

Mitochondrial DNA variability between selected populations
of *Otomys irroratus* (Muridae:Otomyinae)

by

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PREFACE

The experimental work described in this dissertation was carried out in the Department of Biological Sciences, University of Natal, Durban, from February 1991 to December 1993, under the supervision of Dr J.M.Lamb and Mr G-C Contrafatto.

These studies represent original work by the author and has not been submitted in any form to another university. Where use was made of the work of others it has been duly acknowledged in the text.

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Signed: 

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ABSTRACT

An interpopulation study was done on the rodent species *Otomys irroratus* (Muridae:Otomyinae) using restriction fragment length polymorphisms to examine the mitochondrial DNA (mtDNA) of 30 vlei rats (*Otomys irroratus*) from three South African locations and 12 Angoni vlei rats (*O.angoniensis*) from two locations which were included as an outgroup. The three *O.irroratus* populations originated from Karkloof and Kamberg in the Natal midlands and from Rietvlei in the Southern Transvaal. Mitochondrial DNA was extracted and purified by cesium-chloride/ethidium-bromide ultracentrifugation and digested with 19 class II restriction endonucleases. The fragments were end-labelled with ^{32}P -dCTP, separated by electrophoresis on horizontal 1% agarose gels and the bands detected by autoradiography. The resultant individual-specific fragment patterns were analysed using the Restsite analysis program (v 1.1; Nei and Miller, 1990) to obtain a measure of the percent sequence divergences between and within the 3 populations of *O.irroratus* as well as between this species and the outgroup.

The 19 endonucleases detected 19 distinct *O.irroratus* mtDNA maternal lineages and 3 *O.angoniensis* lineages. The *O.irroratus* lineages were clearly geographically structured and most closely reflected the Avise *et al.* (1987) category I (phylogenetic discontinuity with spatial separation). The only exception

was a possible ancestral lineage represented by single individuals from Kamberg and Karkloof. Phylogenetic affinities between the most diverse lineages found at Kamberg and most Karkloof clones appear to be consistent with the finding of Pillay *et al.* (1993) and Contrafatto *et al.* (1992b) that Kamberg *O. irroratus* is an incipient sibling species of Karkloof *O. irroratus*. The mtDNA data indicates that the *O. irroratus* populations at Karkloof and Kamberg last shared a common ancestor approximately 365 000 years ago.

By contrast, *O. angoniensis* showed no evidence of geographic mtDNA structuring and is best described by the Avise *et al.* (1987) category III, which reflects phylogenetic continuity with spatial separation. These classifications must be regarded with caution given the limited distributional range of each species covered by this investigation.

The interspecific mtDNA sequence divergence between *O. irroratus* and *O. angoniensis* of 11.57% substantiates morphological, karyotypic and allozymic evidence that these two sympatric species are also sibling species and they appear to have last shared a common ancestor between 1.2 and 2.4 million years ago.

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CHAPTER 1 : INTRODUCTION

1.1 THE BIOLOGY OF THE VLEI RAT, *Otomys irroratus*

The vlei rat, *Otomys irroratus* is endemic to South Africa and belongs to the rodent family Muridae and the subfamily Otomyinae. The vlei rats are so called because of their association with damp vleis and wet grasslands on the fringes of streams and swamps. As vlei rat biology has been extensively documented by Bronner *et al.* (1988) and by Skinner and Smithers (1990), only the basic points of interest will be mentioned here.

Vlei rats are moderate to large in size, with an average total length of 240 mm and an average body mass of 122 g in males and 114 g in females (Skinner and Smithers, 1990). They have stout bodies, blunt faces, large well-rounded ears and short tails which comprise approximately 60% of the total head to tail body length. The hair is shaggy and long; the colour of the upper body parts varies geographically, but is generally a grizzly dark slate-grey tinged with brown or buff.

Vlei rats occur as an isolated population in the eastern parts of Zimbabwe and adjacent parts of Mozambique, south of the Zambezi river. South of this, this species occurs in the central and

southern parts of the Transvaal, in Natal, the Orange Free State and widely throughout the Cape Province. The species has a wide distribution, as it occurs throughout grassland areas but is more abundant in moist habitats associated with damp soil in vleis, or along streams and rivers or on the fringes of swamps. In montane areas it occurs on grass-covered hillsides and ridgetops, at considerable distances from water (Skinner and Smithers, 1990).

Vlei rats are predominantly crepuscular. They are terrestrial and seldom enter the water except when forced to do so. They are generally solitary but can be found in pairs or family parties. They construct their own saucer-shaped nests on rising dry ground or in clumps of grass. Clearly demarkated runs lead from these nests to the feeding areas which are marked by the short lengths of grass stems discarded during feeding. Davis (1973) found that, in the Transvaal, the mean home range of the males is 1 730 m² and that of females is 1 252 m². The size of the home range decreases in the winter months, but only slightly, in relation to a doubling in the size of the population (Skinner and Smithers, 1990).

Vlei rats are preyed upon by small carnivores, birds of prey, snakes, and in particular by the barn owl, *Tyto alba* and the grass owl, *Tyto capensis*, which live in the same habitat-type as the vlei rat. Vlei rats are wholly herbivorous and show

advanced specialisation of the digestive tract. The teeth bear transverse loths and the caecum is large and complex to allow efficient digestion of ingested material by gut microflora. They have been found to eat nearly all the plant species that occur in their habitat; succulent grass stems, young shoots of the reed *Phragmites*, forbs, exotic plants such as the thistle, *Cirsium vulgare*, as well as grass and other seeds.

Willan and Meester (1989) have found *Otomys irroratus* to be K-selected, maximising the efficiency of resource utilisation and competitive ability by their large size, long lifespan, long gestation period and generation time, small litter size, low reproductive effort and fecundity, well developed parental care of young, comparatively stable population densities and poor colonising ability. *Otomys irroratus* is a shy, aggressive species that exhibits complex threat and communication patterns indicative of an anti-social nature. Although individuals often congregate in suitable mesic habitats and frequently use the same runways, adults are generally solitary (Bronner *et al.*, 1988).

1.2 REVIEW OF LITERATURE ON THE GENUS *Otomys*

The taxonomic status of the subfamily Otomyinae is far from clear; they have been included in the Muridae, Cricetidae and even the

Neomyidae (Contrafatto *et al.*, 1992b). The classification of *Otomys irroratus* as a member of the family Muridae and subfamily Otomyinae is currently most widely accepted (Skinner and Smithers, 1990; Bronner *et al.*, 1988; Pocock, 1976; Meester *et al.*, 1986).

1.2.1 Interspecies relatedness

At the interspecies level, evidence from allozyme electrophoresis (Taylor *et al.*, 1989 a and b), immunoblot analysis (Contrafatto, 1991) and multivariate analysis of kidney morphology (Pillay *et al.*, 1989) shows a clear dichotomy between species occurring in the eastern parts of South Africa, which inhabit more 'mesic' habitats (*O. irroratus*, *O. laminatus* and *O. angoniensis*) and species occurring in the western regions, which inhabit arid and semi-arid habitats (*O. unisulcatus*, *Parotomys brantsii* and *O. sloggetti*).

Within the more 'mesic' group, the two sympatric species, *O. angoniensis* and *O. irroratus*, are considered to be sibling species (Meester, 1988). Morphologically they are almost identical: the only differing feature is the shape of the petrotympanic foramen. In *O. irroratus* the petrotympanic foramen takes the form of a large round hole, whereas in *O. angoniensis* the hole is tiny and lies in a slit-like depression on the inner face of the bulla.

The interrelatedness of these two species is confirmed by both allozyme data (Taylor *et al.*, 1989b) and by immunoblot analysis (Contrafatto *et al.*, 1993 submitted) which clearly indicate that among the Otomyinae, *O.angoniensis* is the closest sister species to *O.irroratus*.

On the basis of karyotype analysis *O.irroratus* and *O.angoniensis* have been found to be distinct. Contrafatto *et al.* (1992b) report that *O.angoniensis* has a diploid number of 56 and a karyotype comprising 28 pairs of acrocentric chromosomes. No variation was detected between specimens originating from Durban and Hazelmere in Natal and Nylsvlei in the Transvaal. This is in sharp contrast to the situation in *O.irroratus*, where both diploid numbers and karyotypes vary between populations. Thus, based on the above evidence, *O.angoniensis* was included in this study as outgroup to *O.irroratus*.

1.2.2. Genetic diversity of O.irroratus

(a) Chromosomal variation

Contrafatto *et al.* (1992 a and b) report the existence of considerable karyotypic variation in 12 South African populations of *O.irroratus*. This variation includes a tandem fusion of

chromosomes 7 and 12 in several populations, extensive variation in the number of heterochromatic short arms between populations, as well as the presence of 1 to 4 B-chromosomes in most populations.

On the basis of their results *O. irroratus* can be divided into three major chromosomal categories. The first group (group A), distributed to the east of South Africa, consists of cytotypes with acrocentric chromosomes. This group can be subdivided into two subgroups on the basis of breeding behaviour (Pillay, 1990) and chromosome rearrangements (Contrafatto *et al.*, 1992b). One of these subgroups is represented by karyotypes containing the tandem fusion of chromosomes 7 and 12. A second group (group B), of western distribution, includes karyotypes with at least seven pairs of large biarmed autosomes, whilst a third group (group C) is represented by cytotypes with four pairs of large biarmed chromosomes and is present on the highlands north of the Drakensberg.

b) Allozyme electrophoresis

Taylor *et al.* (1992) used starch gel electrophoresis to investigate genetic variation in *O. irroratus* at 24 enzyme and protein loci. These authors found that the mean heterozygosity ($H=0.071$) in 12

South African populations of *O. irroratus* was relatively high when compared to the accepted mammalian mean of 0.039. Generally, however, genetic distances between populations were low, (Nei's $D = 0.034$). Cluster analysis of the genetic distances failed to identify groups consistent with geographical position or karyotypic differences between populations. *Otomys irroratus* was found to have a dendritic distribution pattern, where populations are closely tied to suitable moist habitats along the edges of rivers and vleis (Skinner and Smithers, 1990). Thus it could be predicted from the distribution pattern of *Otomys irroratus* that the population genetic structure would be somewhat intermediate on the Wrightian-panmictic continuum. Taylor *et al.* (1992) found the genetic structure of *O. irroratus* to be characterised by high heterozygosity, lack of inbreeding (Wright's $F_{IS} = 0.048$), and extreme local differentiation in allele frequencies (Wright's $F_{ST} = 0.0375$), with genetic similarities approaching the limit of 1.0 (Nei's mean $I = 0.967$). Therefore these parameters were thought to be indicative of an outcrossed population genetic structure having some degree of subdivision and confirmed that the population genetic structure was neither entirely panmictic nor Wrightian but somewhat intermediate.

Taylor *et al.* (1992) also suggested that the population genetic structure of *O. irroratus* was consistent with the population flush

model. According to this model, during high rainfall periods when suitable habitats are expanding and populations are increasing, selective pressures are relaxed, allowing the persistence of novel genetic combinations, and the colonisation of peripheral habitats. During subsequent drought periods, habitats become fragmented and numbers are reduced without particular regard to genotype, thus new populations are formed, some of which may contain genetic novelty (references in Robinson and Elder, 1987). Periods of population fragmentation during drought conditions would favour local allelic differentiation as evidenced by the relatively high F_{ST} in *O. irroratus*, while periods of population expansion would increase gene flow and promote outbreeding, resulting in the low F_{IS} and high H values found in *O. irroratus*. Thus the dendritic distribution pattern of this species and the population flush phenomenon both seem to account for the intermediate population structure of the species (Taylor *et al.*, 1992).

Another important determinant of population structure in mammals is social behaviour. Vlei rats are territorial and show a high degree of aggression towards conspecifics (Davis, 1973). Such factors would be expected to promote dispersal and lead to a reduction in F_{ST} and genetic distance as well as an increase in heterozygosity, a pattern consistent with that found for *O. irroratus* (Taylor *et al.*, 1992).

To explain the lack of agreement between the chromosome- and allozyme-derived divergences between populations of *O. irroratus*, Contrafatto *et al.* (1992b) propose that chromosome rearrangements in *O. irroratus* proceed more rapidly than detectable gene mutations, with the result that they can initiate reproductive isolation and speciation before phenotypic divergence has appeared.

1.2.3 Background to this study

An interesting phenomenon emerged, from this general picture of *O. irroratus*, with respect to the two Natal midlands populations at Karkloof and Kamberg.

Contrafatto *et al.* (1992b) found the two populations to be karyotypically distinct. The cytotypes at Kamberg showed a tandem fusion between autosomes 7 and 12, a feature characteristic of only a few populations. Karkloof specimens were found to have acrocentric chromosomes with two extra pairs of autosomes and no t(7;12). Contrafatto *et al.* (1992b) have also shown that the Karkloof population has a diploid (2n) number ranging from 30 to 32, with 0 to 2 B-chromosomes. Kamberg karyotypes also have 0 to 2 B-chromosomes, but have a 2n within the range of 24 to 27. A population from Rietvlei, in the Southern Transvaal, was included in the present study as it showed both chromosomal and

allozyme divergence from Kamberg as well as from Karkloof. The Rietvlei karyotype was found to have a diploid number of 28, a fundamental number of 36 and 2 B-chromosomes. It belongs to the Contrafatto *et al.* (1992b) group C, which has four pairs of large biarmed chromosomes.

Taylor *et al.* (1992), using starch gel electrophoresis to study allozymes in 12 *O. irroratus* populations, found the highest mean heterozygosity at Kamberg (0.130) and the third highest at Karkloof (0.096). Similarly, the highest level of polymorphism was found at Kamberg (33.3%), whilst Karkloof, at 29.2%, had the second highest. Taylor *et al.* (1992) found Rietvlei to show the second lowest levels of allozyme heterozygosity (0.030) and polymorphism (8.3%).

Previous interbreeding studies between individuals from Hogsback, a population which, like Kamberg, contains the (7;12) tandem fusion, and Karkloof showed a significant reduction in reproductive success and a high pre-weaning mortality rate (Pillay, 1990; Pillay *et al.*, 1992). In crosses between Karkloof and Kamberg animals, Pillay *et al.* (1993) found that intrapopulation pairings between individuals representing different cytotypes gave 100% breeding success whereas fewer than half of the interpopulation pairings produced young. Moreover, the fitness of hybrids was markedly impaired as shown

by increased pre-weaning mortality, inhibited growth in respect of body mass (postnatal development) as well as almost complete sterility of the surviving young. These results were interpreted to indicate the existence of chromosomal and possibly, genetic incompatibility between the two populations.

Further behavioral differences are also noteworthy. The high level of aggression shown in the interpopulation pairings resulted in damaging fights. Consequently half of the Kamberg (male) x Karkloof (female) crosses had to be separated shortly after pairing. Pillay *et al.* (1993) speculates that such high aggression may have been the result of impaired recognition by either or both sexes of the courtship behaviour of the other individual.

Thus the results obtained in this study provide evidence that gene flow between Kamberg and Karkloof *O. irroratus* populations is restricted, through both reduced breeding success and lower hybrid fertility and fitness. As was the case with the Hogsback crosses, the presence of the tandem fusion in the Kamberg karyotype is thought to be particularly significant in causing hybrid sterility, and thus the Kamberg population may be regarded as an incipient sibling species, as predicted by Meester (1988). Pillay *et al.* (1993) also conclude that there is a breakdown in mate recognition between the populations, and thus that pre-mating reproductive

isolating mechanisms are also evolving in the Karkloof and Kamberg *O. irroratus* populations.

Consequently, it was decided to use mtDNA RFLP's (restriction fragment length polymorphisms) to estimate the extent of the genetic diversification between these two Natal midlands populations of *O. irroratus*. The suitability of mtDNA analysis for such an intraspecific study is discussed in section 1.4.

