

# Chapter 5

## Relatedness

## Abstract

Animal societies can be composed of related and/or unrelated individuals. Field observations are difficult especially in subterranean species. Molecular techniques (genetic markers) are proving indispensable in cases where individuals are difficult to observe, mark or follow in the wild and where intensive fieldwork would have been required to achieve the same result. Social bathyergid groups are interesting in that there is usually a single reproductive pair responsible for procreation in each colony. The reproductive pair is assumed to be unrelated individuals in order to ensure genetic heterozygosity. Other colony members are thought to be the offspring of the original reproductive pair. The genetic composition of assumed breeders and other colony members is investigated in this study. The project was initially undertaken in order to test microsatellite primers developed for other bathyergid species on the study species, *Cryptomys hottentotus pretoriae*. Single phalange samples were used to determine inter- and intracolony relatedness and to identify parental individuals. Of nine microsatellite primers tested, six gave positive and scorable autoradiographs. These primers had been developed for use in related species. A universal mammal primer was also used successfully. Conditions for the successful PCR for each primer are reported. It was found that most individuals in each colony were related on a first order level ( $R=0.5$ ), although estimates of relatedness were exaggerated (low sample size). Breeding males identified morphologically were found not to be the paternal contributor of offspring alleles in any of the colonies. The paternal contributor of alleles in offspring was not identified in any of the colonies and it is therefore assumed that he is not present. Morphological identification of breeding females proved to be reliable as those females were identified genetically as being the maternal contributor to offspring alleles. Thus, colony individuals appear to be the offspring of the breeding female and are full-siblings. Evidence of multiple paternity was found in three of the five colonies. Studies are needed to further elucidate aspects of bathyergid social organization.

## Introduction

## History

The arrangement of a social system in animals is intriguing in terms of the way that the social system is formed, how it functions as a cohesive unit as well as which individuals comprise the social unit. The latter aspect of social organisation is interesting in that social units can be made up of related and/or unrelated individuals. Historically, members of a social unit were identified by observing and recording matings and births in the social unit (which neonates belong to which female/male pair and the fate of those neonates). Over several generations, one could reliably predict which individuals would make up a social unit for a particular species and which would be excluded.

However, the use of these procedures requires intensive fieldwork providing that the species is easy to observe in the field and that specific individuals can be identified in each group. The identification of individuals requires individual-specific recognition signatures (marks, scratches, and notches) or the use of a marking procedure. It is also important to add that the fieldwork has to be intensive as if an animal went missing from a group then, without intensive field observations, it would not be clear if the individual left the group willingly, under duress or whether the animal died.

The use of genetic markers has revolutionised biology in numerous ways. In the case of animal social systems, the use of genetic markers has been very useful in identifying specific individuals as well as determining the relatedness of individuals within specific social units (eg Amos *et al.* 1993, Morin *et al.* 1994, Altmann *et al.* 1996, Girman *et al.* 1997). The use of genetic techniques is being increasingly shown to be advantageous in situations where multiple matings are a part of the species reproductive behaviour. While using genetic marker techniques, it has been found that, in baboons, multiple matings in females are far more frequent than ever predicted (Altmann *et al.* 1996).

## **Incompatibility of bathyergid social research and field observations**

Bathyergid social systems range from being asocial (solitary species) to eusocial (highly organised, group living species) (Jarvis 1981, Bennett *et al.* 1988, Jarvis & Bennett 1993, Faulkes *et al.* 1997). The social system of bathyergids is, however, a continuum (Lovegrove & Wissel 1988, Lovegrove 1991) and there are species that can be classified as being loosely social (occasionally group living but not highly organised). The Highveld mole-rat is a loosely social bathyergid (Moolman *et al.* 1998, this study).

Bathyergids occupy a fossorial, subterranean niche (Bennett *et al.* 1988, Faulkes *et al.* 1997) and it is thought that individuals very seldom (if at all) go above ground. It is therefore obvious that intense field observations would prove impossible. The only method that has been adopted to attempt to follow the changing structure of a group of mole-rats in the field is a long-term study (Bennett & Jarvis, pers. comm. 2000) with much effort and resources being employed on an annual basis. This method is carried out by live-trapping all individuals in known colonies and toe-clipping each individual. Annual comparisons are then made with regard to which individuals are new members of each colony as well as which individuals are still present in each colony. However, it is not known, unless a marked individual is captured elsewhere, whether missing individuals have dispersed or have died. Identifying the breeding female is relatively simple but it is assumed that the largest, heaviest male is the breeding male (Bennett pers. comm. 2000). In several studies on other mammalian species, the breeding male identified by morphology has been excluded as a parent through genetic marker techniques (Morin *et al.* 1994).

## **Advantages of genetic marker techniques**

Employing techniques that involve the use of genetic markers would therefore be of considerable advantage to determining the composition of social units of the social (and eusocial) bathyergid species. The innovative approach of using microsatellite DNA as a genetic marker is of utmost importance to bathyergid social research if anything is to be

discovered regarding the composition of social units of social bathyergids. As well as allowing estimates of relatedness to be calculated within colonies, the microsatellite approach also allows the determination of parentage by exclusion analysis; especially the determination of paternity if maternity is already known (Bruford & Wayne 1993, Amos *et al.* 1993, Morin *et al.* 1994, Altmann *et al.* 1996 and Girman *et al.* 1997)

### **Characteristics of Microsatellites**

Microsatellites are tandem repeat units that are widely dispersed throughout eukaryotic genomes (Queller *et al.* 1993). They are also referred to as variable number tandem repeats (VNTRs) and, as this name suggests, consist of tandemly repeated base pairs (Hamada *et al.* 1982, Tautz & Rentz 1984 and Tautz *et al.* 1986). The length of the repeat can vary from 1 base pair to 10 base pairs, but are generally less than 5 base pairs long (Bruford & Wayne 1993). Microsatellites are often highly polymorphic (Queller *et al.* 1993) due to the variation in the number of repeat units (Bruford & Wayne 1993). They are co-dominant and are inherited in a Mendelian fashion (Jarre 1996). They are considered to be neutral (Jarre & Agoda 1996). The use of microsatellites yields a high information content of genetic data such as sexual selection, mating systems and social organisation (Goodnight & Queller 1999). Microsatellites are important due to their abundance and ubiquitous distribution in animal genomes.

