MOLECULAR PHYLOGENY OF THE GENUS *Pronolagus* (MAMMALIA: LAGOMORPHA) AND THE USE OF MORPHOLOGICAL AND MOLECULAR CHARACTERS IN THE DELINEATION OF *P. rupestris*

by

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SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS OF MAGISTER SCIENTIAE: ZOOLOGY

DEPARTMENT: Zoology and Entomology

in the

FACULTY OF BIOLOGICAL AND AGRICULTURAL SCIENCES
UNIVERSITY OF PRETORIA
PRETORIA

March 1995

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ABSTRACT

Molecular and morphological characters were used to clarify phylogenetic relationships within *Pronolagus* and, in particular, within *P. rupestris*. All taxa are characterized by an invariant karyotype while molecular sequence data support the integrity of the conventionally accepted *P. crassicaudatus*, *P. randensis* and two well delimited mitochondrial DNA clades within *P. rupestris*. Cranial morphology was concordant with the molecular results but provided evidence of further subdivision within one of the *P. rupestris* mitochondrial DNA clades. Based on these data, *P. rupestris* is retained while two additional species are proposed: *P saundersiae* (Hewitt) limited to the southern Karoo regions and *P. barretti* (Roberts) which occurs along the eastern Escarpment of southern Africa. Additionally, sequence data from the 12S rRNA gene was utilized to identify the lagomorph sister taxon. Likely sister taxa include elephant shrews, primates and tree shrews; however, a winning sites test could not resolve with certainty the relationships between them.

In the subjects we propose to investigate,
our inquiries should be directed,
not to what others have thought,
nor to what we ourselves conjecture,
but to what we can clearly and perspicuously behold
and with certainty deduce;
for knowledge is not won in any other way.

Descartes, 1628

For my parents

ACKNOWLEDGEMENTS

My sincerest gratitude to my supervisor, Prof. T.J. Robinson for his patience, encouragement and unfailing support.

I am indebted to the following private landowners for permission to collect rock rabbits on their properties and for their hospitality: Mr de Waal (Boshof), Mr & Mrs Liebenberg (Fauresmith) and the Geldenhuys family (Volksrust).

The tree shrew and pika material provided by Dr. L.K. Ammerman (Texas Wesleyan University) and Mary Peacock (University of Arizona) respectively is greatly appreciated. The staff of the Transvaal, Kaffrarian and McGregor Museums are thanked for their assistance with regards to the mammal collections.

Special thanks to Chris Chimimba of the Transvaal Museum without whom the morphometrics would have remained uncharted territory. I also wish to thank Dr. Nico Dippenaar, the members of staff, fellow students and friends in the Department of Zoology and Entomology for their advice, support and understanding during the past few years.

Financial support in the form of a Core Programme Studentship to Prof. T.J. Robinson Foundation of Research Development and the University of Pretoria.

Finally, I wish to thank my parents for their unstinting love, guidance, support and understanding. And Donald for his love, friendship and unwavering support.

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CHAPTER 1

GENERAL INTRODUCTION

LAGOMORPH EVOLUTION AND SYSTEMATICS

The Lagomorphs

Rabbits, hares and pikas were, together with the rodents, originally considered as members of the order Glires (Lopez Martinez 1985). Subsequently however, the lagomorph representatives were reclassified by Gidley (1912) as warranting separate ordinal status. The modern Lagomorpha, comprising 78 extant species (Chapman & Flux 1990), have a cosmopolitan distribution but were introduced by man into Australia, New Zealand, Java, the southern Neotropical region and many oceanic islands (Deblase & Martin 1981).

Lagomorphs represent a conservative mammal order, maintaining a number of modified features for over 45 million years (Lopez Martinez 1985). The group's distinguishing features are a pair of small peglike teeth found directly behind the first upper incisors (Chapman & Flux 1990) and the presence of a fenestrated rostrum. The skull and dentition patterns are similar throughout the taxon. The postcranial skeleton shows little variation, in addition to which the tail is short or rudimentary in all species (Carroll 1988).

Within the order, the two families, Ochotonidae (pikas) and Leporidae (rabbits and hares) are physically distinct from each other. The ochotonids, represented by a single extant genus *Ochotona* (with twenty-three fossil genera), are considerably smaller than the leporids, the fore- and hindlimbs being roughly equal in length; in these species the ears are round, while the skull has no supraorbital bones and the nasals are generally short (Angermann *et al* 1990). In contrast, the leporid hindlimbs are far longer than the forelimbs and the ears are elongated while the skull has conspicuous supraorbital bones and a long nasal region (Angermann *et al* 1990). The Leporidae are represented by twenty-one fossil genera and eleven extant genera: *Lepus* (jackrabbits and hares), *Pentalagus* (Amami Rabbit), *Pronolagus* (Rock Rabbit), *Romerolagus* (Volcano Rabbit), *Caprolagus* (Hispid Hare), *Oryctolagus* (European Rabbit),

Sylvilagus (Cottontails), *Brachylagus* (Pygmy Rabbit), *Bunolagus* (Riverine Rabbit), *Poelagus* (Bunyoro Rabbit) and *Nesolagus* (Sumatran Rabbit) (Chapman and Flux 1990; Figure 1.1). Lagomorphs range in size from the *Ochotona* (130 - 180g) to the largest, *Lepus othus* (3.2 - 6.5kg), with the rabbits somewhat intermediate between these two extremes (Lopez Martinez 1985).