A previous mtDNA RFLP study (A. Rimmer, pers. comm.) analysed 21 *O. irroratus* individuals from nine localities using five restriction enzymes. Fifteen different mtDNA haplotypes were found; Kamberg (n = 5) and Karkloof (n = 4) populations were each found to be represented by four different matrilineages and Rietvlei (n = 1) was represented by one lineage. Rimmer reported average nucleotide diversities between a) Kamberg and Karkloof of 4.02%, b) Karkloof and Rietvlei of 2.68% and c) Kamberg and Rietvlei of 5.52%.

As some of the data was incomplete, these figures may not be entirely reliable.

This study was undertaken to provide further evidence on mtDNA variability within and between Kamberg and Karkloof *O. irroratus* populations with a view to ascertaining whether the extent of the genetic divergence is sufficient to suggest speciation between these allopatric populations.

1.3 VARIETY OF MOLECULAR APPROACHES

The merits of molecular over morphological approaches to systematic and phylogenetic studies has been established and are extensively reviewed by Hillis (1987) and Hillis and Moritz (1990). Despite cost being a major obstacle to the increased use of molecular techniques in systematics, Hillis (1987) states that the value and need for molecular data in systematics is recognised in spite of the expense.

A number of molecular approaches are available to the systematists. These include protein electrophoresis, cytogenetics and nucleic acid analyses such as DNA-DNA hybridisation, restriction site mapping, DNA sequencing and RFLP (restriction fragment length polymorphism) analysis. A detailed review of each is given by Hillis and Moritz (1990).

In the past few years there has been a significant increase in the application of DNA analysis in population genetics and systematics. DNA analysis has a number of integral advantages :

- a) The genotype is assayed rather than phenotype;
- b) One or more specific sequences appropriate to a problem can be selected on the basis of evolutionary rate or mode of inheritance;
- c) The techniques available can generally be applied to most

types of DNA and

- d) No destructive sampling particularly of rare or endangered species is necessary since stable DNA can be obtained from minute amounts of tissue.

There are various approaches for analysing DNA variability. DNA-DNA hybridisation provides an estimate of the sequence divergence between genomes but can produce no discrete character data. Direct sequence comparisons provide a very high level of resolution and yield character data that can be converted to sequence divergence values. An alternative sequence variation assay involves comparison of the number and size of fragments produced by digestion of DNA with restriction endonucleases. These variations are termed restriction fragment length polymorphisms (RFLP). This technique is more laborious and expensive than DNA-DNA hybridisation, but provides information on the nature and extent of the differences between two DNA sequences. RFLP analysis is cheaper and simpler than direct sequencing, but yields less information on sequence evolution (Hillis and Moritz, 1990).

1.4 MITOCHONDRIAL DNA (MtDNA)

1.4.1 Molecular characteristics of Mitochondrial DNA

MtDNA is a cytoplasmic gene system that has become very well characterised at the molecular level (Brown, 1983; Avise and Lansman, 1983) and has been widely used in evolutionary and population genetic studies (see section 1.5). Mitochondrial DNA (mtDNA) is a closed circular molecule encoding 13 messenger RNAs, 22 transfer RNAs and 2 ribosomal RNAs; it has a D-loop region of variable length which contains the origin of replication (Avise, 1986; Brown, 1983; Avise and Lansman, 1983). There are no intervening sequences within genes, and spacers between genes are lacking, as is repetitive DNA. The mitochondrial genome is conservative in size (15.7–19.5 kb) and gene organisation among assayed species as diverse as sea urchins, amphibians, reptiles, birds and mammals (Brown, 1983).

1.4.2 Maternal inheritance of Mitochondrial DNA

MtDNA is maternally inherited (Hutchinson *et al.*, 1974; Hayashi *et al.*, 1978; Giles *et al.*, 1980) and transmitted to progeny through the egg cytoplasm (Avise and Lansman, 1983), and is thus unaffected by recombination in sexually reproducing lines. Maternal

inheritance results in the effective population size for mtDNA being smaller than that for nuclear DNA, as only a subset of the population pass on their mtDNA to the offspring. Thus in mtDNA evolutionary biologists have access to a set of completely linked markers which permit clear definition of maternal genealogies and excellent discrimination between common ancestry and convergence (Harrison, 1989). These features of mtDNA allow reconstruction of matriarchal phylogenies and provide high resolution for distinctions of populations within and between closely related species (Awise, 1986; Awise *et al.*, 1987).

The strict maternal inheritance of mtDNA also allows the maternal form involved in the production of hybrids to be traced and allows the assessment of the direction of hybridisation and introgression (Nelson *et al.*, 1987; Baker *et al.*, 1989; Carr *et al.*, 1986; Lamb and Awise, 1986; Moritz *et al.*, 1989). Awise and Saunders (1984) used a combination of allozyme and mtDNA markers to characterise genetically natural populations of 5 sympatric species of *Lepomis*, a group of fishes renowned for their propensity to hybridise. Ferris *et al.* (1983) also found 'foreign' mtDNA within the Scandinavian house mouse, *Mus musculus*, which they attributed to invasion by introgressive hybridisation. Ferris *et al.* (1983) postulated that the colonisation of Scandinavia by even a single successful *M. domesticus* female could have introduced the foreign

mtDNA type via hybridisation with *M.musculus* males. Thus while the nuclear genome would have become enriched with *M.musculus* DNA with each successive backcross generation, the spreading *M.domesticus* mtDNA would have remained intact. Thus the resulting Scandinavian population may be exclusively *M.domesticus* as far as mtDNA is concerned and predominantly *M.musculus* as regards nuclear DNA.

1.4.3 Nucleotide substitution rates in mitochondrial DNA

According to Brown *et al.* (1979) functional considerations lead to an expectation of slow evolutionary change in the genes of animal mtDNA. Studies have, however, shown that mtDNA evolution occurs 5 to 10 times faster than that of single copy nuclear DNA; the nucleotide substitution rate in mtDNA is estimated at 0.5 to 2% per lineage per million years. These estimates are based on work on hominids (Brown *et al.*, 1979; Ferris *et al.*, 1981), goats and sheep (Brown *et al.*, 1979) and geese (Shields and Wilson, 1987). Within all genomes, however, the rate of change varies for different regions. Mitochondrial ribosomal and transfer RNA genes were found to be more conserved than the rest of the mitochondrial genome whilst the mitochondrial rRNA genes were less conserved than nuclear rRNA genes. Cleavage map comparisons demonstrated that the D-loop region was the site of several deletions and additions, and also the most rapidly changing portion of the mtDNA

(Brown, 1983). The greater levels of variation detectible in the mitochondrial genome permit better resolution of closely related taxa than can be obtained with nuclear DNA (Brown, 1983). Hillis and Moritz (1990) point out, though, that the high evolutionary rate of some mtDNA genes can result in convergence, thus confusing phylogenetic relations even within some species (Aquadro and Greenberg, 1983; Lansman *et al.*, 1983).

Most researchers working on mtDNA have based their divergence time estimates on a substitution rate of 2 percent per million years, including those working primarily on rodents (Yonekawa *et al.*, 1981; Hayashi *et al.*, 1979; Yamagata *et al.*, 1987; Sudman and Hafner, 1992; Yamagata *et al.*, 1990; Ashley and Wills, 1987; Brown *et al.*, 1981; Brown and Simpson, 1981). Harrison (1989) has, however, warned that properties of mtDNA defined for one group of organisms cannot be assumed to hold for all other groups. The possibility that rodent mtDNA divergence could be faster than 2% per million years was first suggested by Kerr (1983), based on the fact that Northern Europe was glaciated and uninhabitable to house mice until 8000 years ago. Yet, in Scandinavia, northern Germany and Scotland, Ferris *et al.* (1983) found apparently unique, derived types of mtDNA. If these variant types originated *in situ* within the past 8000 years, it would be necessary to invoke a higher rate of mtDNA divergence.

Other work on muroid rodents has yielded further information. The pioneering DNA-DNA hybridisation studies of Laird *et al.* (1969) demonstrated that the rate of single-copy DNA change was much higher in rats and mice than in cows and pigs. For mammals, one of the most striking differences in rates of genome change occurs between hominoid primates and muroid rodents, with the largest difference in rates occurring in single-copy nuclear DNA (Britten, 1986) as determined by DNA solution hybridisation experiments. She *et al.* (1990) found that even mtDNA, which is thought by several workers (Wilson *et al.*, 1985) to have the same rate of divergence in organisms as diverse as primates, gallinaceous birds and *Drosophila*, evolves faster in muroid rodents. These authors demonstrated that mtDNA of mice evolves at a rate of 4.8 - 9.7 percent per million years (on average $7.1\% \pm 2.3\%$), a value at least three times faster than the 2 percent per million years currently found in primates and other mammals (Wilson *et al.*, 1985; Harrison, 1989). Catzefflis *et al.* (1992) cautiously propose that these differences in rates of evolution of different mammals may be due to the number of germline replications per generation (and not per year) and different efficiencies of DNA repair mechanisms.

1.4.4 Methodology and data analysis

In recent years a number of authors have studied the genetic variation in mitochondrial DNA within and between species by using restriction endonuclease digestion data. A restriction endonuclease recognises a specific palindromic sequence of nucleotide pairs, generally four to six base pairs in length, and cleaves the DNA within this sequence to produce fragments with either 5' or 3' overhangs or blunt ends. The specificity of these enzymes means that complete digestion of a particular DNA will produce a reproducible set of fragments. Thus, if a circular DNA such as mtDNA has m such restriction sites, digestion will produce m fragments (Hillis and Moritz, 1990).

Changes in the number and/or size of the fragments is caused by sequence rearrangements, addition or deletion of DNA, or base substitutions in cleavage sites resulting in restriction length polymorphisms (RFLP) which can be detected by gel electrophoresis. Inversions across the cleavage site reduce the size of one fragment and increase the size of another by the same amount. Tandem direct duplication including the cleavage site will produce an extra fragment equal to the size of the duplicated sequence. In the event of a gain or a loss of DNA between two cleavage sites, the size of the fragment will either increase or decrease by the amount

added or lost. Base substitutions can result in either the gain or loss of cleavage sites (Hillis and Moritz, 1990).

Numerous studies have demonstrated RFLP analysis to be a powerful tool for assessing the genetic heterogeneity within and among conspecific populations. There are three basic assumptions of RFLP analysis:

- a) Changes in fragment patterns are due to either the gain or loss of cleavage sites;
- b) Changes in mobility of DNA fragments reflect differences in the molecular weight;
- c) There are no structural variations involved if two samples share a particular sized fragment.

Consequently, the greater the similarity of the two DNA sequences compared, the more similar will be the fragment patterns produced.

Thus, on the basis of these assumptions, the proportion of shared fragments (F) can be used to estimate the amount of sequence divergence (d) and the number of nucleotide substitutions (p) between individuals, species or populations (Nei and Li, 1979).

Here it must be stressed that the relationship between F and p is curvilinear and thus small errors in F produce large errors in p . Thus simple fragment comparisons should not be used where less than 25% of the fragments are shared nor where there is structural variation in the mtDNA (Hillis and Moritz, 1990).

Two major conclusions have been reached regarding animal mtDNA variability. Firstly, although mtDNA evolves rapidly at the nucleotide sequence level, individuals usually appear homoplasmic which means that they exhibit predominantly a single mtDNA sequence. Secondly, despite this, genetic polymorphism among conspecifics is extensive but usually takes the form of silent base substitutions and occasional minor insertions and deletions of only a few base pairs (Awise, 1986).

The above assumptions however, apply primarily to mammalian data and may not necessarily be valid for non-mammals. Cases of heteroplasmy in which major length polymorphisms (>500 base pairs) occur have been found in several non-mammalian organisms such as frogs (Bermingham *et al.*, 1986), the American shad (Bentzen *et al.*, 1988), crickets (Rand and Harrison, 1986) and the parthenogenic lizards of the genus *Cnemidophorus* (Densmore *et al.*, 1985).

Bermingham *et al.* (1986) thus suggested that length polymorphisms and heteroplasmy may be more prevalent in the mtDNA of some lower vertebrates than in mammals or birds. Hauswirth and Laipis (1982), however, found heteroplasmy in the mtDNA of a maternal lineage of Holstein cows and Greenberg *et al.* (1983) found weak indications of heteroplasmy in humans, as did Cann and Wilson (1983). Wilson *et al.* (1985) also report similar findings in rats and trout. Both Bentzen *et al.* (1988) and Densmore *et al.* (1985) found from DNA

mapping and hybridisation experiments that these major length polymorphisms occur almost solely in the D-loop region of mtDNA, a view which supports that of Upholt and Dawid (1977) who consider the D-loop region to harbour most of the size-variations in non-avian species.

1.5 MtDNA RFLPs : A LITERATURE REVIEW

The application of animal mtDNA to phylogenetic analysis of congeneric species has been reviewed by Brown (1983), Avise and Lansman (1983), Wilson *et al.* (1985), Birley and Croft (1986), Avise (1986) and Moritz *et al.* (1987). The main problems in such studies arise from the sorting of polymorphisms when recently separated species are compared and from high levels of homoplasy where distantly related species are examined (Hillis and Moritz, 1990). Neigel and Avise (1986) showed that for a haploid marker such as mtDNA, it may take $4N$ generations, where N is the effective population size, before the ancestries of all descendents in recently separated monophyletic sister taxa can be traced to a single founder female. In instances where an ancestral taxon was highly polymorphic and multiple speciation occurred over a short period of time, it is unlikely that the correct topology could be obtained solely from a single sequence such as mtDNA.

Mitochondrial DNA analysis has also been used to examine the speciation process and its relationship to mtDNA differentiation. An apparent discordance between the biological species boundary and mtDNA genotype distribution has been reported by Avise *et al.* (1983) while investigating the relationships between sibling species of the deer mice *Peromyscus maniculatus* and *Peromyscus polionotus*. On the basis of their findings they constructed 3 simple graphical models to illustrate some conceivable relationships of mtDNA differentiation to speciation. In theoretical case I, two reproductively isolated species (A and B) are monophyletic in matriarchal genealogy and the common female ancestor of either species can either pre- or post-date the speciation. In case II, neither species is monophyletic in matriarchal genotype. In case III, species B is monophyletic but forms a subclade within A thus making A paraphyletic in relation to B. Avise *et al.* (1983) found *P.maniculatus* and *P.polionotus* appear to conform to case III. Avise (1986) states that mtDNA genotypes may readily traverse species boundaries through introgression. Barton and Jones (1983) conclude that "mitochondrial DNA is able to penetrate the boundaries between species because it is not closely linked to genes responsible for maintaining reproductive isolation".

Since mtDNA RFLP analysis reveals considerable intraspecific variation among individuals both within and between populations, it has proved to be an effective marker of population structure. MtDNA RFLP analysis has often been used to show intraspecific geographic patterns of differentiation. This revealed historical population subdivisions as well as patterns of dispersal between conspecific populations (reviewed by Avise *et al.*, 1987; Avise and Lansman, 1983; Brown, 1983; Wilson *et al.*, 1985; Avise, 1986; Birley and Croft, 1986; Moritz *et al.*, 1987; Lansman *et al.*, 1983). In the pocket gopher *Geomys* (Avise *et al.*, 1979a), the horseshoe crab *Limulus* (Saunders *et al.*, 1986), the house mouse *Mus domesticus* (Ferris *et al.*, 1983) and in a number of freshwater fishes in the southeastern United States (Bermingham and Avise, 1986), this kind of analysis also revealed that distinct mtDNA lineages occupy different parts of the species' ranges, a phenomenon clearly illustrated when intraspecific phylogenies are overlaid on geographic maps. "Intraspecific phylogeography" is a relatively new discipline based on population genetic principles and systematics. This discipline adds a phylogenetic dimension to studies of population structure, since there is a tendency for geographically separated populations of species, particularly those with limited dispersal capabilities and restricted gene flow, to occupy distinct branches of intraspecific evolutionary trees (Avise, 1986; Avise *et al.*, 1987). This phenomenon is referred

to as phylogeographic structuring. In each case there appears to be a major discontinuity, presumably reflecting current environmental and/or historical influences.

