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MITOCHONDRIAL DNA VARIABILITY AND GEOGRAPHIC POPULATION STRUCTURE IN *PRONOLAGUS RUPESTRIS* AND *P.RANDENSIS* (MAMMALIA: LAGOMORPHA)

MSc **UP** **1993**

Mitochondrial DNA variability and geographic population structure in *Pronolagus rupestris* and *P. randensis* (Mammalia: Lagomorpha)

by

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Submitted in partial fulfilment of the

requirements for the degree of

Master of Science

in the

Faculty of Science

University of Pretoria

Pretoria

February 1993

ABSTRACT

Mitochondrial DNA variability and geographic population structure in *Pronolagus rupestris* and *P. randensis* (Mammalia: Lagomorpha)

by

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Geographic genetic population structure was determined for *Pronolagus rupestris* and *P. randensis*, two allopatric species which have relatively wide distributions in South Africa and are restricted to rocky/mountainous habitat. Restriction fragment length polymorphisms were analysed for 82 individuals from 29 different localities. The fourteen endonucleases used in the analysis resulted in the detection of 43 different maternal lineages.

Two major genetic assemblages, found within *P. rupestris*, were separated by a 7.94 % (±1.40%) sequence divergence. This is one of the highest intraspecific values recorded for vertebrates and is thought to reflect inaccuracies in the existing taxonomy. Demographic influences on mtDNA evolution and past climatic events are put forward as a possible explanation for the phylogeographic pattern observed in *P. rupestris*. Intraspecific mtDNA variation within *P. randensis* was not as pronounced as in *P. rupestris* and little meaningful geographic structure was evident.

dedicated to

Elsie Jacoba Matthee (1936-1992)

ACKNOWLEDGEMENTS

My sincere gratitude to my supervisor, professor Terry Robinson, for his encouragement and unfailing support. His valuable advice and enthusiasm is greatly appreciated.

I am indebted to the following people who assisted in various ways, especially to land owners and conservation agencies who allowed me access to their property; without their generosity this study would not have been possible:

C. Albutt (Kuruman); F.J. van der Merwe (Calvinia); F. Visagie (Carnavon); T. Hookstra (Ladismith); B. Fike (Grahamstown); C. Cloete (Donkerpoort); N. Ferreira (Bloemfontein); G. Jansen van Vuuren (Vredefort); L. Botha (Memel); R.P. Spiers, B. Visser, J.D. Black, D.V. Fly, M.J. Kimber and R.G. Burls (Howick); G. Groenewald, F. Brink and C. Pieterse (Golden Gate); H. Lewis (Marken); B. Harris, W. Gertenbag and B. Pretorius (Kruger National Park); P. Pienaar and K Kotzè (Potgietersrus); J. Fourie, R. de Vos, D. Koen (Suikerbosrand); E. Putzier (Matatiele); M. Loubser, J.H. van der Westhuizen and S.J. van Zyl (Prieska); W. Pretorius (Nieuwoudtville); J. Human and A. Ferreira (Victoria-west); F. Wessels (Verkeerdevlei); N. Bezuidenhout (Rustenburg); P. Marsh (Hilton); C.J. Scheepers (Pietermaritzburg); J. Bornman, H. Bornman and D.P. Nel (Baberton); P. Geldenhys (Volksrust); P. Hitchins and J. Anderson (Songimvelo); D. White (Hilton); H. Erasmus (Rolfontein); P. Serfontein (Vhembi); S. Godschalk (Devision Nature Conservation, S.A.D.F.); T. Middleton and F. Schutte (Blydepoort); F. Visser and W. Roelofse (Lydenburg); I. van der Merwe (Ermelo); J. Greeves (Louis Trichard); M. Ras, L. Ras (Pretoria).

Permits for the collection of specimens were provided by the Transvaal Directorate of Nature and Environmental Conservation, the Cape Chief Directorate Nature and Environmental Conservation, Orange Free State Nature Conservation, Natal Parks Board, National Parks Board and KaNgwane Nature Conservation.

Special thanks to Warren Funston for field assistance which was often given in the most adverse of conditions. Paulette Prinsloo, Gus van Dyk, Stefanie Freitag, Koos Kotzè, Chris Styles, John Malan, Jonathan Bloomer and my father Anton Matthee, also supported me in this respect. I am indebted to professor Koos Bothma (Center for Wildlife Management, University of Pretoria) for the use of a shotgun and to Koos Kotzè for his assistance with various aspects of the computer analysis and to the technical staff, particularly Martin Haupt, Bapsie Potgieter and Simon Maake.

I thank my fellow students and friends at the Mammal Research Institute for their support and friendship throughout the course of this investigation, in particular, Vanessa Bodenstein, Stefanie Freitag, Warren Funston, Bettine Jansen van Vuuren, Koos Kotzè, Debbie Morris, Paulette Prinsloo, Lizel Seesink, Chris Styles, Gus van Dyk and Christina Whiteford.

Financial support was provided by the Foundation for Research Development, University of Pretoria and The Wildlife Society of Southern Africa in the form of the Charles Astley Maberly Memorial Scholarship (1992 Jr.).

Finally I wish to express my sincerest gratitude and appreciation to my parents and sister for their unreserved love, encouragement and understanding.

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CHAPTER 1

Introduction

1.1. Lagomorph evolution and systematics

1.1.1. Palaeontological record

Twelve genera are currently recognized within the order Lagomorpha which, in turn, comprises two families, the Ochotonidae (pikas) and Leporidae (rabbits and hares; Dawson 1979). Rabbits and the ubiquitous hares, genus *Lepus*, comprise 11 genera, subdivided into 53 species (Chapman & Flux 1991). From an evolutionary perspective, there is considerable uncertainty about the precise affinities of lagomorphs to other mammalian taxa (Van Valen 1964). For example, Gidley (1912) states that the development of large scalpriform incisors and similarities in the morphology of the brain and reproductive system in lagomorphs and rodents are not necessarily indicative of close common ancestry, while Wood (1957) holds that lagomorphs originated from the order Condylartha, a primitive ungulate mammal, and Russel (1959), in turn, roots them in the insectivores. The more recent study by Novacek (1992) supports a close association between the Lagomorpha, Rodentia and Macroscelidea.

Interestingly, the first palaeontological record of leporids dates back to the late Eocene (approximately 45 million years ago; Dawson 1979) with the weight of opinion supporting the hypothesis that lagomorphs originated either from the extinct relatives of the tree shrew, the Anagalida or, alternatively, from another unidentified group ancestral to both the Anagalida and Lagomorpha (Dawson 1979).

Quite clearly however, all fossil evidence points to an early North American lagomorph radiation, while the extinct Asian forms are regarded as the more primitive (Dawson 1979). However, due largely to gaps in the fossil record, the early origins of the African leporids are somewhat more problematic (Dawson 1979). Ochotonids are present in the African fossil record from the early Miocene but appear to have become extinct on the continent towards the end of this period (Keast 1972), their present distribution being strictly limited to the Nearctic and the Palearctic mountains and steps (DeBlase & Martin 1981). In contrast, leporids appear as fossils from the early Pliocene and are presumed to represent invaders from the Holarctic (Keast 1972).

Perhaps more pertinent, given the focus of the present investigation, fossil remains of the genus *Pronolagus* are found only in Africa, and date back to the Pleistocene (Dawson 1979). Hibbard (1963) speculated that the extinct *Alilepus dietrichi* (*Pliopentalagus*) is very closely related to *Pronolagus, Pentalagus* and the extinct *Serengetilagus* and might be the representative ancestral taxon. Currently *Pentalagus, Romerolagus* and *Pronolagus* are grouped in the subfamily Paleolaginae (Ellerman & Morrison-Scott 1966) while Dawson (1979), in contrast, illustrates a close affinity between the African rock rabbit, *Pronolagus*, and the Amami rabbit, *Pentalagus*, with both lineages presumably originating during the Pliocene.

1.1.2. Systematics of *Pronolagus*

Both the interspecific and intraspecific classifications of the genus *Pronolagus* lack consensus as is clearly evidenced by the varied taxonomic treatments that follow. Roberts (1951) described six species of *Pronolagus*: *P. rupestris* (Smith 1834), *P. randensis* (Jameson 1907), *P. crassicaudatus* (Geoffroy 1832), *P. curryi* (Thomas 1902), *P. melanurus* (RÜppell 1842) and *P. barretti* (Roberts 1948). Ellerman, Morrison-Scott and Hayman (1953), in contrast, support the recognition only of *P. rupestris, P. randensis* and *P. crassicaudatus*, a classification based primarily on occipitonasal and ear bulla lengths. This is in marked contrast to Lundholm (1955) who regarded *P. randensis* and *P. rupestris* as valid species but viewed *P. crassicaudatus* as a synonym of the former. Since Lundholm's revision, museum collections of *Pronolagus* have grown considerably and new techniques such as multivariate morphometrics, allozyme electrophoresis, comparative cytogenetics and sperm morphology, have all been used in attempts to further resolve the taxonomic affinities within *Pronolagus* (see below; Robinson 1981). In essence, however, these yielded outcomes that concur with previous treatments by Petter (1972) and Meester, Rautenbach, Dippenaar & Baker (1986), and three species are currently regarded as valid (*P. rupestris, P. randensis* and *P. crassicaudatus*). The only recent dissenting opinion has been that of Peddie (1975) who, using measurements of the bulla, skull length and standard external body measurements, concluded that *Pronolagus* is monotypic within South Africa. It should be noted, however, that while consensus has generally been reached on the delineation of species, the status of many of the described races is uncertain and warrants further investigation (Meester *et al.* 1986).

1.1.3. *Pronolagus* systematics revisited

The uncertainty regarding *Pronolagus* systematics has given rise to several recent studies utilizing (i) multivariate skull morphometrics, (ii) allozyme electrophoresis and (iii) comparative cytogenetics.

(i) Morphometric analyses have contributed significantly to the clarification of the species problem within the genus. Robinson & Dippenaar (1983) report that while *P. crassicaudatus* and *P. randensis* have crania of similar overall size, they differ markedly in shape especially with respect to the length of the maxillary premolar toothrow and the width of the mesopterygoid space. *Pronolagus rupestris*, on the other hand, is the smallest of the three taxa and differs in cranial shape as reflected in ratios involving bulla breadth : skull length and bulla breadth : mandibular height. Morphometric analysis are, however, incapable of elucidating past evolutionary events. The high intraspecific variability in standard skull and pelage characters, inherent in the use of these parameters, often causes overlap between species and limits the usefulness of this approach when attempting phylogeny reconstruction (Angermann 1983; Baker, Eger, Peterson and Manning 1983).

(ii) Electrophoretic comparisons of protein variation within and between the *Pronolagus* species were hampered by the limited number of loci used (Robinson & Osterhoff 1983); although the survey indicated intergeneric differences it failed to highlight meaningful interspecific variation in *Pronolagus* (Robinson & Osterhoff 1983).

(iii) Robinson (1980) speculated that given the variation in diploid number with the Leporidae, chromosomal investigations could be useful in assessing interspecific phylogenetic relationships. The major mechanism of chromosome evolution in the Leporidae is centric fusion although heterochromatic additions have also played a role (Stock 1976; Robinson 1980). However, the new world cottontails *(Sylvilagus)* withstanding, cytogenetic analysis failed to reveal meaningful evolutionary associations between species with *Pronolagus* being no exception (Stock 1976; Robinson 1980).

1.2. Comparative morphology and distribution of *Pronolagus*

1.2.1. Smith's red rock rabbit, *Pronolagus rupestris*

Smith's red rock rabbit, is a small species (1.35 - 2.05 kg) characterized by upper principal incisors which are narrow across the face; the species has robust and broad ear bullae, both proportionately and in absolute measurements (Robinson 1982). Externally, *P. rupestris* has cheeks which are greyish-buff but not in marked contrast to the upper parts of the body. The species has a disjunct distribution with specimens occurring in the southern part of the continent, while conspecifics are found in south western Kenya, central Tanzania, eastern Zambia and northern Malawi (Skinner & Smithers 1990; Fig 1). In South Africa, Smith's red rock rabbit is recorded from the southern and south eastern parts of the Transvaal, central and southern Orange Free State, central

and southern Natal and occurs widely in the Cape Province (Skinner & Smithers 1990; Fig 1). That the existing range maps may be incomplete is, however, supported by the fact that specimens from all three species, *P. rupestris*, *P. randensis* and *P. crassicaudatus* (see below), were sampled outside of their previously described distribution areas (Fig 2).

1.2.2. Jameson's red rock rabbit, *Pronolagus randensis*

Jameson's red rock rabbit, is intermediate in size (1.82 - 2.95 kg) with respect to the other recognized species of the genus *Pronolagus*. They have a uniformly whitish chin with the cheeks, side of the neck and lower jaw being light grey in colour and contrasting markedly with the rest of the pelage (Skinner & Smithers 1990). Their occurrence in Africa is, as with *P. rupestris*, disjunct with one geographic assemblage situated in central southern Africa and the other in north western Namibia and southern Angola (Skinner & Smithers 1990; Fig 1). Within South Africa, Jameson's red rock rabbit is limited to the northern part of the Transvaal and ranges from the northern borders of the Orange Free State northwards to the Limpopo including central and western Transvaal (Skinner & Smithers 1990; Fig 1).

1.2.3. Natal red rock rabbit, *Pronolagus crassicaudatus*

The Natal red rock rabbit was not included in this phylogeographic mtDNA study in view of its restricted South African distribution (Fig 1). Nonetheless, for completeness, a brief description of its diagnostic features follow. *Pronolagus crassicaudatus* is the

largest of the three species (2.4 - 3.05 kg). The chin is white to grey and this colour extends along the lower jaw to approach the inferior margin of the nape patch. The cheeks are dark grey and do not contrast with the sides and upper parts of the body (Robinson 1982). Although *P.crassicaudatus* is restricted to Natal, with only marginal extensions into adjacent provinces (Skinner & Smithers 1990), specimens were, surprisingly, collected at Waterval-Boven in the eastern Transvaal a locality which markedly extends the range of the species.

1.3. General biology

Red rock rabbits are nocturnal in habit; they are generally solitary, although females might be accompanied by more than one male in the breeding season. All species feed mainly on grass (Stewart 1971) and their presence in a certain area is revealed by very distinct middens, or latrines, which may be used to mark their home ranges (Skinner & Smithers 1990). Although there is a paucity of reproductive data, all indications are that they breed throughout the year, raising one to three altricial young per litter following a gestation period of 35-40 days (Skinner & Smithers 1990). Considering that the gestation period of the true precocial hares, genus *Lepus*, is 42 days (Skinner & Smithers 1990) and that of the strictly altricial old world rabbit, *Oryctolagus*, is 27 days, *Pronolagus* is characterized by a relatively long gestation period for what is regarded as an altricial species (Cowan & Bell 1986). It seems likely that the increase in gestation length might increase the viability of the young since they are protected for a longer period *in vivo*, thereby consequently reducing their susceptibility to predation while still relatively helpless.

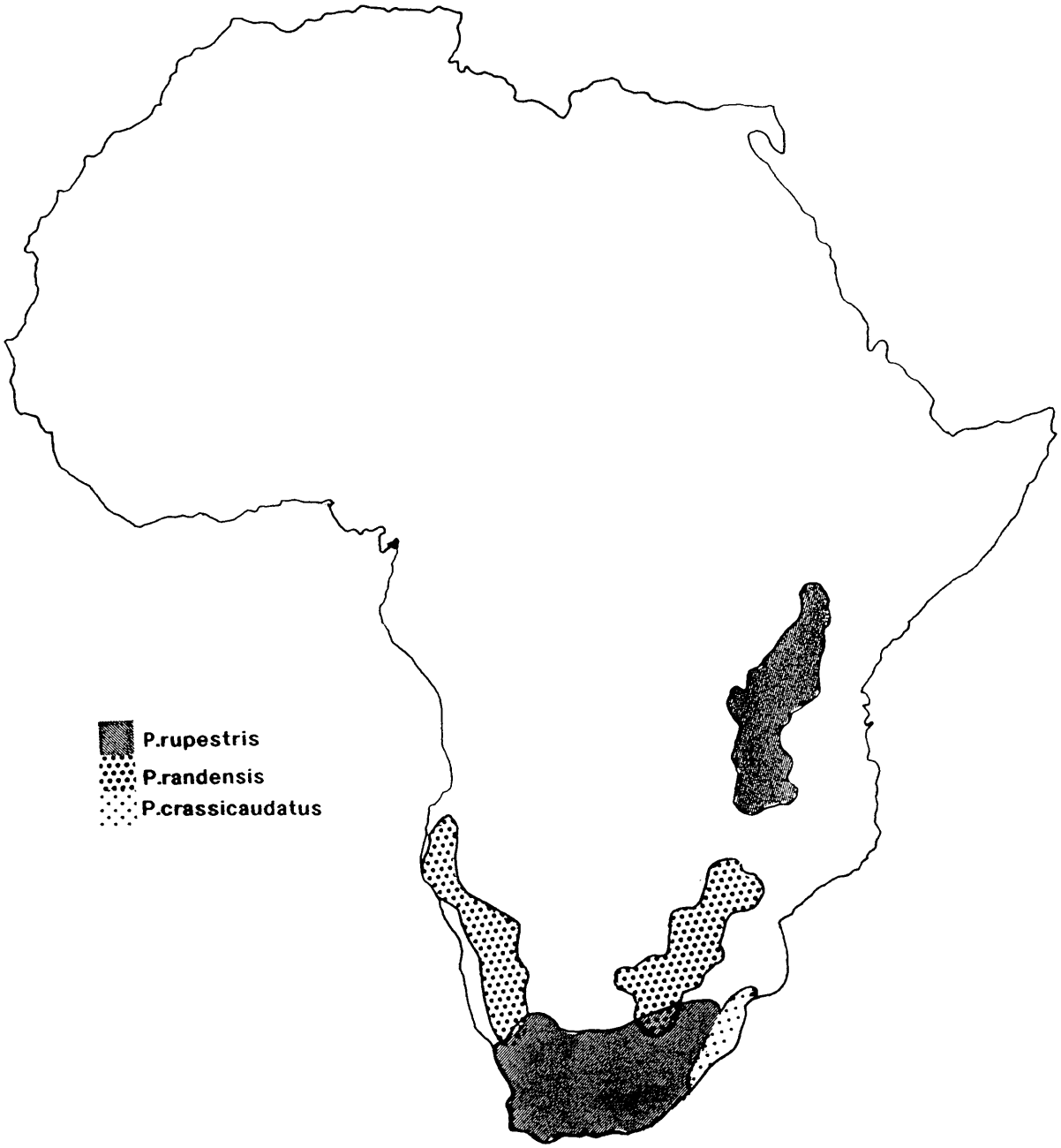

Figure 1: The distribution of *Pronolagus* in Africa (map redrawn from Skinner and Smithers 1990).

