is because initial identification of *C. h. hottentotus* breeding males is generally based on size criteria, where the largest male (>60g) is most likely to be both sexually mature and dominant, and therefore the breeder (Bennett 1989, Spinks *et al.* 2000). However, both behavioural observations of captive mole-rats and physiological assessment of autopsied animals indicate that there may sometimes be more than one reproductive male per colony (Bennett 1989). During the field study (Spinks 1998) a number of large males (>60g and therefore likely to be sexually mature), any one of which may have been a breeder, were captured several times within some of the colonies. This trend was more prevalent at Somerset West, where the number of additional large males ranged from 1 to 9 individuals per colony, compared to 1 to 2 individuals per colony at Steinkopf. Genetic analysis was therefore utilised to assign paternity to the most likely male in order to examine two specific aspects of the social biology of the common mole-rat within the general aims of this chapter:

i) To investigate whether the mating system within colonies of *C. h. hottentotus* comprises one main breeding male or a number of related or unrelated resident males, all with the potential to sire young with the breeding female, and

ii) To determine whether paternity analysis would consistently assign paternity to a resident within-colony male. If this was not found to be the case, candidate adult males within all other sampled colonies were investigated as potential fathers, indicating potential extra-colony paternity of litters.

Simulations to obtain *Delta* criteria for assigning paternity with ≥95% confidence were performed separately for the two sample sets. Paternity was assigned using the following criterion: 99% of loci typed, error rate as determined by simulation for each sample; 90% of candidate within-colony males assumed sampled as a result of intense sampling of colonies over several trapping periods; candidate males in addition to the originally suspected breeding male were those weighing >60g (therefore likely to be sexually mature adult males).

Breeder and mother-offspring pairs

Before determining levels of relatedness within and among colony members at the two sites, breeding and mother-offspring pairs (morphologically identified breeding females and cohorts of small juveniles; <40g and therefore most likely to be that seasons young within a colony) were resolved using CERVUS v2.0 (Marshall *et al.* 1998). Genetically identified mother-offspring pairs were used in both the analysis of paternity of juvenile cohorts and for the calibration of relatedness estimates.

Estimates of relatedness

Relatedness is described as a function of the proportion of genes identical by descent (IBD) between pairs of individuals (Grafen 1985). It is usually estimated by path analysis of pedigrees (Cannings and Thompson 1981) such that parent-offspring pairs and full sibs are characterised by r=0.5 and half sibs by r=0.25, cousins by r=0.125 etc., where r is interpreted within the framework of Hamilton's concept of inclusive fitness (Hamilton 1964a, 1964b) and is representative of the degree to which two individuals are related. However, pedigree construction is often problematic, particularly when sampling a subterranean species like *C. h. hottentotus* where, for example, assignment of parentage is based on morphological characteristics alone.

In order to complement analysis of kinship by pedigrees, Queller and Goodnight (1989) introduced a method for estimating relatedness from genetic markers based on Grafen's (1985) relatedness coefficient. Termed *R*, the measure is interpreted in terms of the degree to which individuals share allelic identity by descent, and differs from the genetic correlation and regression analyses developed by Pamilo and Crozier (1982), Crozier *et al.* 1984 and Pamilo (1984). Queller and Goodnight's (1989) *R* is symmetric ranging from –1 to +1 and is calculated from the equation:

$$R = [p_{i(-j)} - P_{(-i)}] / [p_{ij} - P_{(-i)}]$$

where $p_{i(-j)}$ is the frequency of an allele over all individuals in group $-i$ but not in the individual of interest j, p_{ij} is the frequency of that allele p in the individual of interest j in group i, and $P_{(-i)}$ is the estimate of the allele's frequency in the population. In order to combine allele frequencies over all loci analysed, the numerator and denominator are first summed over all alleles, loci, individuals in colonies and colonies. Queller and Goodnight's (1989) method has several advantages over others in that it accounts for the variance associated with small sample sizes and enables analysis from the population level to that of pairwise dyads of individuals, this last point being crucial to the study of socially interacting species (Pamilo 1989). In so doing, analyses of relatedness from molecular data provide a way of complementing and often completing pedigrees for further analysis.

RELATEDNESS v5.08 (Goodnight and Queller 1999) was used to calculate *R* by combining data from all seven microsatellite loci to provide a single estimate of relatedness for a number of categories. Due to the degree to which Steinkopf and

Somerset West were genetically differentiated, baseline population allele frequencies were calculated separately for each sub-population. Bias-corrected allele frequencies, where members of the same colony as those being analysed were excluded, were used to calculate *R* so as to prevent underestimation of relatedness as a result of the presence of an excess of shared alleles by descent. In addition, estimates of *R* averaged both within and across all colonies at the two sites were calculated by weighting each colony equally. Standard errors for all estimates were calculated by jackknifing over loci (Queller and Goodnight 1989, Sokhal and Rolf 1981).

Calibration of relatedness estimates

Pairwise estimates of *R*, generated from analysis of individuals at Steinkopf, were used to assess whether the estimates of relatedness based on Queller and Goodnight's *R* (1989) provided an accurate assessment of kinship by producing values close to those expected for known categories of relatedness. Values were calculated for dyads of known kinship, based on both field sampling data and the genetic analysis. Due to the considerable constraints on individual dispersal at Steinkopf (discussed in Chapter 3) the qualitative consistency of the data collected at the site was considered more likely to be representative of expected values of kinship.

Comparison of relatedness estimates at Steinkopf and Somerset West

Relatedness values for a number of different categories were calculated for all colonies and individuals at both study sites and compared for any differences using non-parametric tests (Queller and Goodnight 1989, Zar 1984) within the SPSS v9.0 (SPSS Inc. 1998) statistics package.

Correlation analysis

Spearman's rank correlation analysis of the relationship between colony and breeding pair estimates of *R* and colony observed heterozygosity (H_{obs}), and within colony *R* and colony size was carried using SPSS v9.0 (SPSS Inc. 1998).

Mean colony relatedness was calculated for each sampling period and correlated with the corresponding colony H_{obs} for that sampling period. Similarly, pairwise estimates of relatedness for all breeding pairs were determined for each colony per sampling period and correlated with the corresponding colony H_{obs}.

RESULTS

Paternity analysis

The power of the microsatellite data to resolve paternity at the two sites was found to differ. Simulations within CERVUS v2.0 (Marshall *et al.* 1998) predicted that even if all fathers had been sampled within colonies at Somerset West, only 74% of offspring would have paternity assigned with ≥95% confidence (increases to 100% of offspring with ≥80% confidence). This is in contrast to the simulation results at Steinkopf where 100% of offspring were predicted assignment of paternity with ≥95% confidence. Of the total number of offspring actually assigned paternity at Somerset West, only 30.4% (7/24) were assigned paternity with ≥95% confidence. This is in contrast to Steinkopf where 100% (51/51) of assignments were with ≥95% confidence.

Assignment of paternity was not possible for a number of individuals at both sites. Paternity remained unassigned (Table 4.1) for 4.1% and 5.8% of offspring at Somerset West and Steinkopf respectively. Lack of assignment may indicate either the absence of the true father within the sample set, or insufficient genetic power to distinguish the true father from the first and second most likely fathers due to *Delta* criteria requirements based on differences in their respective LOD scores.

Table 4.1 A summary of patemity assignment within colonies at Steinkopf and Somerset West

Assignment category	*Steinkopf (n=51)*	*Somerset West (n=24)*
Assignment to within-colony male:	72.6% (n=34/51)	70.8% (n=17/24)
i. I.D'd breeding male	70.5%	17.6%
ii. different within-colony male	29.4%	82.4%
Assignment to extra-colony male (ECP)	21.5%	25%
Assignment not possible	5.8%	4.1%
Predicted resolving power of loci across sampled colonies[1]	100% of offspring with 95% confidence	74% of offspring with 95% confidence

[1] Percentage of offspring for which CERVUS v (Marshall *et al.* 1998) simulation predicts, based on loci sampled, assignment of parentage with 95% confidence

Assignment of paternity to a within-colony male:

A total of 72.5% and 70.8% of offspring analysed at Steinkopf and Somerset West respectively were assigned patemity to a within-colony male (Table 4.1). Of the offspring at Steinkopf whose patemity was assigned to a within-colony male (n=34), 70.5% (24/34) of these were sired by a male originally identified as the breeder based on size criteria. Therefore, 29.4% (10/34) of these offspring were assigned paternity to a male, other than the assumed breeder, who was present within the colony during the same sampling period. The majority of these males were only slightly smaller, weighing ≥5g less than the originally identified breeding male. In one case however, a cohort of juveniles were assigned paternity with ≥95% confidence to a male who was 32g less, and unrelated to, the largest male present within the colony. At Somerset West however, a number of large potentially unrelated males characterised the composition of colonies, and breeding male status, during the mark-recapture study was generally attributed to the largest of these. Genetic analysis revealed that of the offspring assigned paternity to a within-colony male (n=17) only 17.6% (3/17) of these assignments were to the largest identified male within a colony. Therefore, assignments were generally to an additional large within-colony male (14/17), reflecting the increased presence of large potentially unrelated males within colonies at Somerset West. These males represented a range of weights, weighing from 2-40g less than the heaviest male.

Assignment of paternity to an extra-colony male:

A total of 21.5% and 25% of offspring analysed at Steinkopf and Somerset West respectively were assigned paternity with at least 80% confidence to an extra-colony male, indicating extra-group paternity of litters (Table 4.1).