In contrast, analysis of mtDNA genotypes in populations of the American eel *Anguilla rostrata* (Awise *et al.*, 1986) and humans (Brown, 1980; Johnson *et al.*, 1983) reveals no geographic differentiation. According to Awise (1986) both these cases appear to be illustrative of demographic and life-history characteristics which can significantly influence patterns of mtDNA differentiation. Plante *et al.* (1989) even found mtDNA sequence heterogeneity between trapping lines within an island population of the meadow voles, *Microtus pennsylvanicus*. They consequently found evidence to support the hypothesis that mtDNA diversity can be maintained by population subdivision, rapid population growth rate and dispersal.

Awise *et al.* (1987) identified 5 possibilities when intraspecific phylogenies are geographically overlaid: phylogenetic discontinuities with spatial separation (category I), phylogenetic discontinuities without spatial separation (category II), phylogenetic continuity with spatial separation (category III), phylogenetic continuity without spatial separation (category IV) and phylogenetic continuity with partial spatial separation (category V).

MtDNA restriction site patterns vary within populations (Brown, 1980; Avise and Lansman, 1983; Cann *et al.*, 1984; Avise *et al.*, 1979b) and variants at the intrapopulation level usually show low levels of sequence divergence (< 1%) (reviewed by Avise *et al.*, 1987) but still allow the discrimination of individuals (Kessler and Avise, 1985; Hillis and Moritz, 1990). Higher levels of differentiation are usually found at the interpopulation level, as is the case with African mole-rats (Honeycutt *et al.*, 1987), musk shrews (Yamagata *et al.*, 1990) and parthenogenic lizards (Moritz *et al.*, 1987).

Maternal inheritance and the absence of recombination also make mtDNA an excellent marker for recent historical biogeography (Bermingham and Avise, 1986; Bowen *et al.*, 1989, Avise *et al.*, 1987; MacNeil and Strobeck, 1987) including colonisation (founder) events, introductions and population bottlenecks (Wilson *et al.*, 1985; Ashley and Wills, 1987). Riddle and Honeycutt (1990) used mtDNA haplotype phylogenies of the grasshopper mice (genus *Onychomys*) to reconstruct the historical biogeographical events in the Quaternary and pointed out that the utility of mtDNA variation for such studies depends in part on the ecology and distribution of the species being examined. Birky *et al.* (1983) found mtDNA to be particularly sensitive to population bottlenecks since, as mentioned before (section 1.4.2), the effective population size

for mtDNA is reduced as a result of its strict maternal inheritance. Thus if a population goes through an extreme bottleneck, such a population could lose all of its mtDNA variability but retain a significant fraction of its nuclear variability. Wilson *et al.* (1985) also propose that a founder event would have drastic effects on the number of surviving mtDNA lineages but no direct effect on the number of mutations that accumulated in those lineages. Thus, especially for mtDNA, a founder event may have an enormous and instantaneous effect on genetic distances, as usually calculated between populations, and on the apparent lengths of lineages in a tree relating populations.

Thus in conclusion mtDNA has proved to be an excellent marker to estimate:

- 1) The extent of variation within and among sibling or other closely related species, subspecies and populations.
- 2) Levels of gene flow within and between populations of conspecifics.
- 3) Effective population size.
- 4) Patterns of historical biogeography.
- 5) Parentage and relatedness within populations of conspecifics.

1.6 AIMS AND OBJECTIVES

Based on previously mentioned evidence (section 1.2.3) the Kamberg and Karkloof allopatric populations of *O. irroratus* are karyotypically distinct and genetically incompatible. Furthermore these two populations show higher levels of allozymic heterozygosity and polymorphism than any of the 10 other populations of *O. irroratus* assayed. A previous interpopulation mtDNA study (Rimmer, pers. comm.) produced incomplete data for Karkloof and Kamberg and only five restriction enzymes were used in the study. Thus a study concentrating on these two Natal midlands populations was undertaken using mtDNA RFLP analysis with 19 restriction enzymes. As mentioned previously (section 1.2.3), specimens from Rietvlei, a Transvaal population, were also included in the study as a third population which differs both allozymically and chromosomally from Kamberg and Karkloof *O. irroratus*, and thus served as a negative control. The sibling species, *O. angoniensis* was included as an outgroup.

The objectives of this study were to determine:

- 1) The extent of the interpopulation mtDNA variation between three populations of *O. irroratus* (Kamberg, Karkloof, Rietvlei) and from this to estimate the divergence times between these populations, with special reference to Kamberg and Karkloof.

- 2) The extent of the intrapopulation mtDNA variability at Karkloof, Kamberg and Rietvlei.

- 3) The sequence divergence between *O.irroratus* and its sibling outgroup species, *O.angoniensis*, and thus to obtain an estimated time of divergence of these 2 species.

CHAPTER 2: MATERIALS AND METHODS

2.1 SAMPLE COLLECTION

All *Otomys* specimens used in this study were wild caught. Thirty *O. irroratus* specimens were trapped at Karkloof and Kamberg in the Natal midlands, and Rietvlei in the Southern Transvaal. Twelve *O. angoniensis* specimens were caught at Albert Falls (Natal), Pigeon Valley (Durban) and on the University of Natal, Durban campus. These locations are shown in figure 1. In subsequent references the Pigeon Valley and University of Natal locations will be regarded as a single Durban population since they form part of the same environmental system. Information about sample size and locality of all *Otomys* populations included in this study is given in Table 1. Table 2 contains details of each animal used in this study.

All specimens were collected using polyvinylchloride (PVC) Willan traps (Willan, 1979) and a bait consisting of a blended mixture of oats, currants and sunflower oil. Animals were maintained in the Biology Department of the University of Natal, Durban, in excellent condition in captivity for variable lengths of time since many were used in a concurrent breeding programme (Pillay, 1993). When required the animals were sacrificed and heart, liver and kidney samples were taken for use in mtDNA extractions. If not used

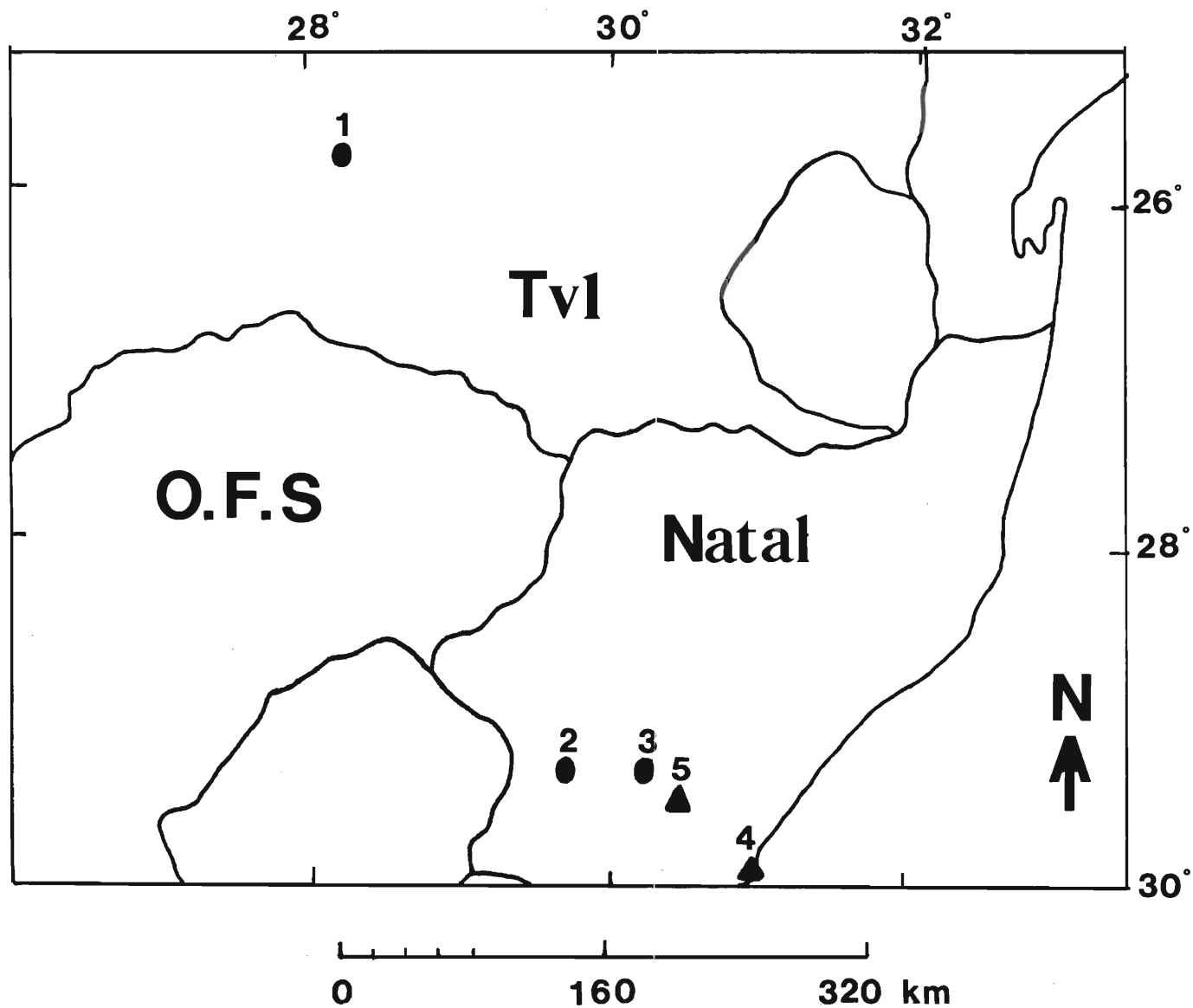


Figure 1

Map of South Africa showing the collection locations of the Otomyinae used in this study.

Locations: 1) Rietvlei (Transvaal)

2) Kamberg (Natal)

3) Karkloof (Natal)

4) Univ. of Natal (Durban)

5) Albert Falls (Natal)

■ *O. irroratus* ▲ *O. angoniensis*

Table 1

Sample size (n) and localities for *O. irroratus* and *O. angoniensis* populations included in this study.

Location	Grid Reference	n	Species
Karkloof (Natal)	29°18'S, 30°11'E	11	<i>O. irroratus</i>
Kamberg (Natal)	29°23'S, 29°42'E	11	<i>O. irroratus</i>
Rietvlei (Transvaal)	25°51'S, 28°18'E	8	<i>O. irroratus</i>
Univ. of Natal (Durban)	29°58'S, 30°57'E	6	<i>O. angoniensis</i>
Albert Falls (Natal)	29°28'S, 30°23'E	6	<i>O. angoniensis</i>

Table 2

Details of wild caught *O.irroratus* (*O.i*) and
O.angoniensis (*O.a*) individuals used in this study.

Location	Animal no.	Species	Sex	Animal no.	Species	Sex
Karkloof	985	(<i>O.i</i>)	f	999	(<i>O.i</i>)	f
	1048	(<i>O.i</i>)	f	1050	(<i>O.i</i>)	f
	1145	(<i>O.i</i>)	m	1189	(<i>O.i</i>)	f
	1311	(<i>O.i</i>)	f	1361	(<i>O.i</i>)	m
	1362	(<i>O.i</i>)	f	1363	(<i>O.i</i>)	f
	1364	(<i>O.i</i>)	m			
Kamberg	1223	(<i>O.i</i>)	m	1058	(<i>O.i</i>)	m
	1062	(<i>O.i</i>)	m	1314	(<i>O.i</i>)	f
	1365	(<i>O.i</i>)	m	1366	(<i>O.i</i>)	m
	1367	(<i>O.i</i>)	f	1368	(<i>O.i</i>)	f
	1396	(<i>O.i</i>)	m	1397	(<i>O.i</i>)	f
	1398	(<i>O.i</i>)	f			
Rietvlei	591	(<i>O.i</i>)	m	598	(<i>O.i</i>)	f
	600	(<i>O.i</i>)	m	601	(<i>O.i</i>)	m
	678	(<i>O.i</i>)	m	792	(<i>O.i</i>)	m
	794	(<i>O.i</i>)	f	795	(<i>O.i</i>)	f
Albert Falls	1154	(<i>O.a</i>)	m	1155	(<i>O.a</i>)	m
	1157	(<i>O.a</i>)	f	1158	(<i>O.a</i>)	f
	1159	(<i>O.a</i>)	m	1160	(<i>O.a</i>)	m
Univ. of Natal	900	(<i>O.a</i>)	f	943	(<i>O.a</i>)	m
	945	(<i>O.a</i>)	m	948	(<i>O.a</i>)	m
	949	(<i>O.a</i>)	f	654	(<i>O.a</i>)	m

immediately, the tissues were stored in an ultracold (-80°C) freezer until processing. The skulls and skins of all sacrificed animals are currently being processed as voucher specimens for deposition in the Durban Natural Science Museum.

2.2 MITOCHONDRIAL DNA EXTRACTION

The method of Lansman, Shade, Shapiro and Avise (1981) was used with minor modifications. Mitochondrial DNA was isolated primarily from heart and kidney tissue. Due to the small size of the study animals, liver samples were often included to increase the overall tissue volume available for extraction.

Homogenization

Two grams of heart, liver and kidney tissue were completely thawed for one hour before being chopped very finely in a sterile petri dish with a sterile scalpel and forceps. This material was then suspended in 2-3 ml of MSB- Ca^{++} buffer (Appendix I) per gram of tissue. The purpose of the Ca^{++} was to reduce nuclear breakage during homogenisation. This suspension was then homogenised using a motor-driven glass teflon homogeniser (10-20 strokes) after which 0.2 M ethylenediaminetetra-acetic acid (EDTA) was added to a final concentration of 10 mM to minimise

nuclease activity and mitochondrial aggregation during differential centrifugation (Lansman *et al.*, 1981).

Differential centrifugation

The homogenate was centrifuged at 700xg (Beckman GPR Centrifuge, 1 900 rpm) for 5 minutes at 4°C in order to remove nuclei and other cell debris. The resulting pellet was discarded and the supernatant centrifuged again as before, to increase the purity of the mitochondrial DNA (mtDNA) preparation. The recovered supernatant was then centrifuged at 20 000xg (Beckman J2-21 Centrifuge, JA 20.1 rotor, 15 000 rpm) for 25 minutes at 4°C in order to pellet the mitochondria. The pellet was washed by resuspension in 10-20 ml MSB-EDTA buffer (Appendix I) and then repelleted by centrifugation at 20 000xg for 25 minutes at 4°C.

Purification

The resulting pellet was suspended in 3 ml STE buffer (Appendix I) per 1-10 g pellet after which the mitochondria were lysed by the addition of 375µl of a 10% solution of sodium dodecyl sulphate (SDS) at room temperature. This caused the solution to clear within 30-60 seconds. Cesium chloride (CsCl) was then added to a concentration of 1.0 M thus inducing precipitation of proteins

which were then removed by centrifugation at 10 000xg (Beckman J2-21 Centrifuge, JA 20.1 rotor, 10 000 rpm) at room temperature for 15 minutes. The supernatant was carefully removed; to this ethidium bromide (EtBr, 10 mg/ml) was added at 200 μ l per 3 ml sample and the density of the gradient was adjusted to 1.56–1.60 g/ml with CsCl. Ethidium bromide is known to intercalate between the bases of the DNA and to fluoresce when viewed under ultraviolet light, thus allowing the DNA to be visualised. The gradients were loaded into Beckman polyallomar Quick-Seal centrifuge tubes, balanced and overlaid with liquid paraffin to remove all airbubbles before being heat-sealed with a Beckman Quick Seal Tube Sealer. These gradients were then centrifuged at 350 000xg (Beckman Optima TL Ultracentrifuge, 100 000 rpm) for 24 hours at 20°C to separate intact mitochondrial DNAs from nuclear DNA, RNA and the remaining protein. The DNA was visualised with ultraviolet light at 365 nm using a Mighty Bright Transilluminator (Hoefer Scientific Instruments, San Francisco).