The mode of evolution of microsatellites is highly contentious. One theory is that microsatellites evolve via a stepwise mutation model, which assumes that mutation either increases or decreases the allele size by one repeat unit (Machugh *et al.* 1994 and Estoup *et al.* 1995a) or due to intra-allelic polymerase slippage during replication (Goldstein *et al.* 1995 and Tautz 1989). This model predicts that allelic length can arise from homology (alleles being identical-by-descent) or size homoplasy (convergence, parallelism or reversion) (Estoup *et al.* 1995b). However, the infinite allele model has also been presented to explain the process of the evolution of microsatellites (Shriver *et al.* 1993 and Di Rienzo *et al.* 1994). This model suggests that any allele arising by mutation is different from those previously present in the species (Machugh *et al.* 1994 and Estoup *et*

*al.* 1995a). There is, as yet, little consensus regarding which model is more appropriate to explain the evolution of microsatellites and neither model takes into account the possibility that mutations may not be asymmetrical (Jarre 1996). However, Forbes (1995) suggests that allele frequency distributions fit the stepwise mutation model more than the infinite allele model.

There are a number of drawbacks in using microsatellites. In particular, the quality of information that can be obtained using microsatellites depends upon the consistency and objectivity of gel-scoring, how accurate observed variation is and how representative the results are of the entire population (Queller *et al.* 1993). Another problem is the occurrence of null alleles. Null alleles are non-amplifying alleles (Paetkau & Strobeck 1995) and are recognised by apparent non-inheritance of parental alleles in known parent-offspring pairs (Pemberton *et al.* 1995). The presence of null alleles cause heterozygotes to be scored as homozygotes. This would result in heterozygote deficiencies relative to Hardy-Weinberg expectations (Brookfield 1996 and Pemberton *et al.* 1995). The expected frequency of null alleles being encountered are low in some species (Brookfield 1996). In other taxa, the occurrence of null alleles is relatively high (Pemberton *et al.* 1995 and Callen *et al.* 1993). Using microsatellites from related taxa, although cost effective, requires caution as the use of degenerative primers may increase the probability of encountering null alleles. Varying the conditions of the Polymerase Chain Reaction as well as the concentration of template DNA mitigates this (Malherbe pers. obs. 2000).

The level of genetic relatedness amongst pairs of individuals ( $R$ ) is defined as the expected proportion of alleles that are identical-by-descent that are shared amongst those individuals (Bourke 1997). Two individuals that are related as parent-offspring will share at least one allele per locus (Palsbøll 1999 and Goodnight & Queller 1999). But this could happen by chance in unrelated individuals and is even more likely for siblings (Palsbøll 1999). To minimise such random matches, it is important to use a sufficient number of loci in the study (Palsbøll 1999). The calculation of the estimate of  $R$  for each relationship is carried out by using excellent software available on the internet (KINSHIP and RELATEDNESS, Queller & Goodnight 1989). Previously, estimates of  $R$  were made

using correlation and regression coefficients (Pamilio & Crozier 1982 and Crozier *et al.* 1984). These methods, however, only allow the estimation of R across groups and are affected by small sample size.

### **Characteristics of social bathyergids**

The highveld mole-rat (*Cryptomys hottentotus pretoriae*) lives in a region of seasonal precipitation (summer rainfall region). Rainfall is thought to be sufficient to allow extensive burrowing to take place when the soil is at its most workable. The species is also a seasonal breeder (Janse van Rensburg 2000) with two breeding peaks in a year; May/July and September (Janse van Rensburg 2000). Between one and three pups are born to a single female in each colony (Malherbe pers. obs. 1999, 2000).

As it is thought that in most social bathyergid systems there is seldom more than a single breeding female present in each colony (Bennett *et al.*, 1999; Faulkes *et al.* 1997; Lovegrove 1991), the breeding female can be identified with a high level of accuracy (using morphological characteristics, in particular the presence of teats). This means that through exclusion analysis paternal alleles can be identified and it can be inferred as to whether there is more than one father in the colony as well as which individuals they are. This is extremely powerful in bathyergid social research as the information can be used to challenge the theories of the social evolution. Other information relating to the causes and consequences of social evolution which are obtainable from parentage and relatedness information includes mating systems, dispersal patterns and gene flow among groups (Burland 1998).

The effect of a small population size (Paetkau & Strobeck 1994) is affected by non-random mating and where there are significant differences in the survival of young (Spencer *et al.* 1995). Non-random mating could play a role in social bathyergid populations.

## Aims of the study

As Girman *et al.* (1997) suggest, one could use molecular genetic data to test hypotheses and predictions that follow from observations of social structure. Microsatellite genotype data will be used in order to identify pedigree and other relationships within colonies of the highveld mole-rat, *Cryptomys hottentotus pretoriae*. The identification of relationships within these colonies will also allow an investigation into possible alternative mating systems and social organisation that may become apparent during the analysis.

This component of the project will serve to complement other research undertaken concerning *Cryptomys hottentotus pretoriae*, specifically answering questions regarding social organisation and the mating system that has evolved in this social bathyergid.

Also, it is hoped that results from this study will allow a deeper understanding of how social units are formed in a loosely social bathyergid and how this species actually fits into the continuum of Cryptomid (and bathyergid) sociality.

It was hypothesised that colonies of the highveld mole-rat consist of a single breeding pair (one breeding female and one breeding male) and that all other colony members would turn out to be the offspring of that reproductive pair.



## Methods and Materials

### General

A total of five colonies were used in this study and named uniquely; L03, L01, GRP, GAS, G42S. These colonies consisted of 13, 10,10,10 and 6 individuals respectively. Of these 49 individuals, one sample was not properly preserved (from L03) and two samples gave impure genomic DNA when analysed for genomic DNA concentration with a spectrophotometer. All other samples (the remaining 46) were properly preserved and contained pure genomic DNA. For further details concerning sample individuals (sex, colony status and genotype) see the Appendix.

Breeding females were identified morphologically due to their size and the presence of conspicuous teats. This method of identification was thought to be accurate as in other bathyergid species. Breeding males were also identified morphologically, purely by size. In other bathyergid species, this method of identification has been shown to be generally accurate (eg *Cryptomys damarensis*, Bennett & Jarvis 1988) although being atypical of eusocial societies (Jarvis *et al.* 1994).

### Genomic DNA sampling

Genomic DNA was obtained from single phalange samples or entire toes samples which were obtained from toe clipping techniques as animals needed to be permanently marked in this way for another study. Phalange samples were preserved at  $-40\text{ }^{\circ}\text{C}$  in a solution of dimethylsulfoxide (DMSO) saturated with sodium chloride (NaCl). Due to NaCl saturation, the samples did not freeze at this temperature. The samples were couriered to the University of London and stored at  $-20\text{ }^{\circ}\text{C}$ .