Relatedness estimates

Calibration of relatedness estimates.

Queller and Goodnight's *R* produced estimates of relatedness in the range expected for dyads of known kinship (Table 4.2). Values for mother-offspring pairs R_{obs} = 0.40 ±0.01 and full siblings R_{obs} = 0.44 ±0.01 were in effect comparable to relatedness estimates expected for outbred, diploid first order relatives (R_{exp} = 0.5). Similarly estimates of relatedness between unrelated individuals were found to be as expected. R_{obs} = 0.03 ±0.009, R_{exp} = 0. Data from the colonies sampled at Steinkopf did not provide the opportunity to generate an estimate of relatedness for half siblings. It appears, therefore, that estimates of Queller and Goodnight's *R* based on the selected samples, provide an accurate assessment of expected kinship values within *C. h. hottentotus*.

Table 4.2 Relatedness estimates for dyads of known kinship. Number of pairs tested in each category is given in parentheses.

Relationship category	R (±S.E)
Mother/offspring (39)	0.40 (±0.01)
Full siblings (75)	0.44 (±0.01)
Unrelated individuals (680)	0.03 (±0.009)

Relatedness estimates at Steinkopf and Somerset West:

The distribution of pairwise *R* estimates within colonies at Steinkopf and Somerset West (Figure 4.1) was found to be significantly different when using X^2 analysis (X^2 =78.68 d.f. 14, p=0.005). Both sites demonstrated a wide range of pairwise values (Steinkopf *R* = -0.4 to 1, Somerset West *R* = -0.6 to 1). The majority of dyads at Somerset West were unrelated or distantly related to the degree of second order relatives (*R* ≤0.25), whilst the majority of dyads at Steinkopf were related to some degree (*R* ≥0.1). In addition Steinkopf was characterised by relatively high frequencies of related individuals in the categories *R* = 0.3 to 0.5, indicative of first and second order relatives.

Mean values of *R* among individuals of *C. h. hottentotus* within and between colonies at Steinkopf and Somerset West (Table 4.3) are presented in Figures 4.2 and 4.3. Mean intra-colony relatedness was not found to differ significantly at Steinkopf and Somerset West, where *R* = 0.28 and 0.23 respectively (Mann-Whitney *U*-test, p>0.05). Likewise, among colony relatedness was found to be similar at the two sites, where *R* = 0.0019 and 0.0038 at Steinkopf and Somerset West respectively (Mann-Whitney *U*-test, p>0.05). The two sites did, however differ with respect to the degree to which colony breeding pairs, identified by parentage analysis, were related to one another. Mean estimated *R* among breeding pairs at Steinkopf, *R* = -0.01, was found to be significantly lower than between breeding pairs at Somerset West, *R* = 0.18 (Mann-Whitney *U*-test, p=0.05). Relatedness between breeding pairs at Steinkopf ranged from R = -0.29 to 0.25, comparable to unrelated individuals to second order relatives. Whereas relatedness between breeding pairs at Somerset West ranged from R = -0.2 to 0.72, comparable to unrelated individuals to first order relatives.

Figure 4.1 Proportion of pair-wise R estimates (%) in each relatedness class among all individuals at Steinkopf (ST) and Somerset West (SW)

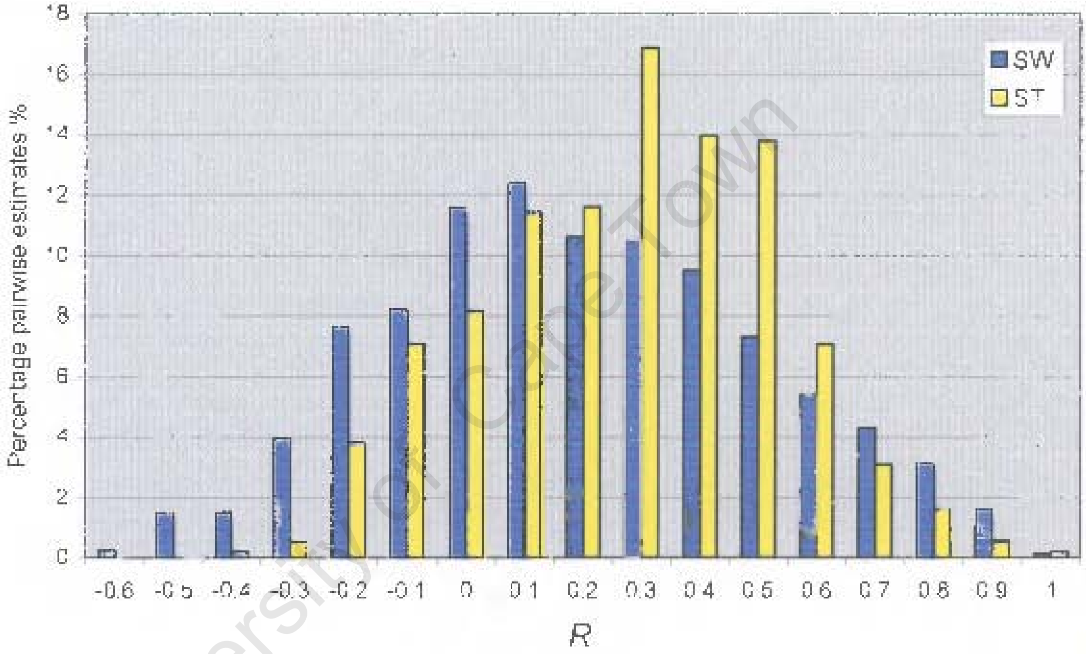
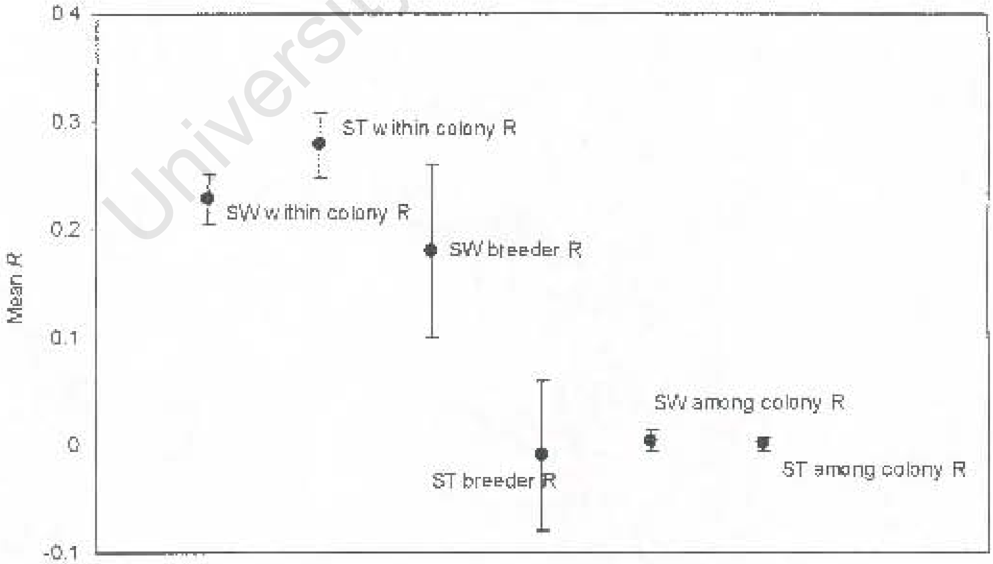


Table 4.3 Summary of mean *R* values ± Standard errors[1] for a number of population categories at Steinkopf and Somerset West.

Category	*Steinkopf*	*Somerset west*
Overall intra-colony *R*	0.28 (±0.03)	0.23 (±0.02)
Overall inter-colony *R*	0.0019 (±0.01)	0.0038 (±0.001)
Breeding pair *R*	-0.01 (±0.07)	0.18 (±0.08)
Intra-colony *R* - females	0.30 (±0.04)	0.23 (±0.03)
Intra-colony *R* - males	0.31 (±0.05)	0.21 (±0.04)
Inter-colony *R* - females	-0.02 (±0.02)	0.01 (±0.01)
Inter-colony *R* - males	0.03 (±0.03)	-0.01 (±0.01)
Intra-colony males V females *R*	0.22 (±0.04)	0.25 (±0.01)
Inter-colony males V females *R*	0.005 (±0.006)	0.002 (±0.009)

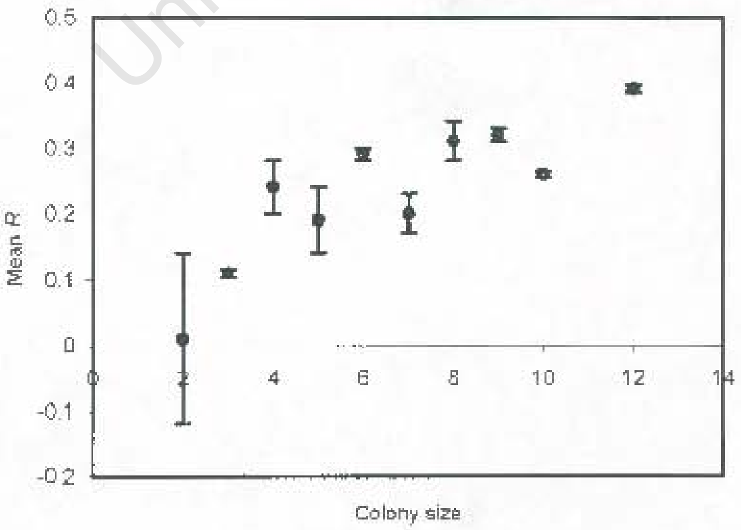
[1] Standard errors calculated by jacknifing over loci.