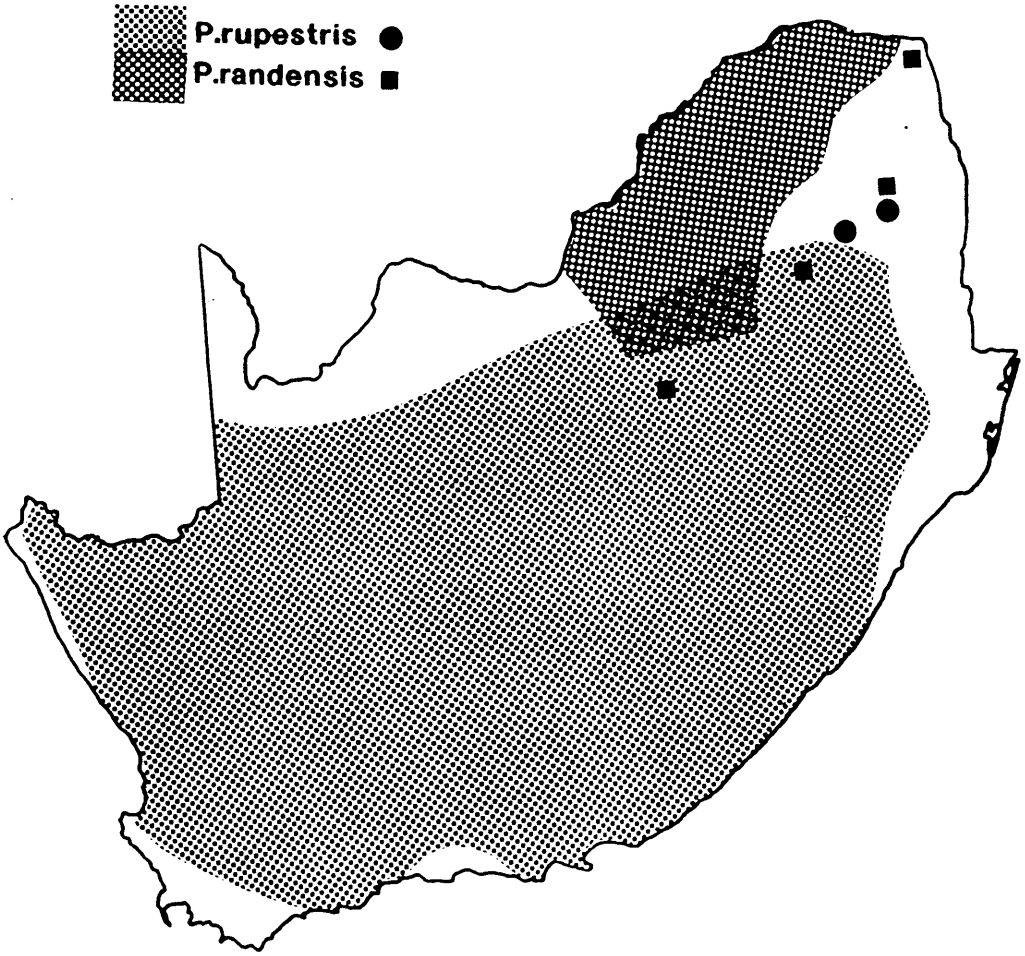

Figure 2: Distribution of *P. rupestris* and *P. randensis* in South Africa (map redrawn from Skinner & Smithers 1990). Collection localities of specimens outside of the previously recognized distributional limits of *P. rupestris* ● and *P. randensis* ■ are also shown.

Although important from a genetic viewpoint, little is known about the dispersal capabilities of *Pronolagus*. Peddie (1975) recorded that *P. randensis,* misclassified as *P. rupestris* (Robinson pers. comm.), rarely ventures any significant distance from its rocky habitat; this perception of limited vagility was further reinforced by Skinner & Smithers (1990) who stated that *Pronolagus* species, while never leaving their rocky habitat, will forage around the base of the koppies in which they live in search for fresh sprouting grasses.

Although both *P. rupestris* and to a lesser extent *P. randensis* are widely distributed throughout South Africa (Fig 2), their presence is highly dependent on the availability of substantial shelter (krantzes, rocky hillsides, boulder-strewn koppies and rocky ravines) which often occurs in the form of disjunct "terrestrial islands" with the constituent populations effectively existing as isolated demes. Furthermore, extensive plains are present within the distribution of *P. rupestris* which might serve as barriers to dispersal. Intuitively, therefore, the intervening unsuitable habitat tends to disrupt gene flow and it could be anticipated that a relatively high degree of genetic differentiation exists between local populations.

1.4. Mitochondrial DNA

It is widely assumed that techniques which manipulate DNA sequences (DNA hybridization, RFLP analysis and sequencing) are more accurate for assessing phylogenies and genetic population structure than protein based methods (allozyme electrophoresis; Hillis & Moritz 1990). In particular, the analysis of the mitochondrial genome, with its unisexual mode of inheritance and high mutation rate, has proved remarkedly useful

allowing inferences on population histories and micro-evolutionary changes over time (Lansman, Avise, Aquadro, Shapira & Daniel 1983, Brown 1983).

Mitochondrial DNA (mtDNA) analyses have been applied to a large variety of taxa in an attempt to clarify phylogenetic relationships (Hillis & Moritz 1990). In respect of the Lagomorpha, however, few of the extant taxa have been studied. Biju-Duval, Ennafaa, Dennebouy, Monnerot, Mignotte, Soriguer, El Gaaied, El Hili & Mounolou (1991) analyzed mtDNA from *Sylvilagus, Oryctolagus* and *Lepus* and found that these three leporids diverged from a common ancestor about 6-8 million years ago; intraspecific mtDNA variation has only been analyzed in *Oryctolagus.* In this investigation, the feral rabbits of Spain and France were found to be delineated by 4.0% sequence divergence (Biju-Duval *et al.* 1991) . The disruptive effects of glaciation were proposed as explanation for the separation of the two races, one in southern Spain and the other in south eastern France. Interestingly, on a finer scale, a mosaic pattern of mtDNA lineages was found implying that local *Oryctolagus* populations from eastern Europe are well separated, at least with respect to mitochondrial transmission and female exchanges (Biju-Duval *et al.* 1991), although support from nuclear markers would clearly strengthen this hypothesis.

1.4.1. Possible outcomes of mtDNA phylogeographic studies

Phylogeographic studies based on geographic patterns of nucleotide sequence divergence in mtDNA indicated a strong correlation between genetic structuring and environmental influences (Avise, Arnold, Ball, Bermingham, Lamb, Neigel, Reeb & Saunders 1987). Variation in mtDNA is usually patterned along geographic lines and tends

to correlate with barriers to gene flow (Avise *et al.* 1987). The observed geographic distribution of mtDNA clades provides a picture of the phylogeographic past of species. While having yielded data on a variety of taxa including representatives of all chordate orders (Avise 1986), the genetic analysis of the mitochondrial genome has been particularly useful in a study on the rock hyrax, *Procavia capensis*, a small African mammal species with similar habitat preference to *Pronolagus* (Prinsloo & Robinson 1992).

Theoretically, intraspecific mtDNA phylogenies overlaid on geographic maps can yield different outcomes (Avise *et al.* 1987). As a result, Avise and co-workers (1987), have proposed five major categories of mtDNA geographic structure ranging from total phylogenetic continuity between populations to major genetic discontinuity due to zoogeographic barriers.

1.4.1.1. Phylogenetic discontinuities

The first two categories described by Avise *et al.* (1987) are concerned with phylogenetic discontinuities found in species with continuous distributions and is perhaps best illustrated by the deer mouse, *Peromyscus maniculatus*. Five major genetic clones were detected within this widely distributed taxon. A geographic overlay of the mtDNA data illustrated strong geographic patterning, reflecting possible long term zoogeographic barriers which, in the evolutionary past, were thought to have prevented deer mice from dispersing. Estimates to common female ancestry for the lineages were in the region of three million years (Lansman *et al.* 1983). Although rarely observed, phylogenetic discontinuities can, theoretically, also be present where no obvious

zoogeographic barrier is effective, however, empirical support for this category is, at best, weak (see Avise *et al.* 1987).

1.4.1.2. Phylogenetic continuities

The other mtDNA phylogeographic categories described by Avise *et al.* (1987) are concerned with limited interpopulational genetic structuring, a case in point being the old field mouse, *Peromyscus polionotus*. In this species spatial separation of populations was present, but, strikingly, this did not appear to influence the dispersal of mtDNA lineages over large geographic areas (Avise, Shapira, Daniel, Aquadro & Lansman 1983). In contrast, a marine organism the American eel, *Anguilla rostrata*, is an example where habitat uniformity (the ocean) is reflected in the mtDNA phylogeographic patterns which showed an absence of genetic divergence among samples from a 4000 km stretch of coastline. Factors responsible for this lack of structure included the extraordinary life history of catadromous eels, which involves a single spawning population, and the subsequent widespread dispersal of larvae by ocean currents (Avise, Helfman, Saunders & Hales 1986). Thirdly, if partial spatial separation of populations occur, one or two mtDNA clones will tend to be widespread throughout the species distribution, while others will be confined to certain areas, an observation borne out by, amongst others, Bermingham and Avise's (1986) study on the bowfin fish, *Amia calva*.

1.4.2. Characteristics of the mitochondrial DNA molecule

Mammalian mtDNA is an extranuclear cytoplasmic circular gene system encoding 13 messenger RNAs, 22 transfer RNAs and two ribosomal RNAs (Brown 1983). In multicellular animals the molecule ranges from 15 600 bp to 19 700 bp in size (Brown 1983) while in mammals it is usually in the order of approximately 16 500 bp (± 200 bp; Brown 1983). Where size variation exists between and within species, this is generally due to deletions or additions of basepairs from the non-coding, or D-loop, region of the mtDNA molecule (Brown 1983). In some instances, size variation can be quite pronounced as is evident in the lizard genus *Cnemidophorus*, which shows genome sizes differing by as much as 1200 bp. (Brown 1981).

Since the sperm contributes almost no cytoplasm to the fertilized egg, mtDNA is almost exclusively maternally inherited (Barton & Jones 1983) making the molecule a particularly sensitive tracer of maternal genealogy (Moritz, Dowling & Brown 1987). Low-levels of paternal leakage (1 molecule per 25 000) have been detected in long-term backcrossing experiments in mice (Lansman, Avise & Huettel 1983), while Gyllensten, Wharton, Josefsson & Wilson (1991) have also demonstrated paternal inheritance of mtDNA in mice litters, albeit at an extremely low frequency relative to the maternal contribution.

Efficient DNA repair mechanisms are absent in mitochondria and, since mtDNA undergoes many more rounds of replication than the nuclear component, this can consequently increase the number of errors produced per cell generation (Brown 1983). The high copy number of mitochondria per cell may also be advantageous since mutations will tend to have little effect on the functionality of the cell itself. It is suggested that

these three factors may, singularly or in concert, contribute to the enhanced mutation rate for the molecule which has been calculated at five to ten times that of single copy nuclear genes (Brown 1983; Lansman *et al.* 1983).

Brown, George & Wilson (1979) calibrated mtDNA substitution rates for 19 pairs of mammalian species against divergence times estimated from fossil or protein data. This study indicated a constant mtDNA mutation rate in vertebrates. The conventional 2% sequence divergence per million years has been accepted in lineages as diverse as the rhinocerosis (George, Peuntes & Ryder 1983), artiodactyls (Uphold & Davis 1977), gallinaceous birds (Helm-Bychowski 1984), salmonid fishes (Gyllensten & Wilson 1986) and rodents (Ferris, Ritte, Lindahl, Prager & Wilson 1983). It should be noted, however, that there are studies that question the universality of this calculation (Avise, Bowen, Lamb, Meylan & Bermingham 1992; Brown, Prager, Wang & Wilson 1982). While no attempt has been undertaken to calibrate a molecular clock for lagomorphs, given the diversity of lineages reviewed by Wilson, Cann, Carr, George, Gyllensten, Helm-Bychowski, Higuchi, Palumbi, Prager, Sage and Stoneking (1985), it is assumed that the conventional 2% per million year similarly holds for this order. However, recent allozyme studies on leporids suggested a temporal acceleration in the rate of allozyme evolution at low taxonomic levels, an observation which is thought to be due to the rapid adaptive radiation of biochemically highly polymorphic taxa (Grillitsch, Hartl, Suchentrunk & Willing 1992).

1.4.3. Heteroplasmy in the Leporidae

Heteroplasmy, the presence of more than one mtDNA type within an individual (Clark 1988), has been detected in the American shad (Bentzen, Leggett & Brown 1988), whiptail lizards (Moritz & Brown 1987), evening bats (Wilkinson & Chapman 1991), Japanese monkeys (Hayasaka, Ishida & Horai 1991) and various other vertebrates (Bermingham, Lamb & Avise 1986; Clark 1988). The observation of heteroplasmy is often explained by a high mutation rate but, without direct estimates of the number of cell divisions per organismal generation, or the number of segregating units per cell, it is not possible to test this prediction (Clark 1988). Heteroplasmy is usually stably inherited from mother to offspring (Wilkinson & Chapman 1991), however biparental inheritance can also give rise to heteroplasmy which can increase in frequency to a dominant position through bottlenecks during oogenesis (Gyllensten *et al.* 1991). Furthermore, previous studies have shown that heteroplasmic species apparently escape the segregation rule of animal mtDNA genetics since stochastic sampling at cell division progressively resolves the cell back to homoplasmy (Biju-Duval *et al.* 1991); therefore, the number of types of mtDNA transmitted to the next generation appears effectively haploid (Wilson *et al.* 1985). Avise (1991) further stated "the thousand to million year timescales involved where mtDNA lineages are concerned are vastly longer than anticipated transitional periods of heteroplasmy and therefore the proportion of intraspecific mtDNA sequence heterogeneity is trivial under natural circumstances".

One of the most recent observations of intra- and interindividual size variation concerns the Lagomorpha. The domestic rabbit, *O. cuniculus,* has a mtDNA genome size that varies between 16.9 and 17.9 kb (Biju-Duval *et al.* 1991) and the cause of size heterogeneity could be assigned to the major non-coding D-loop region (Ennafaa, Monnerot, El Gaaïed & Mounolou 1987). Likewise, *Lepus* and *Sylvilagus* were also found to exhibit genome size variation which ranged between 17.4 - 18.2 kb in the former species, and 17.2 - 17.9 kb in the latter (Biju-Duval *et al.* 1991; Ennafaa *et al.* 1987). This was ascribed to variation in numbers of both short (20 nucleotides) and long repeats (153 nucleotides) in the mtDNA genome (Mignotte, Gueride, Champagne, Mounolou 1990).

1.5. Objectives

The main aim of this study was to assess phylogeographic mtDNA differentiation in *P.rupestris* and *P.randensis*, the two most widely distributed rock rabbit species in South Africa. By including several presumably isolated populations in this study it was anticipated that the data could contribute both to understanding the evolutionary relationships of these two species and their taxonomic affiliations. *Pronolagus*, with its limited dispersal capabilities and fragmented habitat, was considered likely to show deep divisions in its mtDNA gene tree, and it was hoped that the study would, in the broader sense, also contribute to the growing body of literature concerning microevolutionary processes in small mammal species.

Secondly, since the presence of concordant phylogeographic patterns in two independent evolutionary lineages are thought to reflect evidence of similar vicariant histories of population separation (Avise 1992), it was decided to compare the geographic mtDNA structure evidenced in *P. rupestris* and *P. randensis* to that published for the rock hyrax, *Procavia capensis*, a species characterized by similar habitat specificity (Skinner & Smithers 1990). It was felt that should concordant phylogeographic patterns emerge, this may reflect the result of similar episodic changes in the past environmental conditions.

CHAPTER 2

Material & Methods

2.1. Sample collection

In total, 27 *P. randensis* specimens drawn from eight geographically discrete localities in the Transvaal and Orange Free State and 55 *P. rupestris* specimens, representative of 21 localities encompassing all four provinces, were sampled in this study (see appendix I and II; Fig 3). All specimens were collected at night using a spotlight and 12 gauge shotgun. Heart, liver and kidney samples were taken as soon as possible ($\leq$ 1 hr.) after collection and stored in liquid nitrogen in the field. Frozen tissues were subsequently transferred to an ultracold freezer (-70˚C) until processing.

Standard body measurements (sex, mass, HB, T, Hf c/u, E) were routinely taken of every specimen. Skins and skulls of most individuals were deposited as part of the mammal collection of the Transvaal Museum, Pretoria. Accession numbers are listed in Appendix I.

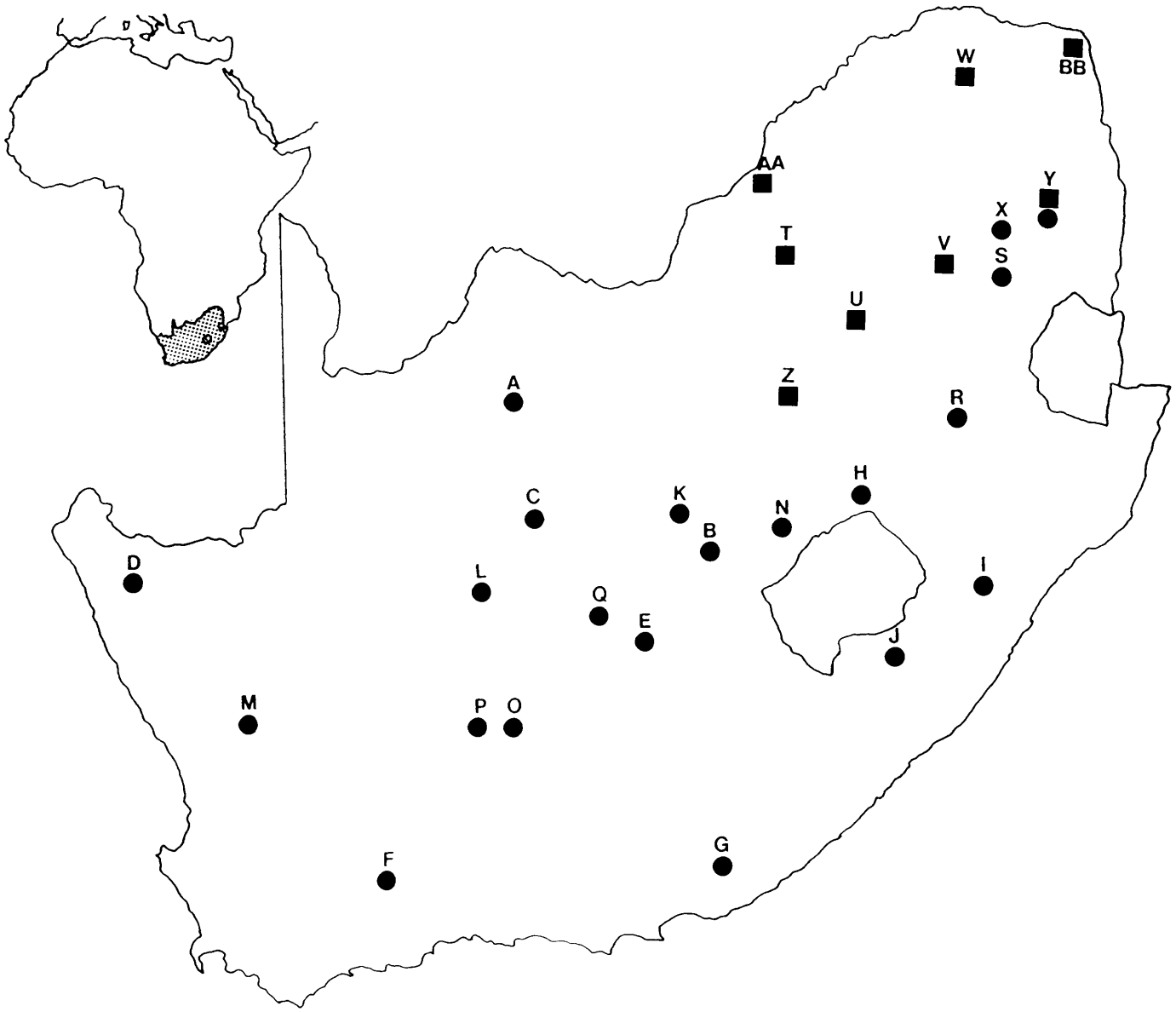

Figure 3: Collection localities of the *P. rupestris* ● and *P. randensis* ■ specimens used in the present study. Kuruman (A), Bloemfontein (B), Campbell (C), Springbok (D), Donkerpoort (E), Ladismith (F), Grahamstown (G), Golden Gate Highlands National Park (H), Howick (I), Matatiele (J), Dealesville (K), Prieska (L), Nieuwoudtville (M), Verkeerdevlei (N), Victoria-West (O), Sandgat (P), Rolfontein Nature Reserve (Q), Volksrust (R), Waterval-Boven (S), Rustenburg (T), Heidelberg (U), Middelburg (V), Louis Trichard (W), Lydenburg (X), Blyderivierspoort Nature Reserve (Y), Vredefort (Z), Zeerust (AA), Kruger National Park (BB). Corresponding map coordinates are presented in Appendix II.

