RESTRICTION PATTERNS OF MITOCHONDRIAL DNA IN NATURAL POPULATIONS OF THE MURID SPECIES *OTOMYS IRRORATUS*

by

Alison Rimmer

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science,

in the

Department of Biology,

University of Natal

Durban 1994

PREFACE

The experimental work described in this dissertation was carried out in the Department of Biology, University of Natal, Durban, from May 1989 to May 1991, under the supervision of Dr J. Lamb and Mr G. Contrafatto.

These studies represent original work by the author and have 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.

Date: 25-2-95 Signed: A J Rimmer

ACKNOWLEDGEMENTS

Sincere thanks are due to the following people:

Dr J. Lamb, my co-supervisor, for help and encouragement and great assistance with the writing of the manuscript;

Mr G. Contrafatto, my co-supervisor for invaluable help with the analysis of the results and the writing of the manuscript;

Professor J. Meester, for valuable discussion and interest;

Dr K. Willan, Dr N. Pillay and Mr S. Shezi, for their dedicated care and maintenance of the experimental animals;

Dr N. Pillay, for his useful advice and help with the writing of the manuscript;

Mr B. Bredenkamp, whose help with techniques made this project feasible;

Dr B. Huckett, for invaluable advice on the handling of DNA, on-hands practical assistance and sharing of equipment;

Professor E. Harley, for accommodating me in his laboratory at the University of Cape Town whilst observing the techniques;

Miss C. O'Ryan, for demonstrating the techniques with thorough care and helpful advice;

S. Govender, K. Govindsamy, K. Appelsamy, A. Grace, J. Wesley-Smith and P. Wright, for their technical assistance;

Members of the Photosynthesis and Nitrogen Metabolism Unit (PNMRU), for assistance with equipment;

The Zoology Department of the University of Port Elizabeth, for generously allowing the use of their computer facilities.

This project was financially supported by the Foundation for Research and Development.

ABSTRACT

Mitochondrial DNA (mtDNA) was isolated from 8 different natural populations of the rodent species *Otomys irroratus* (Muridae: Otomyinae) and from one population of the species *O. angoniensis* occurring in South Africa. MtDNA samples were cleaved with five different restriction endonucleases, end-labelled with phosphorous-32, separated by electrophoresis on horizontal 1 % agarose gels and the resulting fragment bands were detected by autoradiography. The individual-specific fragment banding patterns were analysed and compared among the various populations. The percent sequence divergence among and between the populations was calculated using the formula of Nei (1979). A matrix of sequence divergence values for all intergenomic pairwise comparisons was subjected to a clustering analysis by the unweighted pair group method with arithmetic means (UPGMA, Sneath and Sokal, 1973), using the computer programme NTSYS (Rohlf, 1988). The results of these analyses allowed for a preliminary identification of phenetic groupings in the data set. A matrix generated by scoring the restriction endonuclease fragments as present or absent was used to generate a phylogenetic dendogram using the BIOSYS (Swofford and Selander, 1989) programme.

The overall restriction fragment variation uncovered in this study revealed 15 different mtDNA haplotypes within the 20 individuals examined. This corresponded to a high degree of polymorphism in the populations where more than one specimen was available, as well as within the species *O. irroratus*. There were no clones that were shared between any of the populations sampled.

The intrapopulation sequence divergence values uncovered in this study were high (range 0.35 % to 5.08 %), but also consistent with some other reports in the literature for intrapopulation variation. The outgroup, *O. angoniensis* revealed the highest divergence values when compared to the mtDNA clones found in *O. irroratus*.

The phenetic and cladistic cluster diagrams revealed overall similarity with one another. There appeared to be little correlation between the topology of the mtDNA haplotype phenograms and the geographic distance of the sample localities. There was, however, a marked congruence between the distribution of mtDNA haplotypes and the distribution of three distinct cytotypes occurring over the species range. A possible polyphyletic evolution of populations of *O. irroratus* was inferred from the cladistic analysis.

CONTENTS

LIST OF FIGURES

PAGE

LIST OF TABLES

PAGE

LIST OF PLATES

PAGE

LIST OF APPENDICES

PAGE

CHAPTER ONE

INTRODUCTION

1.1 General biology of *Otomys irroratus*

The genus *Otomys* is a member of the African rodent subfamily, Otomyinae (Muridae). The vlei rat, *O. irroratus* is widely distributed throughout the Cape Province, except in the northwestern, northern and some central areas (Davis, 1974). The species is also found in Natal, Kwazulu, the Orange Free State and the central and southern parts of the Transvaal (Figure 2.1). An isolated population occurs in eastern Zimbabwe and adjacent parts of Mozambique (De Graaff, 1981; Skinner and Smithers, 1990).

O. irroratus is a medium-sized, stocky rodent, which has a shaggy pelage, blunt face, large, yellow, deeply-grooved incisors, and a short well-haired tail. The ears are rounded. The coat colour is essentially buffy-brown dorsally, whilst the throat, cheeks, sides and ventral parts are paler (De Graaff, 1981; Skinner and Smithers, 1990).

The species is closely associated with damp vleis and wet grasslands or with streams and marshes (De Graaff, 1981; Smithers, 1983; Willan, 1982). *O. irroratus* is not, however, restricted to such areas as it also occurs in grass-covered montane areas away from watercourses (Skinner and Smithers, 1990).

O. irroratus usually constructs nests above ground under cover of dense vegetation, or may occupy the abandoned burrows of other small mammals (De Graaff, 1981). These animals utilise runways for access to their feeding areas (De Graaff, 1981; Carlton and Musser, 1984). They are generally solitary, asocial species with quite marked

territoriality, and hence migration among demes is restricted (Willan, 1982). In addition, the dispersal distance of the young is known to be low (Davis, 1973; Brown, 1988).

These rodents are herbivorous, eating mainly grass leaves and stems. They are primarily crepuscular, exhibiting some activity during both day and night (Perrin, 1981). Some populations breed only in the rainy season (Davis and Meester, 1981) while others are reproductively active all year round (Perrin, 1980).

1.2 Current taxonomical and evolutionary research on *O. irroratus*

The taxonomic status of the Otomyinae is uncertain and there have been difficulties in distinguishing between phenotypically similar taxa within the genus (Taylor *et al.*, 1989b; Contrafatto *et al.*, 1994a). Similar taxonomic uncertainties are encountered in the Bathyergidae (Honeycutt *et al.*, 1987). In this family, the determination of species, subspecies and local variants on the basis of morphological variation is ambiguous. Thus it has been suggested that the use of morphological characters to determine species and taxon boundaries be abandoned and/or supplemented with the analysis of genetic characters.

Over the last few years *O. irroratus* specimens from several localities have been collected by the Small Mammal Research Group at the University of Natal Biology Department, Durban. These animals have been the subject of extensive investigations which include general behaviour, reproductive and breeding biology, allozyme electrophoresis, morphometric analyses, karyotyping and immuno-blotting studies (Meester *et al*, 1988).

1.2.1 Chromosomal variation and speciation

It has been suggested that speciation may be initiated by chromosomal rearrangements (White, 1982; Capanna *et al.*, 1985; Bickham and Baker, 1978). Individuals representing populations which are chromosomally different may breed, but offspring resulting from such matings may be sterile and/or non-viable because of chromosomal imbalances. Such populations are thus subject to postmating reproductive isolation barriers. A similar model of speciation in which sibling species may arise following chromosomal rearrangements, before extensive gene mutations can be detected, has been proposed by Meester (1988). Such a model proposes that chromosomal rearrangements can trigger speciation events, by establishing postmating reproductive isolation, before genetic and phenotypic differences become apparent.

Contrafatto *et al.* (1992a, 1992b) report considerable chromosomal variation, both numerical and morphological, in populations of *O. irroratus* with diploid numbers ranging from 2n = 23 to 2n = 32. These authors suggest that a tentative division of *O. irroratus* into three broad 'chromosomal groups' may exist. A first group exhibits eight pairs of biarmed chromosomes and appears to be present in the Eastern Cape Province, whereas a second group includes almost exclusively acrocentric chromosomes and seems to have a more easterly geographical distribution. A third group, which exhibits four pairs of biarmed chromosomes, is to be found in the South West Cape and Orange Free State and Transvaal highveld. Furthermore, within the group of acrocentric cytotypes, three populations (occurring in the Drakensberg) carry a tandem fusion between chromosomes seven and 12 which correlates with severely impaired fertility in the F_1 generation from interpopulation crosses (Contrafatto *et al.*, 1992b; Pillay, 1990: Pillay *et al.*, 1992).

Electrophoretic studies however, reveal little or no significant phenotypic variation of allozymes among populations (Taylor *et al.*, 1992). Genetic distances from such an analysis involving 30 enzyme loci are at the level of local population differences (Nei's D = 0 to 0.117). They suggest no restriction of gene flow (no genotypic and phenotypic differences) between the various chromosomally-defined populations. The allozyme data support a 'dendritic' population structure (F_{IS} = 0.048; Taylor *et al.*, 1992) whereas the chromosomal data suggest that this species consists of a 'Wrightian' (sub-divided) population of small isolated demes (Contrafatto *et al.*, 1992b).

Definite differences based on morphometric methods (Hoffman, 1990) are not obvious and renal morphology is more or less similar between populations (Kearney, 1990). In contrast, however, evidence of reduced interbreeding between some populations (Hogsback, Committee's Drift and Karkloof) has also recently been established by Pillay *et al.* (1992).

From the studies outlined above, there appears to be a measure of reproductive isolation, and consequent occurrence of speciation, in *O. irroratus*. Such events seem to agree with Meester's (1988) model as the populations which are chromosomally distinguishable do not necessarily show gene-flow restriction by allozyme analysis (Taylor *et al.*, 1992, Contrafatto *et al.*, 1992b).

The sibling species *O. irroratus* and *O. angoniensis* are morphologically almost identical, apart from the shape of the petrotympanic foramen (Meester, 1988). The inter-relatedness of these two species is confirmed by both allozyme data (Taylor *et al.*, 1989b) and by immunoblot analysis (Contrafatto *et al.*, 1994) which clearly indicate that

among the Otomyinae, *O. angoniensis* is the closest sister species to *O. irroratus*. Therefore this species was included in this study as an outgroup to *O. irroratus*.

1.3 Approach to the present study

As there was a lack of correlation between cytogenetic and allozyme data (Section 1.2.1) it was decided to investigate further by employing a different and possibly more direct method of assessing genetic variation. Therefore an attempt was made to estimate DNA sequence divergence among the various populations of *O. irroratus*.

Avise *et al.* (1987) introduced a molecular technique whereby DNA divergence between naturally occurring populations can be estimated. This involves the digestion of mitochondrial DNA (mtDNA) by restriction endonucleases in order to compare the fragments of this DNA with those of similar DNA from other individuals. Fragments of mtDNA are obtained using restriction endonucleases which cut DNA at specific sites by recognising specific oligonucleotide sequences, usually four or six nucleotide pairs in length. The fragments that are generated are then separated on electrophoretic gels. The comparison of such mtDNA fragments with DNA fragments of known molecular weight allows the size of the sample fragments to be estimated. Comparison of fragment sizes between individuals can then be used as a basis for assessing variation and/or degree of homology within and between populations. Thus assessment of the sizes of DNA fragments generated by restriction endonuclease cleavage is a measure of DNA sequence divergence among the target sites of the restriction enzymes used in the study.

Information about mtDNA variation in natural populations has resulted primarily from restriction enzyme fragment patterns and site maps (Avise *et al.*, 1987). A typical survey

includes data from 10 or more enzymes and involves 40 to 100 restriction sites per individual (Avise *et al.*, 1987). Simple measures of genetic distance can then be derived from comparisons of restriction fragment patterns (Nei and Li, 1979), and the resulting distance matrices provide the basis for tree or phenogram construction. Alternatively, each fragment can be treated as a character having two states (present or absent), thereby providing a data set appropriate for phylogenetic analysis. This is done using parsimony, character compatibility or maximum likelihood methods.

1.4 The characteristics of mtDNA and its relevance to evolutionary studies

The general molecular properties of mtDNA are well documented (Avise *et al.*, 1987; Moritz *et al.*, 1987; Harrison, 1989). Therefore, this subject is not extensively reviewed in the present section except to highlight features relevant to the present study. Animal mtDNA is typically a covalently closed double-stranded circular molecule exhibiting conservation of gene content. The genome size of mtDNA differs only slightly between major taxonomic groups and, in vertebrates, is generally of the order of 16 kilobases (Brown *et al.*, 1979).

Although certain regions of the mtDNA genome evolve more slowly than others (Brown and Simpson, 1980; Aquadro *et al.*, 1984), there are no large conserved blocks. Mitochondrial DNA sequences diverge, among closely related individuals and species, five to 10 times more rapidly than the observed rate for single-copy nuclear DNA (scnDNA) (Brown *et al.* 1979); the amount of divergence of mtDNA appears to level off to a plateau at approximately 30% after 20 to 40 million years.

There is considerable variability among taxa in relative and absolute rates of sequence divergence (Moritz *et al.*, 1987), so a universal mtDNA molecular clock cannot be assumed. It can, however, be assumed that, within a taxonomic grouping such as a genus, the rate of sequence change is stochastically proportional to the time elapsed since divergence, at least until the plateau of 30% is reached.

Comparisons of individuals within a species show that nucleotide substitution pathways are strongly biased in favour of transitions as opposed to transversions (Aquadro *et al.*, 1984). Initially, the substitutions occur primarily at those sites on the mtDNA molecule which do not result in amino acid replacement (Moritz *et al.*, 1987). When these sites become saturated strong functional constraints on the remaining parts of the coding regions of mtDNA could cause the rate of nucleotide substitution to slow down (Brown *et al.*, 1979; Aquadro *et al.*, 1984). These features of sequence variation and evolution have been deduced by comparisons of mtDNA sequences from a variety of animal taxa (Brown *et al.*, 1979; Avise *et al.*, 1987; Moritz *et al.*, 1987).