Figure 4.2 Mean estimated within and between colony relatedness (±SE) for all individuals and breeding pairs at Steinkopf (ST) and Somerset West (SW). Standard errors calculated by jacknifing over loci.



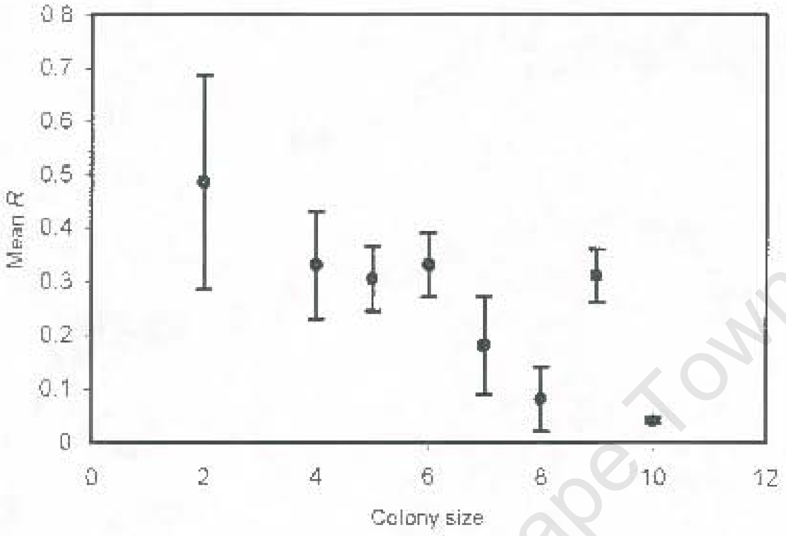
The relationship between colony size and mean colony *R* was analysed using Spearman's rank correlation (r_s) across colonies at both sites and by combining all colonies from Steinkopf and Somerset West to assess whether colony size and mean colony *R* are correlated in *C. h. hottentotus*. When analysed independently, the two study sites were characterised by diametrically opposing relationships between colony size and mean colony *R*. A positive relationship between colony size and relatedness was expected as a function of increased kinship within larger colonies, representative of breeding pairs and several generations of offspring. Indeed this was found to be the case at Steinkopf (Figure 4.4) where correlation analysis indicated a significantly positive relationship between mean colony *R* and colony size (r_s 0.83, $p<0.01$). Contrary to this, correlation analysis at Somerset West (Figure 4.5) indicated a significantly negative relationship between colony size and mean colony *R* (r_s -0.79, $p<0.05$). Consequently, when analysed over all colonies from both study sites (Figure 4.6), correlation analysis failed to detect any relationship between colony size and mean colony *R* (r_s 0.304, $p=0.39$).

Figure 4.4 Variation in mean *R* (±SE) within colonies as a function of colony size at Steinkopf. Regression line equation shown. Standard errors calculated by jackknifing over loci.



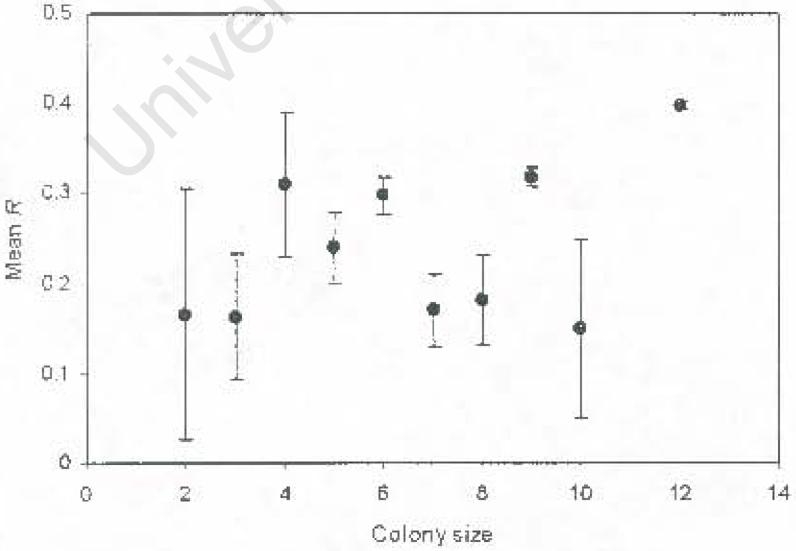
$y = 0.034 + 0.029x$
$r_s = 0.83$ $\alpha=0.05$
p 0.003

Figure 4.5 Variation in mean *R* (±SE) within colonies as a function of colony size at Somerset West. Regression line equation shown. Standard errors calculated by jackknifing over loci.



$y = 0.54 + -0.044x$
$r_s = -0.79^{*\ \alpha=0.05}$
p 0.02

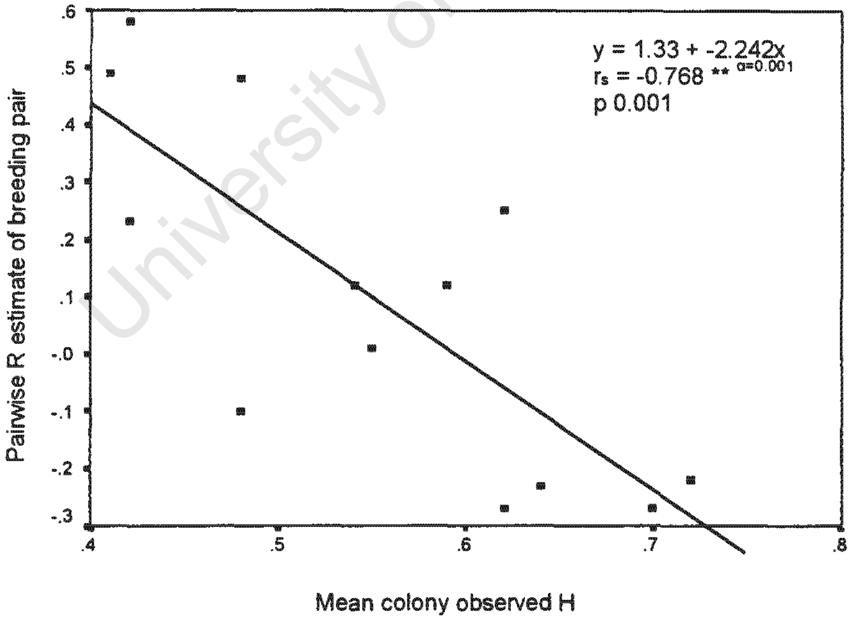
Figure 4.6 Variation in mean *R* (±SE) within colonies as a function of colony size over all colonies sampled. Regression line equation shown. Standard errors calculated by jackknifing over loci.



$r_s = 0.304$
p 0.39

In order to assess the degree to which relatedness among individuals within colonies may be responsible for the lower H_{obs} within colonies at Somerset West (discussed in Chapter 3), the relationship between mean colony *R* and H_{obs}, and mean breeding pair *R* and H_{obs} (Figure 4.7) was examined using Spearman's rank correlation analysis on all sampled colonies and breeding pairs. There did not appear to be any relationship between the degree by which individuals within colonies were related and colony H_{obs} (r_s -0.16 p=0.29). However, there was a significantly negative relationship between breeder pair *R* and colony H_{obs} (r_s -0.76-7, p=0.001) suggesting that colonies at Somerset West, characterized by having relatively low colony H_{obs}, were founded by outbreeding individuals that appear to have an increased chance of sharing allelic identity by descent.

Figure 4.7 The relationship between breeding pair *R* and observed heterozygosity H_{obs} within colonies of *C. h. hottentotus*. Regression line equation shown on graph.



DISCUSSION

Results pertaining to both paternity analysis and genetic relatedness are presented in this chapter. These analyses were performed in order to describe the genetic mating system of *C. h. hottentotus* and discuss its consequences within the context of the detailed behavioural, physiological and demographic accounts of the species.

Marker resolution for paternity assignment

The degree to which paternity could be assigned with confidence differed at the two study sites. Simulations within CERVUS v2.0 (Marshall *et al.* 1998) suggested that had all potential fathers been sampled, 100% of offspring assigned paternity at Steinkopf would be assigned with ≥95% confidence. Observed assignment was in agreement with this estimate, where all individuals assigned paternity, were assigned with ≥95% confidence. At Somerset West simulations suggested that despite the sampling of all potential fathers, only 75% would be assigned paternity with ≥95% confidence. Observed assignment, however, differed from prediction, as only 30.4% of assignments were with ≥95% confidence. The majority of assignments at Somerset West were therefore only with ≥80% confidence, which assumes that 1 in 5 assignments may be incorrect. It is important to note that equal confidence in the reported pattern of social association is clearly an essential prerequisite for comparative analysis of the genetic outcome of a species' mating system in two geographically distinct populations. Intense sampling of colonies at both sites clearly reduced the likelihood of failing to sample all candidate fathers within colonies. Thus the contrasting ability of the loci to resolve paternity with confidence in these two populations is likely to be consistent with a combination of two factors: i) the variability in genetic variation reported at the two sites, and ii) an underestimation of the numbers of candidate males in the system from unsampled colonies (Taylor *et al.* 2000, Worthington Wilmer *et al.* 1999). Colonies at Steinkopf were characterised by a greater number of alleles both per colony and per locus than colonies at Somerset West. This would act to increase the number of possible genotypes and thereby the polymorphic informative content of the loci, in turn increasing the likelihood of paternity assignment with confidence. This then emphasizes the importance of per locus allele frequencies as opposed to number of loci *per se* for paternity studies (Taylor *et al.* 2000). In addition, population assignment tests (reported in Chapter 3) suggested that a larger number of colony members at Somerset West, than at Steinkopf, were indeed immigrants from colonies that were not included in the genetic sample of the site. Somerset West may therefore be characterised by greater number of candidate males than were included in the genetic sample. This suggests that candidate male criteria required for the simulation may have been incorrect, thereby overestimating the power of the data to resolve paternity with confidence (Marshall *et al.* 1998, Worthington Wilmer *et al.* 1999).