2.2. Methods

2.2.1. Mitochondrial DNA extraction

Intact mtDNA was extracted using established protocols (Lansman, Shade, Shapira & Avise 1981). Hearts, frozen in liquid nitrogen and weighing between 1.5-7.0 g, were used as the source tissue for the extraction procedures. The tissue was minced with a sterile scalpel blade, followed by homogenization with a motor-driven glass teflon homogenizer (10-20 strokes) in the presence of MSB-Ca^{++} buffer (0.21 M D-mannitol, 0.07 M sucrose, 0.05 M tris-base, 0.003 M $CaCl_2$; pH 7.5) which reduces nuclear breakage. The nuclei and cell debris were separated from the much smaller mitochondria with two low speed spins (610xg for 5 min) after which, the mitochondria were pelleted in the presence of MSB-EDTA (MSB as described above, 0.01 M ethylenediaminetetra-acetic acid; pH 7.5) at a higher centrifugation speed (15 380xg for 20 min). The EDTA minimizes the aggregation of mitochondria and nuclease activity during differential centrifugation. Thereafter, proteins were removed by centrifugation (15 000xg) in STE (0.1 M NaCl, 0.05 M tris-base, 0.01 M EDTA; pH 8.0) and lysis of mitochondrial cell walls using 10% SDS (Sodium dodecylsulfate) followed. Intact mitochondrial DNAs were isolated from nuclear DNA, and the remaining protein, by CsCl-ethidium bromide gradient ultracentrifugation (225 000xg for 24-48 hr).

Following this step, mtDNA was drawn off the gradient under ultra-violet light (365 nm wavelength) using a syringe and 18 gauge needle. Ethidium-bromide was removed by equal volume iso-amyl alcohol extractions and this was followed by dialysis against $T_{10}E_{0.5}$ (0.01 M tris-base, 0.0001 M EDTA; pH 8.0) to remove the CsCl and excessive

EDTA. Not unexpectedly, mtDNA yields differed between samples and were found to range between 5 -20 μg depending on the amount of starting tissue used. The purified mtDNA was subsequently stored in TE buffer at 4°C for later use.

2.2.2. Restriction digestion

Mitochondrial DNA molecules were screened for restriction site variation using a suite of 14 six-base restriction endonucleases (Promega and Boehringer Mannheim; Table 1). All restriction digestions were carried out in 20 μl reaction volumes in the presence of 5 mM spermidine free-base and sterile distilled water. Supplied incubation buffers were used together with 10 units of the corresponding restriction endonuclease for every 20 ng of mtDNA. Digestion times were dependent on the restriction enzyme used and ranged from 6-15 hr at 37°C.

2.2.3. End-labeling

Mitochondrial DNA fragments produced by the restriction endonucleases were end-labeled using 0.25 μCi ^{32}P-dCTP or -dATP in combination with the other three unlabeled (cold) nucleotides. The Klenow fragment of DNA polymerase I has both polymerase and 3^{l} exonuclease activity with the exonuclease activity being more pronounced. The polymerase will add radioactive nucleotides using the 5^{l} overhang as a template. After terminal bases have been removed by the exonuclease activity of the Klenow enzyme, and subsequently filled in with either a radio-active isotope or one of the other cold nucleotides via polymerization, the reaction was stopped by heating to 65°C. This

was followed by the addition of 2 µl of tracking dye containing 50% glycerol, 0.1% bromophenol blue, 0.1% EDTA.

2.2.4. Gel electrophoresis

The end-labeled mtDNA fragments were almost invariably separated by overnight horizontal, low voltage (2 V/cm) electrophoresis using 1.0% TBE buffered agarose gels (TBE buffer = 0.0089 M tris-base, 0.0089 M boric acid, 0.0002 M EDTA; pH 8.0). Where necessary the resolution of the smaller fragments was increased by increasing the agarose concentration to 1.2%. Molecular weight marker II (Promega and Boehringer Mannheim), consisting of a mixture of fragments derived from *Hind III* cleaved lambda-DNA was used as standard in determining fragment sizes.

2.2.5. Gel drying

The end-labeled gels were vacuum dried (Slab Gel Dryer, Hoefer Scientific Instruments) to Whatman 3 MM chromatography paper for 1.5 hr using standard procedures. The temperature was kept constant at 54°C for the first hr to facilitate the melting of the agarose and the membrane subsequently covered in cling-wrap to prevent desiccation.

2.2.6. Southern blotting

Most end-labeled blots failed to resolve the smaller restriction fragments (< 500 bp) which were usually obscured by lane background caused by nuclear DNA contamination. Southern blotting (Southern 1975) was used to increase the resolution of the mtDNA analysis (Prinsloo & Robinson 1992). Following electrophoresis, gels were denatured for 30 min in a strong base solution (1.5 M NaCl, 0.5 M NaOH). This was followed by two 15 min neutralizing washes in a tris-base - EDTA solution (1.5 M NaCl, 0.001 M EDTA, 0.5 M tris-base; pH 7.8). After soaking the gel in the transfer buffer (10 X SSC) for 10 min it was placed on top of three pieces of Whatman 3MM filter paper presoaked in 10 X SSC and the mtDNA fragments subsequently transferred by capillary action (12-24 hr) to Hybond N nylon membrane (Amersham). Membranes were removed, rinsed in 10 X SSC and baked at 80°C for 2-4 hr; this step facilitates permanent binding of the DNA to the membrane.

2.2.7. Oligolabeling and hybridization of mtDNA probes

Closed circular mtDNA (prepared from specimen No 10: TM 42103) was used as a probe for blot hybridizations. Incorporation of α^{32}P-dCTP was overnight at 37°C in a reaction mix containing ±100 ng mtDNA; 50 µCi ^{32}P-dCTP; 50 mM dATP, dGTP and dTTP; 500 units of random hexanucleotide primers and the Klenow fragment of DNA polymerase I (Feinberg & Vogelstein 1983). The probe DNA was spermine precipitated by adding 475 µl TE^{-4} (1.0 M tris-base, 0.5 M EDTA), 8 µl salmon sperm DNA (10 mg/ml) and 8 µl spermine (100 mM) to a reaction mix containing the labeled mtDNA molecules. The precipitation step was followed by snap chilling on ice (10 min) and

centrifugation at 12 000 g for 10 min at 4°C to pellet the DNA. The DNA was subsequently denatured with 400 µl NaCl (0.5 M NaCl in TE^{-4}) and 40 µl 4N NaOH and the mixture incubated at 37°C for 30 min; the reaction was finally stopped through the addition of 120 µl tris-base (2 M). The radioactivity of the mtDNA probe was determined using a scintillation counter (Packard 1500 tri-carb Liquid Scintillation Counter) and the resulting values used for calculations of percentage incorporation.

Hybridization was carried out overnight at 65°C. Non-specific radioactivity was removed by two low stringency washes: 2 X 10 min with 2 X SSPE (0.36 M NaCl, 0.02 $NaH_2PO_4.H_2O$; pH 7.7) and 0.1% SDS. If necessary, a further wash was performed for 15 min at 65°C with 0.1% SSPE and 0.1% SDS and the hybridization membranes subsequently sealed in plastic bags to prevent desiccation.

2.2.8. Autoradiography

End-labeled mtDNA fragments fixed on the vacuum-dried blots, or radio-labeled mtDNA fragments on the Southern blots, were visualized by autoradiography. The membranes were exposed for 2-10 hr (Fuji RX medical X-ray film) at -70°C. Double intensifying screens were used to shorten exposure times.

2.3. Data analysis

2.3.1. Data interpretation

Alphabetic characters were assigned to the different restriction profiles obtained with each of the 14 restriction endonucleases used. This resulted in a 14 letter code, or composite haplotype, for each individual (Table 2). The composite haplotypes (clones) were subsequently compared and sequence divergence estimates calculated based on the number of shared fragments between them (see below).

2.3.2. Estimates of sequence divergence

The restriction fragment data was analysed using the Restsite v1.1 computer package developed by J.C. Miller (Nei & Miller 1990) which utilizes equations 16 and 17 of Nei (1987). In essence, the analysis involved:

(i) Estimating mtDNA fragment sizes from which the fraction of shared fragments between clones was determined using the equation $F = M_{xy}/M_x+M_y$, where F is the fraction of shared fragments, M_{xy} is the total number of shared fragments between the two clones and M_x and M_y the total number of fragments for clone X and Y respectively.

(ii) Once F was obtained, a value for G was calculated using the iteration formula $G = \{F(3-2G_1)\}^{1/4}$, where G_1 is a trial value of G. The itera-

tion computation was run until G = G_1. It was suggested by Nei (1987) that $F^{1/4}$ be used as the first trial value of G_1. Once G was obtained, the sequence divergence was estimated with d = -2[ln(G2)]/r). This value allows for the calculation of divergence times between clones or major taxonomic groupings. It should be noted, however, that small errors in the estimation of F, due to limited band sharing between the different composite haplotypes, can lead to unrealistically large differences in d (Avise 1986). The Jukes-Cantor correction was applied to all d values in order to avoid under-estimation due to large nucleotide substitution values. This was done using: d = -3/4 $\log_e$[1-(4/3)p], where p = is the proportion of different nucleotides (Jukes & Cantor 1969).

(iii) Genotypic diversity was calculated using $n(1-\Sigma f_i^2)/n-1$ (Nei & Tajima 1981) where f_i is the frequency of the *i*th mtDNA genotype in a sample of size n. Genotypic diversity can range from 0, when all individuals exhibit the same genotype, to 1.0, when each individual is unique (Avise, Bowen & Lamb 1989).

2.3.3. Phenogram construction

Phenograms, illustrating the genetic relationships between different populations, were generated from sequence divergence estimates produced by the Restsite program. First, an UPGMA (unweighted pair-group method using arithmetic averages; Sneath & Sokal 1973) tree was generated. This method is based on equal mutation rates amongst taxa and consecutively joins the two most similar taxa (DeBry 1992). How-

ever, since Jin & Nei (1991) suggest that the UPGMA method is robust only when high sequence divergences are present, the Neighbor-Joining algorithm (NJ; Saitou & Nei 1987) was also included for intraspecific clustering of the different lineages, thereby giving a more accurate grouping when low sequence divergences were involved. The Neighbor-Joining tree builds the phylogeny according to a rigid step-wise procedure and subsequently minimizes the total length of the tree at each step (DeBry 1992). Jukes-Cantor corrections (Jukes & Cantor 1969) were applied to the distance matrix in order to decrease the sensitivity of the NJ tree to rate variation (DeBry 1992). It should be noted, however, that both tree building methods rely on a phenetic approach.

2.3.4. Estimation of mtDNA genetic diversity

In contrast to the preceding quantitative analyses, a qualitative method was also used to construct a phylogeographic network between populations (Avise, Lansman & Shade 1979). This was done by calculating the number of base substitutions between different 3restriction fragment patterns for each enzyme. The different mtDNA clones were compared in a pairwise fashion and the number of mutational changes summed over all enzymes used. Matrilines were subsequently connected in a phylogenetic network which linked composite haplotypes by the minimum number of mutational steps between them.

2.3.5. Analysis of geographic influences

In an attempt to explain the phylogeographic structures within *Pronolagus* and *Procavia*, it was important to examine annual rainfall, altitude and vegetation, all possible parameters that may influence gene flow between populations. This was done with the aid of the geological information surveys (G.I.S) laboratory, centered at the University of Pretoria, by accessing a data base derived from the Water Board (Computer Centre for Water Research, Pietermaritzburg) and Acocks' (1988) description of the veld types of South Africa.

CHAPTER 3

Results

3.1. General

The mitochondrial genome of *P. rupestris* and *P. randensis* was estimated at 18.01 kb (± 164 bp), a value which lies above the expected 17 500 bp suggested by Ennafaa *et al.* (1987) for *Oryctolagus*, the only other leporid for which comparable data are available. The restriction analysis of 82 *Pronolagus* mtDNA samples with 14 endonucleases (Table 1) resulted in 85 different restriction fragment profiles (Fig 4; Appendix III). A total of 4197 fragments were scored for all specimens used in the investigation, resulting in a mean of 51.18 fragments per individual. The survey therefore screened approximately 1.70% of the *Pronolagus* mtDNA genome.

All restriction enzymes, with the exception of *Asp 718* and *Kpn I*, resulted in species specific digestion profiles. Although shared restriction patterns characterized *Asp 718* and *Kpn I*, these enzymes were, nonetheless, informative in the delineation of the two distinct *P. rupestris* clades as were *Bam HI, Pst I* and *Stu I* which, interestingly, showed no variation within the north western clade. The restriction enzyme *Xba I* showed no polymorphisms within *P. randensis* and, additionally, was not informative in the delineation of the two *P. rupestris* clades.

Table 1: The fourteen restriction endonucleases used in this study, together with their recognition sequences and r-values (Nei 1987), are listed below. Also provided are the number of different restriction profiles obtained with each enzyme resulting from the analysis of 82 *Pronolagus* mtDNA samples.

Enzyme	Recognition sequence	r-value	No of Restriction profiles
Apa I	GGGCC/C	6.00	5
Asp 718	G/GTACC	6.00	2
Ava I	C/(G/C)CG(A/T)G	5.33	7
Bam HI	G/GATCC	6.00	3
Cla I	AT/CGAT	6.00	4
Dra I	TTT/AAA	6.00	9
Hind III	A/AGCTT	6.00	10
Kpn I	GGTAC/C	6.00	2
Pst I	CTGCA/G	6.00	4
Sac I	GAGCT/C	6.00	7
Sca I	AGT/ACT	6.00	7
Stu I	AGG/CCT	6.00	9
Sty I	C/C(A/T)(A/T)GG	5.33	10
Xba I	T/CTAGA	6.00	6

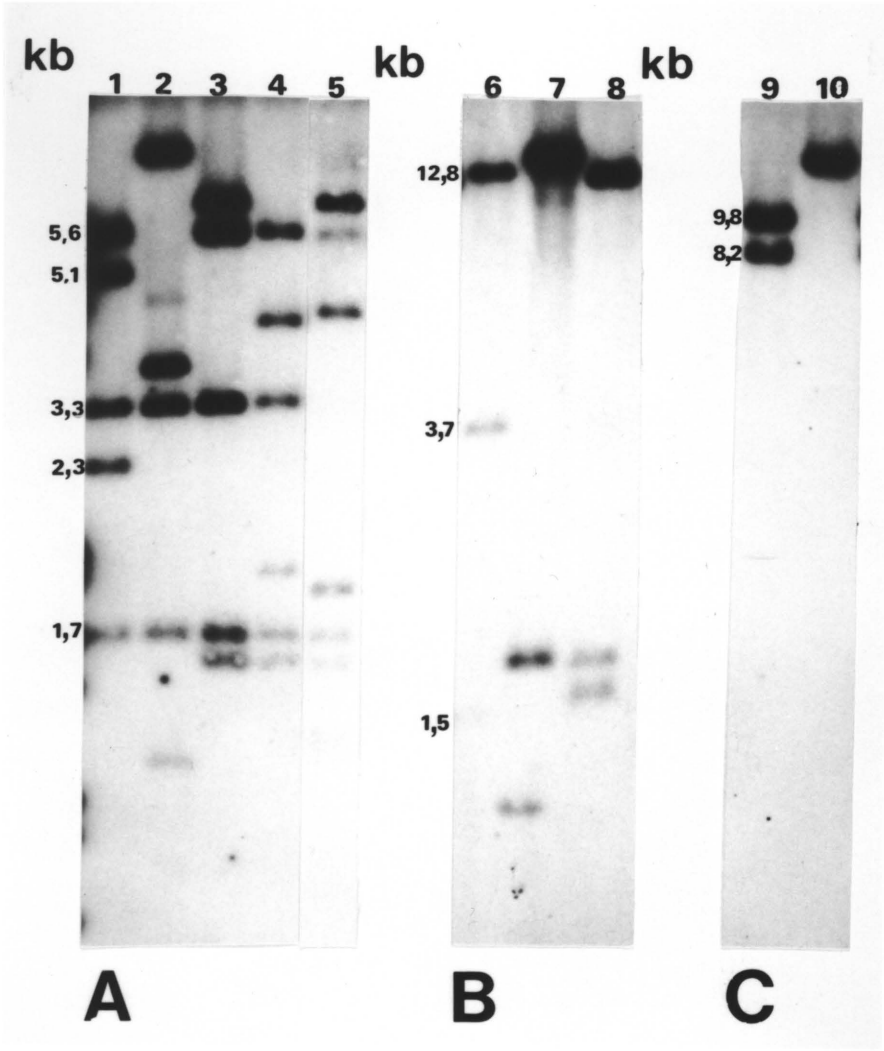

Figure 4: Representative fragment profiles following digestions of *Pronolagus* mtDNA: **(A)** *Dra I*: Lane 1 = type A; lane 2 = type B; lane 3 = type H; lane 4 = type C; lane 5 = type E. **(B)** *Hind III*: Lane 6 = type C; lane 7 = type B; lane 8 = type A. **(C)** *Cla I*: Lane 9 = type B; lane 10 = type D. (See Appendix III for further clarification).