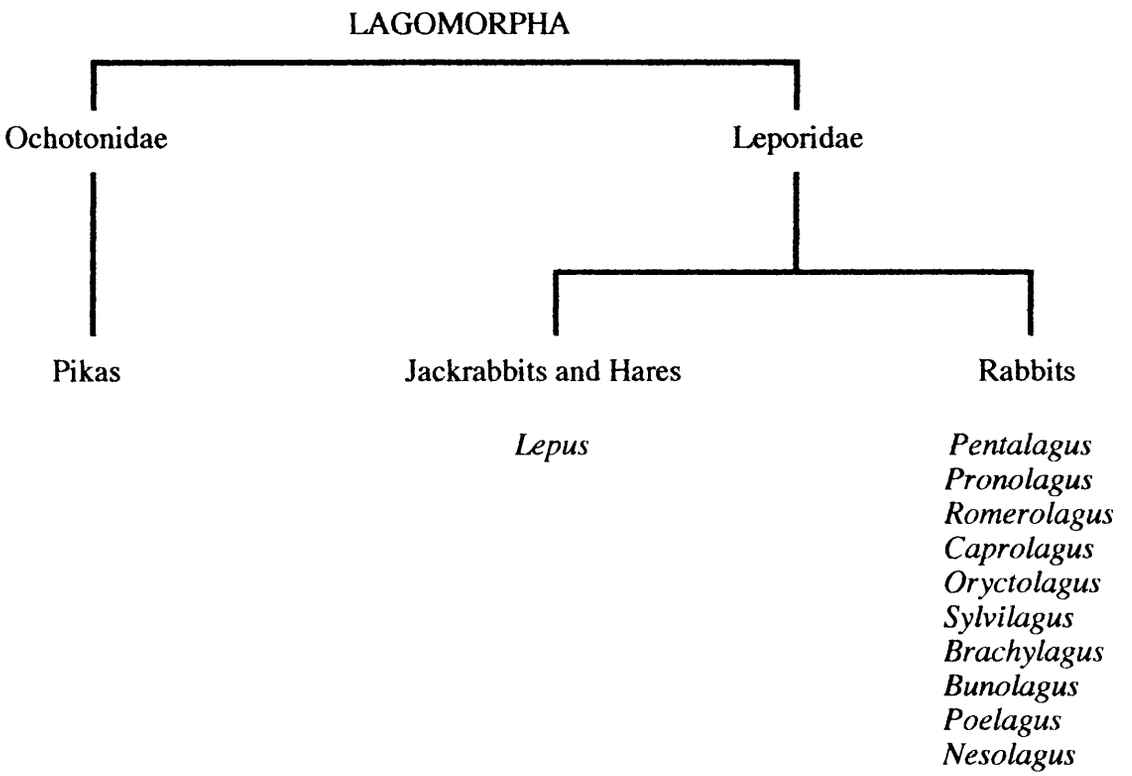

Figure 1.1 The Lagomorphs of the world (Chapman & Flux 1990).

Pikas occur in alpine and steppe environments to which they are adapted. They are strictly territorial and appear to be the only lagomorphs with well developed vocalization (Chapman & Flux 1990). The Leporidae, in contrast, range from being territorial to solitary. Their long hindlimbs are adapted for rapid movement and the large eyes are suited to their crepuscular and nocturnal habits. All lagomorphs groom by licking the body, cleaning the feet and dusting (Chapman & Flux 1990). The herbivorous diet and terrestrial mode of life of the modern leporids appears to have undergone little change during the evolutionary history of the order (Lopez Martinez 1985).

Palaeontological Record

Although the Lagomorpha have been an independent order, separate from their nearest living relatives for at least 60 million years (Dene *et al* 1982), the origin of the group remains obscure. Both Dawson (1979) and Carroll (1988) propose that the Lagomorpha may be related to the Anagalida (extinct relatives of the tree shrews) or, alternatively, have descended from a group ancestral to both taxa. Other suggested ancestors are insectivores or an ancestor shared with the ungulates (Wood 1957; Novacek 1982). The Palaeocene genus †*Mimotona* and the more recent †*Mimolagus* (Family: Mimotonidae) have several features in common with lagomorphs and this has led to suggestions of a close lagomorph/mimotonid relationship (Li & Ting 1985). Dene *et al* (1982), McKenna (1987) and Carroll (1988) are less cautious regarding †*Mimotona*, together with †*Hsiuannania*, as the earliest representatives of the order. The mimotonids form part of the Palaeocene Eurymylidae which also includes the genus †*Heomys*. Additionally, since †*Heomys* is regarded as the mammal closest to the ancestral stem of rodents, it is not surprising that the