It has been stated that restriction fragment comparisons have the following advantages in demonstrating genetic heterogeneity within and among conspecific populations (Avise *et al.*, 1987; Moritz *et al.*, 1987; Harrison, 1989):

- The rapid rate of nucleotide change ensures that a large number of reliable sequence differences accumulate.
- MtDNA is maternally inherited (Francisco *et al.*, 1978), so recombination during gametogenesis is eliminated, and sequence changes arise only from mutation.
- Mutations that are fixed will be reflected as a pure line of inheritance.

- Although polymorphism is high, relatedness among individuals in a population is apparent.
- The method is quick and relatively simple compared to direct sequencing methods and can be very informative when analysing closely related species.

There are also features of mtDNA which make it an unsuitable tool for applied research as noted by Avise *et al.* (1979), Hillis (1987) and Harrison (1989). Firstly, since mtDNA represents a single genetic marker unlinked to the nuclear genome, both random lineage sorting and differential introgression can lead to discordance between patterns of mtDNA variation and those seen for nuclear gene markers. Secondly, the distance measure used in the analysis involves computing the number of shared fragments between individuals (Nei and Li, 1979). Unfortunately this method can only be used for very closely related taxa since the rate of divergence decreases after about 8-10 million years, presumably due to selective constraints (Section 1.4, previous page). This means that the number of shared fragments will be low if taxa are not closely related, and the method will be inaccurate (Harley, 1988). Thirdly, homoplasy, the convergent loss or gain of particular restriction sites during evolution, has to be taken into account (George and Ryder, 1986; Lansman *et al.*, 1983), although at the taxonomic level of this study it was not important.

1.5 Mitochondrial DNA as a genetic marker

The proportion of mtDNA fragments shared between individuals from two populations is expected to be related to the degree of genetic divergence between the two populations. The characteristics of mtDNA listed previously have enabled many workers (Avise *et al.*,

1987; Harrison, 1989) to provide insight into population structure and gene flow, hybridisation, phylogenetic relationships, and ecobiogeography.

For instance, Baker *et al.* (1989) report that two taxa of pocket gophers (*Geomys*), which interact in a narrow hybrid zone, represent discrete mtDNA lineages. Other examples where distinct phylogenetic discontinuities occur within a species are found in the deer mouse, *Peromyscus* (Avise *et al.*, 1987), and the desert tortoise *Xerobates* (Lamb *et al.*, 1989). More recently, mtDNA studies of the teleost fish *Fundulus* (Gonzalez-villasenor and Powers, 1990) indicate discontinuities in mtDNA pattern distribution as presumably reflecting current environmental and/or historical influences.

In contrast, the American eel, (Avise *et al.*, 1986) and some species of crested newts (Wallis and Arntzen, 1989) show homogeneous patterns over their range, revealing no geographical differentiation with respect to mtDNA.

In many cases, relationships defined on the basis of mtDNA comparisons are concordant with relationships defined by morphological, behavioural or allozyme characters. However, there are examples of discordance in which greater similarity in mtDNA genotype has been noted between individuals in different species than between some pairs of conspecific individuals (Avise *et al.*, 1987).

The maternal mode of inheritance and absence of recombination make it possible to trace recent evolutionary history, including colonisation events and population bottlenecks. For example, it has been suggested that the dispersal of grasshopper mice (*Onychomys*) was affected by the same Quaternary climatic changes which have also resulted in fragmentation of arid habitats in western North America (Riddle and Honeycutt, 1990).

Phylogeographic patterns in mtDNA of African molerats, Bathyergidae (Honeycutt *et al.*, 1987), desert tortoises and gopher tortoises, *Xerobates* (Lamb *et al.*, 1989), the artiodactyl tribe Bovini (Miyamoto *et al.*, 1989) and sparrows, *Ammodramos* (Zink and Avise, 1990) all provide insight for reconstructing historical biogeographic and colonisation events.

1.6 Objectives of the study

From the information reported in the previous sections (Section 1.2 and 1.3), it was decided that the aims of the present study were to

- establish a reliable technique for isolating and processing mtDNA in this laboratory;
- estimate restriction endonuclease fragment polymorphisms at an interpopulation level in mtDNA isolated from some populations of *O. irroratus*;
- quantify the mtDNA divergence and make preliminary phylogenetic inferences from these results;
- comment on the relationship of genetic variation as evidenced by mtDNA restriction fragments to previously established estimates of variation in *O. irroratus* by karyotypic and allozymic studies.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Introduction

The greater portion of time and effort spent on this project involved the development of a reproducible experimental protocol for the isolation and purification of mitochondrial DNA (mtDNA). Numerous isolation procedures were attempted with little success. Brief mention of these methods will be made in an appendix to serve as a guide to those attempting future research in this area. Literature on mtDNA surveys of animals has not been found, in the author's opinion, to document such difficulties.

The procedures following the stage at which relatively pure mtDNA is obtained presented no major difficulty and could be routinely followed. One of the most important strategies followed was the use of sterile equipment and non-touch aseptic technique. Only analytical grade chemicals were used and all solutions were made up with deionised water sterilised by membrane filtration and autoclaved prior to use. The compositions of all solutions are listed in Appendix 1.

2.2 Collection and storage of animal specimens

Specimens of *Otomys irroratus* were live-trapped at localities in different parts of South Africa from the extreme south-west to the north-east. Seventeen samples from eight localities were used in order to get representatives over most of the species range. More than seventeen specimens were sampled originally, but owing to experimental difficulty in extracting mtDNA, not all of these were available for analysis. Three specimens of *O. angoniensis* from Pigeon Valley, Durban, were also collected for use as an outgroup in

the cladistic analysis (Section 1.2.1). Geographic positions are indicated in Figure 2.1. The sample composition is listed in Table 2.1.

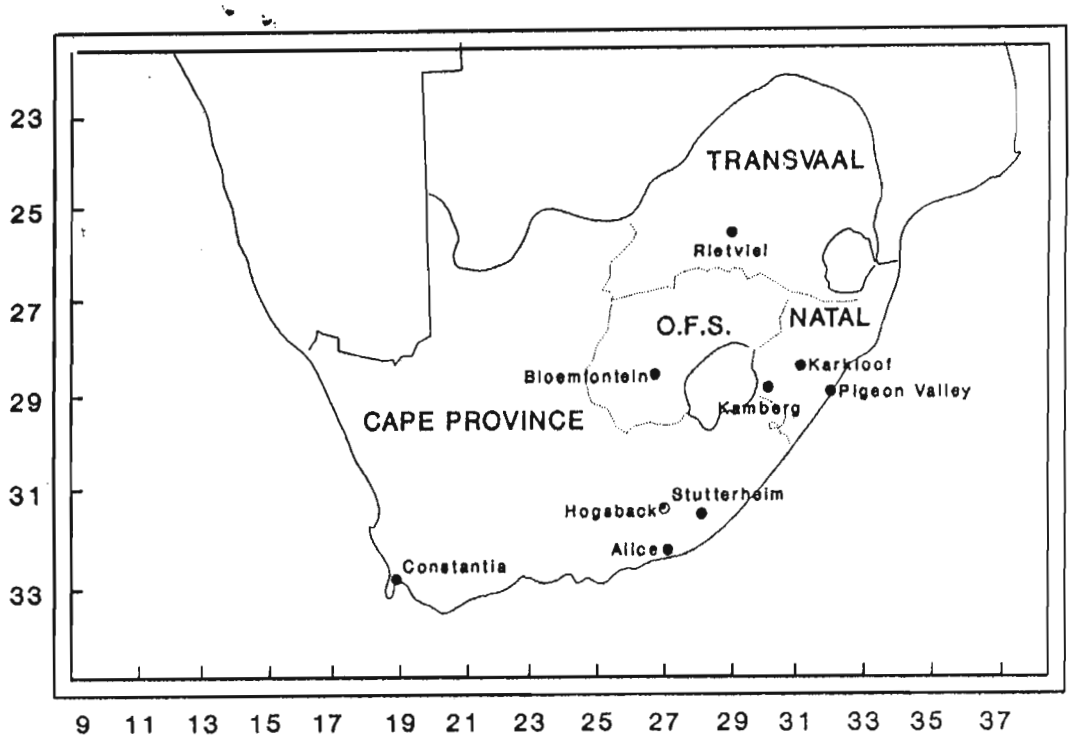

Figure 2.1 Map of southern Africa showing localities of populations of *O. irroratus* where samples were collected. The 3 samples of the outgroup *O. angoniensis* were collected from Pigeon Valley. The remaining localities show where *O. irroratus* specimens were collected. The grid references and sample numbers are shown in Table 2.1.

Table 2.1 Localities and sample sizes (*n*) for *O. irroratus* and *O. angoniensis* (Pigeon Valley) specimens represented in the final analysis.

Locality	Grid reference	(*n*)
Kamberg	29°23'S 29°42'E	5
Karkloof	29°20'S 30°13'E	4
Rietvlei	25°51'S 28°18'E	1
Hogsback	32°33'S 26°56'E	2
Constantia	34°02'S 18°22'E	1
Bloemfontein	29°07'S 26°13'E	1
Alice, UHF Farm	32°47'S 26°50'E	2
Stutterheim	32°34'S 27°26'E	1
Pigeon Valley	29°51'S 30°59'E	3

Animals were held captive for periods of a few days to several months before sacrifice. Upon sacrifice tissues suitable for extracting mtDNA (kidney, heart and liver) were removed from the animals. Tissues were stored in 1 ml cryotubes in liquid nitrogen. Voucher specimens of the animals included in this study were prepared by other members of the Small Mammal Research Group and have been deposited in the mammal collections of the Durban Natural Science Museum (see Appendix 4 for voucher specimen numbers).

2.3 Extraction of mtDNA

2.3.1 Modification of the method of Coen *et al.* (1982) for isolation & purification of mtDNA

Liver and kidney samples (approximately 2 g) were frozen with liquid nitrogen before being crushed into a fine powder using a pestle and mortar. Samples were then homogenised with a hand-held homogeniser using 5 ml of a calcium-containing buffer (CaRSB Buffer, Appendix 1). The resulting nuclei and cell debris were pelleted 3 times by centrifugation at 3000 rpm in a GPR benchtop centrifuge (Beckman, United Kingdom) at 4 °C for 10 min in 5 ml of a mannitol-sucrose buffer (MS Buffer, Appendix 1).

Mitochondria were then collected by centrifugation of the resulting supernatant at 15000 rpm in a Beckman J2-21 centrifuge and JA14 rotor for 20 min at 4 ^{O}C. The pellet, which was expected to contain the crude mitochondrial preparation, was resuspended in 500 µl of Extraction Buffer. Mitochondria were lysed by incubation in 500 µl SDS Buffer for 30 min at 65 ^{O}C.

Following incubation, 100 µl 3 M sodium acetate (pH 5.0) were added to the lysed preparation which was then incubated at 4 ^{O}C for 45 min. This lysate was microfuged at 8000 rpm for 10 min (Beckman, United kingdom). The supernatant was diluted with one volume isopropanol, left at room temperature for 5 min and then centrifuged at 8000 rpm for 5 min in the microfuge. The resulting pellet was left to dissolve in 250 µl H_2O for approximately 30 min. After this 250 µl H_2O, 50 µl sodium acetate (3M, pH 5.0) and 2 volumes (total) absolute ethanol were added. The preparation was placed at -20 ^{O}C for 30 min to allow precipitation of DNA.

The DNA was pelleted in the microfuge for 5 min, after which the supernatant was discarded. The pellet was then left to dissolve overnight in 180 µl of TE buffer at 4 ^{O}C after which the DNA solution could be stored indefinitely at -20 ^{O}C.

2.3.2 Screening of samples by electrophoresis

At this stage each DNA sample was separated by electrophoresis on a minigel apparatus which was designed and made for quick, inexpensive screening of samples (Appendix 2). A 1 % agarose (FMC Bioproducts, United Kingdom) solution was made with TAE Buffer, heated in a microwave oven for a few minutes until the agarose dissolved, and poured into the gel-forming apparatus. A template (comb) inserted into the agarose

allowed 8 wells to be formed when the agarose set. A 10 µl aliquot of each sample was mixed with 1 µl Loading Buffer and loaded in each well. A potential difference of 20 volts (V) was applied for 10 min after which the gel was removed and placed in 150 ml Staining Solution. The gel was viewed on a Hoefer Mighty Bright UV transilluminator in a dark room. The presence of a mtDNA band was verified by its position relative to the two heaviest bands of the marker, a *Hind III* digest of bacteriophage lambda (λ) DNA (Brown *et al.*, 1979; Harley, E., pers. comm.).

Spectrophotometric measurements of the mtDNA concentration were not taken in order to preserve the sample mtDNA as much as possible. An experiment to find out the minimum volume of mtDNA that could be loaded from a typical extracted sample, and still visualised with ethidium bromide-staining, was performed. The recommended volume was 7-8 µl (Bredenkamp, B., pers.comm.), and so 4, 5, 6, 7, 8, 9 and 10 µl of two test samples were loaded in each agarose well respectively, electrophoresed, and visualised with ultra-violet light. A 5 µl aliquot of sample proved to be the minimum volume which could be visualised in this manner.

2.3.3 Purification of mtDNA

Each sample which tested positive for mtDNA was treated with 5 µl of RNAse A (Sigma Biochemicals, USA) at 37 ^{o}C for 45 min. The RNAase A was treated prior to use in order to eliminate any contaminating DNases which may have been present (Maniatis *et al.*, 1982). A chloroform-isoamyl alcohol-chloroform extraction was then performed according to the standard procedure outlined in Maniatis *et al.* (1982) to separate the DNA from the added RNAse. Thereafter 0.1 volumes of sodium acetate solution (3 M, pH 5.2) and 2 volumes of ice-cold absolute alcohol were added to the purified mtDNA

preparation, which was then stored at -20 °C for 30 min in order to precipitate the DNA. The precipitate was collected by centrifugation in a microfuge for 5 min at 8000 rpm and then redissolved in 180 µl TE buffer. This method of extraction and purification proved to be the most efficient of all the methods tested and was used routinely on all the samples selected for the analysis.

2.4 Restriction endonuclease digestion of mtDNA

A typical restriction endonuclease digestion reaction contained the following: 14 µl H_2O, 2 µl appropriate incubation buffer, 3 µl DNA sample, and 1 µl restriction endonuclease.