Table 2: Descriptions of the 43 mtDNA lineages observed in all *Pronolagus* populations sampled. Clones 1-9 are representatives of the *P. rupestris* north western clade, clones 10-32 the *P. rupestris* south eastern clade, while clones 33-43 were found in representatives of *P. randensis*. Capital letters correspond to digestion profiles detected for each enzyme.

	Asp	Bam	Cla	Kpn	Pst	Xba	Sac	Stu	Apa	Sca	Dra	Ava	Hin	Sty
1	A	A	A	A	A	A	A	A	A	A	A	A	A	D
2	A	A	B	A	A	A	A	A	A	A	A	A	A	D
3	A	A	B	A	A	A	A	A	A	A	A	A	A	E
4	A	A	B	A	A	B	A	A	B	B	A	A	A	E
5	A	A	C	A	A	B	A	A	B	B	A	A	A	E
6	A	A	B	A	A	B	A	A	B	A	A	A	A	E
7	A	A	B	A	A	A	A	A	B	A	A	A	A	E
8	B	A	B	B	A	C	B	A	C	A	B	A	B	A
9	B	A	B	B	A	C	B	A	C	A	B	B	B	A
10	B	B	B	B	B	A	C	B	D	C	G	C	C	B
11	B	B	B	B	C	D	C	C	D	C	C	C	C	C
12	B	B	B	B	B	A	C	B	D	C	D	C	C	B
13	B	B	B	B	B	A	C	B	D	C	E	C	C	B
14	B	B	B	B	C	A	C	B	D	C	D	D	C	C
15	B	B	B	B	C	C	C	B	D	D	D	C	D	F
16	B	B	B	B	B	A	C	C	D	D	F	C	E	B
17	B	B	B	B	C	A	C	B	D	C	D	C	F	F
18	B	B	B	B	B	C	D	C	D	C	F	C	G	G
19	B	B	B	B	B	A	C	C	D	C	F	C	C	B
20	B	B	B	B	B	A	C	C	D	C	F	C	C	C
21	B	B	B	B	B	C	C	B	D	D	F	C	H	B
22	B	B	B	B	B	A	C	B	D	D	F	C	E	B
23	B	B	B	B	B	A	C	B	D	C	F	C	E	B
24	B	B	B	B	B	C	C	C	D	C	D	C	C	B
25	B	B	B	B	C	A	C	C	C	D	D	C	F	C
26	B	B	B	B	C	A	E	C	C	D	D	C	F	C
27	B	B	B	B	C	E	C	C	C	D	F	E	F	B
28	B	B	B	B	C	A	C	C	D	C	D	C	F	C
29	B	B	B	B	C	C	C	C	C	D	F	E	F	B
30	B	B	B	B	C	C	C	C	C	D	D	E	F	B
31	B	B	B	B	C	A	C	C	C	D	D	E	F	B
32	B	B	B	B	C	A	C	C	C	D	F	E	F	B
33	B	C	D	B	D	F	F	D	E	E	H	F	I	H
34	B	C	D	B	D	F	F	D	E	E	H	F	I	I
35	B	C	D	B	D	F	F	E	E	E	H	F	J	I
36	B	C	D	B	D	F	F	E	E	E	H	F	J	J
37	B	C	D	B	D	F	G	F	E	F	H	G	J	I
38	B	C	D	B	D	F	F	G	E	E	I	G	J	I
39	B	C	D	B	D	F	F	G	E	E	I	G	J	J
40	B	C	D	B	D	F	G	H	E	G	H	G	J	I
41	B	C	D	B	D	F	F	H	E	G	H	G	J	I
42	B	C	D	B	D	F	F	H	E	G	H	F	J	I
43	B	C	D	B	D	F	G	I	E	E	H	F	I	I

The sizes of the mtDNA restriction fragments obtained after digestion with the 14 different enzymes are given in Appendix III. Heteroplasmy was detected in all *Pronolagus* mtDNAs and was particularly noticeable with *Sty I* digestions (visible as spread out and fuzzy bands). The sizes of these heteroplasmic, or additional bands appear dependent on the enzyme used and size variation ranged between approximately 100-200 bp. The heteroplasmic bands were ignored in calculation of the *Pronolagus* mitochondrial genome size (Avise *in lit.*[1]).

Restriction site mapping has shown that the repeat sequences responsible for the differences in lengths between mtDNA molecules observed in *Oryctolagus* could be assigned to the non-coding D-loop region of the genome (Ennafaa *et al.* 1987). Mignotte *et al.* (1990) stated that the rearangements of the short repeat motives were observed to different extents in the mtDNA from one animal to another. To date similar length heterogeneity has been observed in the mtDNA of several species within the Lagomorpha (including *Pronolagus* - Mignotte *in lit.*[2]) and it is likely to reflect a primitive condition probably present in all extant representatives of the Leporidae (Biju-Duval *et al.* 1991). It should be noted, however, that most enzymes used in this investigation failed to show heteroplasmy, possibly due to the low intra-individual copy number. Furthermore, small length dissimilarities in large fragments are difficult to detect because the distance that a large fragment migrates through the gel is inversely proportional to the logarithm of the molecular weight of the fragment (Maniatis, Fritsch & Sambrook 1982).

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3.2. *Pronolagus rupestris*

Thirty two composite haplotypes were found within *P. rupestris* of which nine (clones 1-9) form part of a distinct clade situated in the north western part of South Africa while 23 composite haplotypes (clones 10-32) are included in a larger south eastern clade (Table 2; Fig 5). These mitochondrially distinct clades were separated by 7.94% (±1.40%) sequence divergence. If the conventional 2% per million years holds for the Leporidae (see section 1.4.2), a divergence time, since common ancestry, of 3.97 million years separates representatives of the two clades (Fig 5).

Sequence diversities of 1.09% (±0.22%) within the north western and 1.69% (±0.34%) within the south eastern *P. rupestris* clades were found. Estimated pairwise nucleotide sequence divergences between the different composite haplotypes were calculated and ranged from 0.16% (clone 1 vs 2) to 12.45% (clone 5 vs 27). Variation within clades was taken into account when these values were computed and the standard errors were derived with 200 bootstrap replications (Table 3). Intrapopulational sequence diversity estimates ranged from 0.00% at Blyderivierspoort to 1.90% at Golden Gate (Table 4). Genotypic diversity was estimated at 0.961 for *P. rupestris* indicating the exclusivity of most composite haplotypes and mirrors the situation for deer mice, *P. maniculatus*, where 61 composite haplotypes were found for 135 individuals resulting in a genotypic diversity of 0.974 (Avise *et al.* 1989). At a finer level, genotypic diversity was calculated at 0.958 for the north western clade and 0.866 for the south eastern clade.

Table 3: Sequence divergences between 32 mtDNA clones of *P. rupestris*. Jukes-Cantor corrected values are presented above the diagonal and standard errors below. Values were derived via 200 bootstrap cycles (see text for details).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1		0.16	0.34	0.92	0.30	0.70	0.52	2.82	3.02	9.75	9.47	10.54	11.30	10.03	10.03	11.22	9.45	11.58	11.15	9.96	12.02	11.22	11.30	11.37	8.90	8.98	12.40	8.98	12.33	11.68	10.95	11.50
2	0.17		0.17	0.71	0.91	0.52	0.35	2.54	2.74	9.12	8.74	9.82	10.47	9.32	9.30	10.40	8.81	10.62	10.33	9.15	11.07	10.40	10.47	10.54	8.25	8.33	11.56	8.33	11.50	10.95	10.31	10.78
3	0.21	0.15		0.53	0.72	0.34	0.17	2.32	2.49	9.22	8.81	9.92	10.56	9.39	9.38	10.49	8.89	10.69	10.42	9.20	11.16	10.49	10.56	10.63	8.30	8.39	11.68	8.39	11.61	11.00	10.43	10.91
4	0.33	0.30	0.28		0.17	0.17	0.35	2.69	2.85	9.71	9.36	10.49	11.23	9.95	9.32	11.16	9.38	10.61	11.06	9.85	11.08	11.16	11.23	10.56	8.81	8.89	11.61	8.89	11.56	11.02	10.96	11.50
5	0.38	0.35	0.37	0.19		0.34	0.53	2.99	3.17	10.42	10.18	11.31	12.19	10.77	10.03	12.11	10.10	11.56	12.02	10.79	12.02	12.11	12.19	11.38	9.53	9.61	12.45	9.61	12.38	11.74	11.68	12.32
6	0.31	0.26	0.24	0.17	0.25		0.17	2.32	2.49	9.78	9.36	10.56	11.31	10.03	9.38	11.23	9.45	10.69	11.16	9.93	11.16	11.23	11.31	10.63	8.89	8.97	11.68	8.97	11.61	11.08	11.02	11.56
7	0.26	0.21	0.16	0.22	0.30	0.16		2.25	2.43	9.16	8.73	9.85	10.49	9.33	9.32	10.42	8.82	10.61	10.35	9.12	11.08	10.42	10.49	10.56	8.25	8.33	11.61	8.33	11.56	11.02	10.39	10.85
8	0.85	0.78	0.74	0.77	0.83	0.77	0.74		0.17	8.77	7.76	8.45	9.39	7.91	6.76	8.39	7.54	7.46	8.77	7.55	7.94	8.39	8.45	7.72	5.95	6.00	8.07	7.03	7.74	7.53	8.07	8.31
9	0.89	0.84	0.77	0.75	0.82	0.80	0.73	0.15		8.89	7.84	8.56	9.50	7.47	6.86	8.50	7.62	7.56	8.89	7.62	8.06	8.50	8.56	7.84	6.04	6.09	10.00	7.12	9.68	9.43	10.00	10.30
10	2.21	2.67	2.71	2.48	2.48	2.48	2.44	1.85	1.92		1.73	3.79	0.50	1.39	1.97	1.92	1.53	2.47	0.97	1.55	1.29	1.19	0.94	1.16	3.40	3.73	3.62	2.38	3.53	3.42	3.08	3.19
11	2.01	1.84	2.04	1.86	1.93	2.11	2.19	1.23	1.18	0.60		1.69	2.19	1.07	2.20	2.13	1.73	1.93	1.27	0.70	3.09	2.93	2.57	1.30	1.59	1.83	3.09	0.88	3.03	2.76	2.52	2.77
12	2.40	2.26	2.44	2.09	2.11	2.19	2.44	1.95	1.87	0.36	0.60		0.49	0.99	1.43	1.87	1.06	2.40	0.94	1.52	1.26	1.16	0.92	0.69	2.64	2.92	3.49	1.81	3.42	2.60	2.34	3.08
13	2.38	2.74	2.85	2.34	2.36	2.19	2.47	1.89	1.87	0.47	0.80	0.45		1.50	2.10	2.07	1.65	2.60	1.10	1.43	1.32	1.07	1.29	1.29	3.54	3.87	3.76	2.51	3.69	3.56	3.23	3.34
14	1.90	2.12	2.35	1.68	2.05	2.00	2.29	1.32	1.16	0.48	0.52	0.44	0.52		1.26	2.97	0.92	2.74	1.99	1.33	2.33	2.21	1.95	1.70	1.93	2.18	4.07	1.18	4.01	3.26	3.02	3.71
15	1.40	1.44	1.24	1.50	1.33	1.54	1.45	1.47	1.64	0.72	0.81	0.67	0.78	0.66		2.41	0.53	2.38	2.76	2.62	1.17	1.69	1.92	1.81	1.57	2.56	1.32	1.32	2.33	1.79	2.04	2.63
16	2.69	2.55	2.41	2.25	1.99	2.38	2.67	1.81	1.32	1.19	0.77	0.97	1.09	0.82	0.71		2.48	1.47	0.72	1.30	1.19	0.50	0.70	1.39	2.19	2.44	1.71	1.91	1.65	2.14	1.88	1.41
17	2.06	1.64	1.60	1.63	1.58	1.72	1.98	1.31	1.63	0.70	0.74	0.67	0.75	0.50	0.34	0.79		2.65	2.22	2.07	1.69	1.75	1.49	1.87	1.32	1.53	2.62	0.70	2.56	2.00	1.79	2.33
18	2.11	2.12	2.03	2.00	2.07	1.88	1.95	1.60	1.70	0.68	0.67	0.70	0.72	0.66	0.70	0.63	0.77		1.26	1.11	1.90	2.15	1.85	1.43	2.80	3.09	2.69	1.89	2.45	2.95	3.31	2.76
19	2.80	2.91	2.69	2.11	2.07	1.88	2.41	1.86	1.17	0.62	0.52	0.62	0.75	0.60	0.83	0.58	0.81	0.59		0.58	1.47	1.36	1.10	0.56	2.51	2.78	2.01	1.69	1.93	2.47	2.20	1.69
20	2.25	2.29	2.46	1.85	1.80	2.13	2.10	1.19	2.02	0.62	0.40	0.68	0.77	0.60	0.82	0.80	0.97	0.50	0.47		2.07	1.95	1.67	1.13	1.95	2.21	2.72	1.13	2.66	3.18	2.91	2.41
21	2.19	2.19	2.29	2.25	2.31	2.23	2.53	2.22	1.82	0.70	0.96	0.70	0.92	0.75	0.47	0.72	0.57	0.77	0.77	0.82		0.58	0.78	1.68	2.93	3.23	2.01	2.57	1.76	2.26	2.62	2.06
22	2.40	2.43	2.52	2.48	2.54	2.10	2.79	1.87	1.87	0.84	0.85	0.62	0.74	0.76	0.62	0.51	0.72	0.66	0.78	0.80	0.44		0.18	2.14	3.00	3.29	2.47	2.64	2.40	2.99	2.69	2.13
23	2.84	2.75	2.58	2.49	2.19	2.60	2.85	1.70	1.86	0.66	0.81	0.69	0.67	0.67	0.70	0.57	0.67	0.68	0.88	0.92	0.51	0.20		1.81	3.29	3.61	2.76	2.32	2.69	3.30	2.99	2.40
24	2.00	2.08	2.13	2.34	1.97	2.42	2.15	2.13	2.11	0.60	0.54	0.54	0.67	0.57	0.82	0.76	0.72	0.57	0.39	0.48	0.91	0.93	0.86		2.14	2.38	2.40	1.41	2.14	1.57	1.84	2.47
25	1.42	1.70	1.59	1.42	1.25	2.15	1.60	1.09	1.21	1.10	0.77	1.13	0.89	1.01	0.58	0.77	0.78	0.92	1.00	1.01	0.72	0.89	0.90	0.93		1.69	1.52	0.53	1.46	1.07	0.91	1.28
26	1.92	1.64	1.62	1.40	1.23	1.30	1.82	1.26	1.12	0.98	0.81	0.98	1.10	1.16	0.70	0.77	0.67	0.97	1.01	0.92	0.60	0.74	0.80	0.92	0.18		1.70	0.71	1.65	1.23	1.07	1.46
27	1.56	2.02	2.02	1.61	1.62	1.38	1.89	1.89	2.41	1.30	0.95	1.18	1.25	1.02	0.64	0.83	0.71	0.73	1.10	1.01	0.83	0.93	1.05	0.96	0.53	0.48		2.13	1.73	0.53	0.60	0.23
28	1.92	1.70	1.85	1.34	1.48	1.72	1.66	1.05	1.07	0.94	0.64	0.91	0.81	0.83	0.57	0.80	0.44	0.70	0.82	0.70	0.66	0.82	0.71	0.73	0.39	0.43	0.67		2.09	1.62	1.43	1.88
29	1.85	1.98	1.75	1.70	1.63	1.62	1.99	2.21	2.66	1.13	1.01	1.21	1.24	0.94	0.65	0.74	0.71	0.93	1.13	0.95	0.84	0.92	1.07	1.02	0.49	0.48	0.18	0.59		0.35	0.54	0.17
30	1.68	1.78	2.00	1.77	1.59	1.93	1.89	1.87	2.22	1.08	0.83	1.20	1.13	1.05	0.59	0.82	0.67	0.90	1.03	1.02	0.78	0.96	0.96	0.85	0.46	0.48	0.37	0.62	0.31		0.17	0.54
31	1.85	2.16	2.03	1.89	1.65	1.98	2.04	2.01	2.68	1.12	0.84	1.06	1.20	0.98	0.67	0.83	0.69	0.88	1.15	0.94	0.85	0.99	1.06	1.11	0.52	0.46	0.44	0.61	0.35	0.17		0.35
32	1.99	1.90	1.96	1.80	1.74	1.85	2.15	2.12	2.42	1.15	0.97	1.05	1.24	1.11	0.60	0.79	0.68	0.92	0.85	0.96	0.69	0.98	0.98	0.99	0.49	0.53	0.24	0.56	0.18	0.39	0.32	

Table 4: Percentage sequence diversity within 12 *P.rupestris* populations. The number of specimens sampled at each locality and the corresponding number of haplotypes detected at each site are also presented. The Jukes Cantor correction was made (see text for details).

Population	No. of individuals	No. of haplotypes	% nucleotide diversity	Std. error
Blyderivierspoort N.R.	7	1	0.00	0.00
Verkeerdevlei	2	1	0.00	0.00
Donkerpoort	4	1	0.00	0.00
Golden Gate Highlands	4	4	1.90	0.54
Grahamstown	4	3	1.30	0.40
Kuruman	2	2	0.17	0.17
Lydenburg	7	3	0.26	0.18
Matatiele	3	2	0.10	0.10
Springbok	3	2	0.17	0.17
Victoria-West/Sandgat	2	2	0.56	0.40
Volksrust	5	2	0.10	0.10
Waterval-boven	3	1	0.00	0.00

The mtDNA restriction data was also subjected to computer analysis to determine the number of mutational steps (base substitutions) between the different composite haplotypes. The analysis showed 1-17 mutational steps separate lineages comprising the *P. rupestris* north western clade (clone 1-9), while, in turn, this assemblage was separated from the south eastern clade (clones 10-32) by 45-62 mutational steps (variation dependent on the clones used in the pairwise comparison; Table 5). A phylogeographic parsimony network was constructed using the minimum number of mutational steps between clones (Table 5) and the resulting data overlaid on a geographic map of sample localities (Fig 5). Using this approach a minimum of 45 mutational steps separate the north western clade's clones 4 and 7 from clone 14 in the south eastern assemblage (Table 5).