As shown in figure 2 the fluorescent mtDNA band (containing covalently closed circular mtDNA molecules) was situated 0.5 cm below the very distinct nuclear DNA band, although it was not always visible. When visible the band was removed with an 18G sterile needle and syringe after a 21G hypodermic needle had been inserted through the top of the sealed polyallomar tube to provide

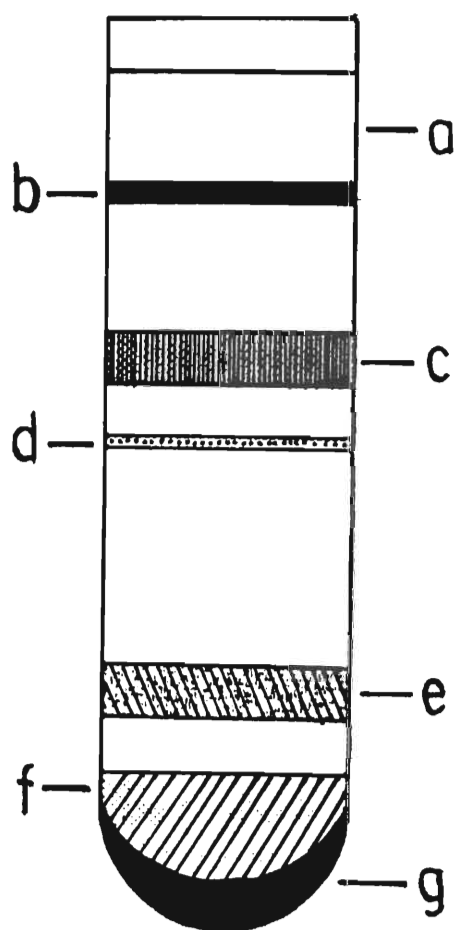


Figure 2

Schematic representation of a CsCl-ethidium bromide gradient visualised under ultraviolet light after ultracentrifugation; a) mineral oil; b) floating precipitate of SDS and protein; c) nuclear DNA band and relaxed forms of mitochondrial DNA; d) covalently closed circular mitochondrial DNA; e) glycogen; f) RNA; g) pellet containing RNA (from Lansman *et al.*, 1981).

an air vent. In cases where the band was not clearly visible (due to lower yield) 0.25 ml was collected in the same manner from a position 0.5 cm below the nuclear band.

The EtBr was removed from the mtDNA fraction by isoamyl alcohol extraction. An equal volume of isoamyl alcohol was added to the mtDNA solution, after which the tube was inverted gently.

The pink alcohol layer, containing EtBr, was then removed with a sterile Pasteur pipette; the process was repeated until the top alcohol phase appeared clear. The isoamyl alcohol was removed and the mtDNA solution (presumed EtBr-free) was dialysed against 1 x TE buffer (Appendix I) for 48 hours with two buffer changes per day to remove any remaining CsCl and excess EDTA. Purified mtDNA samples were stored at 4°C until needed.

2.3 DETERMINATION OF mtDNA CONCENTRATION AND PURITY

The concentration and purity of the mtDNA suspended in TE buffer, was determined from the optical density (OD) of the solution as measured by ultraviolet (UV) absorption spectrophotometry. DNA concentrations were determined by measuring the UV absorbance of the mtDNA samples at wavelengths of 260 and 280 nm. The DNA concentrations, in micrograms per millilitre of sample, was then

determined since it is known that 50 µg/ml DNA gives an OD at 260nm of one. The purity of the samples was determined by calculating the ratio of the OD at 260 nm to the OD at 280 nm. For a pure preparation of DNA the OD₂₆₀/OD₂₈₀ is 1.8. Ratios less than 1.6 indicate contamination of the samples with proteins or any other UV absorbers (Maniatis *et al.*, 1982). Not unexpectedly, mtDNA yields differed between samples and were found to range from 5 to 20 µg depending on the amount of tissue used and the length of time for which the tissues had been stored in a frozen state. Lansman *et al.* (1981) found that the mtDNA yield of frozen tissues is reduced by 50%.

2.4 DIGESTION OF DNA WITH RESTRICTION ENDONUCLEASES

Aliquots of purified mtDNA from each specimen were digested separately with 19 class II restriction endonucleases each having a specific six base recognition sequence. The restriction enzymes used in this study are listed in Table 3 together with their respective recognition sequences and 10X incubation buffers, the components of which are included in Table 4. Digestions were performed in 20 µl reaction volumes in sterile microcentrifuge tubes according to the suppliers' (Table 4) instructions. Usually 10 units of restriction enzyme were used per 20 ng of mtDNA. Five millimolar spermidine free-base (Sigma) was included in the

Table 3

Table of Class II restriction enzymes used and their respective recognition sequences and 10X incubation buffers.

Enzyme	Recognition Sequence	Incubation Buffer (10X)	Supplier
Apa I	GGGCC/C	A	P.
Asp 718	G/GTACC	B	Bo.
Bam HI	G/GATCC	B	Bo.
Bcl I	T/GATCA	C	P.
Bgl I	GCC(N) ₄ /NGGC	D	P.
Bgl II	A/GATCT	M	Bo.
Bst EII	G/GTNACC	B	Bo.
Cla I	AT/CGAT	H	Bo.
Dra I	TTT/AAA	B	P.
Eco RI	G/AATTC	H	P.
Eco RV	GAT/ATC	B	Bo.
Hind III	A/AGCTT	B	Bo.
Pst I	CTGCA/G	H	P.
Pvu II	CAG/CTG	M	Bo.
Sac I	GAGCT/C	A	Bo.
Sca I	AGT/ACT	K	P.
Sty I	C/C(A _T)(A _T)GG	H	Bo.
Xba I	T/CTAGA	H	Bo.
Xho I	C/TCGAG	N	Bo.

Key: P. Promega, Wisconsin, U.S.A.

Bo. Boehringer Mannheim, Germany.

**Composition of Incubation Buffer Set for
Restriction Enzymes.**

Buffer Components	Final Concentration (mM)									
	supplied by Boehringer				supplied by Promega					
	A	B	M	H	C	A	D	B	H	K
Tris acetate	33									
Tris.HCl		10	10	50	10	6	6	6	90	10
Mg-acetate	10									
MgCl ₂		5	10	10	10	6	6	6	10	10
K-acetate	66									
NaCl		100	50	100	50	6	150	50	50	
KCl										150
Diothioerythritol			1	1						
Dithiothreitol	0.5				1	1	1	1		
pH at 37°C	7.9	8.0	7.5	7.5	7.9	7.9	7.9	7.5	7.5	7.4

digestion mixtures to maximise the efficiency of the restriction reaction (Kirby, 1990). Digestion mixtures containing mtDNA, distilled water, 10X incubation buffer, spermidine free-base and restriction enzyme were incubated overnight at the appropriate temperature. This was usually 37°C except in the cases of Apa I, Bcl I and Bst EII, which were incubated at 30°C, 50°C and 60°C respectively.

2.5 ³²P END-LABELLING OF mtDNA DIGESTION FRAGMENTS

Mitochondrial DNA fragments produced by the restriction endonucleases were end-labelled using 2.5 µCi ³²P-dCTP (Amersham, UK) in combination with unlabelled (cold) dATP, dTTP and dGTP. This labelling reaction relied on both the polymerase and 3' exonuclease functions of the large fragment (Klenow) of the *E.coli* DNA polymerase I enzyme. The 3' exonuclease activity results in the conversion of blunt ends and 3' overhangs to 5' overhangs. The polymerase action of the Klenow fragment utilises the 5' single stranded overhangs as templates and synthesises the complementary strands using the radioactive dNTP together with a mixture of cold deoxynucleoside triphosphates (Maniatis *et al.*, 1982).

Two units of Klenow reagent (Boehringer Mannheim, labelling

grade) were added to each digested sample and incubated at 37°C for 20 minutes. Samples digested with Dra I were left at 37°C for 20–25 minutes as this restriction endonuclease produces blunt-ends; conversion of these to 5' overhangs tends to be an inefficient process and requires extra time (Hillis and Moritz, 1990).

A cocktail was prepared in which 2.5 µCi of the radioactive nucleotide (α -³²P-dCTP, Amersham, 10 µCi/µl) was added to a 2 µl aliquot of end-labelling buffer (Appendix I), per sample. Equal aliquots of this cocktail were mixed with each sample of digested mtDNA and then incubated at 37°C for 15 minutes. Digests of Dra I were end-labelled for 30–45 minutes at 37°C. The reaction mixture was incubated at 65°C for 10 minutes, after which it was snap-cooled on ice for 5 minutes to prevent fragments from reannealing. Three microlitres bromophenol blue loading dye (Appendix I) was added to each sample in preparation for electrophoresis. If electrophoresis could not be performed immediately the samples were stored at -20°C until required.

2.6 AGAROSE GEL ELECTROPHORESIS

A gel consisting of 1% agarose (Biorad) in 1 X TAE electrophoresis buffer (Appendix I) was prepared as follows. The agarose

was mixed with 1 x TAE buffer and heated in a boiling water bath until the agarose dissolved. The agarose solution was cooled to 40–50°C before being poured into a casting tray in which was positioned a well-forming comb. The gel was then left at room temperature for one hour to set, after which the comb was removed and the gel totally submerged in 1 x TAE electrophoresis running buffer (Maniatis *et al.*, 1982). Horizontal submarine gels of area 10 x 20 cm² were used; this allowed electrophoresis of 15 samples over a distance of 20 cm at low voltage, for maximum fragment resolution. The end-labelled mtDNA fragments were loaded into the wells and subjected to electrophoresis at 35 V for 18 hours or until the dye front had migrated the total length of the gel.

2.7 GEL-DRYING

After electrophoresis, the end-labelled gels were briefly rinsed in sterile distilled water before being vacuum dried onto Whatman 3MM chromatography paper on a horizontal gel-dryer (Biorad Gel Slab Dryer) for 1.5 hours using standard procedures. The temperature was kept constant at 54°C for the first hour to facilitate melting of the agarose. The dried gels were left at room temperature for 10 minutes to cool, after which they were wrapped in cling-wrap to prevent dessication and labelled in preparation for autoradiography (Maniatis *et al.*, 1982).

2.8 AUTORADIOGRAPHY

Autoradiography was used to detect the location of radioactivity in the gels. Dried gels were placed on unexposed X-ray film (Agfa-Curix medical X-ray film) between two intensifying screens in an X-ray cassette. The cassette was then well closed, sealed in a light-tight bag and exposed at -70°C for the required time. A first film was usually removed after 5-6 hours exposure and developed in order to estimate an optimum exposure time for a second film. The second film was then exposed for the optimum time period.

Films were developed under safelights, for 5 minutes in Agfa X-ray developer (G127C, 1+4 dilution), rinsed in tap water for 2 minutes, fixed in Agfa Rapid X-ray fixer (G334, 1+4 dilution) for 5 minutes and then washed under running water for 5 minutes. The autoradiographs were dried, viewed and scored as described below (section 2.9.1).

2.9 DATA INTERPRETATION AND ANALYSIS

Restriction fragment data were analysed quantitatively to obtain estimates of percentage sequence divergence and qualitatively by geographic overlay (see section 2.9.2) of the mtDNA lineages.

2.9.1 Quantitative Analysis

The molecular weights of all mtDNA digestion fragments were calculated by comparison of their migration distances with the known molecular weights and distances of migration of the Lambda/Hind III marker fragments included on each gel.

This was done by plotting a standard curve of mobility versus the log molecular weight of the marker fragments. Thus based on their migration distances, the molecular weights of the mtDNA fragments could be extrapolated from the standard curve (Maniatis *et al.*, 1982). Restriction fragment patterns were compared for different samples treated with the same restriction enzyme and fragments with the same electrophoretic mobility scored as homologous between individuals.

Restriction enzymes were assigned a numerical value from 1-19 and the mtDNA digestion patterns produced by each enzyme were assigned alphabetical letters where "a" represented the most common restriction pattern. A composite haplotype from all digestions was then constructed for each individual, resulting in a 38 character (numbers and letters) code. Individuals sharing identical composite haplotypes were then grouped into maternal lineages or clones.

Numerous workers, including Upholt (1977), Nei and Li (1979) and Engels (1981) have developed methods for estimating sequence divergence on the basis of restriction fragment data. Most of these yield similar results (Nei, 1987). In this study data were analysed using the Restsite computer package v1.1 developed by Dr.J.C. Miller (Nei and Miller, 1990). This uses the Nei (1987) fragment approach and is based on the following set of equations which estimate sequence divergence:

An estimate of the proportion of shared fragments (F) is given by equation 5.53 in Nei (1987):

$$F = \frac{2m_{xy}}{m_x + m_y}$$

where m_x and m_y are the numbers of mtDNA fragments produced for individuals X and Y respectively, and m_{xy} is the number of fragments shared between the 2 individuals.

A G -value is calculated from this by the following iteration formula (equation 5.54; Nei, 1987):

$$G_2 = [F(3-2G_1)]^{\frac{1}{2}}$$

where G_1 is a trial value of G and G_2 is an estimate of G .

F^* is used as the first trial value of G_1 and a cycle of iterations is performed until $G_2=G_1$. These values are then used to calculate sequence divergence (d) using equation 5.55 (Nei, 1987):

$$d = -2 [\ln (G_2)] / r$$

where r represents the average number of nucleotides of the recognition sequences of all the restriction enzymes used.

The estimates of F can be converted into estimates of nucleotide sequence divergence provided that the following assumptions are valid (Nei and Li, 1979).

- 1) The frequency and distribution of restriction sites is similar to that expected in a random sequence of equal numbers of the 4 bases.
- 2) Non-homologous fragments are not scored as identical due to chance co-migration.
- 3) All fragment changes occur solely by random single base substitutions and not length mutations.

Based on mtDNA RFLP data from other animals, it has been suggested that these assumptions are mostly valid (Upholt, 1977; Nei and Li, 1979; Avise and Lansman, 1983).

Nucleotide sequence divergence estimates (nucleotide substitutions/site) were calculated between all mtDNA lineages, both of *O. irroratus* and *O. angoniensis*, using the approach outlined above. Standard errors were estimated from 200 bootstrapping replicates and the Jukes-Cantor correction (equation 5.3 in Nei, 1987) was applied to all values to correct for multiple hits at the same site.

2.9.2 Qualitative analysis

A composite parsimony network was constructed for the mtDNA clones using the approach of Avise *et al.* (1979b). This was obtained by calculating the number of mutational changes between different restriction fragment patterns for each enzyme. The different mtDNA clones were compared in a pairwise fashion and the number of mutational changes summed over all enzymes used. A single-site mutation was inferred from the appearance of two new fragments in a digestion profile, the sum of whose molecular weights was equal to that of a fragment present in the common original pattern (Avise *et al.*, 1986). In certain instances it was, however, necessary to hypothesise the existence of intermediate fragment patterns (Ball *et al.*, 1988) in estimating the minimum number of mutational steps between different fragment patterns. The assumptions made in order to do this are discussed in section 4.1. The maternal lineages

were subsequently connected in a phylogenetic network which linked composite haplotypes by the minimum number of mutational steps between them.

A phylogeographic network linking the *O. irroratus* and *O. angoniensis* maternal lineages observed in this study was overlaid onto a geographic map of the sample locations. For diagrammatic simplicity, this was reduced to just include the most common lineages at each location and summarised the minimum number of mutational steps between them.

Finally, all the matrilineages were phenetically clustered from a matrix of d-values by the Sneath and Sokal (1973) unweighted pair-group method using arithmetic mean (UPGMA) and a tree constructed using the UPGMA option of the Restsite programme v1.1 (Nei and Miller, 1990). The phenogram produced assumes that equal evolutionary rates exist along all branches.