Within-colony paternity assignment

Paternity was assigned to a within-colony male with similar frequencies at Steinkopf and Somerset West; 72.5% and 70.8% respectively. However, assignment to the originally identified breeding male differed substantially. Within colonies at Steinkopf paternity was assigned to the originally identified breeding male, based on size criteria, for 59.9% of offspring, and for 19.7% to an additional attending male. Ecological constraints on dispersal at Steinkopf appear to have enhanced social cohesion in response to increased costs on dispersal and independent reproduction, such that colonies are predominantly composed of a breeding pair and their offspring (Spinks 1998, Spinks *et al.* 2000, this thesis). Immigration of foreign conspecifics does occur but to a significantly less degree than at Somerset West, thereby increasing the likelihood of assignment to the largest resident male. Colonies at Somerset West, where there are fewer constraints on dispersal and immigration of foreign conspecifics is relatively common, were characterised by the presence of a number of large unrelated males. The largest of these males were initially identified as the potential breeder during the mark-recapture study. Paternity assignment, however, was predominantly to an additional male present within the colony, and accounted for

58.3% of assignments. Whereas males assigned paternity within colonies at Steinkopf weighed ≥5g less than the originally identified breeding male, those at Somerset West represented a range of weights. The weights of reproductive males at Somerset West ranged from 2 to 40g less than the heaviest male present within the colony. It is clear that unrelated adult males of similar size (>60g) at Somerset West, all of whom are likely to be sexually mature, potentially compete for mating opportunities with the breeding female. In addition, Bennett (1989) noted that within a complete field-caught colony, the only males observed mating with the breeding females in captivity were those that were the largest. Therefore, assignment with confidence to a male that is significantly lighter than the heaviest males within the colony, and unrelated to the breeding female, may be representative of sneak matings by lower ranking males.

Furthermore Bennett (1989) noted that colonies might support more than one resident breeding male. Genetic analysis of paternity appears to support this theory because paternity, in a number of colonies, was assigned to a number of different males over subsequent sampling periods, all of which were resident within the colony concurrently. Although there did not appear to be a trend in the number of these males, the frequency with which this did occur was greater within colonies at Somerset West. This disparity between study sites may be as a function of the larger number of additional foreign males present within colonies at Somerset West, thereby increasing the chance of breeding male turnover. Unfortunately it is impossible to comment on whether this is mediated via female-mate choice from season to season, which in turn, would have important implications for measures of female reproductive success and skew, and population genetic variation.

Extra-colony paternity

Paternity was assigned with confidence to extra-colony males at both study sites with comparable frequency. All within-colony candidate males were excluded, indicating that offspring were the result of matings between the breeding female and extra-colony males. Twenty five percent of offspring within colonies at Somerset West were found to be the result of extra-colony paternity (ECP), marginally higher than that found within colonies at Steinkopf (21%). This is of interest as the higher rate of both emigration and immigration, coupled with less aggression displayed to foreign conspecifics at Somerset West (Spinks *et al.* 1998) may be expected to facilitate the incidence of ECP. Reduced ecological constraints on individuals may assist the movement of males between colonies, increasing their chances for opportunistic mating with the resident breeding female. In contrast, lower frequencies of ECP would be expected within the arid adapted colonies at Steinkopf as a result of the significantly increased costs and risks associated with dispersal. Comparative levels of ECP at the two study sites, however, do not support these hypotheses. It is, unfortunately, unclear from the mark-recapture data at either site as to whether these ECP males were active dispersers or merely floater males moving back and forth between their resident colonies whilst opportunistically interacting with neighbouring conspecifics. Or whether ECP is more likely to occur during the initial founding stages of new colonies and then become more rare as the colony becomes established and one individual male becomes the dominant breeding male. Furthermore it is unclear whether some of the ECP's were the result of unsampled recently deceased males who had been members of the colony. Therefore conclusions pertaining to the behavioural dynamics by which ECP's are mediated are difficult to make. The results do, however, suggest that resident breeding females may potentially increase their reproductive fitness by mating with a number of different males over subsequent breeding seasons when compared to females breeding with the same male over an equivalent number of seasons. However the dynamics by which this occurs, e.g. whether it is mediated by male or female mate choice or merely by chance, are far from clear and further detailed sampling of all individual movements are required to provide further insight.

Relatedness estimates at Steinkopf and Somerset West

One of the most important consequences of a species' mating system is the influence that two breeding individuals have on genetic variation in the subsequent generation. Genetic relatedness was calculated for a number of different categories in order to compare relative estimates among colonies and individuals at the two sites.

Both study sites were characterised by comparable *R* values for all categories except that of mean breeding pair *R*. Intra-colony R was found to be marginally higher at Steinkopf where constraints on dispersal have resulted in greater social stability. Though both were essentially zero, overall population *R* was found to be marginally higher at Somerset West where dispersal is possible almost all year round. Accordingly, Steinkopf supported a greater number of pairwise *R* values in the range 0.3 to 0.5, whilst Somerset West was characterised by a greater number in the range 0.2 to 0.1. Furthermore, Spinks *et al.* (2000) reports that both males and females at both sites dispersed from their natal system with equal frequency. This is supported by corresponding measures of relatedness among males, females and males versus females at both sites. Comparative analysis of all categories failed to yield significant differences.

Differences in the lineage-based accumulation of *R*, social stability and recruitment at the two sites are reflected in the respective relationships between *R* and colony size. Spinks *et al.* (2000) notes that recruitment to the colony via immigration of foreign adults at Somerset West significantly exceeded that of Steinkopf. Consequently, more than 95% of colony growth at the arid site could be attributed to the birth of offspring. At Somerset West 26% of all recruitment to colonies occurred via the immigration of foreign adults, facilitated by lower levels of aggressive display, and this is reflected in the significantly negative relationship between intra-colony *R* and colony size. Therefore as the colony increases in size as a function of continued immigration of foreign adults to its carrying capacity, mean colony *R* appears to decrease. The relationship between intra-colony *R* and colony size at Steinkopf however was, as expected, diametrically opposite. Severe ecological constraints on dispersal and limited immigration of foreign adults have resulted in the accumulation of lineage-associated relatedness and this is reflected in the significantly positive relationship between intra-colony *R* and colony size. Therefore as recruitment to colony increases by the birth of offspring, mean colony *R* increases accordingly. Furthermore, it is interesting to note that as the two sites displayed similar levels of ECP, there does not appear to be a contrasting effect on mean colony *R*; ECP at both sites does not appear to influence the site-specific relationships between *R* and colony size.

Measures of population genetic variation, reported in Chapter 3, reveal the disparate effects of dispersal patterns at the two study sites. As a result of these site-specific factors, coancestry and allelic IBD appear to be elevated among colonies at Somerset West. Analysis of the relationship between colony H_{obs} and estimates of breeding pair *R* was performed in order to examine the evolutionarily significant consequences of the species' mating system. The results suggest that variation within colonies, as measured by H_{obs}, can be directly attributed to the degree to which breeding individuals are related by chance. It is important to note that *C. h. hottentotus* is an obligate outbreeder. Strict incest avoidance behaviour within colonies is the principal level of control by which the species prevents the loss of fitness associated with true inbreeding among highly related individuals (Bennett 1989). However, local dispersal patterns at Somerset West appear to increase the chance of unrelated individuals sharing a greater proportion of alleles than at Steinkopf. As a result, genetic variation is decreased by the reduced potential for heterozygote genotypes among offspring. This is perhaps further exacerbated at Somerset West by the occurrence of ECP, such that reduced reproductive skew among males may act to increase the probability of allelic IBD in subsequent generations. This is in contrast to the effect of ECP at Steinkopf where genetic variation may be facilitated by its occurrence.

CONCLUDING REMARKS

Clutton-Brock (1989) notes that mating systems are best viewed as the result of reproductive strategies of individuals rather than populations or species. This is because individuals are driven to maximize personal and reproductive fitness whilst keeping costs to a minimum. There are clearly many costs and benefits to ECP in behaviourally monogamous mammals. Indeed both male and female common mole-rat individuals would appear to benefit from ECP; females increase reproductive fitness both by breeding consistently over a number of seasons. The probability of survival of their offspring is increased, as colonies are already established entities with a number of helpers present. Males also increase their reproductive fitness with minimal costs via ECP, irrespective of whether they are actively dispersing in search of a mate or merely floater males looking for mating opportunities away from their natal burrow systems. This is because they have an increased chance of potentially becoming the breeding male in an already established colony if the previous breeder has died, or merely increasing their reproductive fitness by siring young within a colony to whose maintenance they do not contribute to.