Table 5: Matrix illustrating the minimum number of mutational steps between the 43 composite haplotypes found within the genus *Pronolagus*. Clones 1-32 = *P. rupestris*; clones 33-43 = *P. randensis*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
2	1																																									
3	2	1																																								
4	5	4	3																																							
5	6	5	4	1																																						
6	4	3	2	1	2																																					
7	3	2	1	2	3	1																																			
8	16	15	12	13	14	12	11																																		
9	17	16	13	14	15	13	12	1																																		
10	51	50	49	48	49	49	48	47	48																																	
11	49	48	47	46	47	47	46	48	49	12																																
12	51	50	49	48	49	49	48	49	50	2	12																															
13	55	54	53	52	53	53	52	52	53	6	16	4																														
14	48	47	46	45	46	46	45	47	48	9	7	7	11																													
15	50	49	50	49	50	50	49	46	47	14	15	12	16	10																												
16	59	58	57	56	57	57	56	53	54	15	19	15	19	22	17																											
17	52	51	52	51	52	52	51	50	51	13	14	11	15	9	3	18																										
18	57	56	55	54	55	55	54	50	51	19	16	19	23	19	19	12	18																									
19	57	56	55	54	55	55	54	51	52	8	12	8	12	15	20	7	19	11																								
20	52	51	50	49	50	50	49	47	48	13	7	13	17	10	18	12	17	9	5																							
21	57	56	55	54	55	55	54	48	49	10	22	10	14	17	10	9	13	15	10	15																						
22	57	56	55	54	55	55	54	51	52	11	23	11	15	18	13	4	14	16	11	16	5																					
23	57	56	55	54	55	55	54	51	52	10	22	10	14	17	14	5	13	15	10	15	6	1																				
24	54	53	52	51	52	52	51	50	51	7	9	5	9	12	15	12	16	14	5	10	13	16	15																			
25	49	48	47	46	47	47	46	46	47	23	13	21	25	16	12	18	11	19	21	16	21	22	23	18																		
26	50	49	48	47	48	48	47	47	48	24	14	22	26	17	13	19	12	20	22	17	22	23	24	19	1																	
27	62	61	60	59	60	60	59	52	53	24	26	24	28	29	20	13	21	19	16	21	14	17	18	19	13	14																
28	51	50	49	48	49	49	48	50	51	18	8	16	20	11	9	15	6	14	16	11	18	19	18	13	5	6	18															
29	61	60	59	58	59	59	58	51	52	23	25	23	27	28	19	12	20	18	15	20	13	16	17	18	12	13	1	17														
30	57	56	55	54	55	55	54	51	52	21	21	19	23	24	15	16	16	22	19	24	17	20	21	14	8	9	5	13	4													
31	56	55	54	53	54	54	53	52	53	20	20	18	22	23	16	15	15	23	18	23	18	19	20	15	7	8	6	12	5	1												
32	60	59	58	57	58	58	57	52	53	22	24	22	26	27	20	11	19	19	14	19	14	15	16	19	11	12	2	16	1	5	4											
33	71	70	65	64	65	65	64	65	66	55	58	57	57	57	59	58	62	61	57	55	57	58	59	58	59	60	61	60	60	60	59	59										
34	70	69	64	63	64	64	63	63	64	56	58	58	58	57	58	59	61	60	58	55	58	59	60	59	59	60	62	60	61	61	60	60	1									
35	70	69	64	63	64	64	63	63	64	58	60	60	60	59	58	61	61	60	60	57	60	61	62	61	59	60	62	60	61	61	60	60	3	2								
36	65	64	65	64	65	65	64	64	62	59	62	61	61	61	59	62	62	61	61	59	61	62	63	62	61	62	63	62	62	62	61	61	5	5	3							
37	68	67	62	61	62	62	61	61	63	57	59	59	59	58	57	60	60	59	59	56	59	60	61	60	58	59	61	59	60	60	59	59	6	5	5	8						
38	66	65	60	59	60	60	59	60	62	54	56	58	58	57	56	61	59	60	60	57	58	59	60	61	59	60	62	60	61	61	60	60	9	8	6	9	9					
39	61	60	61	60	61	61	60	58	60	55	58	59	59	59	57	62	60	61	61	59	59	60	61	62	61	62	63	62	62	62	61	61	11	11	9	6	12	3				
40	71	70	65	64	65	65	64	64	66	59	62	61	61	60	59	63	62	62	62	59	61	62	63	63	61	62	64	62	63	63	62	62	7	6	4	7	5	8	11			
41	70	69	64	63	64	64	63	63	65	58	61	60	60	59	58	62	61	61	61	58	60	61	62	62	60	61	63	61	62	62	61	61	6	5	3	6	6	7	10	1		
42	72	71	66	65	66	66	65	65	66	59	62	61	61	60	59	63	62	62	62	59	61	62	63	63	61	62	64	62	63	63	62	62	5	4	2	5	7	8	11	2	1	
43	72	71	66	65	66	66	65	65	66	58	60	60	60	59	60	61	63	62	60	57	60	61	62	61	61	62	64	62	63	63	62	62	5	4	4	7	7	10	13	4	5	4

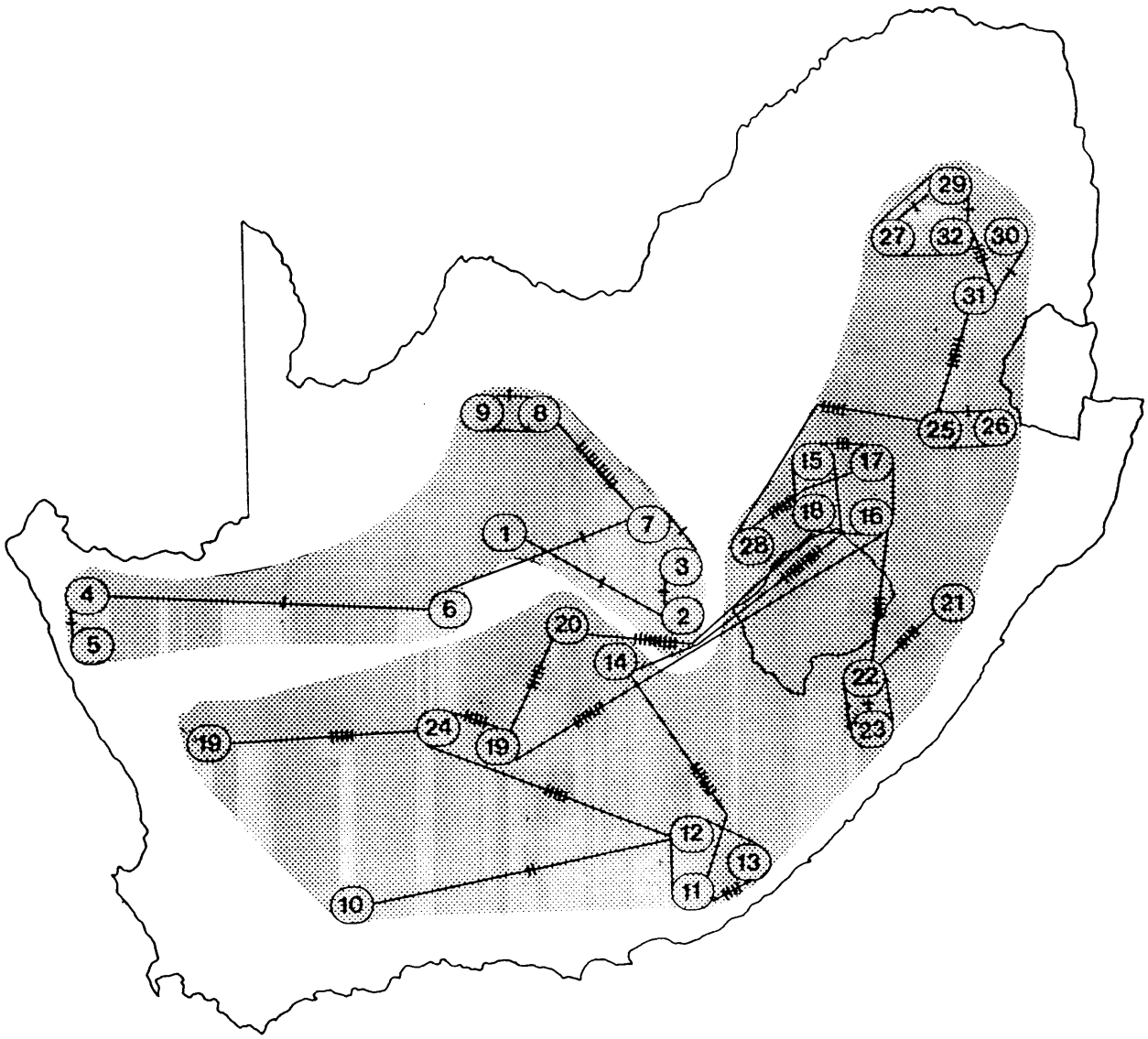

Figure 5: A phylogeographic network based on the minimum number of mutational steps between the 32 maternal lineages found in *P.rupestris*. The number of base substitutions between linked clones are indicated by cross hatching on the connecting branches. Bounded mtDNA clones (eg 27,29 and 32) reflect intrapopulational heterogeneity detected at single sample sites. Shaded areas indicate the geographic limits of the north western and south eastern assemblage as evidenced by this study.

3.3. Pronolagus randensis

Eleven composite haplotypes were found in *P. randensis* (Table 6; Fig 6). A sequence diversity of 0.74% (±0.21%) was estimated for this species, indicating limited intraspecific variation within the taxon, and contrasts markedly with the results documented for *P. rupestris*. Genotypic diversity was calculated at 0.892 indicating the more widespread appearance of shared composite haplotypes within *P. randensis*. Estimated pairwise nucleotide sequence divergences between the different *P. randensis* composite haplotypes ranged from 0.17% (clone 33 vs 34) to 1.63% (clone 39 vs 40; Table 6) and intrapopulational sequence diversity ranged from 0.00% at Middelburg to 0.37% at Rustenburg (Table 7).

A phylogeographic overlay (Fig 6) reflecting the minimum number of mutations between composite mtDNA haplotypes, emphasized the small number of site changes between lineages (Table 5) with only 1 to 5 mutational steps separating geographically isolated clones (Fig 6). Clone 40 is represented in three different localities (AA, BB, V; Fig 3) throughout Transvaal (Fig 6) and may possibly be indicative of an ancestral condition.

Table 6: Percentage sequence divergence between 11 mtDNA clones of *P. randensis*. Jukes-Cantor corrected values are presented above the diagonal and standard errors are below. Values were derived via 200 bootstrap cycles (see text for details).

CLONE	33	34	35	36	37	38	39	40	41	42	43
33		0.17	0.5	0.69	1.08	1.29	1.53	1.26	1.08	0.88	0.61
34	0.14		0.34	0.63	0.89	1.11	1.46	1.09	0.89	0.71	0.45
35	0.22	0.21		0.3	0.76	0.83	1.19	0.68	0.51	0.34	0.57
36	0.29	0.26	0.23		1.11	1.19	0.84	1.04	0.85	0.63	0.86
37	0.38	0.4	0.42	0.33		1.24	1.57	0.52	0.72	0.89	0.74
38	0.53	0.53	0.54	0.52	0.68		0.31	1.31	1.09	1.26	1.37
39	0.54	0.49	0.41	0.53	0.52	0.23		1.63	1.41	1.62	1.73
40	0.44	0.54	0.37	0.31	0.38	0.65	0.57		0.17	0.33	0.67
41	0.4	0.48	0.29	0.28	0.4	0.64	0.58	0.18		0.17	0.88
42	0.42	0.47	0.23	0.26	0.46	0.65	0.53	0.31	0.22		0.69
43	0.35	0.32	0.31	0.35	0.39	0.73	0.59	0.37	0.44	0.31	

Table 7: Percentage sequence diversity within six *P. randensis* populations where more than one specimen was sampled. The Jukes Cantor correction was made (see text for details).

Population	No. of individuals	No. of haplotypes	% nucleotide diversity	Std. error
Blyderivierspoort	5	2	0.17	0.17
Heidelberg	5	2	0.12	0.01
Kruger N.P.	2	1	0.00	0.00
Middelburg	4	1	0.00	0.00
Rustenburg	6	4	0.37	0.15
Vredefort	3	1	0.00	0.00

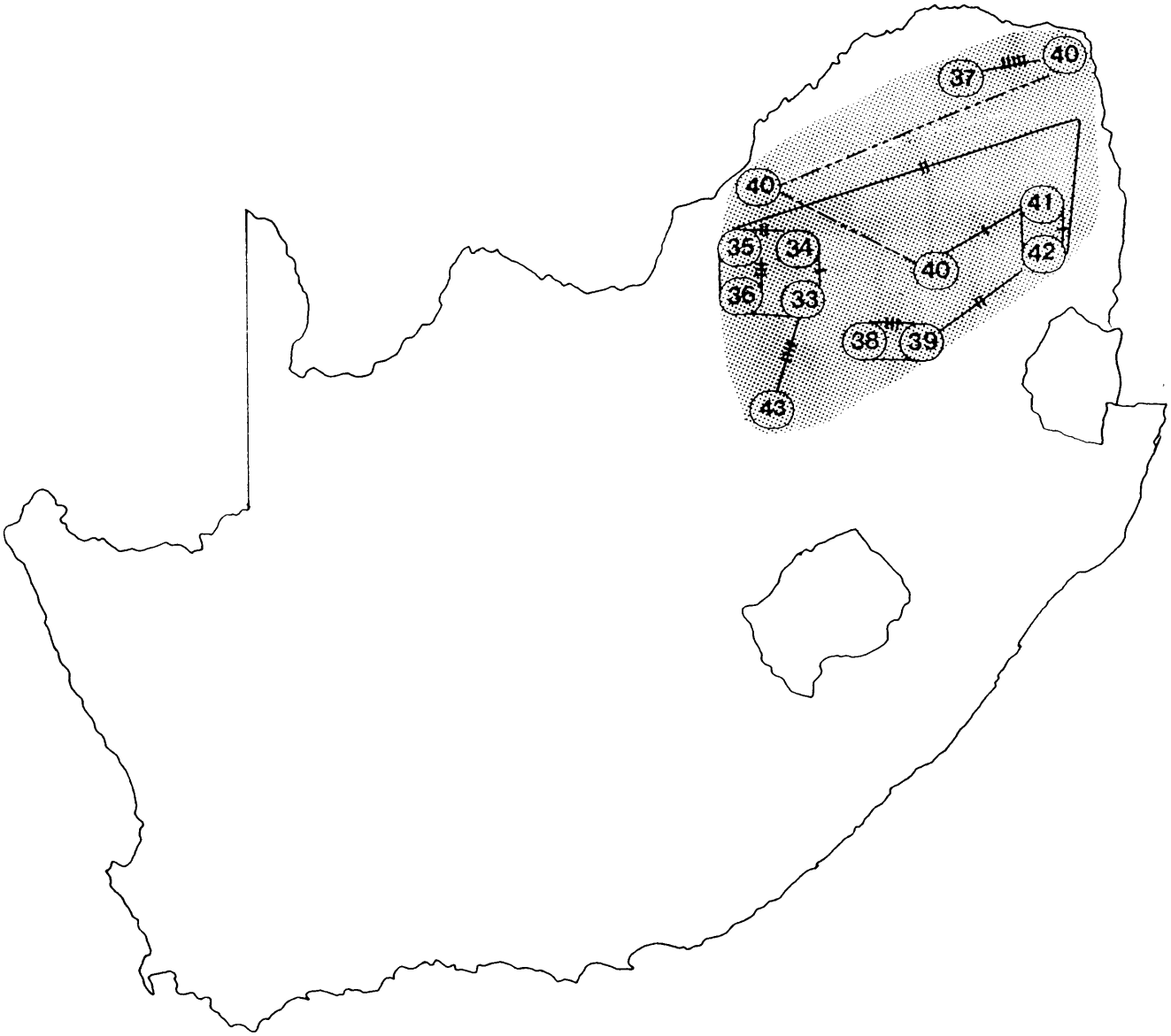

Figure 6: Phylogeographic network based on the minimum number of mutational steps between the eleven composite haplotypes found in *P.randensis*. Clone numbers refer to those given in Table 5. Bounded mtDNA clones (eg 33,34,35 and 36) reflect intra-populational heterogeneity detected at single sample sites.

3.4. Interspecific comparison

Pronolagus rupestris and *P. randensis* are separated by a sequence divergence of 9.30% (±1.70%), implying that they last shared a common female ancestor approximately 4.65 million years ago. Sequence diversities were estimated at 0.74% (±0.21%) and 3.11% (±0.30%) respectively within *P. randensis* and *P. rupestris*. Of the two *P. rupestris* clades detected in this investigation, the south eastern assemblage clearly has a more recent association with *P. randensis* than its north western counterpart, the former separated by a sequence divergence of 9.18% (±1.76%) and the latter by 15.73% (±3.56%).

Mitochondrial DNA relatedness among all 43 *Pronolagus* lineages (*P. rupestris* and *P. randensis*) are depicted in Figures 7 and 8. Tree topologies generated by both the UPGMA and neighbor-joining analyses, using Jukes Cantor corrected sequence divergence values, clearly show that conspecific populations cluster together reflecting closer similarities in haplotypes.

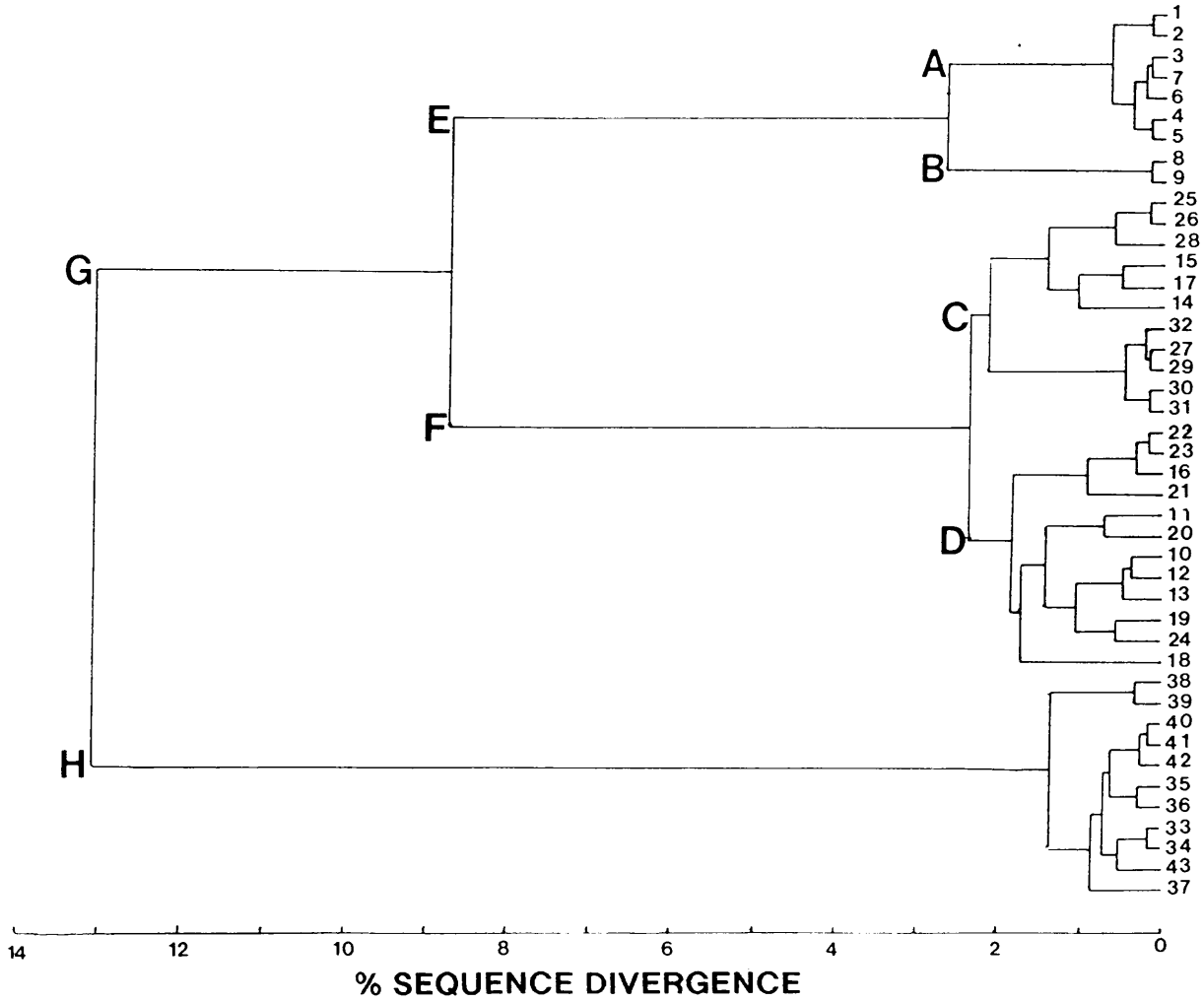

Figure 7: UPGMA tree based on mtDNA sequence divergence between 43 mtDNA lineages found within *P. rupestris* (clones 1-32) and *P. randensis* (clones 33-43). Clone numbers correspond to those in Table 2 and Appendix II.