CHAPTER 3: RESULTS

The 19 restriction enzymes used each yielded 1-7 fragments per specimen, which resulted in a total of 49-58 scored fragments for each individual analysed and revealed an average of 53.4 cleavage sites per rat. This corresponds to approximately 320.4 bp (base pairs) in recognition sequence and represents 1.89 % of the *Otomys* mitochondrial genome. By summing the sizes of the mtDNA fragments produced by individual endonuclease patterns (Table 5), the size of the *Otomys* mitochondrial genome was estimated to be 16.910 kb (± 581 bp).

Identical, invariant fragment patterns were observed among all vlei rats for 3 of the 19 restriction enzymes (Asp 718, Pvu II, Sac I; Table 5). Two variant fragment patterns were detected with Apa I, Bam HI, Cla I and Xho I digestions; three variant patterns with Dra I, Eco RI and Bst EII, four different fragment patterns with Bcl I, Bgl I, Sca I, Hind III, Pst I, Bgl II, five restriction fragment patterns with Sty I and Eco RV and six fragment patterns with Xba I. Of the 19 restriction enzymes used, 14 differentiated between *O. irroratus* and its sibling, outgroup species, *O. angoniensis*. Mean fragment sizes for all fragment patterns are given in Table 5.

Table 5

List of mean fragment sizes for all fragment patterns produced by all 19 restriction endonucleases (RE)

RE	Pattern	Mean Fragment Size (kb)	Total (kb)
Apa I	A	12.40, 4.26	16.66
	B	10.68, 4.26, 1.73	16.67
Asp 718	A	16.88	16.88
Bam HI	A	16.75	16.75
	B	14.44, 2.07	16.51
Bcl I	A	7.14, 4.50, 2.06, 1.50, 1.41	16.61
	B	6.34, 4.50, 2.06, 1.93, 1.50, 1.41	17.71
	C	6.87, 5.54, 4.29	16.70
	D	5.63, 3.75, 2.65, 2.32, 2.23	16.58
Bgl I	A	16.52	16.52
	B	14.89, 2.46	17.35
	C	10.02, 4.13, 2.57	16.72
	D	10.02, 6.11	16.13
Sca I	A	8.16, 5.98, 2.41	16.55
	B	8.16, 4.55, 2.59, 2.41	17.71
	C	8.16, 5.98, 2.96	17.10
	D	6.52, 4.46, 3.31, 2.30	16.59
Cla I	A	16.81	16.81
	B	9.37, 7.09	16.46
Dra I	A	4.54, 3.66, 3.60, 2.26, 1.44, 1.42	16.90
	B	3.66, 3.60, 3.27, 3.22, 2.26, 1.44	17.45
	C	4.81, 4.11, 3.01, 1.98, 1.37, 1.28, 0.70	16.48

Table 5 Continued...

RE	Pattern	Mean Fragment Size (kb)	Total(kb)
Eco RI	A	17.12	17.12
	B	8.60, 5.37, 2.37	16.34
	C	12.21, 3.93	16.14
Hind III	A	8.50, 6.42, 2.16	17.08
	B	5.47, 5.14, 2.58, 2.16, 1.50	16.85
	C	7.70, 6.26, 2.35, 2.03	18.34
	D	12.29, 2.20, 1.94	16.43
Pst I	A	14.06, 2.38	16.44
	B	11.43, 3.80, 2.38	17.61
	C	16.27	16.27
	D	13.11, 4.04	17.15
Pvu II	A	11.93, 5.19	17.12
Bgl II	A	10.48, 5.43	15.91
	B	9.57, 3.96, 3.51	17.04
	C	16.92	16.92
	D	9.57, 7.90	17.47
Sty I	A	4.87, 3.02, 2.35, 2.32, 2.23, 1.34	16.13
	B	5.47, 3.22, 2.47, 2.35, 2.23, 1.34	17.08
	C	4.87, 3.02, 2.29, 2.23, 2.38, 1.34	16.13
	D	4.87, 3.02, 2.54, 2.35, 2.32, 2.23	17.33
	E	8.79, 2.24, 1.70, 1.57, 1.45, 0.84, 0.76	17.35
Xba I	A	4.13, 3.66, 3.27, 2.62, 2.48, 1.58	17.74
	B	8.13, 3.27, 2.48, 2.35, 1.58	17.81
	C	4.70, 3.66, 3.27, 2.58, 1.90, 1.58	17.69

Table 5 Continued...

RE	Pattern	Mean Fragment Size (kb)	Total(kb)
Xba I	D	6.16, 3.43, 3.08, 2.48, 2.35	17.50
	E	6.47, 3.35, 2.66, 2.44, 2.21	17.13
	F	3.29, 2.98, 2.68, 2.42, 2.31, 2.22, 1.54	17.44
Xho I	A	17.21	17.21
	B	9.00, 5.09, 2.78	16.87
Bst EII	A	11.91, 3.24, 1.35	16.50
	B	6.53, 4.29, 3.24, 2.06, 1.52	17.64
	C	9.85, 7.61	17.46
Eco RV	A	16.79	16.79
	B	5.64, 4.33, 3.75, 3.07	16.79
	C	5.64, 4.42, 3.75, 2.56	16.37
	D	5.64, 4.42, 3.75, 3.07	16.88
	E	8.76, 6.99	15.75
Sac I	A	16.70	16.70

Representative digestion profiles obtained after enzymatic mtDNA cleavage with Apa I, Cla I, Hind III and Pst I are given in Figure 3.

On the basis of shared composite haplotypes the 42 specimens could be separated into 19 different *O. irroratus* maternal lineages or clones (A-S; Table 6) and 3 different *O. angoniensis* clones (T-V; Table 6). No fragments of molecular weight less than 500bp could be resolved on the gels used in this study. There was no evidence of heteroplasmy. The mitochondrial genomes of all vlei rats could be unambiguously assigned to composite haplotypes A-V according to results obtained with the 16 restriction endonucleases which gave polymorphisms (Table 6).

The geographic distributions of the lineages are shown in Table 6 and Figure 4. The 11 sample animals from Karkloof were divided into five clones (A, B, C, G, H), all of which, with the exception of B, were found exclusively at Karkloof. The 11 sample animals from Kamberg comprised eleven clones (B, P, K, R, M, L, D, O, Q, N, S), all of which, with the exception of B, were found exclusively at Kamberg. Clone B was found in a single individual from Kamberg and a single individual from Karkloof. The eight sample animals from Rietvlei comprised four lineages (E, F, I, J), each of which was found solely at Rietvlei. The twelve *O. angoniensis* animals were divided into three mtDNA lineages (T, U, V), but

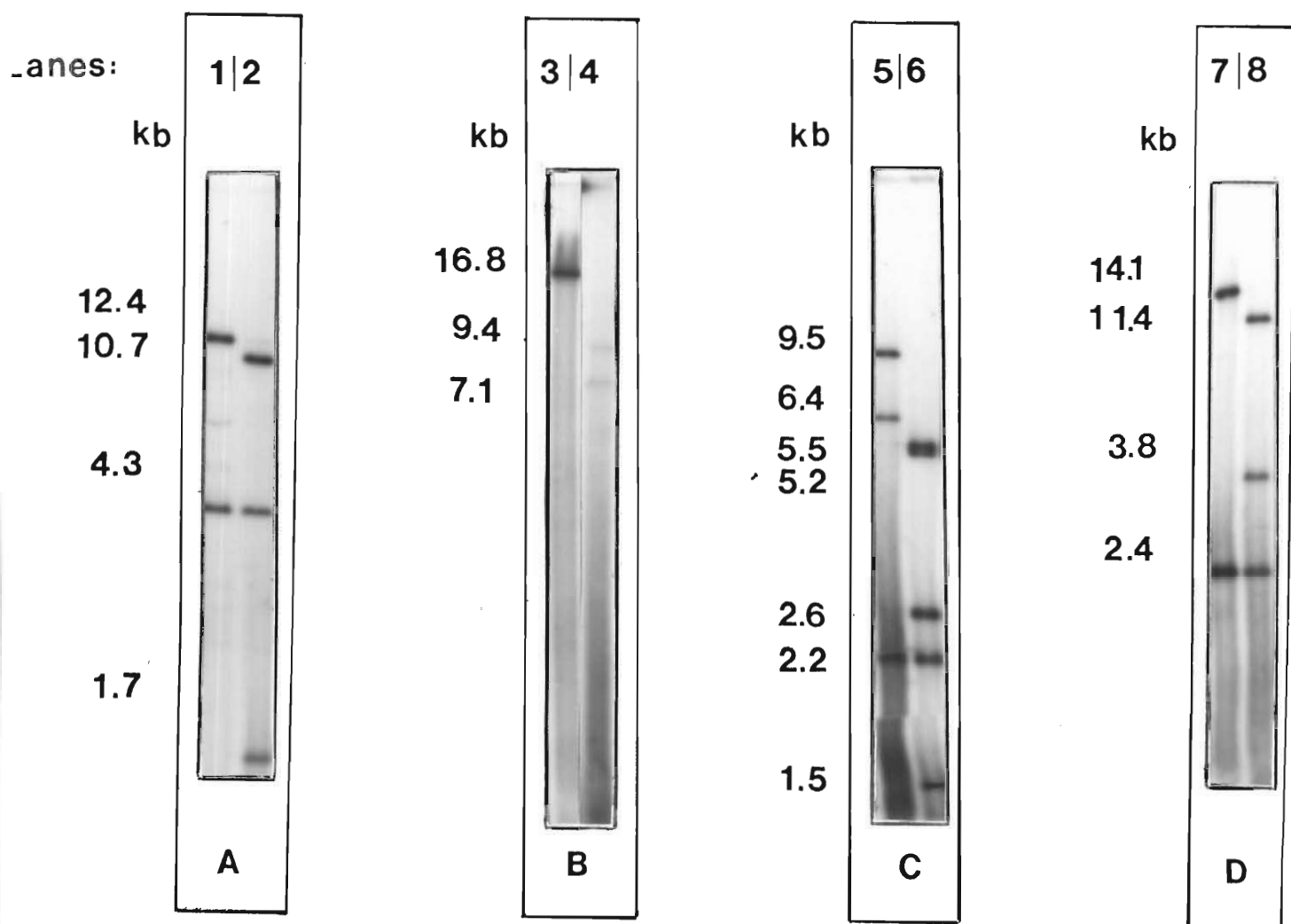


Figure 3

Representative fragment profiles following digestion of *Otomys* mitochondrial DNA.

- A) Apa I: Lane 1 = type A; Lane 2 = type B
 B) Cla I: Lane 3 = type A; Lane 4 = type B
 C) Hind III: Lane 5 = type A; Lane 6 = type B
 D) Pst I: Lane 7 = type A; Lane 8 = type B

Table 6

Composite mitochondrial DNA haplotypes of the vlei rat, *O. irroratus* and the Angoni vlei rat, *O. angoniensis* (*).

Clone	Composite Haplotype																			n	Location
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
A	a	a	a	b	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	7	Karkloof
B	a	a	a	b	a	a	a	a	a	a	a	a	b	a	a	a	a	a	a	1	Karkloof
																				1	Kamberg
C	a	a	a	b	a	a	a	a	a	a	a	a	a	a	a	a	b	a	a	1	Karkloof
D	a	a	a	a	a	a	a	a	b	a	a	a	a	a	c	a	a	b	a	1	Kamberg
E	a	a	a	a	a	a	a	a	b	a	a	a	c	a	a	a	a	d	a	1	Rietvlei
F	a	a	a	a	a	a	a	a	a	b	a	a	b	a	a	a	a	b	a	1	Rietvlei
G	a	a	a	b	a	a	a	a	a	c	a	a	a	d	a	a	a	a	a	1	Karkloof
H	a	a	a	b	a	a	a	a	a	a	a	a	b	a	a	a	b	b	a	1	Karkloof
I	a	a	a	a	c	a	a	a	a	a	a	a	b	a	b	a	a	b	a	4	Rietvlei
J	a	a	a	a	b	b	a	a	b	a	a	a	c	b	a	a	a	d	a	2	Rietvlei
K	a	a	b	a	a	a	a	a	b	a	a	a	c	a	c	a	b	b	a	1	Kamberg
L	a	a	b	a	a	b	a	a	b	a	a	a	c	a	c	a	a	b	a	1	Kamberg
M	a	a	a	a	a	b	a	a	b	a	b	a	c	b	b	a	a	c	a	1	Kamberg
N	a	a	a	a	b	b	a	a	b	b	b	a	a	b	b	a	a	c	a	1	Kamberg
O	a	a	b	a	a	c	a	b	b	b	a	a	a	a	d	a	b	b	a	1	Kamberg
P	a	a	b	a	a	b	a	a	b	b	b	a	a	c	b	a	a	c	a	1	Kamberg
Q	b	a	a	a	a	b	a	a	b	b	b	a	a	b	b	a	a	c	a	1	Kamberg

Clone	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	n	Location
R	a	a	a	a	b	b	a	a	b	b	b	a	c	b	b	a	a	c	a	1	Kamberg
S	b	a	a	c	a	b	a	b	b	b	b	a	b	c	b	a	a	e	a	1	Kamberg
T*	a	a	a	d	d	d	b	c	c	d	a	a	d	e	e	a	c	a	a	3	Alb.Falls
																				1	Durban
U*	a	a	a	d	a	d	b	c	c	d	d	a	c	e	f	b	c	e	a	1	Durban
V*	a	a	a	d	d	d	b	c	c	d	c	a	d	e	e	b	c	e	a	4	Durban
																				3	Alb.Falls

+ Numbers refer to restriction endonucleases used:

1)Apa I; 2)Asp 718; 3)Bam HI; 4)Bcl I; 5)Bgl I; 6)Sca I; 7)Cla I;
 8)Dra I; 9)Eco RI; 10)Hind III; 11)Pst I; 12)Pvu II; 13)Bgl II;
 14)Sty I; 15)Xba I; 16)Xho I; 17)Bst EII; 18)Eco RV; 19)Sac I

Lower case letters designate distinct mitochondrial DNA

restriction fragment patterns obtained with a particular enzyme,

where "a" is the most common pattern.