All the restriction endonucleases (see below) and the accompanying incubation buffers were supplied by Boehringer Mannheim (Germany) and used according to manufacturer instructions with no modification. Each DNA sample was treated with the following restriction endonucleases: *Xba I, Hind III, Pvu II, Dra I, EcoR I, Sal I, Xho I, Mlu I, Ssp I, Sac I, Apa I, Hpa I, EcoR V, Stu I* and *Bgl II*. These restriction enzymes were chosen because they cleave DNA at six base pair recognition sites, so that not too many fragments from each mtDNA sample would be generated. They are also reasonably inexpensive, and they have been commonly used in other mtDNA studies. Unfortunately results for only five of the above restriction enzymes were available for analysis.

Bacteriophage λ DNA (Boehringer Mannheim, Germany) digested with *Hind III* was electrophoresed on each gel to provide a series of molecular weight markers. Each digestion contained 16 µl H_2O, 2 µl Incubation Buffer B 1 µl phage λ DNA, and 1 µl *Hind III*.

All the above digestion reactions were carried out in 1.5 ml microfuge tubes at 37 ^{O}C for 1 h and thereafter immediately placed on ice. One µl of Loading Buffer was added to each reaction mixture before electrophoresis was performed.

2.5 Agarose electrophoresis

A horizontal agarose gel was cast using 1 % agarose in TAE Buffer. One µl EtBr (10 mg ml^{-1}) was added to the gel mixture before the agarose was poured into the gel former in order to allow visualisation of the DNA with ultra-violet light. Twenty-one µl of DNA sample solution resulting from digestions were loaded into wells in the agarose gel and electrophoresed at 90 V for approximately 1.5 h in a Hoefer Slab Gel Unit (USA) using TAE Buffer as the running buffer. The gel was destained by immersion in distilled water for 15 min and the DNA bands were visualised on a transilluminator (Hoefer Mighty Bright, USA). A Minolta camera with a 50 mm objective lens and a red filter was loaded with Ilford (United Kingdom) HP5 monochromatic film and used to photograph the gel. The aperture was set at 5.6, and the film was exposed for 2 seconds. The film was developed for 12 min in Kodak D19 developer (diluted 3-fold) after which it was washed twice in water. Fixation was for 4 min in Ilford Hypam fixer, diluted 4-fold. Negatives were enlarged and printed on Ilford monochromatic paper using an Ilford high-contrast filter (No.5). The paper was exposed for 8 min, developed for 4 min, and fixed according to standard photographic methods.

Initially, restriction endonuclease digestions of samples were performed with the intention of visualizing DNA fragments on EtBr-stained agarose gels. However, it

became apparent that the sensitivity of the method was not great enough to be used, given the small amounts of mtDNA isolated, and the fact that the sensitivity of detection diminishes with decreasing molecular weight of the fragments. It was therefore decided to radiolabel the DNA with phosphorous-32 (^{32}P) in order to allow smaller quantities of DNA to be used per digestion, as radiolabelling increases the sensitivity 100-fold (Harley, 1988). An EtBr-stained sample gel, and a radiolabelled mtDNA gel are illustrated in Plate 2.1 for comparative purposes.

a) b) H λ K C

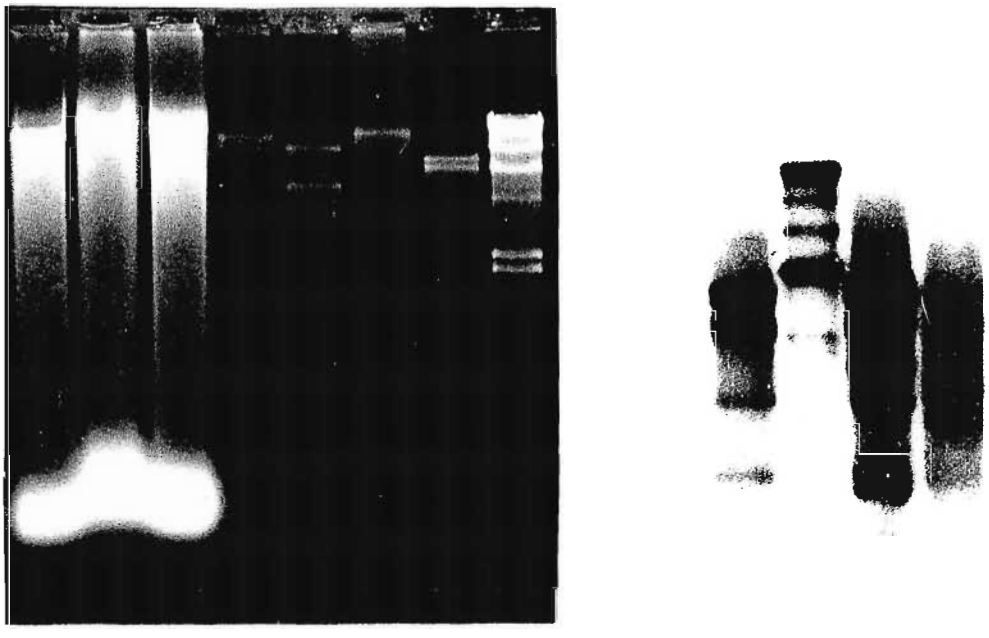

Plate 2.1 Photographs of an ethidium-bromide-stained test gel (a) The 3 lanes on the left-hand side of the photograph show mtDNA samples that were contaminated by nuclear DNA. Lanes 4 and 6 show undigested mtDNA from samples that were free from contamination by nuclear DNA, and lanes 5 and 7 show two samples that were restriction endonuclease digested. Each specimen yielded a different banding pattern. The last lane shows the phage λ DNA digested with *Hind III* which served as a set of molecular weight markers. b.) Autoradiograph showing the phage λ DNA digested with *Hind III* in lane 2 from the left, and three other lanes showing specimens from Hogsback (H), Karkloof (K) and Constantia (C) which were digested with *Eco RI*.

2.6 Radiolabelling of mtDNA fragments

Mitochondrial DNA was first cleaved, and the resulting restriction fragments were labelled with (alpha-^{32}P)dCTP (Amersham, United Kingdom) according to a protocol slightly modified from Maniatis *et al.*, 1982. A radiolabelling laboratory was set up with appropriate safety precautions (according to guidelines from the Directorate of Radiation Control, Department of Population Growth and Development, Pretoria, R.S.A.). A

safety screen 70 cm x 50 cm x 2 cm made of transparent perspex was designed and made for protection at the laboratory bench.

Restriction endonuclease digestions were performed according to the procedure outlined in Section 2.5 except that the volumes were reduced as follows: 1 μl H_2O, 2 μl appropriate incubation buffer, 6 μl DNA sample, and 1 μl restriction endonuclease. At the end of the prescribed digestion time the digested samples were stored at -20 ^{O}C until a fresh batch of (alpha-^{32}P)dCTP, which has a short half-life (14.3 days), was ordered.

2.6.1 Endlabelling of mtDNA with (alpha-^{32}P)dCTP

Klenow enzyme (large fragment of DNA polymerase I) was used to 3'-endlabel the cleaved mtDNA fragments using (alpha-^{32}P)dCTP. One μl of Klenow enzyme (2000 units ml^{-1}, Boehringer Mannheim, Germany) was diluted in 9 μl water and 1 μl of this solution was added to each digested mtDNA sample. The concentration of Klenow enzyme used was sufficiently high to ensure that the endlabelling reaction occurred. The mixture was incubated at room temperature for 15 min to allow the Klenow enzyme to remove bases from the 3' ends, creating 5' overhangs, before the addition of the labelled bases. After this 2 μl of a stock solution containing dATP, dTTP and dGTP (20 μM) (Boehringer Mannheim, Germany) was added to the solution in the microfuge tube, and immediately thereafter the reaction tubes were transferred to the radiolabelling laboratory where 1 μl of (^{32}P)dCTP (~3000 Ci mmol^{-1}, freshly diluted 10 fold in water) was added to each tube. The solution was incubated at room temperature for 30 min to allow the labelling reaction to occur.

2.6.2 Electrophoresis of radiolabelled mtDNA samples

One µl of Loading Buffer was added to the solution in each reaction tube, and the total volume (15 µl) was loaded onto a 1 % agarose gel and electrophoresed in TAE Buffer at 100 V for 1.5 h.

2.6.3 Gel drying, film exposure and development

The gel was carefully removed from the buffer and placed between two thin wads of filter paper (Whatmann No. 3, England) on the surface of a Biorad Slab Gel Dryer (USA). A vacuum pump applied to the dryer at room temperature for 10 min enabled the gel to be dried successfully. The dry gel was wrapped in a single layer of plastic wrap and placed on top of a sheet of Hyperfilm MP (Amersham, United Kingdom) between two intensifying screens (Amersham, United Kingdom) in an autoradiography cassette (Amersham, United Kingdom) in a dark room. The Hyperfilm MP was coated with emulsion on both sides. The cassette was wrapped in a light-tight black bag and the film was exposed at -70 ^{O}C for 1 h. The aforementioned conditions allowed for optimal qualitative resolution of the labelled DNA on the autoradiographs. The film was removed in the dark room, developed for 2 min in G150 Developer (Agfa Chemicals, Germany), diluted 4 times in water and fixed for 2 min in 3 times diluted G334 C Fixative under safety lights (Agfa Chemicals, Germany). The film was washed for 30 min in water prior to air-drying.

2.6.4 Photography of radiolabelled exposures

Autoradiographs, illuminated by incident and transmitted light, were photographed with a Minolta camera (f stop 5.8) loaded with Pan F film (Ilford, United Kingdom). Thereafter the film was developed in D19 developer, diluted 4 fold in water, for 8 min.

Ilford monochromatic paper was exposed for 12 min using an Ilford filter (No.3.5), developed for 4 min, and fixed according to standard processing methods.

2.7 Data analysis

2.7.1 Definition of terms

There are many different terms used in the literature to describe the classification of mtDNA (Brown and Simpson, 1980; Avise *et al.*, 1987; Wallis and Arntzen, 1989, for other terminology). In order to avoid possible confusion in the understanding of the classification of mtDNA patterns in this project, some definitions are given:

RESTRICTION FRAGMENT. A restriction fragment is visualised as a band on an electrophoretic gel. Each restriction endonuclease used in this study cleaves the circular mtDNA, producing a number of fragments which migrate to different positions on the gel according to their molecular size.

HAPLOTYPE. This is the restriction pattern produced by gel electrophoresis of one sample of mtDNA after digestion with a single given restriction endonuclease. Each isolated mtDNA preparation is tested with a number of restriction enzymes, each producing a haplotype, which is then assigned an upper-case Arabic letter. It should also be pointed out that no necessary relationship exists between the haplotypes produced by different enzymes, or by the same enzyme in different individuals, since the letters are assigned arbitrarily.

CLONE. A clone indicates the particular composite pattern of haplotypes produced by a given mtDNA of an individual. Individuals that share a composite code belong to the

same mtDNA clone, although this of course applies only to the particular restriction fragments surveyed.

2.7.2 Haplotype and clonal determination

The relative mobilities of the *O. irroratus* mtDNA restriction fragments were measured and compared with those of the λ DNA in order to estimate their molecular weights. Individuals that shared the same fragment pattern for a particular enzyme were assigned the same haplotype, and individuals were grouped in the same clone when they shared haplotypes for all enzymes.

2.7.3 Divergence estimation

The composite data were also summarised in a presence-absence matrix of all mtDNA fragments (Appendix 2), which was then used to compute divergence (*d*). The proportion of fragments shared by two mtDNA clones is given by

$$F = \frac{2n^{xy}}{n^x + n^y}$$

(Nei and Li, 1979) where n^{xy} is the number of shared fragments between clones x and y, n^x is the number of fragments in clone x, and n^y is the number of fragments in clone y.

A maximum-likelihood estimate of sequence divergence is given by

$$d = \frac{-\ln F}{r}$$

(Nei and Li, 1979), where r is the number of bases in the restriction enzyme oligonucleotide recognition sequence (i.e. six in all cases in the present study).

However, there were cases where the amount of mtDNA was insufficient to be screened by all the restriction enzymes and cases where digestions did not yield interpretable results. A sample was included in the analysis if 50% of the digestions were successful; data for an enzyme was included if 50% of the animals were successfully screened using the enzyme. For any one pairwise comparison, if a haplotype was missing from either sample, this haplotype was eliminated from the analysis of that pair. This was because divergence values would otherwise have been overestimated since this value is based on a ratio between the number of shared fragments and the number of fragments present in each sample (see above) (Nei and Li, 1979).

A matrix of *d* values for all intergenomic pairwise comparisons was subjected to a clustering analysis by the unweighted pair group method with arithmetic means (UPGMA, Sneath and Sokal, 1973), using the computer programme NTSYS (Rohlf, 1989). The results of these analyses allowed for a preliminary identification of phenetic groupings in the data set.

A character state table is shown in Table 2A (Appendix 2) whereby a restriction fragment was scored 1 when it was present in a clone, or it was scored 0 if not present in a clone. These character states were manipulated for use in the BIOSYS computer programme so that a Wagner network could be constructed (using *O. angoniensis* as the outgroup). Double allele genotypes (AA or BB) were used to re-code the presence or absence character states so that they could be accepted for use in the BIOSYS computer programme (Swofford and Selander, 1989). As only 29 of the 53 mtDNA fragments were phylogenetically informative, i.e. were shared by at least two clones, the appropriately reduced matrix was used to generate the Wagner tree.

The resulting Wagner tree was uninformative as some clones, in populations where more than one specimenwas available, did not cluster together or were not resolved at all. Such instability seemed to be caused by the few lineages which were different from the majority of the clones of a given population (i.e. from Karkloof and Kamberg). Therefore, one clone per population was chosen as representative, on the basis that it had all the haplotypes available for analysis, and used to provide a more easily interpretable Wagner tree. This aspect is discussed in Chapter 4, Section 4.2, page 52.

A cladogram was generated from the presence-absence data (Appendix 2) using the computer programme Hennig86 (Farris, 1988) and, in the same way as outlined above, (using *O. angoniensis* as the outgroup), three equally parsimonious trees were generated using a representative clone for each Kamberg and Karkloof population.