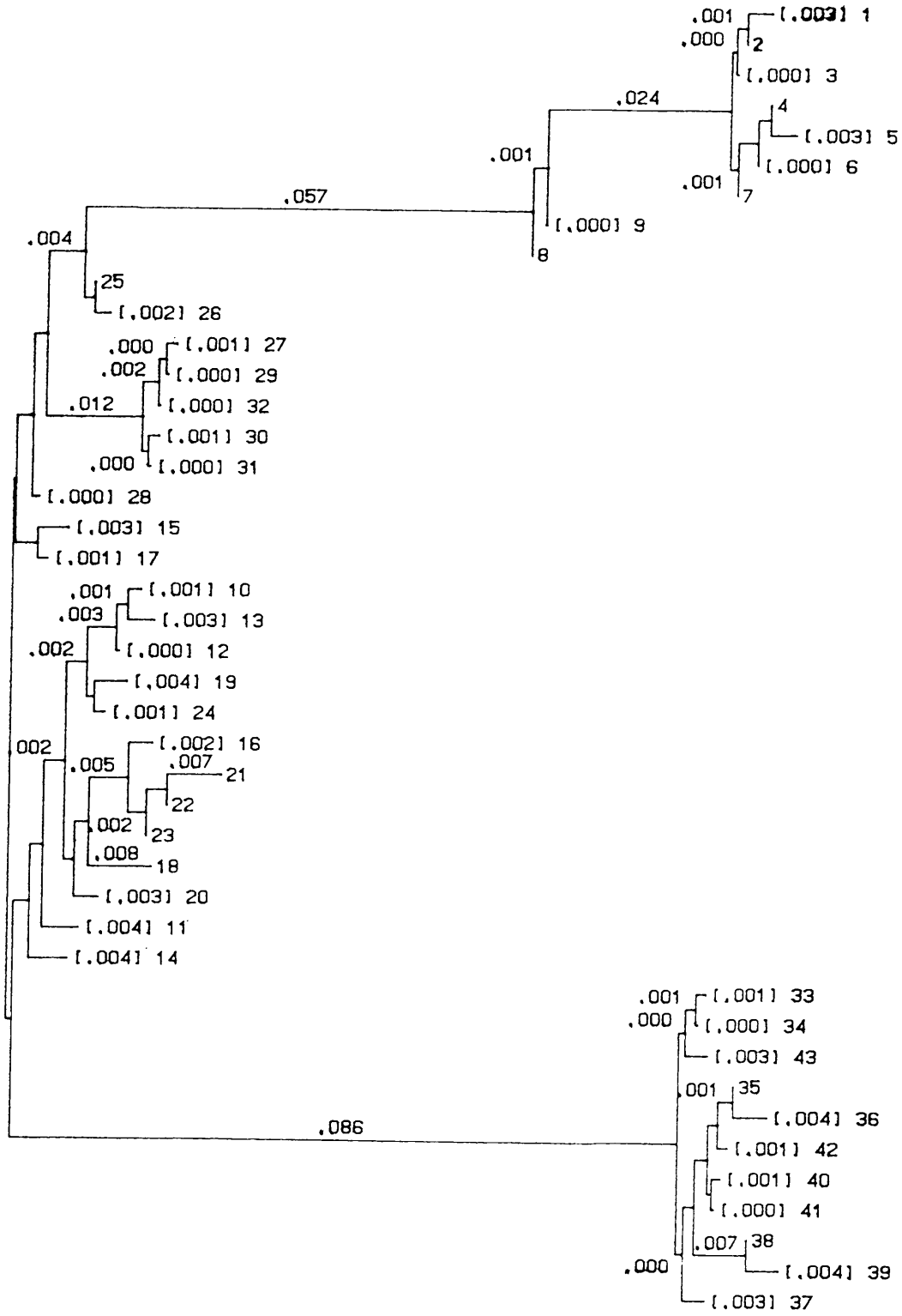

Figure 8: Neighbor-Joining tree based on mtDNA sequence divergences between composite haplotypes of *P. rupestris* and *P. randensis*. Clone numbers correspond to those in Table 2 and Appendix II. The figures in parenthesis over each branch indicate patristic distance.

CHAPTER 4

Discussion

4.1. Correspondence between subspecific taxonomy and the mtDNA structure in *Pronolagus*

Lidicker (1962) defined a subspecies as a relatively homogeneous, and genetically distinct portion of a species which represents a separately evolving, or recently evolved, lineage with its own evolutionary tendencies, inhabits a definite geographical area, is usually at least partially isolated, and may integrate gradually, although over a fairly narrow zone, with adjacent subspecies. Conventionally, subspecies descriptions have been based on one or a few traits (such as pelage color or size) that allow distinction of a high percentage of individuals in a geographic region from those in other areas (Mayer 1969). Criticism of this approach is that it delimits races on the basis of one, or several, of the most obvious characters, and the remainder of the geographical variable parameters are ignored, or if they are considered at all, they are analyzed only in terms of the subspecific units previously described (Avise & Ball 1990).

Based on the Meester *et al.* (1986) treatment of *P. rupestris*, seven subspecies are recognized: *P. r. rupestris* from the Upington district, *P. r. melanurus* from the north western Cape Province, *P. r. curryi* from the western Orange Free State, *P. r. saundersiae* from the Albany district, *P. r. australis* from the south western Cape Province, *P. r.*

fitzsimonsi from the fringes of the escarpment in northern Namibia and *P. r. barretti* from the Ladysmith district in Natal northwards to the south eastern Transvaal (Meester *et al.* 1986). The borders of the subspecies distributions are not well defined but, in broad terms, *P. r. rupestris, P. r. melanurus*, *P. r. curryi*, *P. r. fitzsimonsi* appear to fall within the north western clade, while *P. r. barretti, P. r. australis* and *P. r. saundersiae* cluster within the south eastern clade. At a finer level, however, little congruence exists between the mtDNA data and the seven described subspecies although, clearly, mtDNA lineages might be disconcordant with biological subspecies boundaries owing simply to demographically influenced stochastic patterns of mtDNA lineage survivorship (Avise 1986).

Meester *et al.* (1986) speculate that only two subspecies exist within *P. randensis*. Nevertheless, nine are generally recognized (Meester *et al.* 1986), four of which occur within South Africa: *P. r. randensis* from the southern and south western Transvaal, *P. r. powelli* from the western Transvaal and eastern Botswana, *P. r. makapani* from the Pietersburg district, *P. r. capricornis* from the top of the Soutpansberg. The phylogeographic structure within this species does not correspond to the current subspecies designation and therefore might be more supportive of Meester's *et al.* (1986) hypothesis that there are, in fact, fewer subspecies within *P. randensis* than have previously been recognized.

4.2. Mitochondrial DNA differentiation in *Pronolagus*

4.2.1. General patterns

At the species level there was clear evidence for the recognition of *P. rupestris* and *P. randensis* as two discrete taxa. Additionally, *P. rupestris* showed pronounced intraspecific genetic structure with two well defined, virtually geographically contiguous clades; one situated in the north western Cape Province while the other follows the Great Escarpment extending along the southern and eastern seaboard of South Africa (Figs 5 & 10). Molecular clock calibrations based on mtDNA sequence divergences indicate that *P. rupestris* and *P. randensis* last shared a common female ancestor approximately 4.7 million yrs BP, while the two mtDNA assemblages detected within *P. rupestris* are distinguished by approximately 4 myrs of separation. This phylogeographic profile corresponds to the Avise *et al.* (1987) category I which is defined as reflecting a phylogenetic discontinuity with spatial separation.

The phylogeographic structure of contemporary populations is determined both by current ecology and by historical patterns of vicariance and dispersal (Hayes & Harrison 1992). In this study most mtDNA types were restricted to single localities implying the absence of female exchanges between populations, a situation similar to the European feral rabbit, *Oryctolagus* (Biju-Duval *et al.* 1991), woodrats, *Neotoma* (Hayes & Harrison 1992), meadow voles, *Microtus* (Plante, Boag & White 1987) and deer mice, *Peromyscus* (Lansman *et al.* 1983), all small mammalian species with limited dispersal capabilities. Furthermore, the present investigation also revealed a large number of different mtDNA female lineages, as evidenced by relatively high genotypic diversity

values: 0.974% for *P. rupestris* and 0.892 for *P. randensis*. However, in this respect it should be cautioned that although the genotypic diversities indicated for the two clades within *P. rupestris* may be construed as indicating that the north western clade's populations (0.958) are more divergent than the south eastern clade (0.866) (which appears to be characterized by more gene flow between populations and as a result more individuals share the same haplotype) this does not necessarily hold. The genotypic diversity calculations are based solely on differences in the frequency of lineages, and the degree of relatedness between clones is ignored in the analysis. Therefore, two clones differing by one site change can carry the same weight as two clones which differ by ten. Secondly, the number of specimens sampled at each locality is important as is clearly indicated where three out of the six localities within the north western clade are represented by only one specimen, while three or more specimens were sampled at eight of the 15 localities within the south eastern assemblage. Given the sensitivity of the calculations to these conditions, a more equitable sampling between assemblages (especially larger population sizes for the north western clade's localities) may have resulted in more rigorous assessments of genotypic diversity.

4.2.2. Phylogeographic mtDNA structure in *Pronolagus randensis*

No pronounced intraspecific genetic discontinuities were observed in the survey of *P. randensis* but since only a small portion of the entire distributional range was studied, this may change profoundly following a more extensive survey. The limited genetic structure within *P. randensis* is similarly reflected by the relatively low intraspecific mitochondrial DNA sequence diversity estimate (0.74%). Sequence divergences between clones ranged from 0.17% to 1.57% (Table 6). Five localities are character-

ized by unique maternal lineages (or lineage), while clone 40 was detected at three different localities (Fig 6) and might therefore represent the ancestral mtDNA genotype for this region. Quite strikingly, however, four maternal lineages were detected in the six sample specimens from Rustenburg, a finding which may indicate a relatively old, historically large founder population or, conversely, may simply reflect recent colonization events from elsewhere.

The relatively low number of mutational steps separating the *P. randensis* mtDNA haplotypes (Fig 6) and their close phenetic clustering using the UPGMA (Fig 7) and Neighbor-Joining algorithm (Fig 8) reflects fairly close common female ancestry, possibly due to recent range expansion. Bearing in mind that the geographic genetic structure reflected in *P. randensis* might change considerably through the inclusion of specimens from other portions of its range, the data at hand show that *P. randensis* can be classified as falling into the Avise *et al.* (1987) category V: phylogenetic continuity with partial spatial separation. This type of genetic structure has been reported in taxa with historically intermediate levels of gene flow between geographic populations (Avise *et al.* 1987).

In South Africa *P. randensis* occurs predominantly in the Soutpansberg-Magaliesberg mountain ranges and extends its range to the extreme northern fringes of the Great Escarpment, while the *P. rupestris* south eastern clade occupies the rest of the Great Escarpment (Fig 9). In the eastern Transvaal at Blyderivierspoort Nature Reserve, these two species are found 15 kilometers apart. No distributional overlap between them was detected even though extensive geographic sampling was undertaken in the presumed contact zone, a factor thought to be indicative of competitive exclusion.

Some ecological differences were noted in this contact zone and field observations in this region showed that *P. rupestris* tends to be found at higher altitudes, usually on mountain plateaus which are characterized by less abundant shelter, while *P. randensis* is found on mountain slopes where there is substantial shelter in the form of jumbled boulders and rock crevices. Likewise, *P. randensis* tends to seek refuge in drier habitats (715 mm pa) while *P. rupestris* occurs in wetter areas (1100 mm pa). Although Acocks (1988) does not indicate any vegetational differences between the two localities (possibly reflecting the map's lack of detail), differences in vegetation are to be anticipated in view of the discrepancies in rainfall.

Particularly striking was the degree of congruence reflected in the zone of contact between *P. rupestris* and *P. randensis* (Fig 9A) and the presence, in this region, of two discrete mtDNA clades within the rock hyrax *P. capensis* (Fig 9B), a species of comparable body size and habitat specificity and limited vagility (Skinner & Smithers 1991; Prinsloo & Robinson 1992). These authors suggest that the geographic distribution of the rock hyrax clades (which are separated by almost 4% sequence divergence) reflects historic dispersal along two separate mountain routes (south central clade along the Great Escarpment; northern clade primarily along the Soutpansberg-Magaliesberg axis). Whether the correspondence in distribution patterns between *P. rupestris* and *P. randensis* and the two genetically distinct assemblages detected within *P. capensis* (thought to represent cryptic hyrax species; Prinsloo & Robinson 1992) is merely fortuitous, is subject to speculation. However, Avise (1992) has argued that the presence of concordant phylogeographic patterns in evolutionary independent lineages might reflect similar vicariant events shaping evolutionary processes in phylogenetically unrelated taxa. This hypothesis may hold not only for the species surveyed in this investigation but also for many other mammals with similar habitat and dispersal capabilities, such

as the rock elephant shrew, *Elephantulus myurus*.

Sequence divergence estimates based on the mtDNA data indicate that the events responsible for the divergences within the red rock rabbit and the rock hyrax assemblages occurred, respectively at different times in the evolutionary past (*P. rupestris* vs *P. randensis* = 4 myr c.f. the two clades in *P. capensis* = 2 myr). During the last 50 million yrs global temperature changes of considerable magnitude have occurred and have had the effect of breaking up the African vegetation into a series of discontinuous habitats (Brain 1985). Between 6.5 - 5 million yrs ago a severe temperature plunge was recorded, the terminal Miocene event, resulting in a world-wide rapid and dramatic sea-level drop of over 100 m. Subsequently temperatures in the succeeding Pliocene period appear to have fluctuated, a situation which continued up to approximately 2.6-2.5 million yrs BP. Since then temperatures have oscillated at least 17 times (Brian 1985). Consequently, it is not unlikely that the oscillating temperatures and the concomitant effect on habitat may have prompted speciation, or incipient speciation, at different times in the evolutionary past.

Conversely, it may be that the discrepancies in the divergence times referred to above are, in reality, due to differences in molecular clock calibrations for the two evolutionary lineages (Lagomorpha and Hyracoidae) and therefore the application of the conventional 2% sequence divergence per million years to both tends to obscure true temporal associations between them. Some support for this argument exists since Grillitsch *et al.* (1992) have found a temporal acceleration in lagomorph nuclear DNA mutation rates. Should this hold, it may be that the same vicariant event(s) in the eastern Transvaal was indeed responsible for the phylogeographic structures observed in the two independent lineages, an intriguing possibility that needs further investigation.

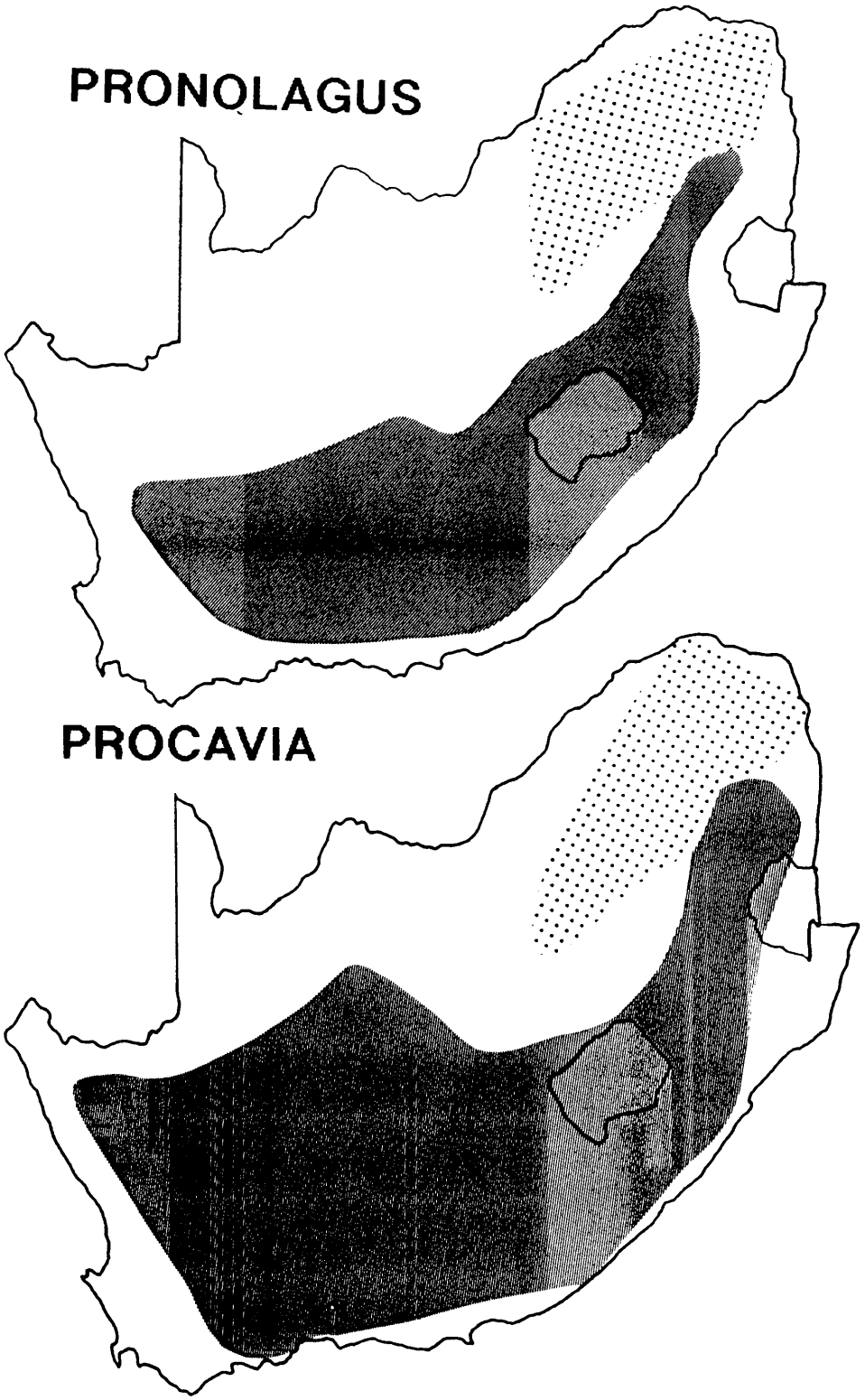

Figure 9: (A) Geographic delineation of the south eastern mtDNA clade in *Pronolagus rupestris* and its contact zone with *P. randensis* in the eastern Transvaal. (B) The geographic extend of the two mtDNA clades in the rock hyrax *Procavia capensis* (with modification from Prinsloo & Robinson 1992).