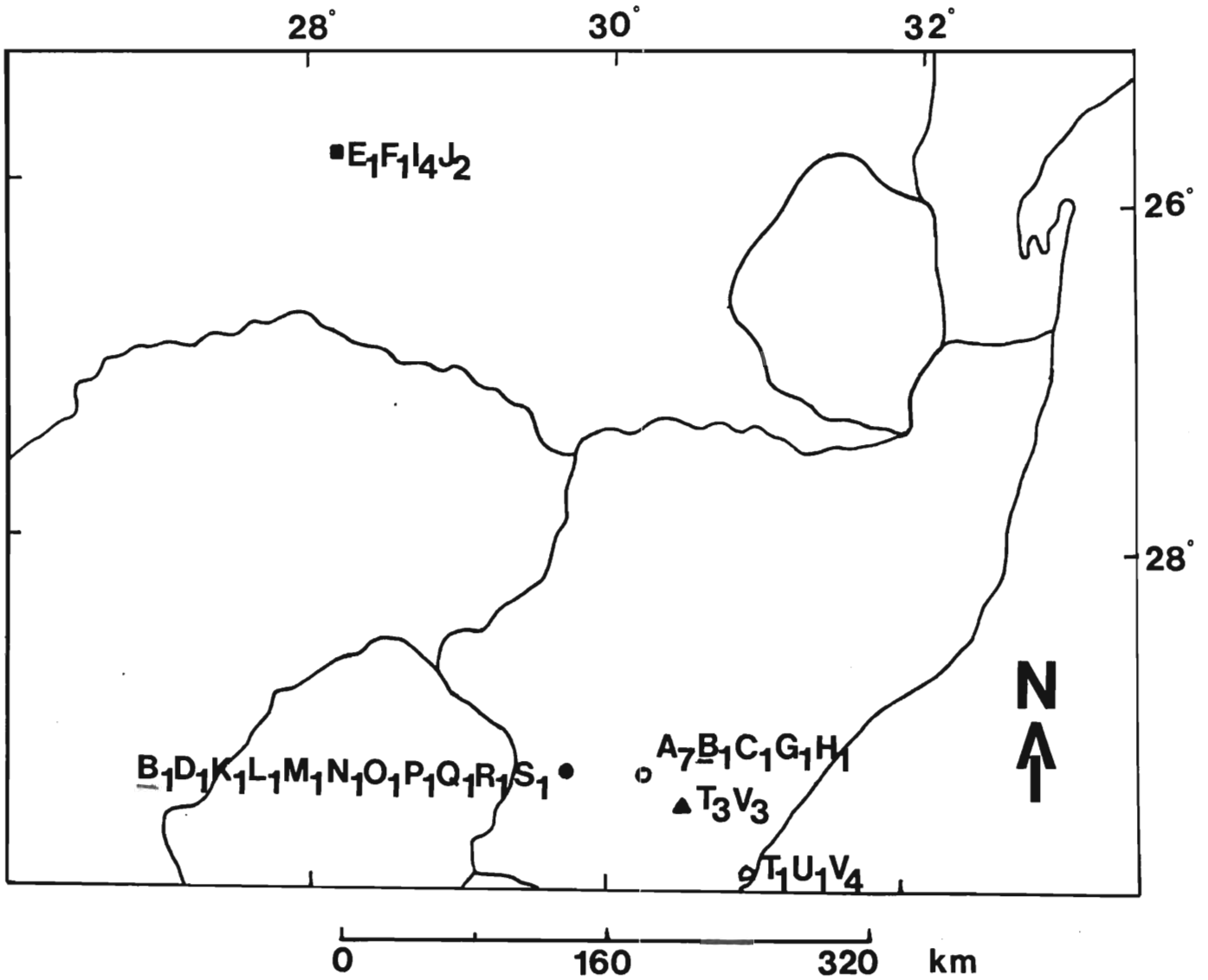


Figure 4

Geographic distribution of the 22 *Otomys* mitochondrial DNA clones sampled in this study. Capital letters designate the clone (as in Table 6) and numerical subscripts refer to the number of specimens of that clone.

O. irroratus locations: ○ Karkloof ● Kamberg ■ Rietvlei

O. angoniensis locations: △ Durban ▲ Albert Falls

showed no evidence of geographic structuring between populations (Table 6 and Figure 4).

Estimated pairwise nucleotide sequence divergences between all 22 mtDNA lineages are summarised in Table 7. The *O. irroratus* interpopulation nucleotide sequence divergences between a) Kamberg and Karkloof lineages (Table 7) ranged from 0.87% (lineage C vs D) to 4.30% (lineage G vs S), b) Karkloof and Rietvlei ranged from 0.61% (H vs F) to 2.44% (G vs J) and c) Kamberg and Rietvlei ranged from 0.62% (D vs E) to 3.62% (S vs J). The *O. irroratus* intrapopulation sequence divergences within a) Kamberg, ranged from 0.15% (lineage N vs R) to 4.35% (lineage K vs S); b) Karkloof, ranged from 0.29% (A vs B) to 1.20% (G vs H); and c) Rietvlei, ranged from 0.67% (E vs J) to 1.80% (I vs J) .

The interspecies nucleotide divergence between *O. irroratus* and the outgroup, *O. angoniensis* ranged from 8.86% (lineage B vs T) to 14.28% (lineage P vs V). The *O. angoniensis* intraspecific sequence divergences ranged from 0.56% (T vs V) to 1.80% (lineage T vs U).

A parsimony network (Figure 5) was constructed for the 19 *O. irroratus* mtDNA lineages to estimate the minimum number of mutational steps separating the clones. The Karkloof lineages differed at 2-22 restriction sites while Kamberg lineages differed at 1-50 restriction sites. The Rietvlei lineages differed at 7-21

Table 7

Percentage sequence divergence (above the diagonal) between 19 clones of *O. irroratus* (A-S) and 3 clones of the outgroup, *O. angoniensis* (T-V). Values below the diagonal correspond to the standard errors obtained by 200 replicates of bootstrapping.

All comparisons are based on data from 19 restriction enzymes.

Refer to Table 6 for clones within each population.

CLONES	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	
A		0.29	0.40	1.10	0.90	1.15	0.53	0.70	1.38	1.73	1.94	1.73	2.15	2.55	2.91	2.84	2.55	2.86	3.77	9.38	11.72	12.57	A
B	0.30		0.70	1.43	0.96	0.81	0.85	0.39	1.02	1.79	2.00	1.79	2.21	2.97	3.36	3.27	2.97	2.91	3.19	8.86	11.78	11.72	B
C	0.29	0.51		0.87	0.81	0.92	0.96	0.27	1.13	1.60	1.51	1.46	2.16	2.55	2.38	2.65	2.55	2.84	4.17	10.21	11.91	12.77	C
D	0.54	0.73	0.42		0.62	1.24	1.73	1.18	1.18	1.37	0.66	0.49	1.60	1.96	1.96	2.22	1.96	2.22	3.56	10.27	11.98	12.83	D
E	0.48	0.54	0.40	0.40		0.92	1.52	0.87	1.13	0.67	0.97	0.80	1.13	1.91	2.22	2.17	1.91	1.71	3.30	10.21	11.11	12.77	E
F	0.62	0.51	0.52	0.66	0.55		1.49	0.61	0.85	1.71	1.76	1.56	2.27	2.02	2.02	2.27	2.02	1.96	2.66	9.70	12.04	11.98	F
G	0.49	0.61	0.62	0.78	0.74	0.80		1.29	2.05	2.44	2.67	2.44	2.93	2.97	3.36	3.27	2.97	3.30	4.30	9.44	11.78	12.63	G
H	0.48	0.34	0.32	0.54	0.47	0.38	0.74		0.80	1.65	1.56	1.51	2.22	2.95	2.77	3.06	2.95	2.90	3.56	9.63	11.98	11.91	H
I	0.63	0.57	0.53	0.68	0.58	0.50	0.93	0.38		1.80	1.71	1.51	1.75	2.27	2.60	2.71	2.43	2.22	3.16	9.06	12.90	11.11	I
J	0.71	0.75	0.58	0.53	0.37	0.76	0.99	0.61	0.67		1.76	1.16	0.73	1.06	3.19	2.43	1.45	0.88	3.62	10.34	12.04	12.90	J
K	0.83	0.82	0.65	0.48	0.55	0.71	0.94	0.62	0.79	0.58		0.48	2.01	2.88	1.50	1.74	2.88	2.65	4.35	11.11	12.10	14.03	K
L	0.64	0.70	0.53	0.35	0.41	0.71	0.88	0.54	0.67	0.51	0.42		1.37	2.17	2.17	1.97	2.17	1.96	3.42	11.05	12.04	13.96	L
M	0.69	0.72	0.58	0.61	0.44	0.71	0.94	0.59	0.66	0.38	0.63	0.66		0.65	3.14	1.50	0.65	0.48	2.61	10.98	11.17	12.83	M
N	0.72	0.88	0.66	0.67	0.63	0.67	0.98	0.73	0.77	0.51	0.85	0.79	0.36		2.48	1.07	0.30	0.15	2.33	11.24	13.15	13.08	N
O	0.98	1.09	0.80	0.91	0.86	0.77	1.05	0.92	0.90	0.90	0.73	0.92	0.80	0.65		1.44	2.48	2.76	3.38	11.24	13.15	14.16	O
P	0.83	0.83	0.73	0.72	0.73	0.66	1.02	0.71	0.92	0.83	0.64	0.66	0.71	0.58	0.49		1.07	1.26	2.12	12.16	13.27	14.28	P
Q	0.70	0.76	0.59	0.66	0.48	0.72	0.97	0.69	0.82	0.61	0.93	0.80	0.40	0.24	0.71	0.54		0.46	1.87	12.04	13.15	14.16	Q
R	0.88	0.78	0.66	0.70	0.56	0.64	1.08	0.76	0.90	0.43	0.80	0.71	0.38	0.16	0.79	0.61	0.29		2.27	11.17	12.16	13.02	R
S	1.05	0.86	0.94	1.01	0.91	0.86	1.15	0.91	1.07	1.10	1.11	1.00	0.89	0.90	1.13	0.87	0.91	0.93		11.05	11.24	11.17	S
T	3.06	2.51	2.72	3.11	3.48	2.97	3.41	3.02	2.54	2.92	3.52	3.98	3.08	3.16	3.70	3.25	2.95	2.97	2.85		1.80	0.56	T
U	3.52	3.61	3.73	3.48	3.40	3.60	3.54	3.79	3.98	3.62	3.58	3.66	3.31	4.05	3.88	3.40	3.39	3.18	3.02	1.06		1.21	U
V	3.14	3.62	3.73	3.76	4.36	3.47	4.08	3.35	2.90	3.75	4.41	4.38	4.15	3.55	4.20	4.58	4.02	3.66	2.88	0.43	0.36		V

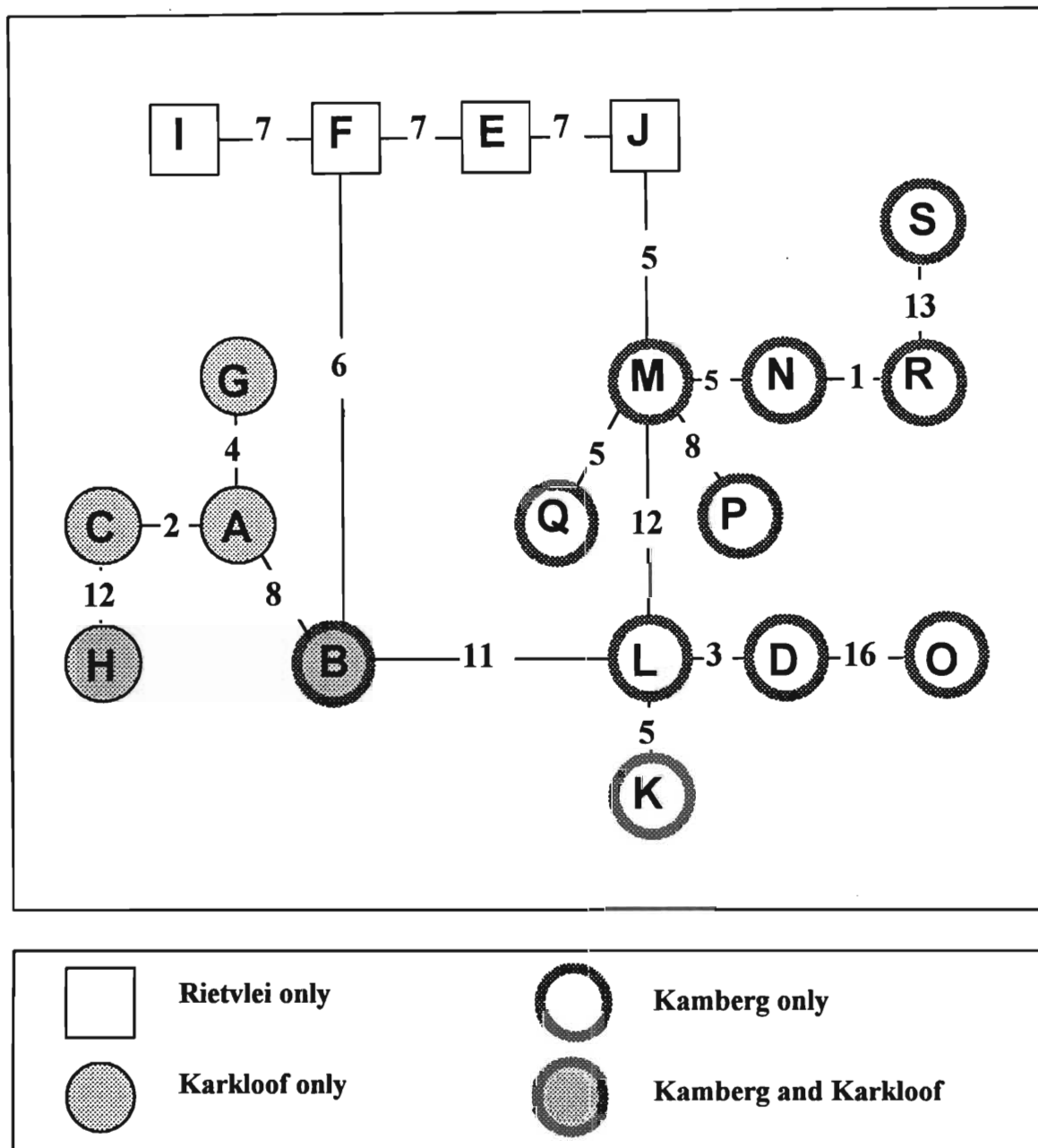


Figure 5

Parsimony network summarising the minimum number of mutational steps between the 19 *O. irroratus* mtDNA clones. Capital letters designate the maternal lineages (as in Table 6) and the numbers on the connecting paths indicate the number of restriction site changes between them.

restriction sites. Lineage B was found to be a direct link between the Karkloof and Kamberg populations. It was also separated by only six mutational steps from lineage F of the Rietvlei population. Lineages L and M at Kamberg were found to form two central points around which all other lineages were clustered. Kamberg lineage M was found to be as closely related to lineage J from Rietvlei as it was to two other (Q and N) Kamberg lineages; it was more distant from all other Kamberg lineages.

A similar network was constructed for the 3 *O. angoniensis* mtDNA lineages (Figure 6), which can be seen to differ from one another by between 4 and 8 restriction site changes.

The geographic overlay in Figure 7 summarises the minimum number of mutational steps between the most common lineages at each location. The outgroup was represented by lineage V since this was the most common lineage at both the Albert Falls and Durban locations. This lineage was indicated at Durban since more individuals at this location showed this composite haplotype than at Albert Falls (Table 6).

The relationships between the 22 mtDNA lineages of both species of *Otomys* are summarised in the UPGMA tree (Figure 8). The dichotomy between *O. irroratus* and the outgroup, *O. angoniensis* as well as

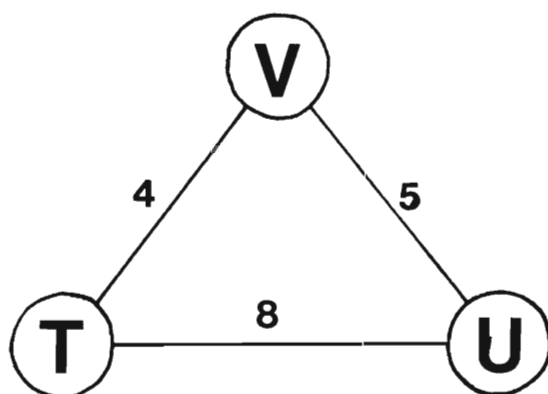


Figure 6

Parsimony network summarising the minimum number of mutational steps between the three *O. angoniensis* clones. Capital letters designate the maternal lineages (as in Table 6) and the numbers on the connecting paths indicate the minimum number of restriction site changes between them. Refer to Table 6 for the distribution of these clones between Albert Falls and Durban.