## Genomic DNA extraction

The samples were subjected to whole genomic DNA extraction techniques. Each sample was cut in a sterile environment so that approximately a single phalange or an entire toe was available for use. Extraction was undertaken by a commonly performed extraction technique, outlined in the Appendix.

## DNA concentration determination

The concentration of genomic DNA was determined accurately on randomly selected samples using a spectrophotometer. Of the samples, only two (individuals 1308 and 1333) showed impure extracts but were used in further analysis so as to compare the results of impure extractions with those of pure extractions as well as to see what results an impure extraction gave. However, the results of the scoring of these samples were not used in any analytical procedures.

## DNA amplification

The success of extraction was determined by a 1% agarose gel checking procedure (see Appendix for recipe and method).

Once it was clear that there was sufficient genomic DNA in the sample, the DNA was amplified using the Polymerase Chain Reaction (PCR). Each sample was subjected to separate PCR's for each primer that was tested a total of nine primers being tested. The primers that were tested consisted of three developed for *Cryptomys hottentotus hottentotus* (VB11, DD4 and EF12; Burland *et al.* in press.), five developed for *Cryptomys damarensis* (DMR2, DMR3, DMR4, DMR5, and DMR7; Burland *et al.* in press.) and one universal mammal primer (NCAM; Moore *et al.* 1998). The DMR primers were made using an enriched technique while the EF12 primer was made using a non-enriched technique (pers. comm. Burland 2001). Unfortunately, due to time constraints it was impossible to develop primers specific to the study species. Also, it did

not seem necessary to develop primers specific to the study species as there were a number of primers already available for other closely related species.

Several primers produced excellent results without any modification of original DNA whereas other primers needed PCR conditions and/or DNA dilution(s) to be altered for positive results. Three primers (DMR2, VB11 and DD4) were excluded from further analysis as, after several attempts, their use proved to be unsuccessful.

Final PCR conditions and template DNA concentrations that resulted in positive, scorable banding patterns for each locus are presented in Table 2 (Results section).

### **Microsatellite allele size determination**

The products of the PCR were run out on 6% polyacrylamide gels in order to determine the presence and size of alleles at each microsatellite locus. Polyacrylamide gels are used instead of agarose gels for size determination as an agarose gel has a poor resolution of small fragments (Weber 1990).

Polyacrylamide gels were vacuum dried, placed on autoradiograph film and left for varying time periods depending on the level of radioactivity and the type of film used. The film was processed using an automated process and scored. Size markers were used to determine the absolute sizes of alleles for each locus. For DMR3 a size standard could not be run and the sizes of the alleles were named arbitrarily.

### **Scoring of gels**

The banding of each individual for each locus was compared to its relevant size standard (except for DMR3). For individuals where banding was unclear, the PCR products were run on a separate gel again and usually resulted in the individual being successfully scored. Unclear banding could be due to several reasons (Weber 1990). Strands can migrate at different mobilities or the Taq polymerase enzyme (*Thermophilus aquaticus*)

could add a single non-complementary base to the 3' end of a fraction of the newly replicated models resulting in bands differing by a single base pair. A third reason could be due to Taq polymerase skipping or occasionally adding repeats during elongation. Another situation where scoring could become difficult arises when heterozygotes differ by one base pair and when these individuals are compared to homozygotes with skipping as described above.

### **Calibration of R estimates**

KINPOP software estimates the number of loci necessary to provide consistent and robust estimates of relatedness (Altmann *et al.* 1996, Girman *et al.* 1997). The software accomplishes this by a rarefaction analysis that is carried out in the following way (Girman *et al.* 1997). Relatedness is calculated from the microsatellite genotype data from a single locus selected at random. A second locus is selected at random and, without replacement of the first locus already used, relatedness is re-calculated. This relatedness value is thus based on the microsatellite genotype data of both loci. This process of addition of loci without replacement is continued until all loci have been added. The difference between successive relatedness values is expressed as a function of the total number of loci used. This process is repeated 100 or 1000 times and the mean difference values of relatedness are calculated for each locus addition. A curve can then be constructed of mean difference (dependant axis) versus number of loci (independent axis). If it is observed that the curve asymptotes towards the independent axis, it is evident that even if several more loci are used the estimate of relatedness would not improve significantly for there to be sufficient reason to use more loci. This allows cost and time saving.

In the rarefaction analysis in this study, genotype data was used from 46 individuals from five colonies of the highveld mole-rat. Both 100 and 1000 simulations were carried out in order to determine whether the number of simulations used altered the outcome of the

analysis. The mean difference in relatedness ( $\pm$  SD) between  $n$  and  $n+1$  loci was expressed as a function of  $n+1$  loci for 100 and 1000 repetitions respectively.

### **Identification of mother- and father-offspring pairs**

The KINSHIP 1.3 software package assigns parentage and other hypothesised pedigree relationships to individuals using microsatellite genotype data via exclusions (Goodnight & Queller 1999). Single locus markers (microsatellites) can be consistently scored across gels, and are used for unbiased estimates (for example; estimates of relatedness) and likelihood methods can be used to identify parentage and other relationships (Goodnight & Queller 1999). KINSHIP 1.3 codes each type of pedigree relationship in terms of the probability that the individuals in question share an allele identical-by-descent from the maternal or paternal line (Goodnight & Queller 1999).

It was thus possible to identify mother-offspring as well as father-offspring pairs. The KINSHIP software package uses microsatellite genotypes to calculate the likelihood that a dyad is related by a specified pedigree (Goodnight & Queller 1999). KINSHIP also calculates a likelihood ratio for any pair of hypothesised pedigrees (Goodnight & Queller 1999). The program attaches statistical significance to the results by executing a given number of simulations. Type II error rates (false negatives) are also calculated (Goodnight & Queller 1999). This refers to the rate of dyads which are of the defined pedigree relationship are not identified as such by KINSHIP 1.3 (Goodnight & Queller 1999).

KINSHIP allows one to set hypothesised  $r_m$  and  $r_p$  values for each hypothesis. The  $r_m$  value refers to the probability that half of the focus individual's alleles are identical by descent to the putative mother, while the  $r_p$  value refers to the probability that half of the focus individual's alleles are identical by descent to the putative father. In order to identify mother-offspring pairs, the null hypothesis was set at  $r_m=0$ ,  $r_p=0$  while the primary hypothesis was set at  $r_m=1.0$ ,  $r_p=0$ . This analysis made it possible to determine the number of paternal alleles within a colony (among the mother's offspring which were

identified from the maternal analysis above) that in turn gave evidence in determining whether multiple paternity had occurred.