CHAPTER THREE

RESULTS

3.1 Restriction fragment variation

Each extracted sample of *Otomys irroratus* mtDNA was initially tested by electrophoresis on a minigel to confirm the presence of mtDNA by estimating its molecular weight. The expected molecular weight of the mtDNA was 16.5 kilodaltons (Section 2.3.2), and the extracted mtDNA samples always migrated to the same position, with respect to the marker DNA fragments, on the gel. This position corresponded to a molecular weight in the region of 16 kilodaltons.

The five restriction enzymes used each yielded between 1 and 7 fragments per specimen, which resulted in a total of 20 to 25 scored fragments for each individual and therefore revealed an average of 22.5 cleavage sites per rat. This corresponds to 135 base pairs (bp) in recognition sequence and represents 0.84 % of the mitochondrial genome of *Otomys*, using the estimated size of 16 kilobase pairs.

Five restriction enzymes and 20 individuals representing nine localities were used in the final analysis. The overall restriction fragment variation uncovered in this study revealed 15 different mtDNA haplotypes within the individuals examined (Section 2.7.1, for terminology). The relationship between the haplotypes and the individual animals is shown in Table 3.1.

Table 3.1 Mitochondrial DNA clones observed in samples of *O. irroratus* and the outgroup *O. angoniensis*. Alphabetical letters, from left to right refer to haplotypes produced by digestion with *Xba I, Hind III, Pvu II, Dra I* and *EcoR I* respectively. *n* = number of individuals, a dash = missing data.

Clone name	composite mtDNA haplotype	Locality	*n*
KAM1	AAAAA	Kamberg	2
KAM2	BAAB-	Kamberg	1
KAM3	CAAAA	Kamberg	1
KAM4	ABAAA	Kamberg	1
KAR1	B-AA-	Karkloof	1
KAR2	B-AAB	Karkloof	1
KAR3	BAAAA	Karkloof	1
KAR4	BB-AA	Karkloof	1
OUT	DCACC	Pigeon Valley	3
RIE	BD-AB	Rietvlei	1
HOG	BAAAD	Hogsback	2
CON	E-AD-	Constantia	1
BLM	-DA-B	Bloemfontein	1
ALI	FE-EB	Alice,UHF Farm	2
STU	GAAAA	Stutterheim	1

A common mtDNA haplotype (AAAAA) was observed in 2 individuals from Kamberg, while three other individuals from Kamberg were found to have unique haplotypes. The mtDNA clones for the individuals from Kamberg were designated KAM1, KAM2, KAM3 and KAM4 respectively.

The samples from Karkloof also differed considerably from one another with respect to mtDNA cleavage patterns; four clones were apparent. These mtDNA clones were designated KAR1, KAR2, KAR3 and KAR4 respectively. Unfortunately some data were missing and these clones may not reflect accurate groupings of mtDNA haplotypes. The samples representing the sibling species, *O. angoniensis*, all shared identical cleavage patterns and the clone was designated OUT (Table 3.1). The haplotypes of the individuals from the remaining localities that were sampled were all unique with respect to locality and were designated names in a similar manner (Table 3.1).

One restriction enzyme (*Pvu II*) revealed a single mtDNA digestion pattern for all individuals. Patterns of mtDNA digested with *Xba I* showed the greatest variation; seven haplotypes were present among the 20 individuals. *Xba I* cleaved the sample mtDNA into many fragments which were not visualised as discretely as the other digestion profiles. *EcoR I* yielded four haplotypes; *Hind III* and *Dra I* each yielded five different haplotypes among the individuals examined (Table 3.1).

3.2 MtDNA divergence between *O. irroratus* clones

Estimates of nucleotide sequence divergence between 14 *O. irroratus* clones and one *O. angoniensis* clone are presented in Table 3.2.

Table 3.2 Matrix of percent nucleotide sequence divergence estimates (after Nei & Li, 1979) between 14 mtDNA clones of *O. irroratus* and one *O. angoniensis* clone. For clonal designations see Table 3.1.

Nucleotide diversity (%)

mtDN clone	STU	ALI	BLM	CON	HOG	RIE	OUT	KAR4	KAR3	KAR2	KAR1	KAM4	KAM3	KAM2	KAM1
STU	0.00														
ALI	8.66	0.00													
BLM	8.09	0.00	0.00												
CON	10.5	13.9	0.00	0.00											
HOG	2.82	4.08	12.9	9.06	0.00										
RIE	5.52	3.97	0.00	13.9	7.10	0.00									
OUT	12.4	22.0	8.98	10.5	9.71	16.2	0.00								
KAR4	4.64	6.24	18.3	13.1	7.65	5.94	20.9	0.00							
KAR3	0.37	6.24	8.09	9.06	2.32	4.79	9.71	3.93	0.00						
KAR2	0.58	3.97	0.00	9.06	3.29	0.00	11.5	1.69	0.43	0.00					
KAR1	4.08	6.04	0.00	9.06	0.00	0.00	9.91	0.00	0.00	0.00	0.00				
KAM4	1.82	5.30	10.3	9.58	6.40	6.35	14.9	0.43	3.54	1.75	0.54	0.00			
KAM3	0.37	4.79	8.09	11.0	3.18	5.94	12.7	5.08	0.74	2.28	0.58	3.54	0.00		
KAM2	0.95	10.1	9.79	8.50	0.45	4.60	9.32	6.19	0.45	0.58	0.58	5.08	1.40	0.00	
KAM1	0.74	5.30	8.09	9.06	2.68	5.20	11.5	4.37	0.35	1.75	0.54	3.00	0.35	0.90	0.00

The divergence values between some mtDNA clones were zero as a result of the the method used to treat missing data (Section 2.7.3). The overall divergences range from 0.35 % to 22.00 % (Table 3.2). The interspecific nucleotide divergence between *O.irroratus* and the outgroup *O. angoniensis* ranged from 8.98 % and 22.00 %. Pairwise comparisons of mtDNA clones from individuals collected in Natal (Kamberg and Karkloof) revealed lower intrapopulation divergence estimates than those among haplotypes from localities elsewhere in southern Africa (Table 3.2, KAR4 and KAR2 versus KAR4 and ALI or KAR4 and BLM)).

Pairwise divergence estimates obtained for the western Cape clone (CON) were notably higher (mean divergence 10.17 %, range 8.5 % to 13.9 %) compared to other clones. This was also true for the Bloemfontein clone (BLM, mean divergence 10.56 %, range 8.09 % to 18.3 %). This divergence value of 10.56 % for the BLM clone appears to be over estimated as there were 4 pairwise comparison data points missing from the data (Table 3.2). The divergence estimates for the STU clone collected from Stutterheim in the Eastern Cape were of similar magnitude to those of the Natal clones (Table 3.2).

The intrapopulation nucleotide sequence divergences within the Kamberg population ranged from 0.35 % (clones KAM1vs KAM3) to 5.08 % (clones KAM2 vs KAM4), whilst within the Karkloof population, the range was from 0.43 % (clones KAR2 vs KAR3) to 3.93 % (clones KAR3 vs KAR4). The interpopulation sequence divergences between Kamberg and Karkloof populations of *O.irroratus* ranged from 0.43 % to 6.19 %. A condensed divergence matrix where the divergences of the 4 Kamberg clones and the 4 Karkloof clones were averaged and called KAM and KAR clones respectively, is presented in Table 3.3.

Table 3.3 Matrix of nucleotide sequence divergence percentages (after Nei & Li, 1979) between 9 mtDNA clones of *O. irroratus* and one clone of *O. angoniensis*. For clonal designations see Table 3.1

Nucleotide diversity (%)

mtDNA clone	STU	ALI	BLM	CON	HOG	RIE	OUT	KAR	KAM
STU	0.00								
ALI	8.66	0.00							
BLM	8.09	0.00	0.00						
CON	10.5	13.9	0.00	0.00					
HOG	2.82	4.08	12.9	9.06	0.00				
RIE	5.52	3.97	0.00	13.9	7.10	0.00			
OUT	12.4	22.0	8.98	10.5	9.71	16.2	0.00		
KAR	2.42	5.62	6.60	10.1	3.32	2.68	13.0	0.00	
KAM	0.97	6.37	9.07	9.54	3.18	5.52	12.1	4.02	0.00

This matrix revealed similar trends to those shown in Table 3.2.

3.3 Cluster analyses

The analyses that were undertaken should be viewed with some caution since the number of restriction enzymes used in the final analysis was smaller than is routinely used in such analyses.

3.3.1 Distance phenograms

The entire original matrix of *d* values (Table 3.2) was used to construct an UPGMA phenogram for the 15 mtDNA clones. The clustering procedure yielded more than 25 trees using the 'find' option in the computer programme NTSYS (Rohlf, 1988). This meant that all tied values (when present) were alternately clustered resulting in more than one tree being found. It thus appeared that the trees were unstable, and the apparent cause of tree instability was due to inconsistencies in the higher order branching levels; i.e. the Natal populations (Kamberg and Karkloof).

In order to deal with the apparent instability it was decided to average each of the four Kamberg and four Karkloof divergence estimates respectively. This was expected to give a preliminary idea of the underlying patterns of mtDNA in *O. irroratus* populations. The resulting three UPGMA phenograms are presented in Figure 3.3.