4.2.3. Phylogeographic mtDNA structure in *Pronolagus rupestris*

Pronolagus rupestris is characterized by deep divisions in the intraspecific gene tree. The mtDNA sequence divergence (7.94%) calculated between the south eastern and north western clades is amongst the highest recorded in the literature, and is interpreted as probably reflecting inaccuracies in the currently accepted taxonomy of this species. In support of this, a survey by Avise *et al.* (1987) documenting intraspecific sequence divergences for 15 vertebrates, yielded values ranging from 0.1% in the American eel, *Anguilla rostrata*, a species with no geographic structuring to 4.0% in the deer mouse, *Peromyscus maniculatus*, which has a pronounced genetic structure, similar to *P. rupestris*. Clearly the 7.94% sequence divergence detected between the two *P. rupestris* clades gives cause for thought. In this respect it is striking that the sequence divergence separating the two *P. rupestris* clades approximates that distinguishing the two well delineated cogenors, *P. rupestris* and *P. randensis* (9.30%).

The phylogeographic profiles of the two *P. rupestris* clades differ markedly from each other. The north western clades' matrilines are generally separated by few mutational steps (even with respect to those that are geographically distant; Fig 5). In contrast, the south eastern clade is characterized by deep divisions between populations as is reflected by the large number of mutational steps linking closely allied haplotypes (Fig 5).

***Pronolagus rupestris* north western clade:** The north western *P. rupestris* assemblage comprises two discrete subclusters which are reflected in both the UPGMA and Neighbor-Joining tree topologies (Figs 7 and 8) as well as by the relatively high number of mutational steps (11) separating them (Fig 5). The sequence divergence estimates for seven of the lineages (clones 1-7; Table 3) are low and are thought to reflect recent

common female ancestry due to colonization from a source population in the recent evolutionary past. The specimens comprising the one subcluster were all sampled at Kuruman (clones 8 and 9) and differ from the haplotypes constituting the other north western subcluster (clones 1 to 7) by a mean sequence divergence of 2.66% indicating at least one million years of separation between them and the other specimens in this assemblage (Table 3). Although the reasons for the marked difference in sequence divergence are not immediately apparent, it probably reflects the greater age and degree of isolation of the Kuruman population.

Interestingly, the specimens from the Kuruman district (representing the geographically most northern collection locality in this clade; Fig 3) show a greater phylogenetic affinity to the clones comprising the south eastern assemblage than to other representatives of the north western cluster, a relationship clearly indicated by the Neighbor-Joining tree (Fig 8). Based on the magnitudes of sequence divergence estimates, it would seem that the Kuruman population was, from an evolutionary perspective, in contact with the south eastern assemblage for at least one million years longer than the other seven lineages found within the north western assemblage (Table 3). This more recent contact is further underscored by the fact that several Kuruman restriction profiles are shared with lineages in the south eastern assemblage, an observation in marked contrast to that illustrated by other north western clones where nearly all restriction profiles were clade specific (Table 2). It is interesting to speculate on the divergence of the two mtDNA clades and whether the past colonization of the Kuruman district was from the present day Volksrust area of the far south eastern Transvaal. If this were so, the data seem to suggest that the Kuruman population represented the focus of the dispersal of specimens which "founded" the extant north western clade. It is also evident, given the low sequence divergences separating the Kuruman lineages from all other north west-

ern haplotypes, that subsequent range expansion must have occurred relatively recently.

***Pronolagus rupestris* south eastern assemblage:** The south eastern assemblage, in contrast to the north western clade, is characterized by high sequence divergences between the sampled mtDNA lineages (Table 3) which suggest that the constituent populations have been isolated from each other for long periods, thus allowing for the accumulation of more site changes. Sequence diversity estimates within populations were invariably low, the only exceptions being Grahamstown (1.9%) and Golden Gate National Park (1.3%) which were characterized by three and four lineages respectively. Noteworthy was the fact that, in several instances, a closer affinity was shown to haplotypes from other localities rather than to those from the same locality (for example 10 and 12; Fig 5). The presence of several discrete lineages within populations can, however, also reflect evolutionarily old populations which may have served as core regions from where colonization into the adjacent areas may have occurred. Most other populations sampled within the south eastern clade were distinguished by single, or two closely related, lineages. These were usually separated by fairly substantial sequence divergences from other populations, and it is likely that, in contrast to the north western clade, the south eastern assemblage has not been subject to recent bottlenecks and large-scale range expansions. In essence, given the strong geographic mtDNA partitioning between different populations one can infer that historic gene flow between populations has been limited.

Generally, nucleotide divergences increase with increasing geographic distance between sample points (Avise, Giblin-Davidson, Laerm, Patton & Lansman 1979; Tegelström 1987). In this study, however, geographic distance *per se* does not contribute

significantly to the genetic structuring within the *P. rupestris* south eastern clade. This is clearly illustrated by the parsimony analysis, where animals from Ladismith (clone 10) differ by only two restriction site changes from specimens collected at Grahamstown (clone 12), localities which are separated by nearly 500 km (Fig 5). On the other hand, specimens drawn from Howick (clone 21) and Matatiele (clone 22) differ by five site changes although, in this instance, the two localities are only 150 km distant (Fig 5). Strikingly, both areas fall within a continuum of mountains which form part of the Great Escarpment. Therefore, it seems more likely that the availability of suitable habitat, which appears disjunct within the Great Escarpment (Fig 10), may be of greater significance in determining the genetic structure within the *P rupestris* south eastern assemblage.

Possible geographic influences on the genetic structure of *P. rupestris*: At the outset of this investigation it was hypothesized that extensive plains which lack suitable habitat (for example the Knersvlakte and Orange Free State plains; Fig 10) may have served as barriers to dispersal since the *Pronolagus* species are presumed to be highly vulnerable when leaving the protection of their rocky refuges. Results from this study are, however, in conflict with this thesis since populations from the north western *P. rupestris* which straddle extensive plains (for example the Bushmanland plain separating Springbok and Prieska; Fig 3), show little genetic divergence between them.

In an attempt to further identify possible extrinsic factors which may have disrupted gene flow and contributed to the intraspecific genetic structure within *P. rupestris*, representative localities in the contact zones of the two genetic clades were included in a geographic analysis (G.I.S) of rainfall, altitude and vegetation. The analysis centered on Nieuwoudtville and Springbok (Fig 3), two localities which span the "Knersvlakte"

(Fig 10) and three geographically closely allied populations which define the south eastern (Verkeerdevlei and Donkerpoort; Fig 3) and north western (Bloemfontein; Fig 3) clades respectively. From the G.I.S. analysis it was evident that all localities have similar elevations and mean annual rainfalls (Fig 11).

Likewise, the G.I.S. analysis showed Nieuwoudtville and Springbok to have similar vegetation types with both being described as Karoo-type vegetation by Acocks (1988). Although Donkerpoort has a false upper karoo veld type, the similarity in vegetation between the Verkeerdevlei and Bloemfontein populations (*Cymbopogon-Themeda* veld-type; Acocks 1988), suggests that vegetation does not serve as an effective barrier to dispersal between populations comprising the two clades. Consequently, although it was not possible to implicate selected environmental factors (and the effects that these may have on generating differences in habitat) in the maintenance of the genetic integrity of the two *P. rupestris* clades, this clearly does not mean that these do not exist. It is quite possible that the choice of parameters was poor and/or the interaction between them too complex to readily detect.

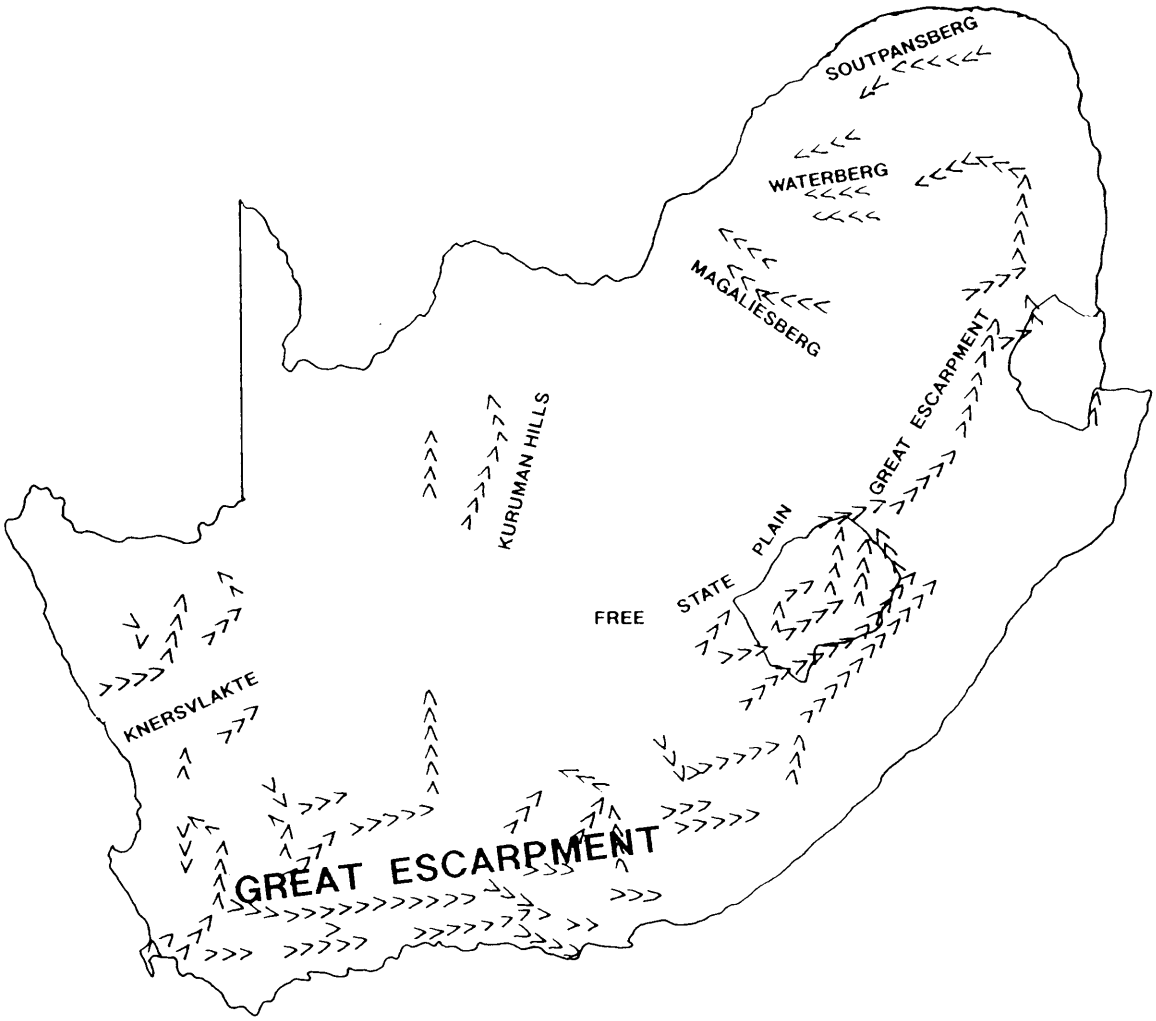

Figure 10: Mountain ranges of South Africa (redrawn from D. Bristow 1985, *Mountains of Southern Africa*. C. Struik, Cape Town) showing the major plains separating the Great Escarpment from the other isolated mountains.

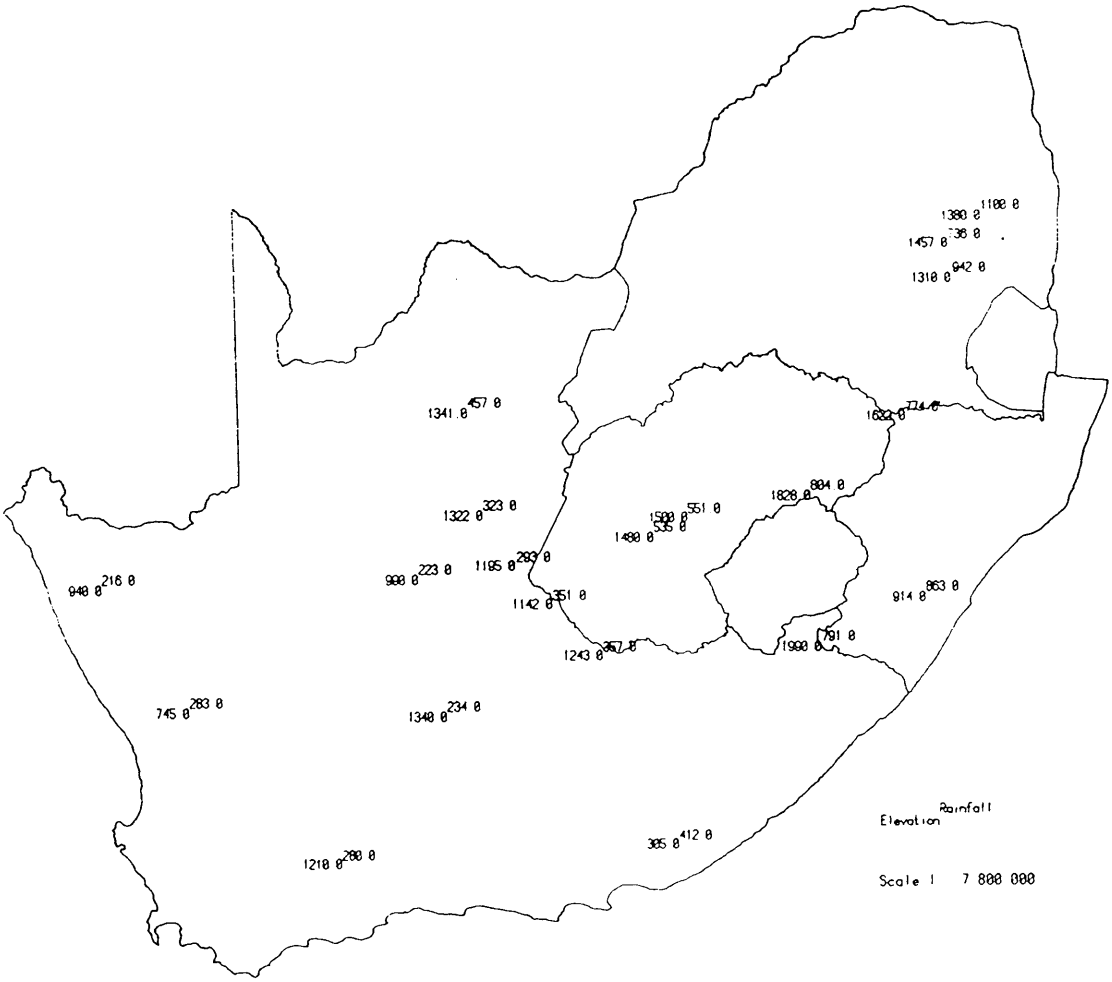

Figure 11: Elevation and annual rainfall of *P. rupestris* collection localities. Refer to Fig. 3 for locality description.

In the absence of disruptive extrinsic factors, however, it can be argued that the clade specificity of the mtDNA haplotypes, in fact, reflects an absence of gene flow, ascribable not to zoogeographic barriers, but to competitive habitat exclusion (Begon & Mortimer 1986) and the inability of the representatives of the two clades to interbreed. Since no obvious morphological differences are apparent between the specimens of the north western and south eastern clades it can be argued that they may, in fact, represent cryptic species. Mayr (1970) stated that when species designations are based merely on morphological differences this overlooks the strictly secondary role morphology plays. Quite clearly, the primary criterion of species rank rest on reproductive isolation.

In conclusion therefore, although the two clades within *P. rupestris* cannot be delineated on obvious morphological differences, the mtDNA data indicate that the two have a strictly parapatric distribution with no overlap in mtDNA haplotypes across their zone of contact (Fig 5). This tends to support the hypothesis that the constituent populations are reproductively isolated from each other and points to the existence of two hitherto undetected biological species, in what has conventionally been recognized as a single taxon, *P. rupestris*.

SUMMARY

Phylogeographic mitochondrial DNA (mtDNA) population structure was determined for *Pronolagus rupestris* and *P. randensis*, two species of red rock rabbit occurring in South Africa. The study revealed that *P. randensis* mtDNA patterns reflect phylogenetic continuity with partial spatial separation between populations, although this should be viewed with caution given the limited distributional range covered by the investigation. Eleven composite haplotypes were found in the 27 *P. randensis* specimens sampled. The relatively low sequence diversity of 0.74% (±0.21%) was interpreted as reflecting limited intraspecific variation within the taxon, and is thought to be indicative of fairly recent range expansion.

In the eastern Transvaal (Blyderivierspoort Nature Reserve) *P. rupestris* and *P. randensis* are found approximately 15 kilometers apart. No distributional overlap was detected even though extensive geographic sampling was undertaken in the presumed contact zone, a pattern which is thought to reflect competitive exclusion. An interesting observation was that previously documented mtDNA phylogeographic patterns recorded in the rock hyrax, *Procavia capensis*, coincide with the observed zone of contact between *P. rupestris* and *P. randensis*. The rock hyrax is a mammalian species of comparable body size which occurs sympatrically with *Pronolagus* and is similarly limited to outcrops of rock in the form of krantzes, rocky koppies or rocky hillsides. It is argued that similar vicariant events may have been implicated in shaping the evolutionary processes in these independent lineages. This hypothesis may hold for not only the taxa surveyed in this investigation, but for other mammal species with similar habitat and dispersal capabilities.

The 55 *P. rupestris* specimens sampled showed pronounced intraspecific genetic structure with two well defined, virtually geographically contiguous clades; one situated in the north western Cape Province while the other follows the Great Escarpment extending along the southern and eastern seaboard of South Africa. These mitochondrially distinct clades were separated by 7.94% (±1.40%) sequence divergence, a relatively high value which is thought to reflect inaccuracies in the currently accepted taxonomy of *P. rupestris*. The phylogeographic profiles of the two *P. rupestris* clades differ markedly from each other. The north western clades' matrilines are separated by few mutational steps between isolated populations, while those of the south eastern clade are characterized by deep divisions. Disruptive extrinsic factors between the two assemblages could not be identified and the clade specificity of the mtDNA haplotypes is thought to be due to competitive habitat exclusion and possibly the inability of the representatives of the two clades to interbreed.