Once the maternal contributor of alleles had been identified, an extra column was added to the original input file to give identified offspring a label referring to the identity of their mother. This input file was used in the subsequent likelihood analysis that would identify father-offspring pairs. For this analysis, the null hypothesis was not changed from the maternal analysis but the primary hypothesis became;  $r_m = 0$ ,  $r_p = 1.0$ .

### Calculating Pairwise Relatedness

The RELATEDNESS 5.0.7 software package weights each individual allele by its frequency in the population (Girman *et al.* 1997) using microsatellite genotype data. This means that rare alleles receive a relatively higher weight than more common alleles.

Output values from RELATEDNESS 5.0.7 can range from +1 to -1. A relatedness output value of 0.5 is expected for outbred first order relationships such as mother-offspring and full sibling dyads (Table 1). The output of RELATEDNESS is based on the following expression (Girman *et al.* 1997).

$$R_{\text{dyad}} = \frac{\sum \sum (P_y - P^*)}{\sum \sum (P_x - P^*)}$$

Where:  $P_x$  and  $P_y$  = the frequency of each allele in individual x and y respectively.

$P^*$  = the population frequency of each allele excluding the compared individuals.

Using the RELATEDNESS 5.0.7 software package it was possible to calculate pairwise relatedness estimates between each individual (dyad) in each colony.

**Table 1: Expected relatedness estimates for outbred pedigree and other relationships at Hardy-Weinburg equilibrium (after Rasmuson 1993).**

Relationship	Relatedness Estimate
Mother-offspring	0.5
Father-offspring	0.5
Full siblings	0.5
Half siblings (maternal allele shared)	0.25
Half siblings (paternal allele shared)	0.25

### Calculating Mean Relatedness

Mean relatedness was calculated within colonies using the RELATEDNESS 5.0.7 software package. Mean estimates of relatedness were calculated for the following categories of colony members: amongst males, amongst females, amongst offspring, amongst all colony members, amongst mother-offspring pairs, males vs females and mother vs not-offspring,. These individuals were identified from the KINSHIP analyses. Not-offspring refers to those individuals in the colony that were not identified as offspring of the breeding female using KINSHIP analysis.

## Results

### Polymerase Chain Reaction

**Table 2: Results of Polymerase Chain Reaction condition manipulation. The conditions outlined below resulted in successful amplification of microsatellite genomic DNA for further analysis.**

Locus Tested	Target Taxon (Degree of Sociality)	Dilution of original* DNA	PCR Conditions	Number of alleles identified
EF12	<i>Cryptomys hottentotus hottentotus</i> (social)	1 in 10	Initial denaturation @ 94 °C (3 min) 5 cycles of: Denaturation @ 94 °C (45 s) Annealing @ 57 °C (1 min) Extension @ 72 °C (1 min) Then 25 cycles of: Denaturation @ 94 °C (45 s) Annealing @ 55 °C (1 min) Extension @ 72 °C (1 min) Final extension @ 72 °C (10 min)	6
DMR3	<i>Cryptomys damarensis</i> (eusocial)	Original	Initial denaturation @ 94 °C (3 min) 30 cycles of: Denaturation @ 94 °C (45 s) Annealing @ 55 °C (1 min) Extension @ 72 °C (1 min) Final extension @ 72 °C (10 min)	16
DMR4	<i>Cryptomys damarensis</i> (eusocial)	1 in 5 or 1 in 10**	As DMR3	11
DMR5	<i>Cryptomys damarensis</i> (eusocial)	Original	As DMR3	8
DMR7	<i>Cryptomys damarensis</i> (eusocial)	Original or 1 in 10**	As DMR3	8
NCAM	Mammalia	Original	As DMR3	4

\*where original refers to the concentration of DNA after extraction and resuspension in tissue quality water. No further dilution was made.

\*\* sample dependant.



## Parentage Analysis

It was assumed that there was a single reproductive female in each colony. This was verified by morphological characteristics, with the reproductive female having enlarged teats as well as, occasionally, penetrated vaginas. Reproductive females were therefore identified morphologically. Using the reproductive females as a known factor, it was possible to determine whether the reproductive female was the maternal parent of any of the other colony members. In all of the five colonies, it was apparent that most individuals in each colony were offspring of the reproductive female that was identified. Across all five colonies, there were only seven exceptions to this. These were individuals 1284, 1285 (from colony L01); 1316 (from colony GRP); 1326, 1328 (from colony GAS) and 1332, 1334 (from colony G42S) (see Appendix). All mother-offspring relationships that were identified, in each colony, were significant at  $p < 0.001$ . Of the seven individuals that did not appear to be offspring of the reproductive female, six individuals shared alleles with the reproductive female at all but one locus. The seventh exception, individual 1326, only shared maternal alleles at three of the six loci.

The paternal parent was also morphologically identified. This identification was then compared to paternal analyses using the maternal alleles already identified. In all cases, the male that was identified morphologically turned out not to be the breeding male (see Relatedness section). This was evident by the null hypothesis not being rejected in all cases where the putative father was compared with offspring that were identified during the maternal analysis. All of the males morphologically identified as the breeder in the colony were also identified by KINSHIP as being offspring of the breeding female in that colony.

## Paternal allele analysis

The paternal allele analysis showed evidence of multiple paternity for all colonies except L01 and GAS. Multiple paternity was identified using manual exclusion analysis, basing the analysis on the identified maternal alleles. If more than two alleles (excluding those

of the mother) were present in a colony, for a particular locus, it was assumed that the extra alleles came from a second father (Table 3).

**Table 3: Loci identified, for each colony, in which there were more than two paternal alleles (evidence of multiple paternity).**

Colony	Locus giving evidence of multiple paternity	Number of alleles identified not of breeding female (possible paternal alleles) for each locus giving evidence of multiple paternity
LIDIA01	None	-
LIDIA03	DMR3	3
GLENRP	DMR4, NCAM	4, 3
GLENAS	None	-
GLEN42S	DMR3	4

### Rarefaction Analysis

It was shown by rarefaction analysis (Figure 1) that as the number of loci used to calculate estimates of relatedness increases, so the mean difference in the estimate of relatedness decreases. Note that the actual difference between successive additions of loci also decreases. Using five loci to calculate an estimate of relatedness results in the difference in the mean estimate of relatedness being around 0.08 while when using six loci, the difference in the mean estimate of relatedness is around 0.06. The standard deviations related to each estimate of the difference in mean relatedness also decrease as the number of loci being used increase. In this analysis, the estimates were based on whether 100 or 1000 repetitions were used in order to calculate the mean  $\pm$  SD. Whether 100 or 1000 repetitions were invoked, there was negligible differences between the two curves (Figure 1).