Fig. 3.3a

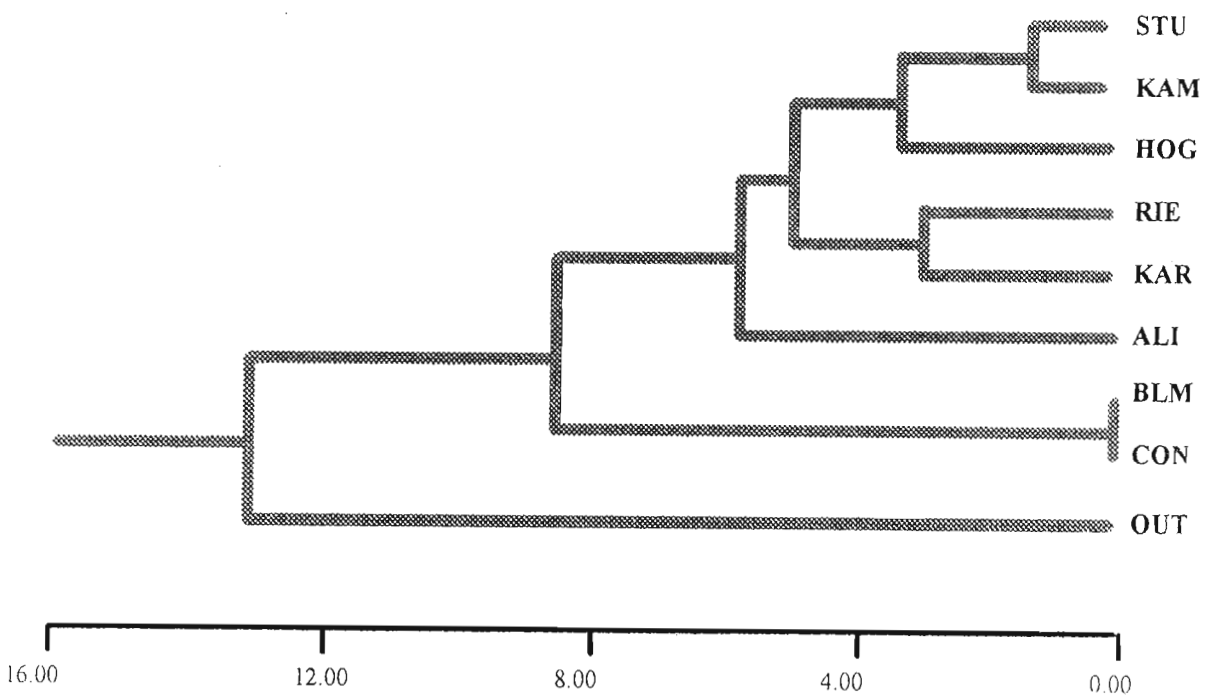

Figure 3.3a,b and c contd

Fig. 3.3b

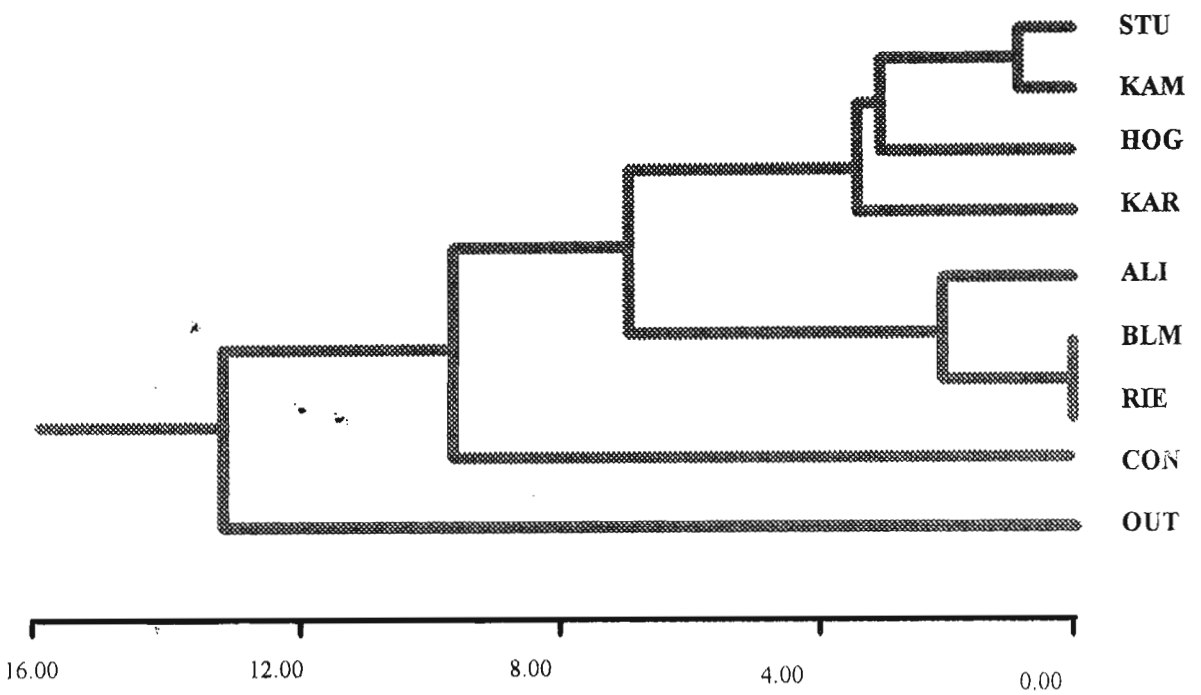

Fig. 3.3c

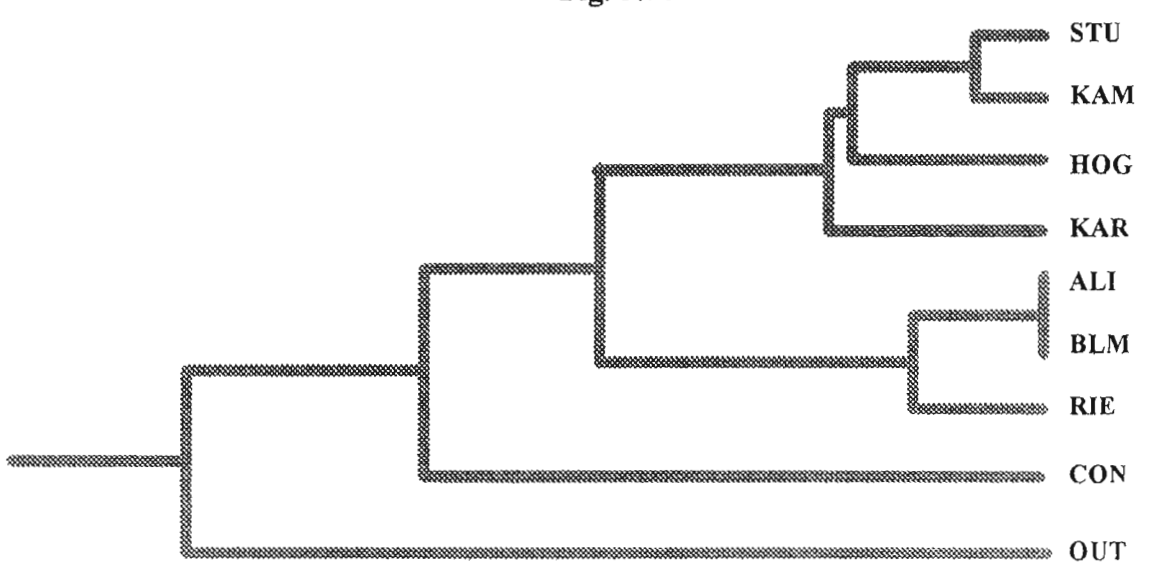

Figure 3.3a,b,c UPGMA phenograms clustering the distance matrix of sequence divergence among *O. irroratus* and *O. angoniensis* mtDNA clones (KAM is the average of the divergences of KAM1 to KAM4, KAR is the average of the divergences of KAR1 to KAR4). For clonal designations see Table 3.1.

A consensus tree was obtained using the 'majority rule' option in the NTSYS computer programme (Rohlf, 1988). This is presented in Figure 3.4. The *O. angoniensis* clone clustered in the way it did in the previous analyses (Figs 3.3a,b and c). Likewise, the CON clone separated sharply from all other populations in two of the three equivalent phenograms. The dichotomies between clones ALI and RIE and between clones BLM and CON were not resolved, while a cluster was formed by the Natal populations together with STU and HOG. When the 'Stinebrickner' option was used in the NTSYS computer programme, the BLM clone separated out from the ALI and RIE cluster of clones, but the remaining clones separated in a similar way to the aforementioned tree.

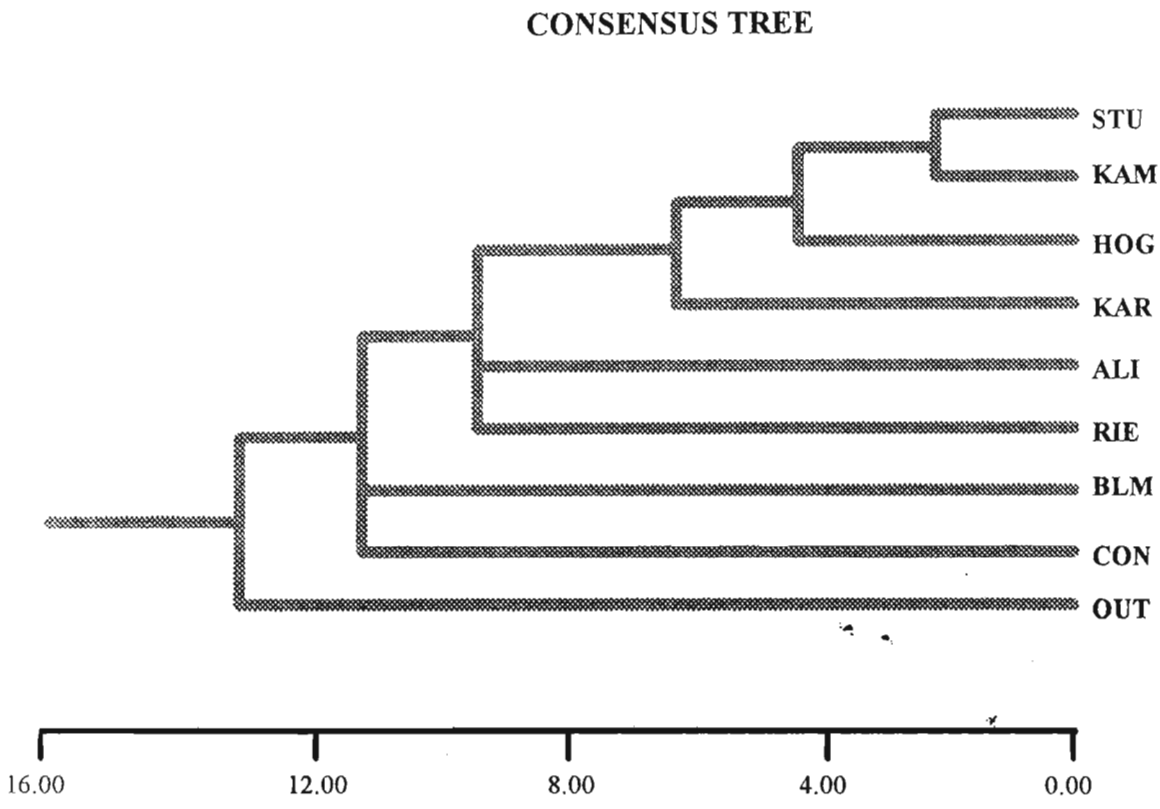

Figure 3.4 The consensus tree obtained using the 'majority rule' option in the NTSYS computer programme (Rohlf, 1988). For clonal designations see Table 3.1.

3.3.2 Cladistic analysis

A Wagner tree was obtained from a condensed matrix of a modified version of the data in Table 3A (Appendix 3) so that it was accepted by the BIOSYS programme (Swofford & Selander, 1989) (Section 2.7.3.). This tree is shown in Figure 3.5.

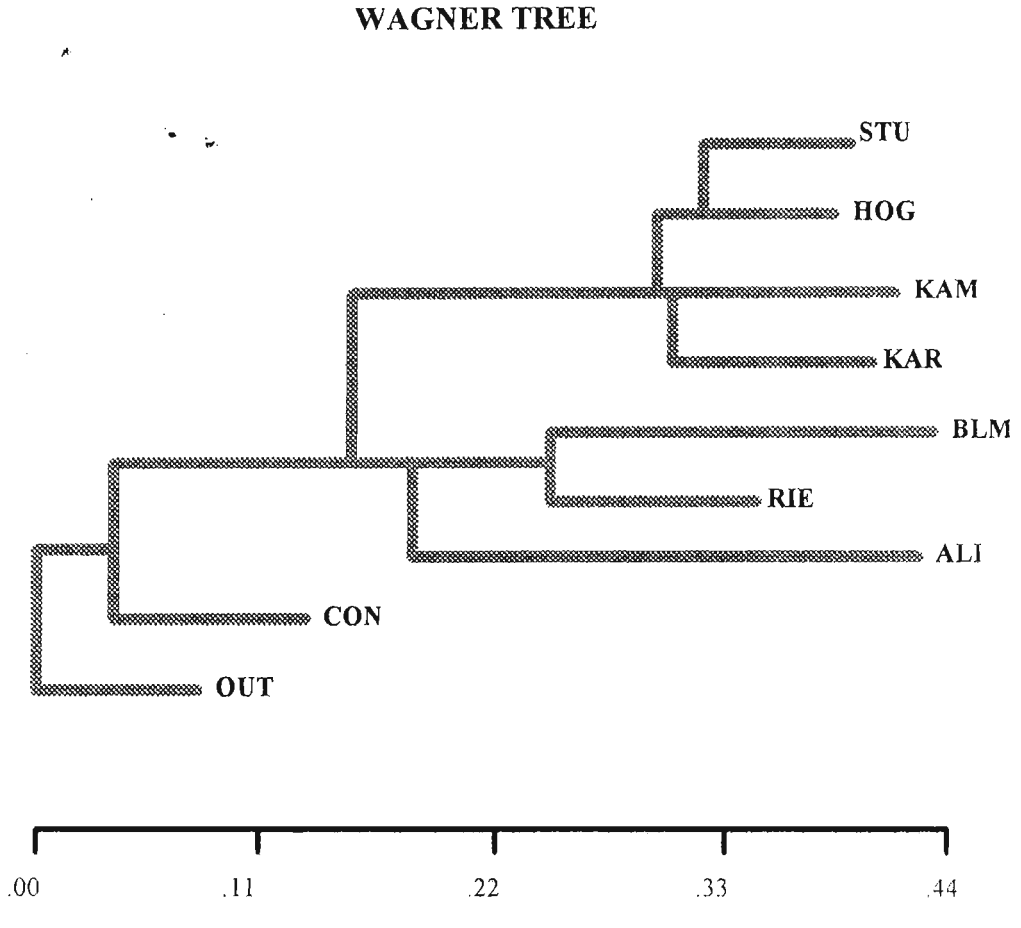

Figure 3.5 Optimized Wagner network generated from the modified presence-absence mtDNA fragment matrix, and rooted using outgroup. Cophenetic correlation is 0.888. For clonal designations see Table 3.1.

The Wagner tree showed a similar branching pattern to that of the 'majority rule' consensus phenogram (Figure 3.4).

Three cladograms, and a Nelson consensus tree, were produced using the Hennig86 (Farris, 1988) computer programme. These trees were the same as the trees produced using the divergence estimates with the NTSYS method of tree construction.

CHAPTER FOUR

DISCUSSION

4.1 A critical assesment of methods

4.1.1 Discussion of experimental techniques

A variety of methods of isolation and subsequent purification of mitochondrial DNA (mtDNA) were attempted during the course of the experimental study (Section 2.3 and Appendix 3). In many cases the yield was too low to be detected with an ethidium bromide-stained gel; in other situations the mtDNA band was very faint owing to the small amount of DNA loaded onto the gel, and once digested, the resulting restriction fragments could not be resolved.

Attempts at separating mtDNA on a cesium chloride-ethidium bromide gradient would, presumably, have been more efficient had a greater mass of starting material been available or had the starting material not been frozen. Crosetti, D. (pers. comm.) reported that freezing dramatically decreases the yield of mtDNA obtained. This would have allowed visualisation of the closed circular mtDNA band with ultra-violet light which, in turn, would have facilitated its recovery from the centrifuge tube.

As the amount of starting material was fixed, it followed that a more suitable technique for mtDNA extraction under the present circumstances was required and investigations for obtaining a better yield of mitochondria were pursued (Bredenkamp, B., pers. comm.). This led to a successful extraction protocol whereby a visible mtDNA band was observed in the screen test gel.

The various techniques for purifying mtDNA by recovery from agarose gels (Maniatis *et al.*, 1982, Appendix 3) were unsuccessful for two reasons. Firstly, the yield was low since the ethidium bromide-stained mtDNA band was very faint after electrophoresis. Secondly, the efficiency with which DNA is extracted from agarose gels is a function of its molecular weight (Maniatis *et al.*, 1982); in this instance the mtDNA molecular weight is relatively high for this technique, resulting in a low rate of recovery (Maniatis *et al.*, 1982).

The most successful protocol (Coen *et al*,. 1979) that was established during the present study did not yield totally consistent results. One of the critical stages in this procedure was the separation of mitochrondria from other cellular components (Section 2.3.1). In order to obtain autoradiographs that showed clear mtDNA bands, uncontaminated by nuclear DNA, it was desirable that the mitochondrial pellet be pure at the outset. The greatest purity was achieved by washing the mitochondria in the supernatant three times (as opposed to once or twice initially) in a stabilising buffer (MS Buffer, Appendix 1). It was found that the degree of contamination of mtDNA with linear (sheared nuclear DNA) increased with fewer washes and it appeared that this step was critical in determining the purity of the mtDNA sample. This was done at the risk of decreasing mitochondrial yield, as presumably some mitochondria were being pelleted along with other larger cellular components during each precipitation.

With hindsight, the following improvements might have enhanced the yield and purity of mtDNA obtained:

- The use of fresh tissue only. Tissue damage during freezing and thawing in liquid nitrogen could have caused lysis of mitochondria as well as allowing access of

nucleases to the mtDNA (Crosetti, D., pers. comm.). Raubenheimer, J. (pers. comm.) working on *Otomys* tissue, found that fresh tisssue extractions yielded mtDNA bands visible on cesium chloride-ethidium bromide (CsCl-EtBr) equilibrium density gradients. Experiments using frozen tissue gave greatly reduced yields. Furthermore, the longer the tissue had been frozen, the poorer the result.

- Further attempts at improving the CsCl-EtBr equilibrium gradients. This method is the most commonly employed separation method in studies such as the present investigation (see Avise *et al.*, 1987, Harley, 1988). Many of these studies advocate the use of greater starting volumes of sample tissues or the use of fresh tissues, which was impractical in the present study.
- Hybridisation of a highly radioactive mitochondrial DNA probe to Southern blots of fragments separated by electrophoresis might have provided the sensitivity (Southern, 1975) to compensate for the low amounts of DNA available for the study. The problem of sample contamination with nuclear DNA is also avoided with this method.
- Liver tissue is not optimal owing to its high enzyme content (Robinson, T., pers. comm.). Better results would have been obtained if only heart and kidney tissue had been used, although this would have yielded a low starting mass.