A number of the important evolutionary consequences of a species' mating system are well illustrated by these two sub-populations of *C. h. hottentotus*. Whilst dispersal patterns, gene flow and social stability have been revealed to differ in response to site-specific ecological conditions, the mating system of the species appears to be similar. Corresponding levels of both within- and extra-colony paternity were revealed by parentage analysis, contributing to concordant measures of relatedness for all but one category. In addition, analysis of genetic relatedness, specifically that of breeding pairs, appears to account for the lower levels of H_{obs} within colonies at Somerset West. Such that the increased probability of sharing alleles by decent, facilitated by localised dispersal patterns, is manifest in decreased levels of H_{obs} when compared to colonies at Steinkopf.

The results reported in this chapter describe some of the genetic consequences of the mating system of *C. h. hottentotus*. Of particular interest is that extra-colony paternity appears to be characteristic of the species, and was found to occur at comparable frequencies within colonies at the two study sites. The identification of extra-colony paternity within *C. h. hottentotus* provides further evidence that its incidence may be more widespread, than originally thought, in species conventionally described from field and laboratory studies as displaying social monogamy.

CHAPTER 5

General Synthesis and Conclusions

A number of well-studied species, ranging from invertebrates to mammals, demonstrate high levels of intraspecific variation in social behaviour and organisation within and among populations. Facultative sociality is reported in species as diverse as social carpenter bees *Xylocopa spp.* (Stark 1992, Hogendoorn and Velthuis 1993), ant-eating chats *Myrmecocichla formicivora* (du Plessis *et al.* 1995), European badgers *Meles meles* (Kruuk and Parish 1987, da Silva *et al.* 1993) and wild rabbits *Oryctolagus cuniculus* (Cowan and Garson 1985, Fuller *et al.* 1997). Variability in social organisation may be maintained by natural selection, such that species are characterised by genetic and developmental systems that enable the expression of solitary or social behaviour in response to temporally varying environmental conditions (Eickwort 1996, Wcislo and Danforth 1997).

Spinks' intraspecific study of social structure and dispersal within two populations of the common mole-rat (Spinks 1998, Spinks *et al.* 2000) demonstrated that increased environmental constraints on dispersal in arid habitats, relative to a mesic habitat, promote a greater degree of social cohesion between individuals within colonies. In so doing, the study provided further support for the aridity food-distribution hypothesis as an explanation for the variation in sociality observed across species of the Bathyergidae (Ebensperger 2001).

The main aim of this thesis was to address the population genetic consequences of this variation in social structure in *C. h. hottentotus* using a suite of microsatellite markers. Within this framework, I have attempted to investigate a number of hypotheses relating to the relationship between gene flow, dispersal and genetic variation, the roles of mutation and drift in promoting genetic structuring, and the role of genetic parentage in the species' mating system. The immense value of a study such as this is that by analysing gene flow and its attributes in relation to the spatial scale over which the populations differ, we are provided with a link between the evolution of the species and its ecology (Bohonak 1999). Indeed Bossart and Prowell (1998) note that a combination of the direct assessment of movement among contemporary population units and its analysis at the genetic level provides the only valid approach to the interpretation of gene flow in an ecological context.

Social structure, dispersal and the genetic properties of populations

Many socially structured species are characterised by high levels of both within-group genetic relatedness and among-group genetic variance as measured by F_{st} (Chesser 1983, Dobson *et al.* 1997, 1998, Painter *et al.* 2000, Pope 1992). Indeed, over time, genetic differentiation between social groups results in an increase in relatedness within groups, relative to that of two individuals drawn from a population at random (Painter *et al.* 2000, Storz 1999). This is exemplified by the highly inbred nature of the eusocial naked mole-rat, *Heterocephalus glaber*, for which mean within-colony relatedness estimates of 0.81 (Reeve *et al.* 1990) and DNA fingerprinting band sharing co-efficients of 0.93 to 0.99 (Faulkes *et al.* 1997b) have been reported. These genetic features of socially organised species are of particular significance to co-operatively breeding species such as *C. h. hottentotus* because of the potential influence population genetic structure has on both the evolution and maintenance of co-operative and altruistic behaviours. This is because the evolution of such traits requires some form of genetic subdivision for their initial establishment within populations (Uyenoyama 1984, Wade 1996, Wade and Breden 1987). The continued persistence of these social traits is then dependent upon the co-evolution of the social and genetic properties of breeding groups (Chesser *et al.* 1993b), mediated by their associated costs and benefits at the different levels of population structure (Chesser 1998).

Within the two populations of *C. h. hottentotus* at Steinkopf and Somerset West, variation in social structure was found to be the result of divergent ecological constraints on dispersal behaviour (Spinks 1998, Spinks *et al.* 2000). Measures of dispersal between social groups are highly informative because they often result in gene flow. Subsequently, gene flow impacts upon the respective roles of natural selection and random genetic drift in determining genetic structure, by influencing the spatial distribution of variation within populations (Barton and Clark 1990). As a result, dispersal plays an important role in the evolution of co-operation and altruism, their maintenance by kin selection, and hence the development of socially structured species (Crozier and Pamilo 1996, Hoelzel 1998, Painter *et al.* 2000, Ronce *et al.* 2000, Ross 2000).

As hypothesised, ecological factors limiting dispersal of individuals at Steinkopf have resulted in reduced levels of gene flow between colonies, relative to those at Somerset West. In accordance with the AFDH, low and unpredictable rainfall at Steinkopf appears to delay the movement of individual mole-rats, relative to that in mesic conditions, resulting in significant sub-structuring at the colony level. The long-term effects of reduced dispersal and colony stability at Steinkopf are apparent in measures of R_{st}, reflecting the divergent role of mutation in promoting sub-structuring at the two sites. Social structuring within colonies of *C. h. hottentotus* at both sites was, however, sufficient to counteract the diluting effects of gene flow on population differentiation as measured by F_{st}. This is as a result of the spatially localised accumulation of lineage-specific allelic similarity that is characteristic to socially structured species (Chesser 1991b). Reported measures of F_{st} therefore accurately reflect the influence of current population dynamics on changes in allele frequencies at the two sites.

Colonies at Steinkopf and Somerset West were found to differ significantly in both allelic richness and heterozygosity. Whilst Steinkopf was characterised by a greater number of alleles and an excess of heterozygote individuals, Somerset West was found to support fewer alleles and an excess of homozygote individuals. Castric *et al.* (2001) report on the potential effect that extrinsic features may have on dispersal and gene flow in the brook charr *Salvelinus fontinalis*. Analysis of 30 separate populations demonstrated the impact local landscape features have in shaping genetic diversity, both allele richness and measures of heterozygosity, at various levels of the population. Within *C. h. hottentotus* highly localised dispersal patterns in response to colony and food resource densities appears to have promoted the accumulation of allelic identity by descent (IBD) among individuals at Somerset West. As a result, correlation analysis indicated an unexpectedly negative relationship between mean monthly rainfall and colony H_{obs} across the species range. The causes and consequences of this are discussed in further detail in the following section.

Analysis of paternity, mating systems and relatedness

A species' mating system contributes directly to the arrangement of genetic variation within and among individuals and social groups (Goosens *et al.* 2001). This is because the degree to which mated pairs share genetic similarity is as a result of the combined influence of long-term mating patterns, dispersal behaviour and effective gene flow (Peacock and Ray 2000). This relationship is evident in the significantly negative correlation that exists between H_{obs} within colonies at Steinkopf and Somerset West and genetic relatedness of breeding pairs. Subsequently, an excess of homozygote individuals within colonies at Somerset West appears to be the result of apparent increased genetic relatedness between breeding pairs. Subsequently, this has direct bearing on the establishment of genetic variation within the Somerset West population and its ensuing spatial arrangement. Within *C. h. hottentotus* the effect of this important dynamic is evident in the reduced levels of genetic variation and positive measures of F_{is} within colonies at Somerset West. Both the incidence of decreased allelic diversity and heterozygosity appears to be the result of highly localised dispersal patterns in response to colony and food resource densities. As a result unrelated individuals have an increased chance of sharing a greater proportion of alleles than those at Steinkopf. Long term spatial isolation of genetic lineages and the rarity of dispersal events at Steinkopf has promoted genetic variation within individual mole-rats, thereby reducing the probability of breeding pairs sharing an excess of alleles by descent. It is important to note that this apparent 'inbreeding' within colonies at Somerset West is simply the result of chance allelic IBD as *C. h. hottentotus* is an obligate outbreeder, displaying strict incest avoidance behaviour within colonies.

Paternity analysis of offspring indicated that extra-colony paternity (ECP) does indeed occur within colonies of this socially monogamous mammal. Of particular interest is that it was found to occur with corresponding frequencies within colonies at both study sites. Taylor *et al.* (2000) report that variation in the structural dynamics of widely distributed species is often reflected in corresponding flexibility in their mating systems. Within *C. h. hottentotus* however, the variation in social stability and dispersal dynamics that enable the species to adapt to the range of environmental conditions in which they occur is not reflected in a flexible mating system. Molecular analysis of population structure and parentage reported within this thesis indicates that the social unit of *C. h. hottentotus* is not identical to that of the reproductive unit. Instead *C. h. hottentotus* appears to be characterised by a mating system that encompasses both social monogamy and genetic polygamy across the broad ecological range of the species.

Extra-colony paternity within *C. h. hottentotus* may also impact upon the levels of genetic variation reported at the two study sites. Whilst it appears to contribute to genetic variation at Steinkopf, ECP within colonies at Somerset West appears to further promote the accumulation of allelic IBD.