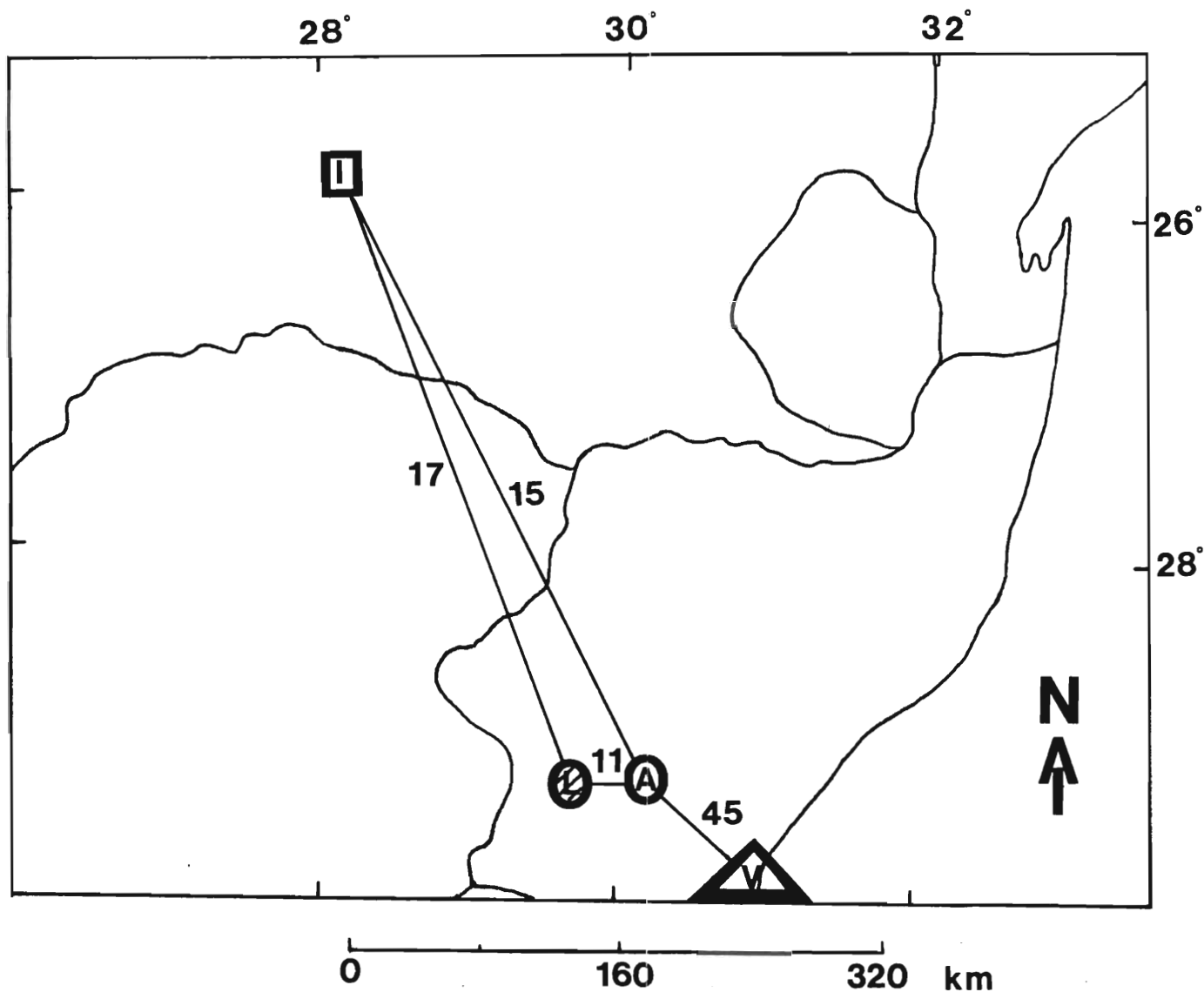


Figure 7

Geographic overlay of the minimum number of mutational steps between the most common mitochondrial DNA lineage of each population of *O. irroratus* and *O. angoniensis*.

For Kamberg, each individual studied produced a distinct lineage. Lineage L is used as a representative clone as it appears to be closely related to many other Kamberg clones.

O. irroratus locations: ○ Karkloof ◐ Kamberg □ Rietvlei

O. angoniensis locations: ▲ Durban and Albert Falls

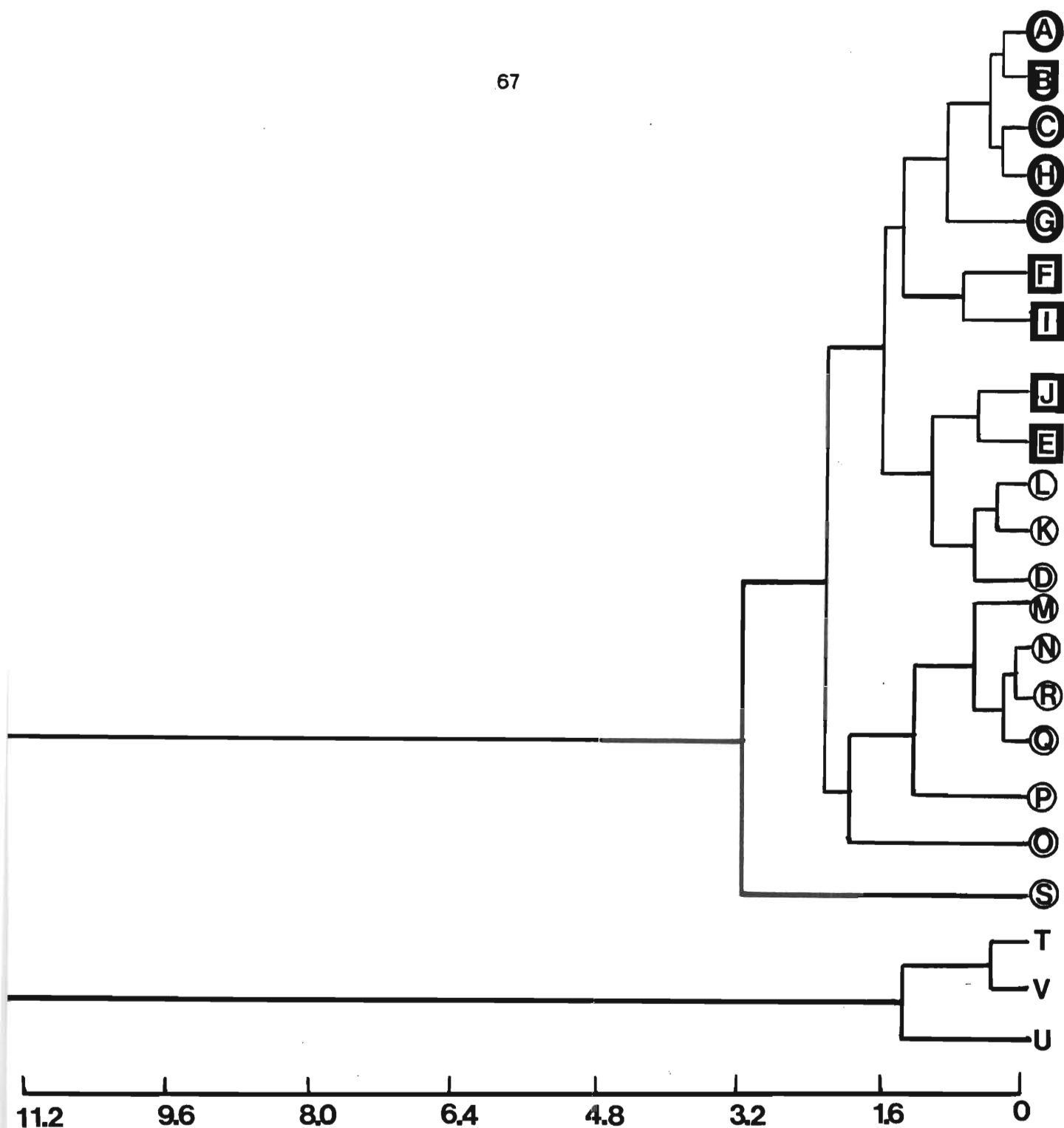


Figure 8

UPGMA tree summarising the relationships among the mtDNA clones detected in *O. irroratus* (A-S) and *O. angoniensis* (T-V). The scale represents percentage nucleotide sequence divergence.

The origins of the *O. irroratus* clones are designated as follows:

○ Karkloof ○ Kamborg ◻ Rietvlei

◻ Kamborg & Karkloof

between the Kamberg and Karkloof populations of *O. irroratus* is apparent. As is also indicated by the parsimony network (Figure 5), Rietvlei lineages I and F are more closely clustered with the Karkloof lineages and Rietvlei lineages E and J are clustered as intermediates between the two Kamberg subclusters. Once again as shown by the parsimony network, lineages L and M at Kamberg form the bases of these two subclusters. This tree also illustrates the greater diversity within the Rietvlei and Kamberg populations relative to that at Karkloof.

CHAPTER 4: DISCUSSION AND CONCLUSIONS

4.1 CRITICAL ASSESSMENT OF METHODS AND RESULTS

The choice of mtDNA RFLP analysis as an approach for detecting genetic divergence between conspecific populations is well supported by the literature (see sections 1.4 and 1.5).

Numerous methods are available in current literature for the extraction of mtDNA, but a definite preference is shown for protocols which ensure high purity mtDNA yields. Hillis and Moritz (1990) state that for maximum purity the use of neutral cesium-chloride equilibrium gradients with intercalating dyes such as ethidium bromide are best if end-labelling detection methods are used. Consequently, a slightly modified form of the Lansman *et al* (1981) extraction method was chosen, as it incorporates the above mentioned purification steps.

Direct radioactive-labelling of the mtDNA was chosen as the means of detection because of the relative simplicity and efficiency of the method. The only disadvantage to this approach is that any contaminating nuclear DNA that may be present will also be labelled to the same degree as the mtDNA and consequently may obscure mtDNA band detection, particularly of the low molecular weight mtDNA fragments. Since predominantly fresh tissue was used throughout

this study, mtDNA bands were clearly visible on the CsCl-EtBr gradients and consequently nuclear DNA contamination was minimised. The smallest digestion fragments that were resolved in this study were approximately 500 base pairs in length. The result of this was however, that relatively large discrepancies were found between digestion profiles when the molecular weights of digestion fragments were summed. This is clearly visible in Table 5. However, the overall effect of this is reduced when the digestion profiles of all 19 restriction enzymes are taken into account and the overall mtDNA genome size was estimated to be 16.91 kb (\pm 581 bp). Although this is a slight underestimation as it takes no account of fragments of less than 500 bp, it is consistent with the range of 16.00 – 16.80 kb reported for other rodents (Yonekawa *et al.*, 1981; Yamagata *et al.*, 1987; Plante *et al.*, 1989; Ashley and Wills, 1987).

Some difficulties were encountered when the digestion profiles were used to estimate the minimum number of mutational steps between individual haplotypes of different restriction enzymes for the parsimony network (Figure 5). For 58% of the total different digestion patterns produced, single-site mutations could be inferred from the appearance of two new fragments in a digestion profile, the sum of whose molecular weights was equal to that of a fragment present in the original profile (Awise *et al.*, 1979b; Awise *et al.*, 1986). For the remaining 42% of the digestion

profiles it was necessary to hypothesise the existence of intermediate fragment patterns in estimating the minimum number of mutational steps between them (see Ball *et al.*, 1988). Certain assumptions were made in order to do this.

Firstly, in instances where there were discrepancies between total molecular weights of digestion patterns, it was assumed that one or more small fragments were present but were not detected since the smallest detected fragments were only 500 bp in length. This would for example, explain the 8% difference in total molecular weight between Pst I haplotypes B vs C (Table 5) as well as smaller differences such as in the case of Eco RV profiles B vs C (Table 5). Here profile C shows a fragment of 4.42 kb but profile B shows only a single corresponding fragment of only 4.33 kb. Once again it was assumed that profile B did actually contain a fragment of 0.09 kb but that this was not detected due to resolution limitations imposed by nuclear DNA contamination. The second assumption made was that the gain or loss of a single restriction site is more likely than two or more gains or losses (Hillis and Moritz, 1990). Thus again using the example of Pst I haplotypes B vs C, it appeared more likely that one restriction site would be lost at a given time rather than two simultaneously. Here the two haplotypes were estimated to be separated by two mutational steps rather than one. In such cases the estimate for the

number of mutational steps between differing profiles of a specific restriction enzyme may not always be the minimum and thus the resultant network may not be the shortest (Ball *et al.*, 1988). A third assumption, made in complex cases such as that of the restriction enzyme Bcl I, was that a reduction in the size of one fragment could result in the increase of the size of another by the same amount (Hillis and Moritz, 1990).

Although these assumptions do not invalidate the information obtained from such an exercise, it does mean that these qualitative estimates and the resultant network may not be the most parsimonious and as such should be regarded as tentative.

Nevertheless, the chosen approach allowed all 42 studied individuals to be unambiguously assigned to each of the 22 different maternal lineages identified by this study. The nineteen restriction enzymes used produced an average of 53.4 cleavage sites/fragments per individual and a total of 2 243 assayed fragments. The restriction fragment data were converted to sequence divergence estimates using the Restsite analysis program based on the approach of Nei (1987) and phenetically clustered to produce a UPGMA tree reflecting the interrelationships between all 22 mtDNA clones. Hillis (1987) points out that in using such a rate-dependent approach in which character-state data is converted into single numerical summaries or "genetic distances",

considerable information is lost. Furthermore, Hillis feels that rate-dependent clustering algorithms, such as UPGMA, are inappropriate for reconstructing phylogenies since they assume that equal evolutionary rates exist along all branches. Nevertheless, they are widely used because of their computational simplicity. In this instance, however, all that was required was a method which could cluster the closest taxa together. The above assumption seemed to be adequately met by the fact that all taxa analysed here belong either to the same species, *O.irroratus* or are congeners in the case of the outgroup, *O.angoniensis*.

4.2 MITOCHONDRIAL DNA DIFFERENTIATION BETWEEN *O.irroratus* and *O.angoniensis*

At the species level there was clear evidence for the recognition of *O.irroratus* and *O.angoniensis* as two discrete taxa. The nucleotide sequence divergence of 11.57% between *O.irroratus* and *O.angoniensis* is not inconsistent with the value of 15% found between the sibling species, *Peromyscus polionotus* and *P.maniculatus* (Avice *et al.*, 1979b). This value is also similar to that found by Rimmer (*pers. comm.*) who reported a sequence divergence of 12% between *O.irroratus* and *O.angoniensis*. The geographic overlay (Figure 7) shows that as expected the 2 sibling species of *Otomys* are separated by a high number (45) of

mutational steps. If the accepted norm of 2% sequence divergence per million years is used, it can be calculated from the average nucleotide sequence divergence between the two species, that they last shared a common ancestor approximately 5.8 million years ago. There is, however, evidence to suggest that muroid rodents show a mtDNA evolution rate of $7.1 \pm 2.3\%$ per million years (She *et al.*, 1990; Catzefflis *et al.*, 1992). Based on these figures, the two sibling species last shared a common ancestor between 1.2 and 2.4 million years ago. The latter figure appears to be supported by a number of other studies as discussed below.

Chevret *et al.* (1993) found on the basis of fossil evidence and molecular clock data that the *Otomys* lineage split from the other Murinae approximately 7 million years ago, during the Upper Miocene. Taylor *et al.* (1989a), using the electrophoretic clock, found that the genetic distance between the two most divergent lineages among 4 species of Otomyines (including *O. irroratus* and *Parotomys* species) corresponded to a radiation among the Otomyinae 6.4–5.5 million years ago. The estimated divergence of *O. irroratus* and *O. angoniensis* of 1.2–2.4 million years ago also agrees with similar estimates of Chevret *et al.* (1993). Allozyme (Taylor *et al.*, 1989a) and immunological studies (Contrafatto *et al.*, 1993 submitted) indicate that these are among the most recently diverged species within the sub-family (see section 1.2.1).