4.1.2 Discussion of methods of analysis

A comparison of restriction patterns between each sample pair should ideally have been carried out on a complete data set, but unfortunately some data points were missing. For certain pairwise comparisons, when a haplotype was missing for either pair of individuals it was eliminated totally from the analysis of that pair (Section 2.7.3). The corresponding divergence value for that pairwise comparison was not affected, whereas if these points had been included, divergences would have been overestimated. Occasional pairwise divergence estimates were accidentally zero as a result (Tables 3.2 and 3.3) since in some cases the only bands left after eliminating those corresponding to missing data happened to be identical in both individuals. These divergence values would have influenced the cluster analysis by producing clustering levels lower than expected for the populations whose clones, listed in Table 3.2, had values of zero.

The number of restriction enzymes used in this study were fewer than in most other investigations of this nature (Section 3.1). It is generally recommended that 60 to 100 restriction fragments, derived from digestion with 10 or more enzymes, are included in the analysis (Avise *et al.*, 1987) in order to obtain a sample representative of the entire genome. It follows that the greater the number of restriction enzymes employed, the larger the portion of the mitochondrial genome sampled. For example, divergence value estimates have been shown to be affected when a larger cohort of enzymes have been employed in the analysis (Avise *et al.*, 1983). The larger the cohort of enzymes, the higher is the likelihood of including monomorphic loci which then need to be eliminated as they are phylogenetically uninformative. However, by using more enzymes, the chance of including synapomorphic characters also increases resulting in more accurate estimates of divergence. These facts underline the need for caution in the interpretation

of absolute levels of mtDNA sequence divergence based on information from small numbers of restriction enzymes (Li, 1981).

The data set was analysed by both phenetic and cladistic methods in order to assess the accuracy. Dendograms from both techniques were relatively similar to one another, so it may suggest a somewhat accurate estimate of divergence with the given data set.

4.2 Restriction fragment variation

Four clones out of four individuals studied, were shown to exist at the Natal locality of Karkloof, and four clones out of five individuals studied were shown to exist at Kamberg. Such high polymorphism has been found in other mtDNA studies (Avise *et al.*, 1983). This confirms the findings of Raubenheimer (1993) of a high degree of intrapopulation restriction fragment length polymorphism (RFLP) in *O. irroratus* at both localities. It is also of interest that, in the same study, a lower degree of polymorphism was observed in the outgroup species *O. angoniensis* than in these two populations of *O. irroratus* as was the case in the present study.

According to Harley (1988) it is normal for mtDNA lineages to be relatively rapidly lost in small populations, which could result in low polymorphism, therefore, the high polymorphism found at Karkloof and Kamberg could be taken as an indication that these two populations are large. To date, however, we have no reliable data on population size in this species to confirm such a hypothesis. An alternative explanation, not incompatible with the previous one, for this high degree of polymorphism could be that because mtDNA is maternally inherited, mutations become fixed more rapidly due to a lack of recombination. On the other hand it could be argued that the polymorphism is

due to these populations being ancient and had, therefore, the opportunity to accomulate new lineages. This hypothesis does not seem likely for the following two reasons. Firstly, if these populations were sufficiently ancient to have accumulated a great number 0of new mtDNA clones, they would also have had the opportunity to accumulate enough nuclear DNA mutations to the point that high polymorphism could be detected by allozyme electrophoresis. However, a high polymorphism wasa not detected in samples from Kamberg and Karkloof by allozyme analysis (Taylor *et al.* 1992). Secondly, the Kamberg/Karkloof dichotomy has been dated at about 400 000 yr ago based on mtDNA studies of three populations of *O. irroratus* by Raubenheimer (1993).

A further point to be stressed is the fact that the species is structured in such a way that no clones are shared between any of the localities (populations) studied. This is confirmed by Raubenheimer (1993) who, in a study of populations at Karkloof, Kamberg and Rietvlei, found that only one out of 19 clones was shared between Kamberg and Karkloof. This indicates lack of gene flow between the populations studied.

4.3 Genetic divergence

There are a number of possible explanations for the presence of the gene flow barriers mentioned in the previous section. One possibility is the behavioural and social structure of *O. irroratus* which has been described by Willan (1982). This species has been shown to have a high level of territoriality thus implying resistance to immigration which would lead to limited gene flow. Because of this, fixed mutations are more likely to become clones peculiar to a particular locality as observed in this study and particularly that of Raubenheimer (1993). Post-zygotic reproductive barriers are a further possible explanation as these have been shown to exist in this species (Pillay *et al.*, 1992;

Contrafatto *et al.*, 1992b). Other possible isolating factors can be geographic and/or climatic changes which can lead to high divergence values as discussed by Honeycutt *et al.* (1987) in their work on mole rats (Bathyergidae).

A wide range of intraspecific sequence divergence estimates has been reported in the literature. For example, 2 % in *Peromyscus polionotus*, 4 % in *P. leucopus* and 0.3 % in *Drosophila pseudobscura* (Coen *et al.*, 1982) as well as higher values such as 9 % in fresh water fish populations in the USA (Bermingham and Avise, 1986) and 15 % between the two species of *P. polionotus* and *P. maniculatus* (Avise *et al.*, 1983). Although the results presented here show high values of divergence, they are not inconsistent with some of those reported above. Furthermore, they are entirely consistent with those presented by Raubenheimer (1993) of 2.6 % for the *O. irroratus* populations of Karkloof, Kamberg and Rietvlei. Moreover, the sequence divergence between *O. angoniensis* and *O. irroratus* was 12.1 % in this study and 11.5 % in Raubenheimer's study.

In several vertebrate groups mtDNA sequences have been shown to diverge at an initial rate of about 2 % for every million years (Wilson *et al.*, 1985). From this it can be calculated that *O. angoniensis* and *O. irroratus* last shared a common ancestor 6 million years (myr) ago. However, She *et al.* (1990) have evidence to suggest that muroid rodents show a mtDNA evolution rate of 7.1 % per myr. Based on this estimate, therefore, these two species diverged 1.7 myr ago. The latter figure is compatible with paleontological evidence and molecular clock data, both of which put the divergence of the Otomyines from the other murines at approximately 7 myr ago (Chevret *et al.*, 1992, Taylor *et al.*, 1989a,1989b).

4.4 Analysis of restriction fragment data

There is no clear consensus as to which of the numerous methods available is most appropriate for the analysis of restriction fragment patterns. Such methods are divided into two groups: distance phenograms (i.e phenetic analyses) and phylogenetic inference (i.e. cladistic analyses).

Although the phenograms provide information on the genetic relatedness of the taxa they are not considered a valid method to infer phylogenies because they are rate dependent (Sneath and Sokal, 1973). This is because the assumption of a constant rate of fixation of mutations is not necessarily valid. However, it is generally regarded that UPGMA trees are useful for summarizing average genetic distances among taxa (Avise *et al.*, 1987; Nei and Li, 1979).

A more beneficial approach, however, would be that of comparing results from the rate independent methods of cladistic analysis with those from phenetic methods.

4.4.1 Cluster and cladistic analyses

The outgroup, *O angoniensis*, consistently separated, as would be expected, with a high distance from the other *O. irroratus* populations (Figs 3.3, 3.4, 3.5). The Western Cape sample of Constantia was most frequently separated from the other populations. Furthermore, a cluster including Alice, Bloemfontein and Rietvlei was evident as well as another cluster including Stutterheim, Kamberg, Hogsback and Karkloof.

The separation of the Constantia sample could be suggestive of geographic differentiation because of the relatively great distance between this locality and the closest locality to it (Alice). This suggestion cannot, however be made about the other clusters evident from the phenograms, thus showing an overall lack of geographic differentiation between the species. This is in contrast with the well documented information that different populations within a highly polymorphic species usually show marked geographic differentiation (Avise *et. al.*, 1983). However, as mentioned in Section 4.3, the genetic structure of the species is indicative of well separated populations and restricted gene flow. It can be concluded, therefore, that the barriers likely to have caused such restriction in gene flow are unlikely to be geographic.

Although there seems to be no clear cut correlation between geographic distances and the topology of the phenograms, there is, to an extent, a correlation between topology and cytotypes as discussed in Section 4.5 below.

The Wagner network (Figure 3.5) generated from the presence-absence matrix (Appendix 2) analyzed using the BIOSYS programme (Swofford and Selander, 1989) shows an overall similarity with the phenograms generated by NTSYS (Rohlf,1988). On the basis of this network the Constantia population appears to be the most ancestral whereas the most derived populations seem to be the two Natal (Karkloof and Kamberg) and the two Eastern Cape (Stutterheim and Hogsback) populations. The topologies of the dendograms discussed so far were confirmed by cladistic analysis using the programme Hennig86 (Farris, 1988) (Section 3.3.2). The branching pattern is suggestive of a polyphyletic evolution of this species. This suggestion is, however, not confirmed

because of the finding of an identical pattern for the enzyme *Pvu II* across all populations, which reflects an ancestral panmictic population.

4.5 Comparative studies

The apparent congruence of the mtDNA- and karyotype-derived phylogenies could be explained in terms of the reproductive isolation produced by the presence of the tandem fusion in the Hogsback and Kamberg populations and the presence of two extra pairs of chromosomes at Karkloof, which also is associated with a measure of reduced fertility as shown by Pillay *et al.* (1992). Since both chromosome variants present barriers to gene flow, derived characters evolved after the establishment of such barriers should generate phylogenetic relationships similar to those derived from chromosomal data (Contrafatto *et al.*, 1994b). On the other hand, the chromosome rearrangements found in the other populations (Contrafatto *et al.* 1992b) are not of a type capable of causing meiotic malsegregation and, therefore, unlikely to represent gene flow barriers. Thus the agreement between the mtDNA and chromosomal phylogenies regarding these populations remains unexplained.

Carson's population flush hypothesis as discussed by Robinson and Elder (1987) could possibly apply to *O. irroratus*. According to this model, selective pressures are relaxed during periods of favourable climatic conditions when suitable habitats are expanding and population sizes increase. This allows for the persistence of novel genetic combinations, and the colonization of marginal habitats. During subsequent periods when unfavourable conditions prevail, habitats become fragmented and numbers are reduced, giving rise to new populations in which novel mutations have been fixed. The population flush model is consistent with the population genetic structure of *O. irroratus* as derived from

patterns of mtDNA and chromosome variation, since on this basis one would expect to find many divergent populations. The fact that mtDNA divergence between populations is high, may be further evidence to support the above argument.

Furthermore, Taylor *et al.* (in press) have shown a strong correlation between the distribution of chromosomal races of this species with climatic conditions. There are two extreme groups: one which includes all the cytotypes with seven pairs of biarmed chromosomes, found at low altitudes in hot and dry climates; and one including the cytotypes with all acrocentric chromosomes which are found at higher altitudes in cold and wet climates. There is an intermediary group with four pairs of biarmed chromosomes which is found in intermediate climatic conditions. Climatic unpredictability and seasonality seem to be important features in discriminating between cytotypes. Because of the agreement between phylogenies, it is likely that mtDNA sequence divergence patterns, found in the populations in this study, will correlate with the same climatic features.

Taylor *et al.* (1992), from their allozyme data on 12 populations of this species, found high heterozygosity, low genetic distances between populations, low F_{ST} and low F_{SD} values. Moreover, cluster analysis failed to identify groups consistent with geographic distribution or karyotypic differences. Overall, this was consistent with a panmictic genetic structure of *O. irroratus*. On the other hand, both mtDNA and chromosomal analyses indicated a 'Wrightian' genetic structure of the species consisting of a number of isolated populations.

Lack of correlation between the data sets derived from mtDNA and allozyme markers can be explained in terms of the relative resolution of the methods employed. MtDNA evolves much more rapidly than nuclear DNA, hence, the allozyme analysis may well be reflecting a more ancient pattern of population genetic structure than that observed in the mtDNA analysis. The level of sensitivity of starch gel electrophoresis does not equal that of restriction analysis of mtDNA (Ayala, 1975). Furthermore, allozymes are phenotypic expressions of the genetic code and many mutations which occur may not be expressed as changes in the amino acid sequence of the proteins. It appears, therefore, that the indications from the mtDNA analysis are more acceptable, in the context of this study, as this method is more likely to reveal more recent evolutionary changes.

4.6 Summary and conclusions

The populations of *O. irroratus* that were sampled in this study showed a high level of polymorphism. One explanation could be that the population sizes are large, as Harley (1988) has shown that mtDNA lineages tend to be rapidly lost from small populations (which would imply lower polymorphism in small populations). Another explanation could be that the high level of polymorphism is a reflection of the more rapid rate of fixation of mutations in maternally-inherited mtDNA.

There were no shared clones of *O. irroratus* between localities, this being suggestive of the existence of barriers to gene flow between populations. This was confirmed in the Natal populations and the Transvaal population of Rietvlei, by the study of Raubenheimer (1994). Previously mentioned explanations for these barriers to gene flow include the behavioural and social structure of the species. *O. irroratus* is a shy, territorial species, and would thus be likely to show resistance to migration.

Additionally, it has a low dispersal distance. Thus mutations fixed at one locality would be unlikely to spread to distant localities. In the case of the Karkloof, Kamberg and Hogsback populations, barriers to gene flow may be due to chromosomal rearrangements.

The sequence divergence values obtained in this study were high, but were not inconsistent with other reports at the same taxonomic level. The high divergence values can possibly be explained in terms of behavioural, climatic and geographic factors (Section 4.3 and 4.5) leading to the existence of gene flow barriers.

The ancestral population of the species was probably monophyletic, as all sample animals were monomorphic for the *Pvu II* haplotype. The population genetic structure of *O. irroratus,* as defined by mtDNA clone cluster analysis, appears subdivided or 'Wrightian' in nature.

The mtDNA-derived phenograms of this study correspond with cytogenetical phenograms in the karyotypic studies of *O. irroratus* populations (Contrafatto *et al.*, 1992a). This would at least partly be explained by the occurrence of chromosomally-driven speciation at Hogsback, Karkloof and Kamberg although the low clustering level of Stutterheim, a cytotype without a tandem fusion, somewhat blurs the picture.