The incidence of ECP within *C. h. hottentotus* has additional implications for future studies that aim to estimate male and female reproductive success, skew and fitness. This is because analysis of the relative costs and benefits of ECP to either sex should take into account a number of potentially influential behavioural and ecological factors; for example male dispersal behaviour and female reproductive status, potential female mate choice, behaviour-related predation risk and sex-dependent dispersal distances. Analysis of paternity within *C. h. hottentotus* provides additional confirmation that both non-resident and subordinate males of socially structured species can adopt opportunistic mating strategies that overcome the social mating system (reviewed in Hughes 1998). These results provide further evidence for the incidence of extra-pair paternity at a higher frequency than originally assumed within apparently socially monogamous mammals, and suggest that behavioural dynamics within wild populations may be more complex than previously perceived. Indeed *C. h. hottentotus* could better be described as being representative of facultative monogamy.

It is clear then that, in addition to mutation and variable local selection, both dispersal patterns and mating systems can influence the spatial arrangement of genetic variation via the processes of gene flow and random genetic drift. This is because both have the potential to reinforce or moderate the spatial aggregation of relatives within socially structured species. In so doing, both directly influence structuring by genetic differentiation.

Climate change and variation in social structure

Regional variation in ecological, behavioural and life history traits of species clearly contribute to their success in a diversity of environments. This is exemplified by the success of *C. h. hottentotus* across its broad ecological range. The AFDH proposes that sociality in mole-rats has evolved in response to a number of ecological constraints associated with arid environments. However, *C. h. hottentotus* is found to be sympatric with a number of strictly solitary species[1] (Bennett and Faulkes 2000). In addition, though rainfall is sufficient to promote dispersal of all potentially sexually active young adults at Somerset West, not all individuals do disperse. This suggests that it is not only constraints on dispersal that ultimately shape genetic structuring within *C. h. hottentotus*, but also the accumulation of genetic relatedness within social lineages. As a result, within-colony relatedness may act as a mechanism whereby selection favours individuals who continue to forego dispersal and independent breeding opportunities, despite their availability. Phylogenetic analysis of the Bathyergidae by Faulkes *et al.* (1997a) has demonstrated the

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[1] These solitary species are *Georychus capensis, Bathyergus suillis, Bathyergus janetta*

relationship between ecological constraints, aridity and the evolution of sociality within the family. However, phylogeographic analysis of *C. h. hottentotus* mitochondrial cytochrome-*b* sequences across its ecological range has not been able to provide a conclusive answer as to whether the species' ancestral origins are associated with arid areas (Bennett and Faulkes 2000). This is further hampered by the rarity of cryptomid remains within the fossil record (D. M. Avery pers. comm.) and the difficulties associated with determining precise estimates of palaeoclimatic conditions in southern Africa (reviewed in Tyson 1999). The application of present-day climatic models to the late Quaternary environment in southern Africa does provide some indication of the changes in rainfall and temperature that the region has experienced. Indeed palaeoclimatic reconstruction of rainfall, temperature and vegetation distributions for a number of periods within the last 25,000 years indicate that major changes to the ecology of southern Africa have occurred at regular intervals. Perhaps most notable is that abrupt changes, often over decades, appear to be a feature of the climatic record for southern Africa (Tyson *et al.* 2001). Additionally, higher temperatures, as a consequence of the present-day rate of global warming, have been predicted over the whole of South Africa. Rutherford *et al.* (2000) predict that by as soon as 2050, the effects of current climate change in South Africa will be apparent in decreased botanical diversity in regions as diverse as the fynbos, Karoo grasslands and coastal forests as a result of increasing aridity. The distribution of *C. h. hottentotus* within mesic areas might then be indicative of strong selective pressures that have favoured sociality in response to long-term climatic change. Indeed their future survival and success, in areas predicted to experience increasingly arid conditions, may be further enhanced by high within-colony relatedness and the evolution of flexible dispersal regimes.

Limitations of project rationale

Despite strong support for the population genetic data presented within this thesis, analysis of only two demographically representative populations of *C. h. hottentotus* inevitably produces problems of replication. This in turn hinders the interpretation of the observed results, in that it brings into question whether the findings are unique to the two studied populations, or global to the species across its ecological range. Accordingly, though current data presented within this thesis is congruent with a number of the assumptions of the Aridity-Food distribution hypothesis, further corroboration or contradiction, by replicating the data within the framework of this study, would be ideal.

CONCLUDING REMARKS

The extent to which variation in social structure and dispersal patterns influences overall genetic structuring in co-operatively breeding mammals, from within the breeding group to the level of the population, is largely unknown. Indeed the intraspecific analysis of population genetic structure within a co-operatively breeding mammal along an environmental gradient cannot to be found within current molecular ecology literature. This thesis has attempted to assess variation in genetic structure as a correlate of substantial differences in the ecology and social systems of two populations of *C. h. hottentotus*. Within the sample populations at Steinkopf and Somerset West social structure and dispersal patterns have evolved in response to divergent ecological constraints on successful foraging and dispersal. These constraints have affected the spatial distribution of genetic variation amongst colonies at the two sites in a complex manner that is influenced by a number of socio-ecological factors. As a result, local population genetic structuring within co-operatively breeding species may have long-term consequences for the evolution of social behaviours. Furthermore, these socio-ecological factors may influence the future distribution of the species in the event of potential changes to future climatic conditions in southern Africa.

Kalisz *et al.* (2001) note that rigorous assessments of the causes and consequences of spatially significant genetic structuring within populations are rare in the literature; despite the central role it undoubtedly plays in the evolution of socially structured species. Furthermore, Kalisz *et al.* (2001) remark that this is the result of a dependence on genetic markers alone for the inference of its causes and consequences. Indeed only a handful of these types of studies have been substantiated by ecological data. This study on *C. h. hottentotus* therefore makes a significant contribution to studies that aim to account for patterns of genetic variation within wild populations in that it is supported by invaluable long-term demographic data.

It is then clear that the combination of behavioural, demographic and molecular techniques within ecological and evolutionary studies, will continue to provide researchers with the impetus to re-examine theories accounting for the evolution of mating systems, co-operative breeding and sociality. This approach promises to be particularly enlightening to the study of subterranean taxa that, by their very nature, impede our direct enquiries into their behavioural and population dynamics in the wild.

CHAPTER 6

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APPENDIX I

Standard buffers used in the isolation and characterisation of microsatellite loci

All buffers as in Ausubel *et al.* (1989) Current Protocols in Molecular Biology.

SOC

0.5% yeast extract
2% tryptone
10mM NaCl
2.5mM KCl
10mM $MgCl_2$
10mM $MgSO_4$
20mM filter sterilised glucose

20 x SSC (stock solution)

3M NaCl
0.3M Na_3citrate.$2H_2O$
adjust to pH 7.0 with 1M HCl

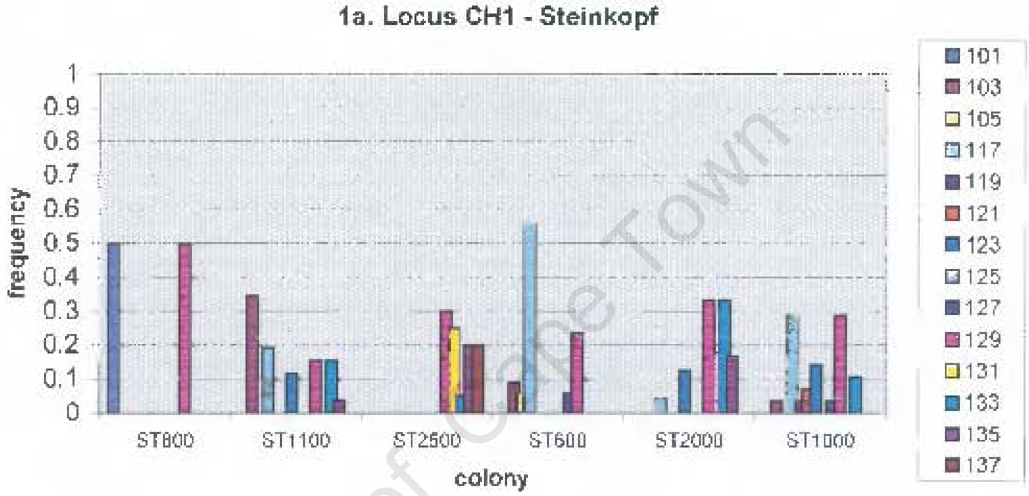
10 x TBE (1litre stock solution)

108g Tris base
55g Boric acid
40ml 0.5M EDTA pH 8.0

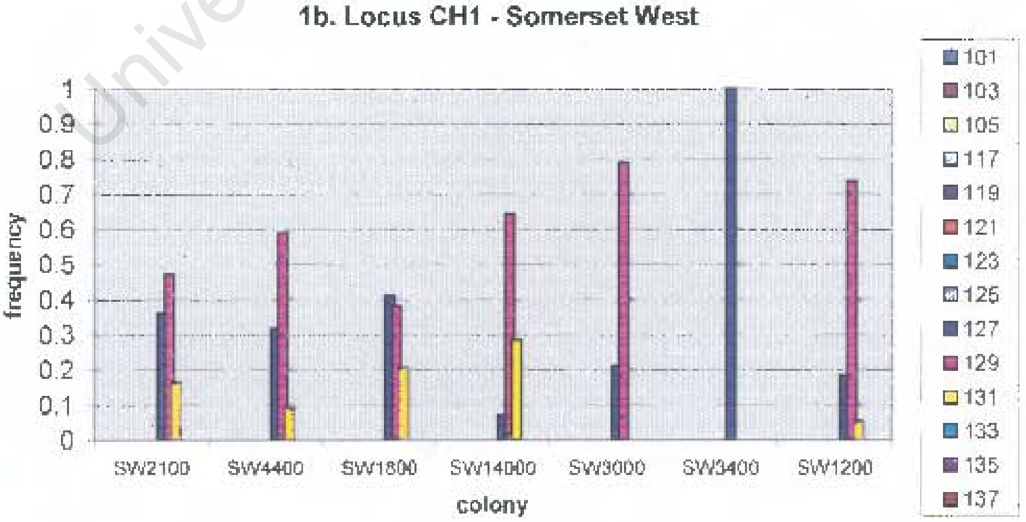
APPENDIX II

Allele frequency histograms for all colonies at Steinkopf and Somerset West, at all loci analysed