4.3 MITOCHONDRIAL DNA DIFFERENTIATION WITHIN *Otomys irroratus*

4.3.1 General Patterns

There is evidence to show that levels of phylogenetic structuring, (see section 1.5) based on mtDNA restriction data, among conspecific vertebrate populations are negatively correlated with species' dispersal capabilities (Ball *et al.*, 1988). Thus taxa with moderate to high female dispersal levels are expected to show little geographic structuring of mtDNA genotypes. The results of this study are consistent with the above finding. In this study most clones were restricted to single locations, implying the absence of female exchanges between populations, a situation similar to that found with woodrats (Hayes and Harrison, 1992), meadow voles (Plante *et al.*, 1989) and deer mice (Lansman *et al.*, 1983), all species with limited dispersal capabilities. There was, however, one exception; lineage B was represented by a single individual at Kamberg and a single individual at Karkloof. According to the results of this study, the *O. irroratus* phylogeographic profile cannot be classed as belonging to any of the Avise *et al.* (1987) categories although it most closely resembles category I (a phylogenetic discontinuity with spatial separation) as is suggested by all other mtDNA lineages of *O. irroratus*. It must, however, be borne in mind that the geographic genetic structure reflected here might change

considerably through the inclusion of specimens from other regions of the species' range.

There are at least three possible explanations for the presence of lineage B at both Kamberg and Karkloof. One explanation is that of retention of ancestral lineages. According to this explanation lineage B could have been a common lineage in an ancestral *O. irroratus* population from which both the Karkloof and Kamberg populations were derived. This explanation is both the most likely and the most parsimonious since it only requires that lineage B be maintained at both locations as is shown by the mtDNA data (Table 6). This argument is also supported by the parsimony network (Figure 5) which shows that in addition to lineage B being present at both Kamberg and Karkloof, lineage B is also the closest lineage to the Rietvlei population, lineage F in particular. Thus it appears that lineage B could also be ancestral to the Rietvlei population. This is an intriguing possibility since Rietvlei is separated from Kamberg and Karkloof by the Drakensberg mountain range, which would be expected to be a major dispersal barrier between these populations. Thus many questions are raised as to how these three populations arose from an ancestral population and as to where such a population may have been located.

A second possible explanation is that convergent evolution produced a mtDNA clone common to Karkloof and Kamberg. The likelihood of this occurring can be estimated from the parsimony network (Figure 5) by calculating the number of mutations necessary to obtain lineage B from its closest sister clone within each population. As is seen from figure 5, A is the closest Karkloof clone to lineage B, being separated by eight mutational steps. The closest Kamberg clone to B is L from which it is separated by 11 mutational steps. As the average number of mutational steps separating clones within a population is 9.5, this explanation would be possible in terms of the number of mutation steps. The likelihood of these mutations being of the same type and at the same DNA site is much lower, however. Thus convergent evolution is an unlikely explanation.

A third explanation is that of female recruitment. In this case, a single female or a group of females may have either moved from Karkloof to Kamberg or vice versa. Subsequent mating with resident males would produce offspring with "new" mtDNA lineages, thus adding to the Kamberg genetic pool. Furthermore, since the distribution limits of these two populations are as yet undetermined, the possibility also exists that such lineages originated from other populations, not necessarily Kamberg or Karkloof. This explanation seems unlikely, however, in view of the almost complete reproductive barrier shown to exist between these two populations. Pillay *et al.* (1993) also

found that hybrid offspring resulting from such Kamberg/Karkloof reproductive crosses showed high pre-weaning mortality rates and almost complete sterility of any surviving young.

Generally speaking, the intraspecific sequence divergence values ranged from 0.27% to 4.35%. These values are of the same magnitude as those quoted by Avise *et al.* (1979 a and b) who found a divergence of two percent within *Geomys pinetis*, one percent within *Peromyscus polionotus* and three percent within *Peromyscus maniculatus*. On the other hand, the *O. irroratus* values are higher than those of Ferris *et al.* (1983) in which the intraspecific sequence divergence of *Mus domesticus* was found to be between 0.05% and 1.55%, with an average of 0.77%. The Kamberg values are particularly noteworthy since this population was found to show intrapopulation sequence divergence levels which are higher than those of the other two study populations. This point will be discussed further in section 4.3.2.

The mtDNA data agrees with the chromosomally-derived information on the relationships among the three *O. irroratus* populations in that the phenetic clustering of the mtDNA lineages generally defines the three populations as being separate from one another just as each population was shown to have a distinctive karyotype (Contrafatto *et al.*, 1992 a and b). The Rietvlei lineages are an exception, however, as 50% are clustered with the Karkloof lineages and the remainder with the Kamberg lineages. This point will be

discussed further in section 4.3.4. This contrasts with the results of Taylor *et al.* (1992) who reported that the allozyme findings for 12 studied populations of *O. irroratus* were not consistent with geographic or karyotype groupings.

4.3.2 Kamberg mtDNA structure

As seen from the parsimony network (Figure 5) and the UPGMA tree (Figure 8), the mtDNA-derived phylogenetic structure of the Kamberg population is complex, with both a high number of clones represented and a higher number of mutational steps between these clones than was the case within the other two studied populations. The number of mutational steps ranged from one between clones N and R to a maximum of 50 between clones O and S. By contrast the two most divergent clones at Karkloof (B and H) are separated by only 22 steps and at Rietvlei (I and J) are separated by 21 steps. Furthermore, the Kamberg intrapopulation sequence divergences, which range from 0.15–4.35% are of the same magnitude as the interpopulation divergences between Kamberg and Karkloof, which range from 0.87–4.30% (Table 7). These high intrapopulation sequence divergences together with the fact that each individual studied at Kamberg represents a separate lineage suggest that Kamberg may be a relatively older population as it has accumulated more site changes than the other two populations.

There are a number of possible explanations for the fact that 11 sample animals formed 11 clones. One possibility is that there may be restricted female mobility within this population. If the animals are territorial within specific habitats or demes and migrate little throughout their lifetime, this would allow site changes to accumulate in many different lineages. These conclusions are consistent with the limited dispersal capabilities and the behavioural profile of *O. irroratus* as documented by Davis (1973) and Willan and Meester (1989).

Another explanation for the high clone:individual ratio is that the sampled deme could have contained females recruited from neighbouring demes or their progeny. This explanation, however, seems less likely in view of the high degree of aggression shown by vlei rats towards conspecifics (Davis, 1973). The allozyme results of Taylor *et al.* (1992), however, show Kamberg to have the highest levels of heterozygosity and polymorphism of all 12 populations of *O. irroratus* studied (see section 1.2.3). These high levels of heterozygosity suggest that intermingling between demes or between populations may have occurred at this locality.

Another possible explanation is that there appears to have been a higher degree of lineage retention at Kamberg than at the other two localities. This implies that a number of the identified

lineages may be ancestral remnants and not only clone B. It may be possible to speculate that lineages M and L may be cases in point, since both the UPGMA tree (Figure 8) and the parsimony network (Figure 5) show that these two lineages appear to form the centres of the two Kamberg subclusters. A more extensive interpopulation study may reveal that these lineages also occur at other locations.

Another possible reason for the high number of clones present at Kamberg could be that this population has a higher mutation rate than the other two populations. Unfortunately no other data is available to substantiate or negate this theory but the possibility nevertheless exists.

4.3.2.1 Kamberg: An incipient species of Karkloof?

It must be pointed out however, that in a diverse population like Kamberg, a lot of vital population genetic information is lost if conclusions are based only on the overall picture. The most divergent clones (O and S) within this population provide an interesting discussion point. Lineage S shows both the maximum intrapopulation divergence (4.35%, versus lineage K) as well as the maximum interpopulation divergence (4.30%, versus Karkloof lineage G) within the *O. irroratus*. Similar figures can be quoted for lineage O, which appears to be the second oldest lineage within this population. Thus it seems possible that speciation

may be occurring within this population as similar divergence figures have been quoted between other rodent subspecies.

Yonekawa *et al.* (1981) found the percent sequence divergence between subspecies of *Mus musculus* to be in the range of 3–7.1%, and Yamagata *et al.* (1990) found a divergence of 3.3% between subspecies of the musk shrews, *Suncus murinus*.

More direct evidence for speciation however, comes from the findings of the breeding trials between Kamberg and Karkloof animals, and the differences in behaviour from which Pillay *et al.* (1993) suggested Kamberg to be an incipient sibling species of Karkloof *O. irroratus* (see section 1.2.3). This is further supported by the karyotypic studies, since the presence of the (7;12) tandem fusion allows one to predict meiotic mal-segregation, which would lead to the production of unbalanced gametes (Contrafatto *et al.*, 1992 a and b). A possible result of this could be the impaired hybrid fertility reported by Pillay *et al.* (1993).

The average mtDNA sequence divergence data shows that Kamberg and Karkloof last shared a common ancestor approximately 365 000 years ago, thus indicating that the tandem fusion, present at Kamberg but not at Karkloof, must have been established during the last 365 000 years.

4.3.3 Karkloof mtDNA structure

The Karkloof population is characterised by five lineages (Table 6) which are separated by relatively few mutational steps (2-22, Figure 5). Possible lineage extinction could be cited as a reason for the lower clone:individual ratio found at Karkloof. The UPGMA tree (Figure 8) shows that these lineages are phenetically closely clustered. The close relationship between the clones is also evident from the mtDNA sequence divergence values which range from 0.29% (clone A vs B) to 1.20% (G vs H). These also show that the most diverse Karkloof clones diverged from a hypothetical ancestor merely 169 000 years ago, which is much more recent than the separation of the most diverse Kamberg lineages which occurred 612 000 years ago. This represents further evidence to the effect that Kamberg can be considered the evolutionarily older of the two populations.

Allozyme results of Taylor *et al.* (1992) reveal that Karkloof shows the third highest levels of heterozygosity and the second highest levels of polymorphism, respectively, of the 12 populations of *O. irroratus* studied (see section 1.2.3). Once again, as for Kamberg, these heterozygosity results suggest high levels of interbreeding between demes and continuous gene flow. This allozymic similarity between Karkloof and Kamberg is in sharp contrast to the markedly different mtDNA profiles found for these two populations.

4.3.4 Rietvlei mtDNA structure

The Rietvlei population was found to be represented by four different mtDNA matrilineages, the most common of which is representative of 50% of the assayed population (Table 6, Figure 4). The mtDNA results obtained are surprising because although Rietvlei is geographically the most distant from Kamberg and Karkloof, and is separated from them by the Drakensberg mountain range, this population is more closely related to each of the Natal midlands populations than they are to one another. Thus the clustering of Rietvlei clones I and F with the Karkloof lineages and that of lineages E and J with the Kamberg clones (Figure 8) is unexpected. A similar relationship is revealed by the parsimony network (Figure 5) which shows that Rietvlei lineage F is separated by six mutational steps from Kamberg and Karkloof lineage B and Rietvlei lineage J is separated by five mutation steps from Kamberg lineage M. Thus it appears that Rietvlei lineage I is the closest to ancestral lineage B. The geographic overlay (Figure 7) also shows Rietvlei as being "intermediate" between Kamberg and Karkloof, since clone I, which is the most common at Rietvlei, is separated from the most common clones at Karkloof (A) and Kamberg (L) by approximately the same number of mutational steps (15 and 17, respectively).

This unusual grouping of the Rietvlei lineages is further supported by the interpopulation sequence divergence values and divergence time estimates. It can be calculated that Kamberg and Rietvlei last shared a common ancestor between 87 000 and 510 000 years ago (average = 300 000 years) and that Karkloof and Rietvlei last shared a hypothetical ancestor between 86 000 and 344 000 years ago (average = 209 000 years). These events were thus more recent than the estimated divergence between Karkloof and Kamberg which appears to have occurred between 122 000 and 605 000 years ago (average = 365 000). Thus it does not seem unlikely that Rietvlei is more closely related to both Kamberg and Karkloof, than these are to one another.

4.4 MITOCHONDRIAL DNA DIFFERENTIATION WITHIN *Otomys angoniensis*

Unlike the situation with *O. irroratus*, there is no evidence of mtDNA phylogeographic structuring in *O. angoniensis*. Lineage T is represented by 3 individuals originating from Albert Falls and one individual from the University of Natal Campus in Durban (UND). Lineage V is represented by 3 individuals from Albert Falls and 4 individuals from UND. Another individual from UND can be seen to be clearly separate from the previously mentioned lineages (Table 6, Figure 8) and is the sole representative of lineage U.

On the basis of the mtDNA phylogenetic data for *O. angoniensis*, this species is best described as Avise *et al.* (1987) category III, phylogenetic continuity with spatial separation.

4.5 CONCLUSIONS

This study has confirmed that *O. angoniensis* and *O. irroratus* are indeed sibling species as suggested by morphological and chromosomal data. The mtDNA sequence divergence of 11.57% found between them is consistent with values cited in current literature for other rodent sibling species and indicates that these two taxa last shared a common ancestor between 1.2 and 2.4 million years ago. *O. angoniensis* showed no evidence of geographic structuring of mtDNA clones and can thus be classed as Avise *et al.* (1987) category III.

By contrast, the *O. irroratus* mtDNA clones were restricted to single locations. The only exception to this was lineage B which was represented at both Kamberg and Karkloof. The most parsimonious explanation for this phenomenon was that clone B is an ancestral lineage retained in both populations. Intraspecific sequence divergence values for the Rietvlei and Karkloof populations are within the same range as those quoted in current literature. The Kamberg population was, however, an exception since the

intrapopulation divergence estimates were of the same magnitude as those found between the Karkloof and Kamberg populations. This population was also characterised by a high clone:individual ratio. The most likely explanation for this appeared to be restricted female mobility. The sequence divergence values recorded for the most diverse clones of the Kamberg population seemed to substantiate the Pillay *et al.* (1993) hypothesis, that Kamberg is an incipient species of Karkloof *O.irroratus*. The MtDNA results also indicated that Karkloof and Kamberg last shared a hypothetical ancestor 365 000 years ago.

Rietvlei mtDNA data revealed an unusual phenomenon which requires further investigation. Rietvlei was found to be more closely related to both the Kamberg and Karkloof populations than these are to one another. This is particularly unusual since Rietvlei is separated by the Drakensberg mountain range, from the other two localities. The only explanation which seemed reasonable was that the Rietvlei population may have retained a number of ancestral lineages. Rietvlei is also shown to be the "youngest" of the three populations having last shared a common ancestor with Karkloof approximately 209 000 years ago and with Kamberg approximately 300 000 years ago.

Thus from these mtDNA results, it can be concluded that the *O. irroratus* phylogeographic profile most closely resembles Avise *et al.* (1987) category I.

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APPENDIX I**COMPOSITION OF BUFFERS AND STOCK SOLUTIONS**BROMOPHENOL BLUE LOADING DYE

0.1% bromophenol blue

0.1 M EDTA

50% glycerol

sterile distilled water

PREPARATION OF DIALYSIS TUBING

Boil tubing for 10 min in a large volume of 2% Na-bicarbonate,

1 mM EDTA

Rinse in distilled water

Boil for 10 min in 1 mM EDTA

Cool and store at 4°C in 50% ethanol

END-LABELLING BUFFER (ELB)

Solution A: 0.0417 M Tris-HCl

0.00417 M MgCl₂

pH 8.0

Solution B: 400 μ M dATP
400 μ M dTTP
400 μ M dGTP
3 mM Tris-HCl
0.2 mM EDTA
pH 7.0

To make ELB: 750 μ l solution A
250 μ l solution B
0.45 μ l β -mercaptoethanol

Store at -20°C

MSB-Ca⁺⁺ BUFFER

0.21 M D-Mannitol
0.07 M Sucrose
0.05 M Tris-base
3.0 mM CaCl₂
pH 7.5

MSB-EDTA BUFFER

0.21 M D-Mannitol
0.07 M Sucrose
0.05 M Tris-base
0.01 M EDTA
pH 7.5

STE BUFFER

0.10 M NaCl

0.05 M Tris

0.01 M EDTA

pH 8.0

10xTE STOCK

0.1 M Tris

10 mM EDTA

pH 8.0

Diluted 10 fold for use in dialysis.

10xTAE STOCK

2 M Tris-base

5.71 % v/v glacial acetic acid

0.5 M EDTA

pH 8.0

Diluted 10 fold for use in agarose electrophoresis.