## Relatedness

Figure 2 shows that mean relatedness across colonies is very high for most relationships investigated. All relationships investigated had mean relatedness estimates of around 0.6, except for the mother-not offspring relationship. Not-offspring refers to those individuals in the colony that were not identified as offspring of the breeding female using KINSHIP analysis.

The relatedness values estimated are nearer that expected for first-order relatives such as full sibling relationships or mother-offspring relationships (see Table 1). These estimates (except mother-not offspring) are all very close to the value estimated for the mother-offspring relationship (0.66 in this study) that is a first-order relationship. However, pairwise estimates of relatedness ranged from  $-0.1$  to  $0.8$ ; the majority of comparisons however occurred in the  $0.5 - 0.6$  range (62 %). This is what is expected for mother-offspring relationships (first-order relatives).

Similarly, when the relatedness estimates of offspring are compared to each other (Figure 2) it is evident that relatedness is estimated at 0.65. Using pairwise relatedness estimates, a range of relatedness values are obtained from 0.1 to 0.9. However, the majority of pairwise estimates (64 %) lie between 0.5 and 0.7.

When comparing putative mothers to offspring that were identified through KINSHIP not to be offspring of the putative mother ( $n=7$ ), most relatedness estimates occur between 0.4 and 0.6 (67 %) when analysing pairwise relatedness estimates. Mean estimates of relatedness are for mother-not offspring relationships. Most estimates therefore fall in a range typical to that expected for first-order relatives.

All of the estimates of relatedness of male individuals lie between 0.5 and 0.7, with the majority of estimates around the 0.5 relatedness category (Figure 2). The range in relatedness estimates (majority) for females was the same as that for males, although not all estimates were within this range. Several estimates occurred between  $0.8 - 0.9$  and  $0.3$

– 0.4 (Figure 2). The majority of estimates therefore were within the range expected for first-order relationships. Comparing males to females (or vice versa) it is evident that a similar trend exists although the range of the majority of estimates of relatedness appears to expand to 0.4 to 0.7 (Figure 2).

### Possible incidence of mutation and null alleles

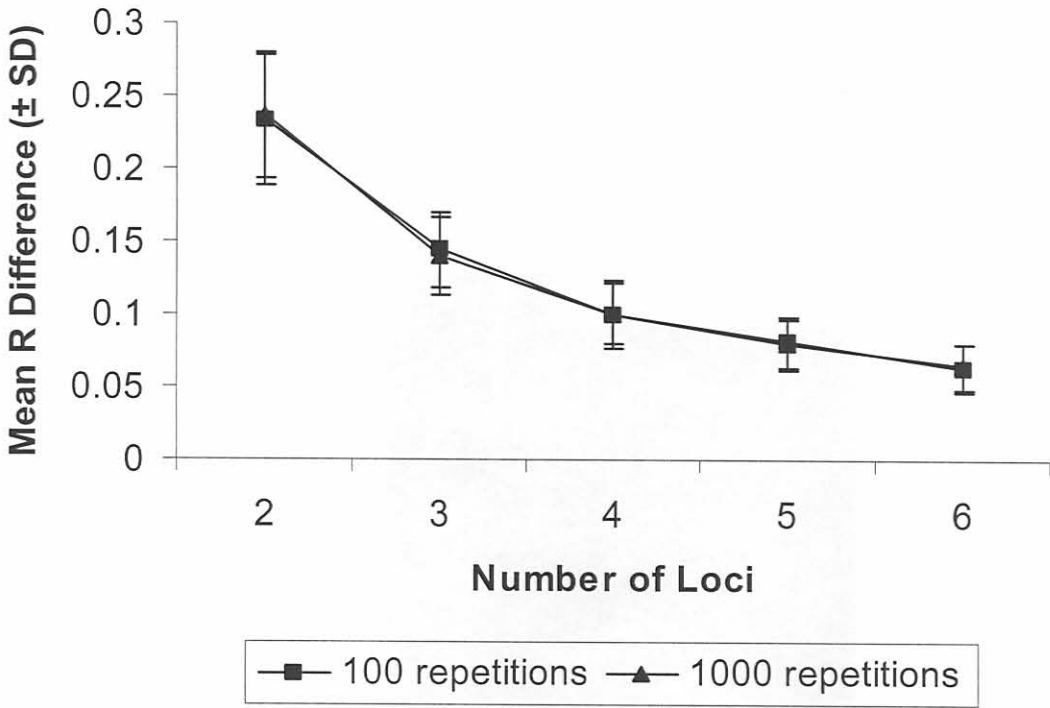
Due to the fact that six of the seven individuals excluded as offspring of the reproductive female shared her alleles at most loci (and returned high relatedness estimates when compared to her), it is possible that these individuals were actually her offspring, but were excluded as a result of a mutation event or a null allele.

**Table 4: Possible explanation for individuals not showing relatedness to the breeding female.**

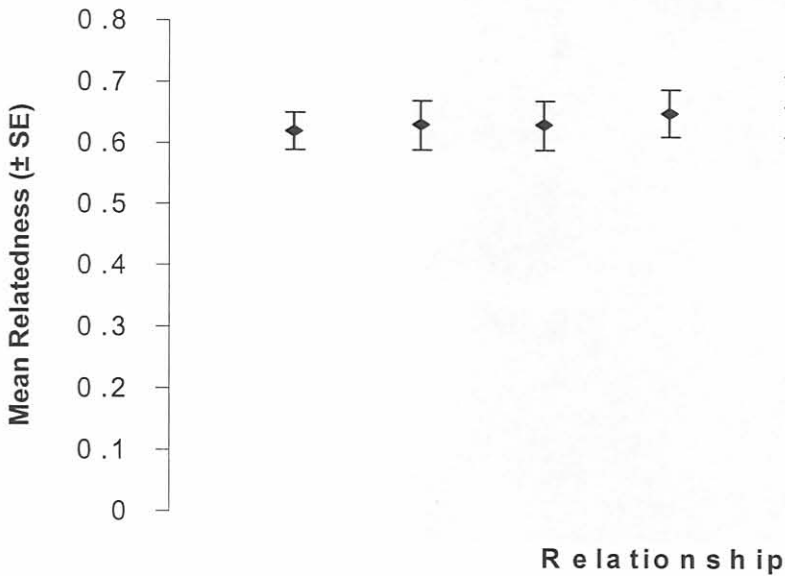
Colony	Individual	Possible reason for disparity (other than being unrelated)	Locus	Alleles involved
LIDIA01	1284	Null allele	EF12	E
	1285	Null allele	EF12	E
GLENRP	1316	Mutation	DMR4	H mutating to J
GLENAS	1326	None	DMR4	K mutating to L
	1328	Mutation		
GLEN42S	1332	Mutation	DMR3	C mutating to D OR D mutating to E
	1334	Mutation	EF12	C mutating to E