Figures 1a and b: Allele frequencies at Locus CH1

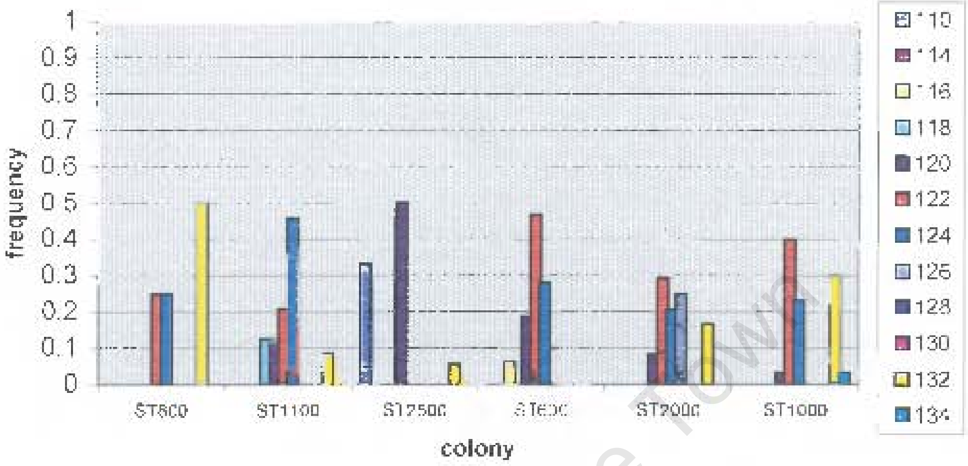


1a. Locus CH1 - Steinkopf

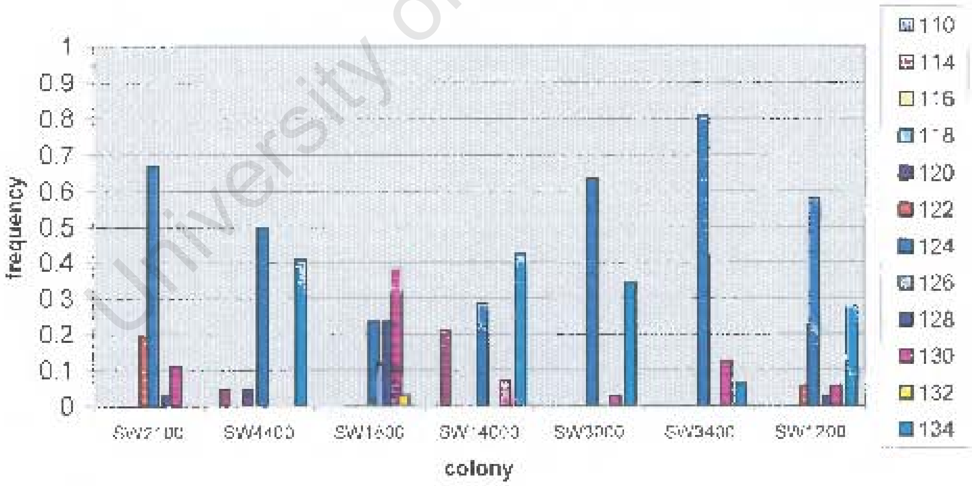


1b. Locus CH1 - Somerset West

Figures 2a and b: Allele frequencies at Locus CH2

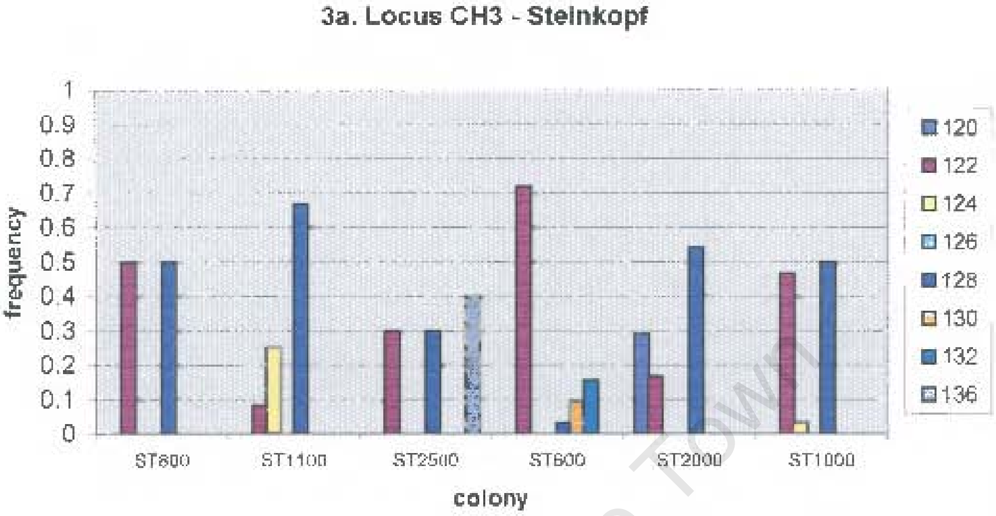


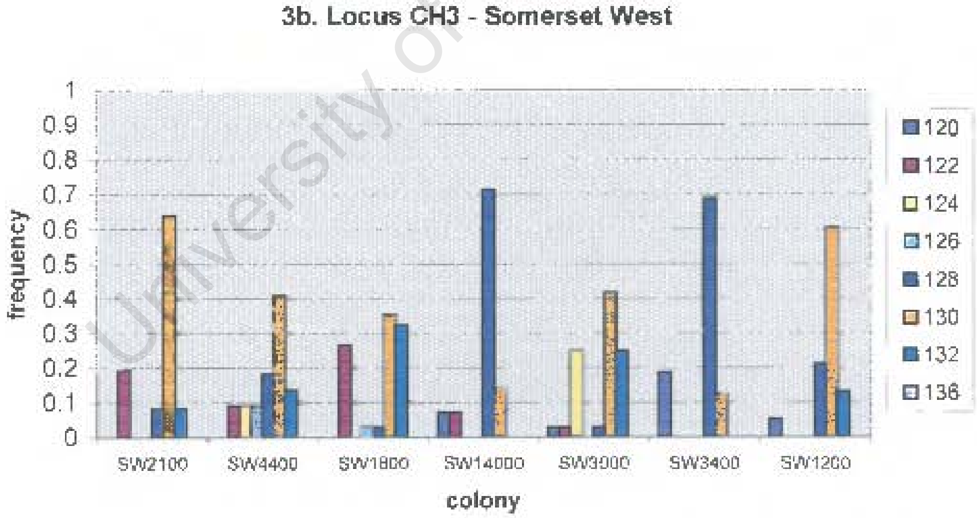
2a. Locus CH2 - Steinkopf



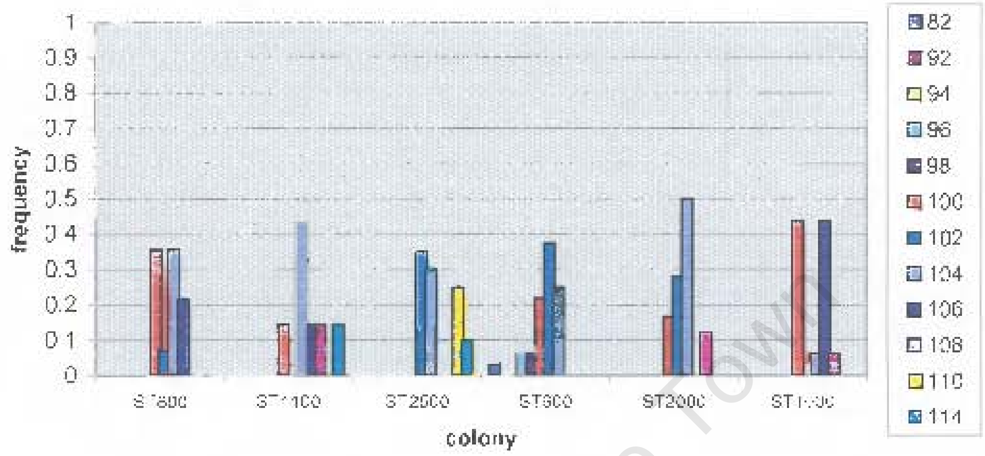
2b. Locus CH2 - Somerset West

Figures 3a and b: Allele frequencies at Locus CH3

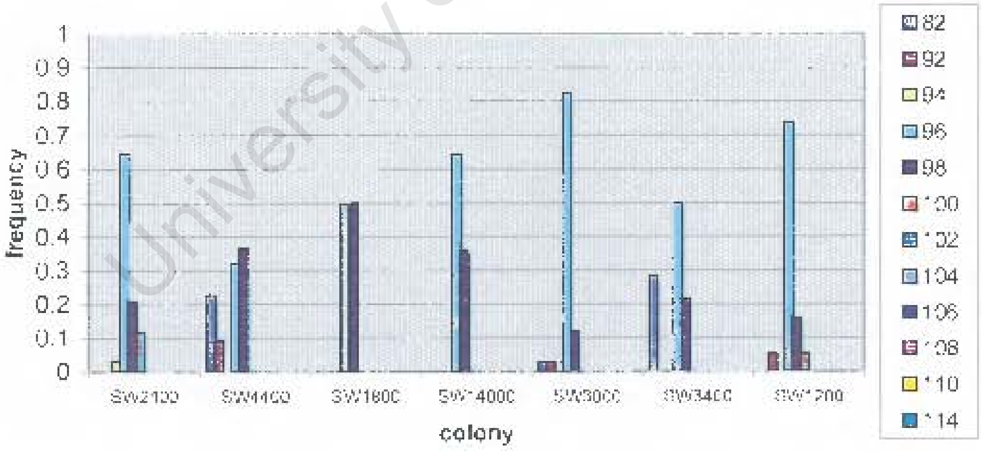




Figures 4a and b: Allele frequencies at Locus CH4