REFERENCES

AQUADRO, C.F., KAPLAN, N. and RISKO, K.J. 1984. An analysis of the dynamics of mammalian mitochondrial DNA sequence evolution. *Mol.Biol.Evol.* 1(5):423-434.

AVISE, J.C., ARNOLD, J., BALL, R.M., BERMINGHAM, E., LAMB, T., NEIGEL, J.E., REEB, C.A. and SAUNDERS, N.C. 1987. INTRASPECIFIC PHYLOGEOGRAPHY: The Mitochondrial DNA Bridge Between Population Genetics and Systematics. *Ann.Rev.Ecol.Syst.* 18:489-522.

AVISE, J.C., HELFMAN, G.S., SAUNDERS, N.C. and HALES, L.S. 1986. Mitochondrial DNA differentiation in North Atlantic eels: Population genetic consequences of an unusual life history pattern. *Proc.Natl.Acad.Sci.USA.* 83:4350-4354.

AVISE, J.C., SHAPIRA, J.F., DANIEL, S.W., AQUADRO, C.F. and LANSMAN, R.A. 1983. Mitochondrial DNA Differentition during the Speciation Process in *Peromyscus*. *Mol.Biol.Evol.* 1(1):38-56.

AYALA, F. J. 1975. Genetic differentiation during speciation. *Evol. Biol.* 8:1-78.

BAKER, R.J., DAVIS, S.K., BRADLEY, R.D., HAMILTON, M.J. and VAN DEN BUSSCHE, R.A. 1989. Ribosomal-DNA, mitochondrial-DNA, chromosomal, and allozymic studies on a contact zone in the pocket gopher, *Geomys*. *Evolution.* 43(1):63-75.

BICKHAM J.W. and BAKER, R.J., (1979). Canalization model of chromosomal evolution. *Bull.Carnegie.Mus.Nat.Hist.* 13:70-84.

BERMINGHAM, E. and AVISE, J.C. 1986. Molecular zoogeography of freshwater fishes in the southeastern United States. *Genetics*. 113:939-965.

BREDENKAMP, B. Department of Chemical Pathology, Medical Research Council, Durban, Natal.

BROWN, E.D. 1988. Comparative socio-ecology of *Otomys irroratus* and *Otomys unisulcatus*. M.Sc. thesis, Univ. Fort Hare, Alice. 184 pp.

BROWN, G.G. and SIMPSON, M.V. 1980. Intra- and interspecific variation of the mitochondrial genome in *Rattus norvegicus* and *Rattus rattus*; Restriction enzyme analysis of variant mitochondrial DNA molecules and their evolutionary relationships. *Genetics*. 97:125-143.

BROWN, W.M., GEORGE, M. and WILSON, A.C. 1979. Rapid evolution of animal mitochondrial DNA. *Proc.Natl.Acad. Sci.USA*. 76:1967-1971.

CAPANNA, E., CORTI, M.,, NASCETTI, G. and BULLINI, L. 1985. Pre- and post-isolating mechanisms in the speciation of the European long-tailed house mouse *Mus musculus domesticus*. *Acta Zool.Fennica.* 170:155-170.

CARLTON, M.D. and MUSSER G. 1984. Muroid Rodents. In "Orders and families of Recent mammals of the world. II". edit. S. Anderson and J. Knox-Jones Jr, pp 289-379, Wiley, New York.

CHEVRET, P., DENYS, C., JAEGER, J-J., MICHAUX, J. and CATZEFLIS, F. 1992. Molecular and paleontological aspects of the tempo and mode of evolution in *Otomys* Otomyinae: Muridae: Mammalia). *Biochem.Syst.Ecol.* 21(1):123-131.

COEN, E.S., THODAY, J.N. and DOVER, G. 1982. Rate of turnover of structural variants in the rDNA gene family of *Drosophila melanogoster*. *Nature*. 295:564-568.

CONTRAFATTO, G., MEESTER, J., WILLAN, K., TAYLOR, P.J., ROBERTS, M.A. and BAKER, C.M. 1992a. Genetic variation in the African rodent sub-family Otomyinae (Muridae). II: Chromosomal changes in some populations of *Otomys irroratus*. *Cytogenet.Cell Gen.* 59:293-299.

CONTRAFATTO, G., MEESTER, J., BRONNER, G., TAYLOR, P. and WILLAN, K. 1992b. Genetic variation in the African rodent sub-family Otomyinae (Muridae). IV: chromosome G-banding analysis of *O. irroratus* and *O. angoniensis*. *Isra.J.Zool.* 38:277-291.

CONTRAFATTO, G., DAVID, D. and GOOSEN-LE-CLERQ,V. 1994a. Genetic variation in the African rodent subfamily Otomyinae (Muridae) VI: Phylogeny Inferred by immuno-electrotransfer analysis. *Durban Mus. Novit.* 19:1-7.

CONTRAFATTO, G., VAN DEN BERG, J.R., and GRACE, J.H. 1994b. Genetic variation in the African rodent subfamily Otomyinae (Muridae). VI: Immuno-electrotransfer of liver proteins of some *Otomys irroratus* (Brants, 1827) populations. Submitted.

CROSETTI, D. Department of Human and Animal Biology, First University of Rome, Rome.

DAVIS, D.H.S. 1974. The distribution of some southern African small mammals (Mammalia: Insectivora, Rodentia). *Ann.Transv.Mus.* 29:135-184.

DAVIS, R.M. 1973. The ecology and life history of the vlei rat, *Otomys irroratus* (Brants, 1827), on the Van Riebeeck Nature Reserve, Pretoria. Ph.D. thesis, Univ.of Pretoria, Pretoria.

DAVIS, R.M. and MEESTER, J. 1981 Reproduction and postnatal development in the vlei rat, *Otomys irroratus*, on the Van Riebeeck Nature Reserve, Pretoria. *Mammalia.* 45:99-116.

DE GRAAFF, G. 1981. The rodents of southern Africa. Butterworths, Durban.

FARRIS, J. S. 1988. Hennig86, version 1.5. Copyright James S. Farris 1988.

FRANCISCO, J.F., BROWN, G.G. and SIMPSON, M.V. 1978. Further studies on types A and B rat mtDNAs: cleavage maps and evidence for cytoplasmic inheritance in mammals. *Plasmid.* 2:426-436.

GEORGE, M. and RYDER, O.A. 1986. Mitochondrial DNA evolution in the genus *Equus.Mol.Biol.Evol.* 3(6):535-546.

GONZALEZ-VILLASENOR, L.I. and POWERS, D.A. 1990. Mitochondrial-DNA restriction-site polymorphisms in the Teleost *Fundulus heteroclitus* support secondary intergradation. *Evolution.* 44(1):27-37.

HARLEY, E.H. 1988. DNA approaches to molecular taxonomy. *Trans.Roy.Soc.S.Afr.* 46(4):283-290.

HARLEY, E. Department of Chemical Pathology, University of Cape Town, Cape Town.

HARRISON, R.G. 1989. Animal mitochondrial DNA as a genetic marker in population and evolutionary biology. *Tree.* 4(1):6-11.

HOFFMAN, T.L. 1990. A craniometric analysis of *Otomys irroratus*. Unpubl. Hons project report, Univ. of Natal, Durban.

HONEYCUTT, R.L., EDWARDS, S.V., NELSON, K. and NEVO, E. 1987. Mitochondrial DNA variation and the phylogeny of African mole rats (Rodentia: Bathyergidae). *Syst.Zool.* 36(3):280-292.

HUCKETT, B. Sugar Experimental Research Station, Mount Edgecombe, Natal.

KEARNEY, T. 1990. Comparative renal morphology of the vlei rat *Otomys irroratus* (Brants, 1827) from nine southern African localities. Unpubl. Hons project report, Univ. of Natal, Durban.

LAMB, T., AVISE, J.C. and GIBBONS, J.W. 1989. Phylogeographic patterns in mitochondrial DNA of the desert tortoise (*Xerobates agassizi*), and evolutionary relationships among the North American gopher tortoises. *Evolution.* 43(1):76-87.

LANSMAN, R.A., AVISE, J.C., AQUADRO, C.F., SHAPIRA, J.F. and DANIELS, S.W. 1983. Extensive genetic variation in mitochondrial DNAs among geographic populations of the deer mouse, *Peromyscus maniculatus. Evolution.* 37:1-16.

LI, W.-H. 1981. A simulation study on Nei and Li's model for estimating DNA divergence from restriction enzyme maps. *J.Mol.Evol.* 17:251-255.

MANIATIS, T., FRITSCH, E. F. and SAMBROOK, J. 1982. Molecular cloning. A laboratory manual. Cold Spring Harbour Laboratory, USA. 545 pp.

MEESTER, J. 1988. Chromosomal speciation in Southern African small mammals. *S.Afr.J.Sci.* 84:721-724.

MEESTER, J., TAYLOR, P.S., CONTRAFATTO, G., WILLAN, K., LAMB, J.M. and PILLAY, N. (1992). Chromosomal speciation in Southern African Otomyinae (Rodentia: Muridae): a review. Durban Mus. Novitae. 17:58-63.

MIYAMOTO, M.M., TANHAUSER, S.M. and LAIPIS, P.J. 1989. Systematic relationships In the artiodactyl tribe Bovini (Family Bovidae), as determined from mitochondrial DNA sequences. *Syst.Zool.* 38(4):342-349.

MORITZ, C., DOWLING, T.E. and BROWN, W.M. 1987. Evolution of mitochondrial DNA: Relevance for population biology and systematics. *Ann.Rev.Ecol.Syst.* 18:269-292.

NEI, M. and LI, W.-H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc.Natl.Acad.Sci.USA.* 76(10):5269-5273.

PERRIN, M.R., 1980. The breeding strategies of two co-existing rodents, *Rhabbdomys pumilio* (Sparrman, 1974) and *Otomys irroratus* Brants,1827 in relation to rainfall and reproduction. *Acta.Oecol.* 1:387-414.

PERRIN, M.R. 1981. Notes on the activity patterns of 12 species of southern African rodents and a new design of activity monitor. *S.Afr.J.Zool.* 16:248-258.

PILLAY, N. 1990. The breeding and reproductive biology of the vlei rat *Otomys irroratus*. Unpubl. M.Sc thesis, Univ. of Natal, Durban.

PILLAY, N., WILLAN, K. and MEESTER, J. 1992. Post-zygotic reproductive isolation in the African vlei rat *Otomys irroratus* (Muridae: Otomyinae). *Isra.J.Zool.* 38:307-313.

RAUBENHEIMER, J. 1993. Mitochondrial DNA variability between selected populations of *Otomys irroratus* (Muridae: Otomyinae). Unpubl. M.Sc thesis, Univ. of Natal, Durban.

RAUBENHEIMER, J. Department of Zoology, University of Durban-Westville, Natal.

ROBINSON, T. Department, University of Pretoria, Pretoria.

RIDDLE B.R. and HONEYCUTT, R.L. 1990. Historical biogeography in North American arid regions: An approach using mitochondrial-DNA phylogeny in grasshopper mice (*Genus Onychomys*). *Evolution.* 44(1):1-15.

ROBINSON, T.J., and ELDER, F.F.B. 1987 Extensive genome reorganisation in the African rodent genus *Otomys. J.Zool.Lond.* 211:735-745.

ROBYT, J.F. and WHITE, B.J. 1987. Chapter 5. In "Biochemical Techniques. Theory and Practice". Brooks/Cole Publishing Company, Monterey, California.

ROHLF, F. J. 1988. NTSYS-pc. Numerical taxonomy and mutivariate analysis system. Applied Biostatistics Inc., Setauket, New York.

SHE, J.X., BONHOMME, F., BOURSOT, P., THALER, L. and CATZEFLIS, F 1990. Molecular phylogenies in the genus *Mus:* Comparative analysis of electrophoretic, scnDNA hybridisation, and mtDNA RFLP data. *Biol.J.Linn.Soc.* 41:83-103.

SKINNER, J.D. and SMITHERS, R.H.N. 1990. The mammals of the Southern African region. 2nd Edition. University of Pretoria 757 pp.

SMITHERS, R.H.N. 1983. The mammals of the Southern African subregion. Univ. of Pretoria, Pretoria. 736 pp.

SNEATH, P.H.A. and SOKAL, R.R. 1973. Numerical taxonomy. W.H. Freeman, San Francisco.

SOUTHERN, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J.Mol.Biol.* 98:503-517.

SWOFFORD, D.L. and SELANDER, R.B. 1989. BIOSYS-1: A computer program for the analysis of allelic variation in population genetics and biochemical systematics. David L. Swofford, Illinois Natural History Survey, Release 1.7.

TAYLOR, P.J., CAMPBELL, G.K., MEESTER, J., WILLAN, K. and VAN DYK, D. 1989a. Genetic variation in the African rodent sub-family Otomyinae (Muridae). I: allozyme divergence among four species. *S.Afr.J.Sci.* 85:257-262.

TAYLOR, P.J., CAMPBELL, G.K., VAN DYK, D., MEESTER, J., and WILLAN, K. 1989b. Phenetic and phylogenetic analyses of seven species of vlei rats (Rodentia: Muridae: Otomyinae) based on allozyme electrophoresis. Fifth International Theriological Congress, Rome. (Abstract).

TAYLOR, P.J., CAMPBELL, G.K., VAN Dyk, D., MEESTER, J. and WILLAN, K. 1992. Genetic variation in the African vlei rat *Otomys irroratus*. (Muridae: Otomyinae). *Isra.J.Zool.* 38:293-305

TAYLOR, P. J., CONTRAFATTO, G. and WILLAN, K., 1994. Genetic variation in the African rodent sub-family Otomyinae (Muridae).V: Climatic correlates of chromosomal varaints of the vlei rat (*Otomys* irroratus) (In press).

WALLIS, G.P. and ARNTZEN, J.W. 1989. Mitochondrial-DNA variation in the crested newt superspecies: limited cytoplasmic gene flow among species. *Evolution.* 43(1):88-104

WHITE, M.J.D. 1982. Rectangularity, Speciation, and Chromosome Architecture. In "Mechanisms of Speciation" edit. Alan R. Liss, Inc., New York. pp 75-103.