It is possible that the genotypes of individuals 1316, 1328, 1332 and 1334 at loci DMR4, DMR4, DMR3 and EF12 respectively are consistent with expectations of allele mutation events. In these cases, the allele that has been scored is only a single base pair different from putative offspring (of the breeding female) genotypes. Individuals 1284 and 1285 had genotypes that appear to have arisen from a null allele belonging to the breeding female whose genotype (at locus EF12) is ‘EE’. Individual 1326 appeared to be very

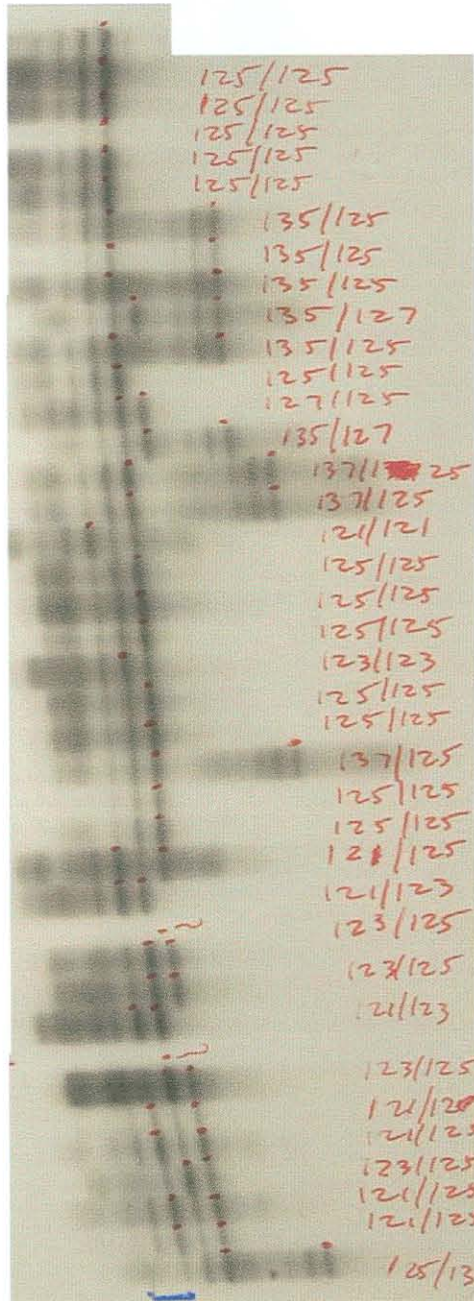
different from the breeding female's genotype; mutation or the presence of a null allele are therefore unlikely. This individual's presence in the colony is interesting.



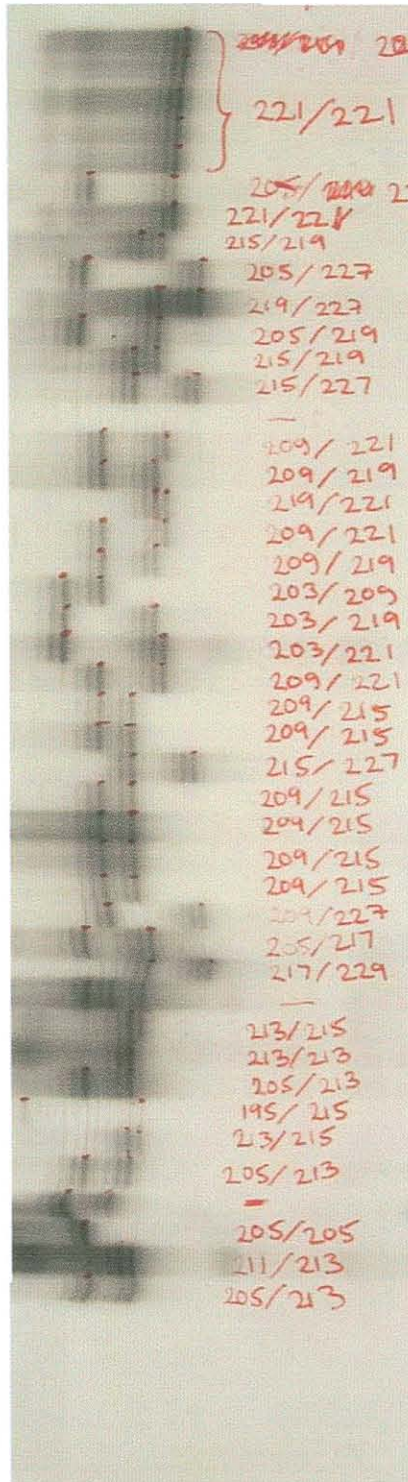
**Figure 1: Results of the rarefaction analysis carried out on the estimates of relatedness in order to establish whether six loci were sufficient to carry out further analysis.**



**Figure 2: Mean relatedness estimates for each type of relationship investigated in this study. All relationships are near that hypothesized for first-order relatives except mother-not offspring relationships.**