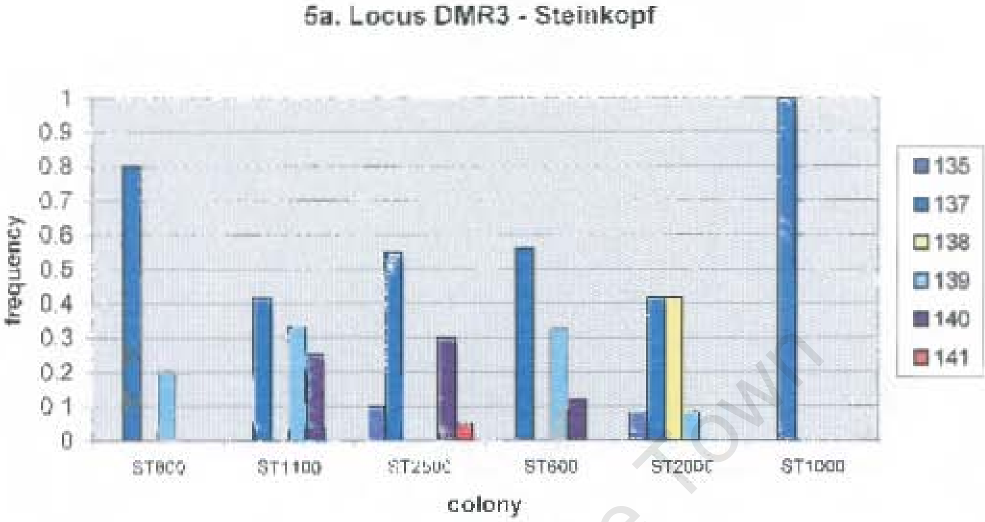


4a. Locus CH4 - Steinkopf

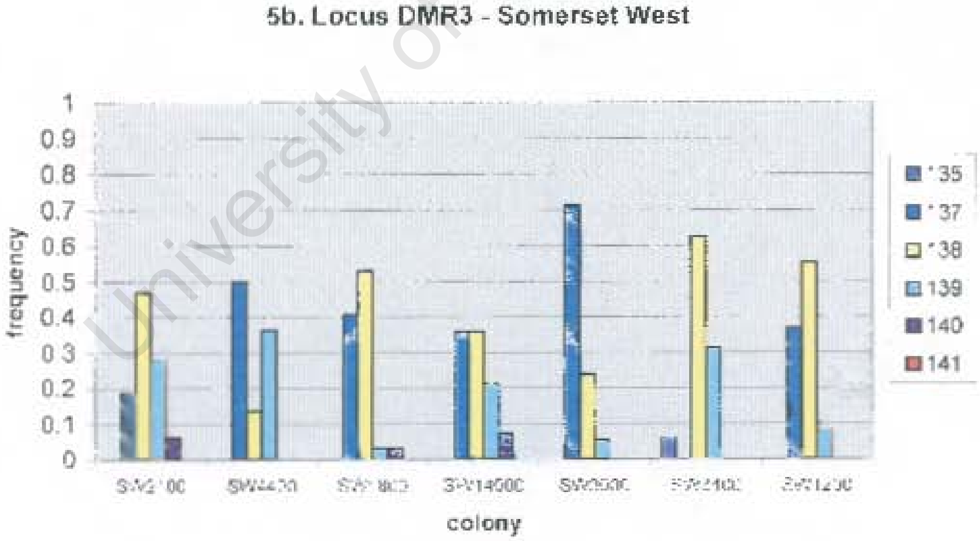


4b. Locus CH4 - Somerset West

Figures 5a and b: Allele frequencies at Locus DMR3



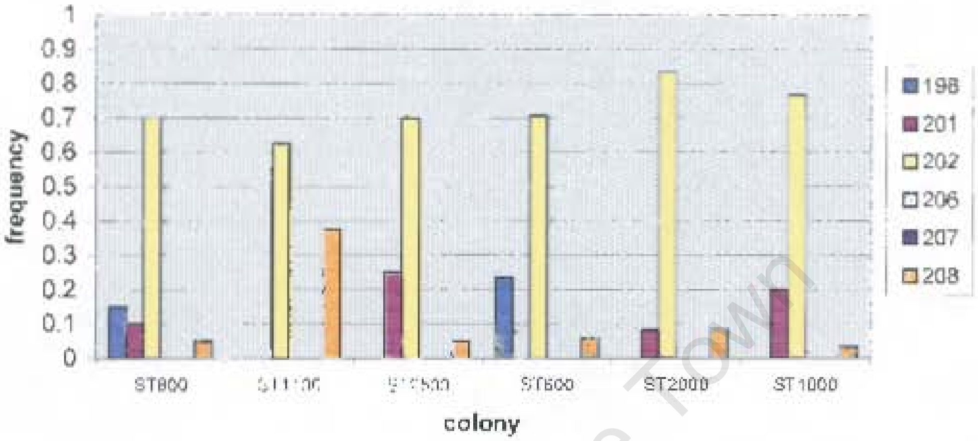
5a. Locus DMR3 - Steinkopf



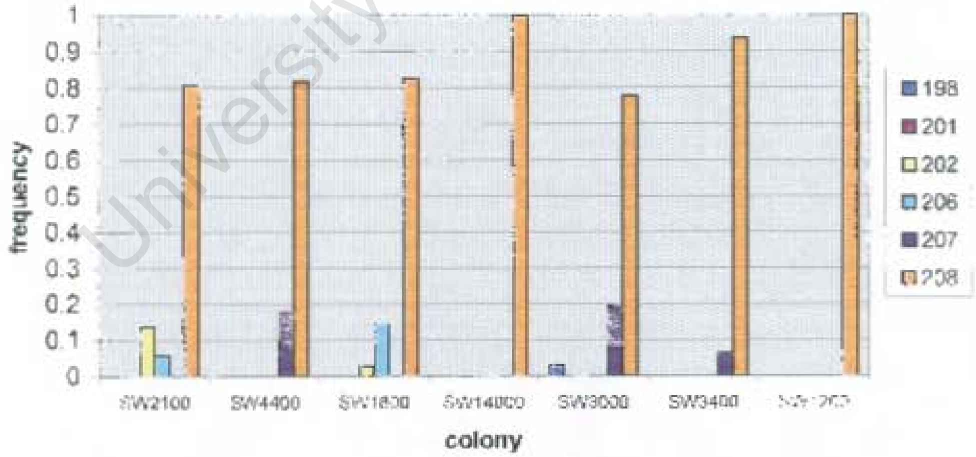
5b. Locus DMR3 - Somerset West

Figures 6a and b: Allele frequencies at Locus DMR4

6a. Locus DMR4 - Steinkopf

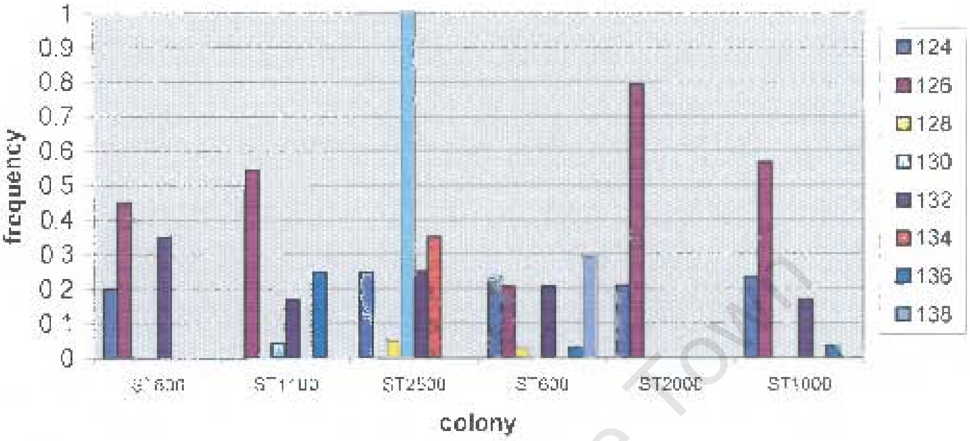


6b. Locus DMR4 - Somerset West



Figures 7a and b: Allele frequencies at Locus DMR7

7a. Locus DMR7 - Steinkopf



7b. Locus DMR7 - Somerset West