OPSOMMING

Die filogeografiese mitochondriale DNA (mtDNA) bevolkingstruktuur is bepaal vir *Pronolagus rupestris* en *P. randensis*, twee rooi klipkonynspesies wat in Suid Afrika voorkom. Die studie het aangetoon dat *P. randensis* se mtDNA struktuur 'n filogenetiese kontinuum illustreer met gedeeltelike ruimtelike skeiding tussen bevolkings. Dit moet egter met versigtigheid gehanteer word, aangesien 'n beperkte verspreidingsgebied deur die studie gedek is. Elf saamgestelde haplotipes is gevind in die 27 *P. randensis* eksemplare wat ingesamel is. Die relatiewe lae nukleotied-opeenvolging-diversiteit van 0.74% (±0.21%) is geinterpreteer as 'n aanduiding van 'n beperkte intraspesifieke variasie binne die takson en dit is die vermoede dat 'n redelike onlangse gebiedsuitbreiding plaasgevind het.

In die Oos Transvaal (Blyderivierspoort natuurreservaat) is *P. rupestris* en *P. randensis* ongeveer 15 kilometer vanaf mekaar versamel. Geen oorvleueling is gevind nie ten spyte van intensiewe geografiese monsterneming in die kontaksone, 'n patroon wat vermoedelik toegeskryf kan word aan die kompeterende uitsluiting beginsel. 'n Interessante waarneming was dat mtDNA filogeografiese strukture wat opgeteken is vir die klipdassie, *Procavia capensis* ooreenstem met die waargenome kontaksone tussen *P. rupestris* en *P. randensis*. Die klipdassie is 'n klein soogdier spesie met vergelykbare liggaamsgrootte wat simpatries met *Pronolagus* voorkom asook op soortgelyke wyse beperk is tot bergagtige gebiede, in die vorm van kranse, klipperige koppies en klipperige hellings. Die voorgelegde argument is dat 'n soortgelyke vikariante gebeurtenis dalk verantwoordelik was vir die vorming van die evolusionêre prosesse in hierdie twee onafhanklike lyne. Die hipotese mag nie net gebeurtenisse in hierdie twee taksons

verklaar nie maar kan moontlik geëkstrapoleer word na ander soogdierspesies met soortgelyke habitat en verspreidingsvermoë.

Die 55 *P. rupestris* eksemplare wat ingesamel is het duidelike intraspesifieke genetiese struktuur vertoon met twee goed gedefinieërde, feitlik geografies aangrensende groeperings; een geleë in die noord westelike Kaapprovinsie terwyl die ander een hoofsaaklik in die Groot Eskarpement geleë is, al langs die suidelike en oostelike kuslyn van Suid Afrika. Hierdie mitochondriaal bepaalde groeperings word verdeel deur 'n nukleotied-opeenvolging-diversiteit van 7.94% (±1.40%); 'n relatiewe hoë waarde wat dalk aandui dat die aanvaarde taksonomie van *P. rupestris* nie korrek is nie. Die filogeografiese profiele van die twee *P. rupestris* groeperings verskil duidelik van mekaar. Die noord westelike groepering se moederlyne word geskei deur slegs 'n paar mutasie stappe tussen die geisoleerde bevolkings terwyl die van die suid oostelike groepering gekenmerk word deur diep skeidings. Ontwrigtende ekstrinsieke faktore tussen die twee groeperings kon nie geidentifiseer word nie en die groeperings spesifisiteit van die mtDNA haplotipes mag dalk wees as gevolg van kompeterende habitat uitsluiting asook die moontlike onvermoë van die verteenwoordigers van die twee groeperings om te teel.

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APPENDIX I

PRONOLAGUS **COLLECTION RECORD - Specimens deposited in the mammal collection of the Transvaal Museum.** ***(P. rup.*** **=** ***Pronolagus rupestris: P. ran.*** **=** ***Pronolagus randensis*****; Nw = north western mtDNA clade: Se = south eastern mtDNA clade)**

Specimen number	Species	Clade affiliation	Accession number	Material type
01	*P.rup.*	Nw	--------	-----
02	*P.rup.*	Nw	--------	-----
03	*P.rup.*	Nw	--------	-----
04	*P.ran.*	--	TM 42115	Skull
05	*P.ran.*	--	TM 42101	Skull
06	*P.ran.*	--	TM 42184	Skull
07	*P.ran.*	--	TM 42099	Skull
08	*P.ran.*	--	TM 42098	Skull
09	*P.ran.*	--	TM 42100	Skull
10	*P.rup.*	Nw	TM 42103	Skull, skin
11	*P.rup.*	Nw	TM 42102	Skull, skin
12	*P.rup.*	Se	TM 42104	Skull, skin
13	*P.rup.*	Se	TM 42107	Skull, skin

Continued/

Specimen number	Species	Clade affiliation	Accession number	Material type
14	*P.rup.*	Se	TM 42108	Skull
15	*P.rup.*	Se	TM 42114	Skull
16	*P.rup.*	Se	TM 42105	Skull, skin
17	*P.rup.*	Se	TM 42106	Skull, skin
18	*P.rup.*	Se	TM 42110	Skull, skin
19	*P.rup.*	Se	TM 42111	Skull, skin
20	*P.rup.*	Se	TM 42112	Skull, skin
21	*P.rup.*	Nw	--------	-----
22	*P.rup.*	Nw	TM 42113	Skull, skin
23	*P.rup.*	Nw	--------	-----
24	*P.rup.*	Se	TM 42183	Skull, skin
25	*P.ran.*	--	TM 42252	Skull, skin
26	*P.cra.*	--	TM 42355	Skull
27	*P.cra.*	--	TM 42354	Skull
28	*P.ran.*	--	TM 42374	Skull, skin

Continued/

Specimen number	Species	Clade affiliation	Accession number	Material type
29	*P.ran.*	--	TM 42375	Skull
30	*P.ran.*	--	TM 42376	Skull
31	*P.ran.*	--	TM 42377	Skull, skin
32	*P.ran.*	--	TM 42378	Skull, skin
33	*P.rup.*	Se	TM 42379	Skull, skin
34	*P.rup.*	Se	TM 42380	Skull
35	*P.rup.*	Se	TM 42381	Skull, skin
36	*P.rup.*	Se	TM 42397	Skull
37	*P.cra.*	--	TM 42487	Skull
38	*P.cra.*	--	TM 42419	Skull, skin
39	*P.cra.*	--	--------	-----
40	*P.rup.*	Se	TM 42422	Skull, skin
41	*P.rup.*	Se	TM 42423	Skull
42	*P.rup.*	Se	TM 42420	Skull, skin
43	*P.rup.*	Se	TM 42421	Skull, skin
44	*P.rup.*	Nw	TM 42449	Skull, skin

Continued/

Specimen number	Species	Clade affiliation	Accession number	Material type
45	*P.rup.*	Se	TM 42450	Skull, skin
46	*P.rup.*	Nw	TM 42451	Skull
47	*P.ran.*	--	TM 42463	Skull
48	*P.ran.*	--	TM 42464	Skull, skin
49	*P.ran.*	--	TM 42465	Skull, skin
50	*P.ran.*	--	TM 42466	Skull, skin
51	*P.rup.*	Se	TM 42467	Skull, skin
52	*P.rup.*	Se	TM 42468	Skull, skin
53	*P.rup.*	Se	TM 42469	Skull, skin
54	*P.ran.*	--	Skukuza	Skull
55	*P.ran.*	--	Skukuza	Skull, skin
56	*P.ran.*	--	TM 42651	Skull, skin
57	*P.rup.*	Se	TM 42652	Skull, skin
58	*P.rup.*	Se	TM 42653	Skull, skin
59	*P.rup.*	Se	TM 42654	Skull, skin

Continued/

Specimen number	Species	Clade affiliation	Accession number	Material type
60	*P.ran.*	--	--------	-----
61	*P.rup.*	Se	TM 42883	Skull, skin
62	*P.rup.*	Se	TM 42884	Skull
63	*P.ran.*	--	TM 42882	Skull, skin
64	*P.ran.*	--	TM 42881	Skin
65	*P.ran.*	--	--------	-----
66	*P.rup.*	Se	--------	-----
67	*P.rup.*	Se	TM 43329	Skull, skin
68	*P.rup.*	Se	TM 43328	Skull, skin
69	*P.rup.*	Se	TM 43330	Skull
70	*P.rup.*	Se	TM 43331	Skull
71	*P.rup.*	Se	TM 43332	Skin
72	*P.rup.*	Se	TM 43333	Skull, skin
73	*P.cra.*	--	TM 43389	Skull, skin
74	*P.cra.*	--	TM 43390	Skull
75	*P.rup.*	Se	TM 43391	Skull, skin
76	*P.rup.*	Se	TM 43392	Skull, skin

Continued/

Specimen number	Species	Clade affiliation	Accession number	Material type
77	*P.rup.*	Se	TM 43393	Skull, skin
78	*P.ran.*	--	TM 43394	Skull
79	*P.rup.*	Se	TM 43409	Skull
80	*P.rup.*	Se	TM 43410	Skull, skin
81	*P.rup.*	Se	TM 43411	Skull
82	*P.rup.*	Se	TM 43412	Skull
83	*P.ran.*	--	TM 43413	Skull, skin
84	*P.ran.*	--	TM 43414	Skull, skin
85	*P.ran.*	--	TM 43415	Skull
86	*P.rup.*	Se	TM 43416	Skull, skin
87	*P.rup.*	Se	TM 43417	Skull, skin
88	*P.rup.*	Se	TM 43418	Skull, skin
89	*P.rup.*	Se	TM 43419	Skull, skin

APPENDIX II

GAZETTEER

Collection locality	Map co-ordinates	Clone designation	Specimen number
*A=Kuruman	(27°35'S 23°26'E)	8,9	10,11
*B=Bloemfontein	(29°06'S 26°14'E)	2,3	2,21,22
*C=Campbell	(28°50'S 23°40'E)	1	1
*D=Springbok	(29°40'S 17°53'E)	4,5	3,23
*E=Donkerpoort	(30°40'S 25°30'E)	14	17,18,19,20
*F=Ladismith	(33°22'S 21°27'E)	10	12
*G=Grahamstown	(33°06'S 26°45'E)	11,12,13	13,14,15,16
*H=Golden Gate	(28°30'S 28°35'E)	15,16,17,18	24,33,34,35
*I=Howick	(29°45'S 30°23'E)	21	40
*J=Matatiele	(30°28'S 28°50'E)	22,23	41,42,43
*K=Dealsville	(29°30'S 24°10'E)	7	46
*L=Prieska	(29°40'S 22°52'E)	6	44
*M=Nieuwoudtville	(31°20'S 19°08'E)	19	45
*N=Verkeerdevlei	(28°50'S 26°45'E)	28	71,72

*_Pronolagus rupestris_

Continued/

Collection locality	Map co-ordinates	Clone designation	Specimen number
*O=Victoria West	(31 24 S 23$_{o}$07$_{l}$E)	19	36
*P=Sandgat farm	(31°17'S 22°45'E)	24	62
*Q=Rolfontein	(30°00'S 24°43'E)	20	61
*R=Volksrust	(27°25'S 29°52'E)	25,26	66-70
*S=Waterval-Onder	(25°35'S 30°23'E)	31	75,76,77
*X=Lydenburg	(25°08'S 30°35'E)	27,29,30,32	51-53,86-89
*Y=BlyderiversN.R.	(24°43'S 30°52'E)	30	57-59,79-8
T=Rustenburg	(25°43'S 27°11'E)	33,34,35,36	4,5,6,7,8,9
U=Suikerbosrand	(26°30'S 28°15'E)	38,39	28-32
V=Middelburg	(25°45'S 29°33'E)	40	47-50
W=Louis Trichard	(23°02'S 29°45'E)	37	25
Y=Blyderivers N.R.	(24°36'S 30°48'E)	41,42	56,60,83-85
Z=Vredefort	(27°07'S 27°15'E)	43	63,64,65
AA=Zeerust	(25°30'S 26°05'E)	40	78
BB=Kruger N.P.	(22°35'S 31°10'E)	40	54,55

*_Pronolagus rupestris_

APPENDIX III

RESTRICTION FRAGMENT DATA

Enzyme	Type	Fragment sizes
Apa I	A	11.0 : 3.8 : 1.7 : 1.5
	B	11.0 : 5.3 : 1.7
	C	10.2 : 5.5 : 1.7 : 0.6
	D	12.0 : 3.5 : 1.7 : 0.8
	E	12.5 : 2.8 : 1.7 : 1.0
Asp I	A	15.7 : 2.3
	B	18.0
Bam HI	A	10.5 : 7.5
	B	18.0
	C	12.8 : 5.2
Cla I	A	8.2 : 5.6 : 4.2
	B	9.8 : 8.2
	C	9.8 : 5.6 : 2.6
	D	18.0

Continued/

Enzyme	Type	Fragment sizes
Ava I	A	4.5 : 4.1 : 4.0 : 2.6 : 1.8 : 1.0
	B	4.3 : 4.1 : 4.0 : 2.6 : 1.8 : 1.0 : 0.2
	C	5.2 : 4.5 : 4.3 : 4.0
	D	5.2 : 4.3 : 4.0 : 2.3 : 2.2
	E	5.2 : 4.5 : 4.4 : 3.9
	F	10.5 : 3.8 : 3.6 : 0.1
	G	10.5 : 3.9 : 3.6
Dra I	A	5.6 : 5.1 : 3.3 : 2.3 : 1.7
	B	8.2 : 3.5 : 3.3 : 1.7 : 1.3
	C	5.6 : 3.8 : 3.3 : 2.1 : 1.7 : 1.5
	D	4.3 : 3.8 : 3.3 : 2.1 : 1.7 : 1.5 : 1.3
	E	6.0 : 4.2 : 1.9 : 1.7 : 1.5 : 1.4 : 1.3
	F	7.5 : 3.8 : 3.3 : 1.9 : 1.5
	G	5.9 : 5.6 : 3.3 : 1.7 : 1.5
	H	6.0 : 5.4 : 3.3 : 1.7 : 1.5
	I	5.0 : 4.3 : 3.1 : 1.8 : 1.7 : 1.5 : 0.6

Continued/

Enzyme	Type	Fragment sizes
Pst I	A	12.7 : 5.3
	B	16.2 : 1.8
	C	11.9 : 4.3 : 1.8
	D	6.9 : 6.2 : 3.3 : 1.6
Hind III	A	12.3 : 2.0 : 1.9 : 1.8
	B	14.7 : 2.0 : 1.3
	C	12.8 : 3.7 : 1.5
	D	9.0 : 3.9 : 3.1 : 2.0
	E	9.0 : 3.5 : 2.6 : 1.6 : 1.3
	F	9.0 : 3.2 : 3.1 : 2.0 : 0.7
	G	12.5 : 3.9 : 1.6
	H	9.0 : 5.9 : 3.1
	I	5.7 : 4.2 : 3.7 : 3.1 : 1.3
	J	5.7 : 5.0 : 4.2 : 3.1
Kpn I	A	15.4 : 2.6
	B	18.0

Continued/

Enzyme	Type	Fragment sizes
Sac I	A	15.5 : 2.5
	B	15.5 : 1.9 : 0.6
	C	10.1 : 5.2 : 1.9 : 0.8
	D	10.1 : 3.1 : 2.1 : 1.9 : 0.8
	E	5.2 : 5.1 : 5.0 : 1.9 : 0.8
	F	13.8 : 2.3 : 1.9
	G	13.3 : 2.3 : 1.9 : 0.5
Sca I	A	8.9 : 4.7 : 2.3 : 2.1
	B	13.6 : 2.3 : 2.1
	C	12.0 : 6.0
	D	18.0
	E	6.8 : 6.0 : 3.2 : 2.0
	F	12.2 : 3.2 : 2.0 : 0.6
	G	12.8 : 3.2 : 2.0

Continued/

Enzyme	Type	Fragment sizes
Stu I	A	6.8 : 5.3 : 4.0 : 1.9
	B	8.8 : 4.0 : 2.0 : 1.5 : 1.0 : 0.7
	C	5.2 : 4.2 : 4.0 : 2.1 : 2.0 : 0.5
	D	7.9 : 5.1 : 2.7 : 2.3
	E	7.9 : 3.9 : 2.7 : 2.3 : 1.2
	F	13.0 : 2.7 : 2.3
	G	7.9 : 3.9 : 3.5 : 2.7
	H	6.9 : 3.9 : 2.7 : 2.3 : 1.2 : 1.0
	I	6.9 : 3.9 : 2.7 : 2.3 : 2.2
Xba I	A	13.8 : 3.3 : 0.9
	B	11.1 : 3.3 : 2.7 : 0.9
	C	7.5 : 6.3 : 3.3 : 0.9
	D	13.8 : 1.8 : 1.5 : 0.9
	E	6.3 : 4.0 : 3.5 : 3.3 : 0.9
	F	16.7 : 1.3

Continued/

Enzyme	Type	Fragment sizes
Sty I	A	3.50 : 2.25 : 2.1 : 2.05 : 1.55 : 1.5 : 1.2 : 1.0 : 0.9 : 0.8 : 0.6 : 0.55
	B	4.65 : 2.8 : 2.1 : 1.9 : 1.65 : 1.35 : 1.3 : 0.85 : 0.82 : 0.58
	C	5.75 : 2.1 : 1.9 : 1.65 : 1.55 : 1.35 : 1.3: 0.95 : 0.85 : 0.6
	D	5.55 : 3.05 : 2.1 : 1.55 : 1.5 : 1.2 : 1.0 : 0.9 : 0.6 : 0.55
	E	5.55 : 2.25 : 2.1 : 1.55 : 1.5 : 1.2 : 1.0 : 0.9 : 0.8 : 0.6 : 0.55
	F	4.65 : 2.65 : 2.1 : 1.9 : 1.65 : 1.35 : 1.3: 0.95 : 0.85 : 0.6
	G	2.80 : 2.5 : 2.0 : 2.1 : 1.9 : 1.65 : 1.35 : 1.3 : 0.95 : 0.85 : 0.6
	H	2.37 : 2.21 : 2.1 : 1.65 : 1.64 : 1.4 : 1.22 : 1.16 : 1.03 : 0.95 : 0.85 : 0.70 : 0.63 :0.09
	I	2.37 : 2.3 : 2.1 : 1.65 : 1.64 : 1.4 : 1.22 : 1.16 : 1.03 : 0.95 : 0.85 : 0.70 : 0.63
	J	2.48 : 2.37 : 2.3 : 2.21 : 2.1 : 1.22 : 1.16 : 1.03 : 0.95 : 0.85 : 0.7 : 0.63