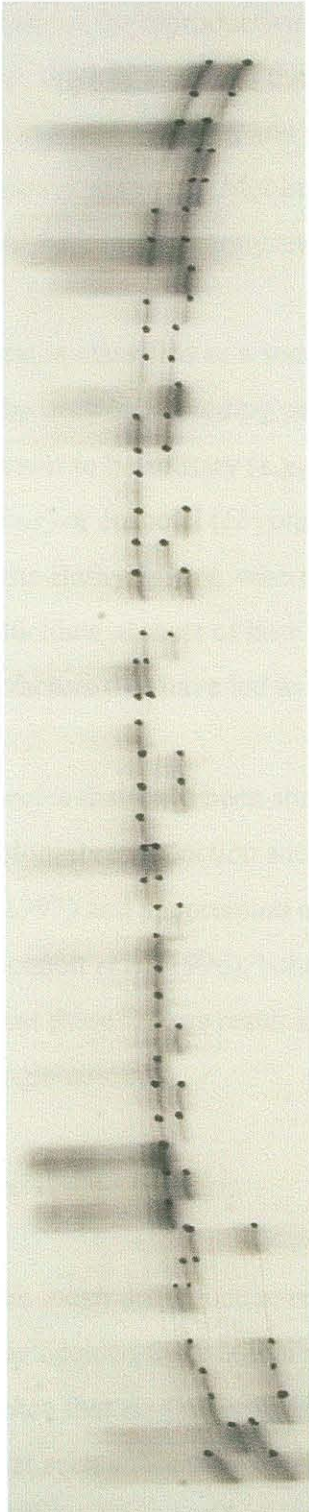


**Plate 1: The resulting autoradiograph for the EF12 primer (*C. h. hottentotus*) which was successfully tested at the PCR conditions outlined in the results section. Most bands (which represent individuals) are clearly scorable resulting in homozygous or heterozygous allele sizes being identified. Size standards were run on separate gels.**



**Plate 2: The resulting autoradiograph for the DMR4 primer (*C. damarensis*) which was successfully tested at the PCR conditions outlined in the results section. Most bands (which represent individuals) are clearly scorable resulting in homozygous or heterozygous allele sizes being identified. Size standards were run on separate gels.**





**Plate 3: The resulting autoradiograph for the DMR5 primer (*C. damarensis*) which was successfully tested at the PCR conditions outlined in the results section. Most bands (which represent individuals) are clearly scorable resulting in homozygous or heterozygous allele sizes being identified. Size standards were run on separate gels.**

## Discussion

Very little is known about the reproductive biology of the highveld mole-rat, *Cryptomys hottentotus pretoriae*. What is known is that the species is a seasonal breeder (Janse van Rensburg 2000) and induced ovulator, and that colonies are made up of a very loose social system/dominance hierarchy (Moolman *et al.* 1998) with no significant correlation in terms of individual rank in the colony and sex or mass.

The highveld mole-rat is classified as a social bathyergid species as it is group living and there is assumed to be only one breeding pair in the colony. Other members of the Bathyergidae are known to be solitary (e.g. *Georychus capensis*), social (e.g. *Cryptomys hottentotus hottentotus*) or eusocial (*Cryptomys damarensis* and *Heterocephalus glaber*). In a family such as the Bathyergidae, where a continuum of sociality exists, it would be very rewarding to elucidate aspects of how social units are comprised and formed in order to understand factors that have led to the evolution of sociality and eusociality.

Other bathyergid species that have been studied to date show that there can be several important cues to colonial reproduction such as incest avoidance (*Cryptomys damarensis*; Rickard & Bennett 1997) and suppression of reproduction (*Cryptomys damarensis*; Bennett 1994 and Bennett *et al.* 1996); behaviourally, chemically or a combination of the two. It is assumed that these factors result in a single breeding pair being responsible for reproduction in a single colony.

### Number of loci used

As the estimate of the mean difference in relatedness estimates started to asymptote at six loci (Figure 1), it was decided that six loci would be sufficient for the purposes of this study. Further evidence that six loci would be sufficient for the study was that the standard deviations at each successive locus addition were becoming negligible and that the number of repetitions above 100 had no effect on the estimates (Figure 1). It was concluded that six loci would provide robust estimates of relatedness.

## Calibration of relatedness estimates

Mean pairwise relatedness estimates suggest that individuals within colonies are related in a way that approximates first-order relatives (parent-offspring or full siblings). Known first order relationships (mother-offspring pairs identified using KINSHIP) were calculated, using RELATEDNESS, to be 0.66. All other relationships investigated (except mother-not offspring relationships) resulted in relatedness estimates very close to this value. Knowing that our data suggests that first-order relationships approximate 0.6 in our samples, it can be assumed that other first-order relationships might also approximate this value.

It can be concluded that most relationships investigated approximate the estimate for relatedness of a known first-order relationship (0.5; Rasmuson 1993, Bourke 1997). The low relatedness estimate for mother-not offspring comparisons may be due to the inclusion of individual 1326 that appears to be very different, in terms of its genotype, to any other colony member.

## Overestimation of relatedness estimates

The overestimation of relatedness estimates can be explained in terms of sample size and assumptions regarding mating system and gene flow.

Although microsatellite primers developed for use on a specific species are considered to be effective in amplifying genomic DNA in other related taxa (Bruford 1999, Schlotterer *et al.* 1991 and Moore *et al.* 1998), three of the nine primers tested resulted in unsuccessful amplification of genomic DNA from the highveld mole-rat. The nine primers were developed from the eusocial *C. damarensis* and the social *C. h. hottentotus*. Thus, only six microsatellite primers were used in this study. Although from rarefaction analyses it appears clear that six loci were sufficient for reliable estimates of relatedness, it also appears that six would be the minimum number of loci acceptable for reliable

estimates of relatedness (Figure 1), as although the mean difference in relatedness estimates is low at six loci (0.06), the curve is only beginning to asymptote. The highveld mole-rat is colonial (Moolman *et al.* 1998 and Malherbe pers. obs. 1999) and it was only possible to investigate five colonies (consisting of 49 individuals). A low number of loci, a low number of individuals and colonies contribute to a relatively low sample size. Furthermore, colonies were caught from geographically separate areas, up to 30 km apart. As above-ground dispersal has seldom been recorded for any social bathyergid (pers. comm. Bennett), it is unlikely that gene flow between areas would be likely. A combination of the low sample size and geographically dispersed sampling may mean that insufficient background data on allele frequencies may be available for these analyses.

It is not likely that these factors affected the results of the KINSHIP analyses, as all mother-offspring relationships were significant at  $p < 0.001$ . However, it is possible that unrepresentative allele frequencies may have affected the relatedness estimates. This is because there is certainty (from KINSHIP analyses) surrounding the mother-offspring relationships being correct and the estimate of relatedness, which is 0.66, should, as a first-order relationship, be estimated as 0.5. The slightly elevated estimate of relatedness for first-order relatives may also be due to the effects of inbreeding (Shields 1983 and Shields 1993). It is apparent that there is evidence of much homozygosity amongst individuals which may be attributed to a mating system where close relatives breed.