WILLAN, K. 1982. Social ecology of *Otomys irroratus, Rhabdomys pumilio* and *Praomys natalensis*. Ph.D. thesis, Univ. of Natal, Pietermaritzburg.

WILLAN, K. and MEESTER, J. 1989. Life history tactics of Southern African *Mastomys natalensis, Otomys irroratus* and *Rhabdomys pumilio* (Mammalia, Rodentia). In "Alternative life history styles of animals" edit. M.N. Bruton, Kluwer, Dordrecht. 421-439.

WILSON, A.C. 1979. Rapid evolution of animal mitochondrial DNA. *Proc.Natl.Acad.Sci.USA*. 76:1967-1971.

WILSON, A.C., CANN, R.L., CARR, S.M., GEORGE, M., GYLLENSTIEN, U.B., HELM-BYCHOWSKI, K.M., HIGUCHI, R.G., PALUMBI, S.R., PRAGER, E.M., SAGE, R.D. and STONEKING, M. 1985. Mitochondrial DNA and 2 perspectives on evolutionary genetics. *Biol.J.Linn.Soc* 26:375-400.

ZINK, R.M. and AVISE J.C. 1990. Patterns of mitochondrial DNA and allozyme evolution in the avian genus *Ammodramus*. *Syst.Zool.* 39(2)148-161.

APPENDIX 1

The following solutions were made according to instructions in the relevant references and all were prepared freshly with analytical grade chemicals on a monthly basis.

- CaRSB Buffer (Bredenkamp, B., pers. comm.)

  10 mM NaCl, 10 mM Tris, 1.5 mM $CaCl_2$, pH 7.5

- MS Buffer (Bredenkamp, B., pers. comm.)

  420 mM mannitol, 140 mM sucrose, 10 mM Tris, 10 mM EDTA, pH 7.5

- Extraction Buffer (Coen *et al.*, 1982)

  10 mM Tris, 60 mM NaCl, 5 g sucrose, 10 mM EDTA in 100 ml, pH 7.8

- SDS Buffer (Coen *et al.*, 1982)

  1.25 g SDS, 300 mM Tris, 5 g sucrose, 0.372 g EDTA in 100 ml, pH 9.0

- TE Buffer (Maniatis *et al.*, 1982)

  10 mM Tris, 1 mM EDTA, pH 7.4

- Loading Buffer (6x) (Maniatis *et al.*, 1982)

  0.25% (w/v) bromophenol blue, 40% (w/v) sucrose in water

- TAE Buffer (Maniatis *et al.*, 1982)

0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0

- Staining Solution (Maniatis *et al.*, 1982)

  1 µl EtBr (10 mg ml^{-1}) in 150 ml TAE Buffer, pH 8.0

- RNAse A solution (Maniatis *et al.*, 1982)

  1 mg RNAse A (Boehringer Mannheim, Germany) in 1 ml sterile water, boiled for 15 min

- Kodak D19 Developer (3x)

  2.2 g 'Elon' (Kodak, South Africa) 144 g sodium sulphite, 8.8 g hydroquinone, 130g sodium carbonate, 4 g potassium bromide in 1 l water

APPENDIX 2

Table 2A Character states for 53 restriction fragments in the 15 mitochondrial DNA clones found in *Otomys irroratus* and the outgroup (OUT) *O. angoniensis* Presence = 1, absence = 0 and undetermined = ?.

MtDNA Fragment	CLONE NAME														
	KAM1	KAM2	KAM3	KAM4	KAR1	KAR2	KAR3	KAR4	OUT	RIE	HOG	CON	BLM	ALI	STU
Xba I 1	0	0	0	0	0	0	0	0	1	0	0	1	?	0	0
2	1	1	1	1	1	1	1	1	0	1	1	0	?	0	1
3	1	1	1	1	1	1	1		0	1	1	1	?	1	1
4	1	1	1	1	1	1	1	1	0	1	1	1	?	1	1
5	0	0	0	0	0	0	0	0	1	0	0	0	?	1	0
6	1	1	1	1	1	1	1	1	1	1	1	1	?	0	0
7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
8	1	0	1	1	0	0	0	0	0	0	0	0	?	1	0
9	0	0	0	0	0	0	0	0	0	0	0	1	?	1	0
10	1	1	1	1	1	1	1	1	1	1	1	0	?	0	1
11	0	0	0	0	0	0	0	0	0	0	0	1	?	0	0
12	1	1	1	1	1	1	1	1	0	1	1	0	?	1	1
Hind III 1	1	1	1	0	?	?	1	0	0	0	1	?	0	0	1
2	1	1	1	0	?	?	1	0	0	0	1	?	0	1	1
3	1	1	1	0	?	?	1	0	1	1	1	?	1	1	1
4	1	1	1	0	?	?	1	0	1	1	1	?	1	1	1
5	0	0	0	0	?	?	0	0	0	1	0	?	1	0	0
6	0	0	0	0	?	?	0	0	0	1	0	?	1	0	0
7	0	0	0	0	?	?	0	0	0	1	0	?	1	0	0
8	0	0	0	0	?	?	0	0	1	1	0	?	1	0	0
9	0	0	0	0	?	?	0	0	0	0	0	?	0	1	0
10	0	0	0	1	?	?	0	1	0	0	0	?	0	0	0
11	0	0	0	1	?	?	0	1	0	0	0	?	0	0	0
12	0	0	0	1	?	?	0	1	0	0	0	?	0	0	0
13	0	0	0	1	?	?	0	1	0	0	0	?	0	0	0
Pvu II 1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Table 2A continued

MtDNA Fragment	CLONE NAME														
	KAM1	KAM2	KAM3	KAM4	KAR1	KAR2	KAR3	KAR4	OUT	RIE	HOG	CON	BLM	ALI	STU
Dra I 1	0	0	0	0	0	0	0	0	0	0	0	1	?	0	0
2	1	0	1	1	1	1	1	1	0	1	1	0	?	1	1
3	1	1	1	1	1	1	1	1	0	1	1	0	?	1	1
4	0	0	0	0	0	0	0	0	1	0	0	0	?	0	0
5	0	0	0	0	0	0	0	0	0	0	0	1	?	0	0
6	0	0	0	0	0	0	0	0	1	0	0	0	?	0	0
7	1	1	1	1	1	1	1	1	1	1	1	1	?	1	1
8	0	0	0	0	0	0	0	0	1	0	0	0	?	0	0
9	0	0	0	0	0	0	0	0	1	0	0	0	?	0	0
10	0	0	0	0	0	0	0	0	0	0	0	1	?	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0	?	1	0
12	0	0	0	0	0	0	0	0	0	0	0	1	?	0	0
13	1	1	1	1	1	1	1	1	0	1	1	0	?	1	1
Eco RI 1	1	1	1	1	?	1	1	1	0	1	0	?	1	1	1
2	1	1	1	1	?	1	1	1	0	1	0	?	1	1	1
3	0	0	0	0	?	1	0	0	0	1	0	?	1	1	0
4	0	0	0	0	?	1	0	0	0	1	0	?	1	1	0
5	1	1	1	1	?	1	1	1	0	1	0	?	1	1	1
6	1	1	1	1	?	0	1	1	0	0	0	?	0	0	1
7	0	0	0	0	?	0	0	0	0	0	1	?	0	0	0
8	0	0	0	0	?	0	0	0	0	0	1	?	0	0	0
9	0	0	0	0	?	0	0	0	0	0	1	?	0	0	0
10	0	0	0	0	?	0	0	0	1	0	0	?	0	0	0
11	0	0	0	0	?	0	0	0	1	0	0	?	0	0	0

APPENDIX 3

3A Other methods that were attempted:

3A 1. Extraction and isolation of mtDNA by centrifugation to equilibrium in a cesium chloride-ethidium bromide gradient

Total cellular DNA was extracted and mitochondrial DNA (mtDNA) was isolated from this according to procedures described by Brown *et* al. (1979) as modified by Harley, E. (pers. comm.).

The method of Maniatis *et al.* (1982) for purification of closed circular DNA was followed. For every millilitre of DNA solution, exactly 1 g of CsCl was added and mixed gently until it was dissolved. For every 10 ml of this solution, 0.8 ml of a 10 mg ml^{-1} EtBr solution was added and mixed well. The density of the solution was measured using a densitometer (Atago, Japan) and adjusted to a final density of 1.55 gml^{-1}. This solution was transferred, using a Pasteur pipette, to a Beckman Quickseal centrifuge tube. Care was taken to avoid bubble formation which may have caused shearing of circular DNA (Maniatis *et al.*, 1982). Tubes were sealed using the Beckman Quicksealer and loaded in a Beckman Vti 65 vertical rotor. The tubes were centrifuged at 55000 rpm at 20 ^{O}C for 18 h in a Beckman L8-55M ultracentrifuge.

A bottom band of DNA (i.e. the presumed closed circular mtDNA band) was not observed under ultra-violet light, presumably because the concentration of mtDNA was too low in relation to the sensitivity of this method. Acting on this assumption, therefore, an aliquot was withdrawn from the position where this band was expected to be (i.e. approximately 1 cm below the top band, Harley, E., pers. comm.).

A piece of Scotch tape was placed on the outside of the tube, as a marker of the expected band position. With the tube held in a retort stand, a vent was made by inserting a hypodermic needle (27 gauge) into the neck of the tube. Another needle connected to a 2 ml syringe was used bevel-side up to puncture the tube through the tape. A 1 cm measure of the solution was collected with this syringe. Care was taken not to contaminate the aliquot with the main DNA band which could be clearly observed under ultra violet light. The aliquot was transferred to a 1 ml Eppendorf microfuge tube. During this procedure care was taken not to force the solution out of the plunger of the syringe as this is expected to result in the shearing of circular DNA.

The EtBr contained in this aliquot was removed as follows: An equal volume of isoamyl alcohol was added to the solution and mixed. The resulting upper pink layer was then discarded using a clean Pasteur pipette. The procedure was repeated 3-4 times until no traces of the pink stain remained in the lower aqueous layer (Maniatis *et al.*, 1982).

The lower aqueous layer was diluted with 1 volume of sterile water, and the DNA was precipitated by the addition of 2 volumes of ice-cold absolute ethanol. This was placed at -20 ^{O}C for 1 h. The DNA precipitate thus obtained was collected after centrifugation of the solution at 8000 rpm in a microfuge (Haraeus Sepatech, Switzerland) for 10 min at 4 ^{O}C. The resulting pellet was washed with 70 % ethanol, recentrifuged under the above conditions, and allowed to dry in a vacuum desiccator. The dry pellet, which was not directly visible, was dissolved in 150 µl TE Buffer at room temperature for approximately 18 h and then stored at -20 ^{O}C.

At this stage each DNA sample was electrophoresed on a minigel as outlined in Section 2.3.2. After numerous attempts failed to yield a visible mtDNA band at this position, and these difficulties were known to be shared by the team working with this procedure in Cape Town, it was decided that another isolation procedure should be tried.

3A 2. Isolation of mtDNA (after Coen *et al.*, 1982)

Liver and kidney samples (approximately 2 g) were frozen with liquid nitrogen before being crushed into a fine powder using a pestle and mortar. Samples were then treated according to the method of Coen *et al.* (1982).

Each sample was screened for the presence of mtDNA by electrophoresis in an EtBr-stained agarose minigel as outlined in Section 2.3.2. The yield obtained from this procedure was insufficient to allow visualisation on ethidium bromide-stained gels, so certain modifications were introduced which allowed a workable yield to be obtained (Section 2.3). It has been suggested (Bredenkamp, B., pers. comm.) that the underlying reason for low yields of mtDNA could be that the method of Coen yielded a low number of intact mitochondria in the initial stages. Steps were therefore taken to protect the mitochondria osmotically, and so possibly enhance the yield of both mitochondria and mtDNA. These modifications were performed during the initial stages of the protocol (Section 2.3.1). The addition of 2 buffers (CaRsB and MS Buffers, Appendix 1) to help stabilise the mitochondria osmotically during the initial stages of the isolation resulted in the test gels showing the presence of mtDNA.

3A 3. Purification of mtDNA

Mitochondrial DNA was initially isolated (Coen *et al.*, 1982, see Section 2.3.3) and then purification by several procedures of electroelution documented by Maniatis *et al.* (1982) was attempted.

Another method of recovery was also attempted, whereby the mtDNA-containing band in the agarose gel was cut out using a sharp scalpel, placed in dialysis tubing and frozen (Huckett, B., pers. comm.). The agarose was then 'squeezed' between the thumb and fore-finger and the resulting DNA solution was recovered in a microfuge tube and concentrated by alcohol precipitation. Insufficient yields of mtDNA were obtained from the above methods.

A phenol-chloroform-isoamyl alcohol extraction of mtDNA was also attempted (Maniatis *et al.*, 1982). The mtDNA was first isolated following the procedure of Coen *et al.* (1982) up to the stage where the SDS Buffer was added (see Section 2.3), and then it was extracted with phenol-chloroform-isoamyl alcohol.

The commercially available 'Circleprep' kit (Boehringer Mannheim, Germany) was then used according to the manufacturer's instructions. 'Circleprep' allows the separation of closed circular plasmid DNA (mtDNA) from linear DNA (sheared nuclear DNA). The procedure includes a series of alkaline denaturation/renaturation steps to remove non-supercoiled linear DNA. This procedure was unsuccessful when applied to mtDNA, which is a very large circular molecule present in relatively low concentrations.

Isolation of mitochondria and subsequent purification, using a sucrose gradient, was performed according to Robyt and White (1987). It was, however, found that the

mtDNA was contaminated with nuclear DNA when visualised on an ethidium bromide-stained gel.

APPENDIX 4

List of voucher specimen numbers of 20 samples of *Otomys irroratus* and *O. angoniensis* which were used in the present study and are stored at the Durban Natural Science Museum.

DM2025

DM2586

DM2622

DM2623

DM2625

DM2653

DM2665

DM2666

DM2675

DM2753

DM2766

DM2770

DM2807

DM2825

DM2907

DM3041

DM3052

DM3074

DM3087

DM3100