Other relationships investigated using the RELATEDNESS analyses included (offspring, males, females, males vs females) all suggest that individuals within colonies are first-order relatives. Although the estimates of relatedness, for the above relationships, are higher than expected (0.5), the estimates approximate that of mother-offspring pairs whose elevated estimate were explained.

The relationship between mothers and individuals not identified as their respective offspring, although also falling in the 0.4 – 0.6 range of estimates, appears to be lower than the expected estimate for first order relatives. Given that the relatedness of first-

order relatives are overestimated in this study, one can assume that this estimate of relatedness is also overestimated. There appears to be individuals within the colonies to which the respective mother is not related ( $n=7$ ). However, reaching this conclusion can be misleading as if one analyses the manual exclusion analysis performed on each individual and locus, it is apparent that the cause of these individuals not being related to the breeding female may be due to the occurrence of null alleles at certain loci or due to point mutation during the polymerase chain reaction. Thus, these individuals may be the offspring of the breeding female but due to errors in the PCR process are evident as unrelated individuals. These individuals may also be the full-siblings of the breeding female.

### **Null alleles and mutation**

Most individuals were identified as being offspring of the reproductive female that was identified morphologically (using results from KINSHIP analyses). The majority of exceptions to this (six of seven individuals) were related to the breeding female, and are either the breeding female's offspring or her full-siblings (using estimates calculated by RELATEDNESS). These exceptions can be explained by the presence of a null allele (individuals 1284 and 1285) or by mutation by a single repeat unit (individuals 1316, 1328, 1332 and 1334). Individual 1326 had a very different genotype to any other individual in the colony and its presence in the colony is interesting. This individual may be an immigrant of the colony; possible a male (although the sex of the individual is unknown) and may be the type of individual that is responsible for multiple paternity in colonies.

### **Origin of paternal alleles**

It appears that the paternal alleles are obtained from males external to the colony, as putative fathers morphologically identified were not responsible for the alleles in

offspring of the reproductive female. As no putative fathers were identified in any of the colonies sampled, the identification of the breeding male by morphological characteristics is not reliable in this species. It would be of great value to investigate this aspect of the species reproduction more intensely in order to understand how future fathers come into contact with the breeding female of each colony, as well as how long they remain in colonies. Multiple paternity may also be occurring within colonies (evident by  $>2$  paternal alleles being identified) although pairwise relatedness estimates suggest that the majority of offspring of the reproductive female are full siblings.

### **Social organisation of colonies of the highveld mole-rat**

Colonies of the highveld mole-rat appear to be made up of first-order relatives, specifically mother-offspring relationships. No paternal contributor to offspring allele combination was identified genetically in any of the colonies that were studied. It appears likely that males may migrate into already existing colonies in order to reproduce. However, analogous to this is the fact that known offspring of the reproductive female appear generally to be full siblings of each other – suggesting that they had been fathered by a single male. Studies dwelling specifically on this aspect of the social organisation of the highveld mole-rat would do much to elucidate intricacies of social mole-rat reproductive biology.

### **The highveld mole-rat's position amongst subterranean rodents**

The highveld mole-rat is expected to resemble other subterranean rodents in that the truly subterranean niche typically has a limited capacity for gene flow among populations (Bennett & Faulkes 2000). Population genetics studies of *Spalax*, a blind, solitary myomorph rodent mole of the Middle East, have revealed that limited gene flow has led to the evolution of numerous local forms (Bennett & Faulkes 2000 and Nevo *et al.* 1995). Along with a co-operative breeding strategy, the influence on genetic components and relationships within populations of the highveld mole-rat is thought to be significant in

terms of the effects these factors have on the genetic relatedness of individuals of the species.

There appear to be two main hypotheses regarding genetic patterns evident in subterranean mammals. The first hypothesis concerns stochastic processes that arise from limited gene flow, fluctuating population sizes and genetic drift (e.g, Sage *et al.* 1986). The second hypothesis (Nevo 1979) concerns niche-width-variation. It is thought that the reduced genetic variation seen in subterranean mammals (when compared with small mammal species living above ground, Nevo 1990) results from the narrow subterranean niche which is stable and predictable.

Knowledge of the genetic structure of populations and within groups is crucial if we are to understand the factors involved in the evolution and maintenance of social systems (Bennett & Faulkes 2000), as the social structure of co-operative breeders can potentially influence the genetic structure of mole-rat populations.

In bathyergid rodents that have varying degrees of sociality and reproductive skew, one would expect varying patterns of relatedness in the different species (Bennett & Faulkes 2000). Three bathyergid species have been studied genetically but only the eusocial naked mole-rat *Heterocephalus glaber* (Reeve *et al.* 1990, Faulkes *et al.* 1990 and 1997) has been studied in terms of its genetics relatedness.

The naked mole-rat (*Heterocephalus glaber*) was shown to have a mean intra-colony relatedness of 0.81. The highest mean coefficient of inbreeding that has been reported for a natural mammal population was found for the naked mole-rat (Reeve *et al.* 1990). Other bathyergids to date have been shown to be obligate outbreeders and a lower mean relatedness is expected.

Studies on both the Damaraland mole-rat and the common mole-rat (*Cryptomys hottentotus hottentotus*) give evidence that does not support the niche-width-genetic-variation hypothesis. The three bathyergid species (including *H. glaber*) show patterns of

genetic variation more explicable in terms of social structure, effective population size and stochastic processes like genetic drift.

## Summary

Although relatedness estimates are slightly higher than expected for outbred first-order relatives, it can be stated with reasonable confidence that colonies of the highveld mole-rat consist of first-order relatives (mother-offspring/full siblings). Colonies are composed of highly related individuals, although it is unlikely that they are simply monogamous family groups, it is more likely that colonies are composed of extended family groups.

The higher than expected estimates of relatedness can be explained by low sample size (in terms of the number of individuals, colonies and loci used in the study), limited gene flow or a hypothesised mating system where close relatives breed.

The identification of a reproductive female in colonies by morphological characteristics is reliable in this species, while the identification of the breeding male is unreliable when using morphological characteristics. The breeding male(s) do not appear to be resident in the colonies; when and where mating takes place is unknown at this time.

Offspring of the reproductive female appear, in most cases to be full siblings, although multiple paternity was identified in a number of colonies. Whether multiple paternity is within or between litters is unknown at this time. Individuals which were identified as not being the offspring of the reproductive female generally are thought to be her offspring or full siblings but were mistyped or had a null allele or had genotypes that differed from the expected alleles by a single base pair (evidence of mutation).



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