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† extinct taxa

Rodentia have also been proposed as the sister taxon to Lagomorpha (Li & Ting 1985; Hartenberger 1985; Chuan-Kuei *et al* 1987). Whichever is correct, the connections between the Palaeocene Eurymylidae and the recent lagomorphs is, however, equivocal. The Asian representatives of the order which are present from the middle to late Eocene, and those of the North American late Eocene, have a dental structure from which both the Leporidae and the Ochotonidae may have evolved. The beginning of the Oligocene saw the spread of lagomorphs (the Palaeolaginae and their progressive descendants, the Archaeolaginae) into Europe and by the Pliocene both ancestral ochotonids and leporids had reached Africa (Carroll 1988).

The first true leporid, †*Alilepus* (descended from the Archaeolaginae) appeared in the late Miocene while modern leporids, with the occasional intermediate step, can be traced from †*Alilepus dietrichi* (*Pliopentalagus*). The genera representative of the early leporid stocks (*Pronolagus*, *Pentalagus*, *Nesolagus*, *Brachylagus* and *Romerolagus*) appear to have had their geographic distributions restricted by the more vigorous and closely related genera of *Lepus*, *Sylvilagus* and *Oryctolagus* (Hibbard 1963; Dawson 1979).

Lagomorph Evolutionary Relationships

As noted previously, lagomorphs may be related to ungulates, tree shrews or insectivores, while Novacek (1982) is of the opinion that rodents cannot be excluded from this assemblage. Indeed, Novacek (1982, 1992), Novacek and Wyss (1986) and Chuan-Kuei *et al* (1987) regard the Rodentia and Lagomorpha as having a closer common ancestry with each other than with any other Recent eutherian group, and place macroscelideans (elephant shrews) as the nearest living outgroup. Morphological evidence from both living and fossil taxa suggests that of possible eutherian superordinal groupings, that of lagomorphs and rodents is strongly supported (resurrecting the Glires concept). In support of this Shoshani (1986) using 182 non-dental morphological characters of various extant and extinct mammalian species, similarly found rodents and lagomorphs to be allied with macroscelideans as the nearest outgroup.

In contrast, Lopez Martinez (1985) is of the opinion that the similarities between lagomorphs and rodents are the result of parallel evolution, and that the lagomorph sister taxon should rather be sought among other mammalian taxa. Although morphology provides strong support for the monophyly of Glires, molecular studies propose several novel relationships:

(i) Goodman *et al* (1982) and Dene *et al* (1982) suggest a close relationship between the Lagomorpha and Scandentia (tree shrews) and that genealogically the lagomorph-tree shrew group are placed closer to the primates than to rodents,

(ii) lagomorphs and rodents are grouped in a superorder together with primates (Miyamoto & Goodman 1986) and

(iii) a relationship between primates and lagomorphs to the exclusion of rodents (Goodman *et al* 1985; Springer & Kirsch 1993).

In general, however, a weak aspect of most molecular analyses is the omission of two frequently suggested sister taxa - the elephant shrews and the tree shrews.

Systematics of the genus *Pronolagus* Lyon 1904

In an early definitive work Roberts (1951) described nine *Pronolagus* species within the southern African region: *P. crassicaudatus* (Geoffroy 1832: five subspecies), *P. rupestris* (Smith 1834: four subspecies), *P. melanurus* (Rüppell 1842), *P. curryi* (Thomas 1902), *P. barretti* (Roberts 1948), *P. caucinus* (Thomas 1929), *P. kobosensis* (Roberts 1938: three subspecies), *P. randensis* (Jameson 1907: four subspecies) and *P. whitei* (Roberts 1938).

Ellerman *et al* (1953) synonymized many of the taxa and recognized only three well delineated species: *P. rupestris, P. randensis and P. crassicaudatus.* This reductionist approach was extended by Lundholm (1955) who in turn retained only *P. randensis* and *P. rupestris*. Peddie (1975), on the other hand, regarded *Pronolagus* as monotypic within South Africa.

The *Pronolagus* species currently recognized by Meester *et al* (1986) are those based on morphometric analysis by Robinson and Dippenaar (1983). Three species enjoy their support: *P. rupestris* (Smith 1834: seven subspecies), *P. randensis* (Jameson 1907: nine subspecies) and *P. crassicaudatus* (Geoffroy 1832: five subspecies). Additionally, Kingdon (1974) recorded *P. rupestris vallicola* as occurring in East Africa and therefore extralimital to this study.

Species Description and Distribution

As is evident, a great deal of uncertainty exists both as far as the systematic relationships within *Pronolagus* is concerned as well as with the higher order evolutionary history of the order. Nonetheless, for the purposes of this investigation I follow Meester *et al* (1986) and regard *Pronolagus* as comprising:

(i) Pronolagus crassicaudatus (Geoffroy 1832) Natal Red Rock Rabbit.

Pronolagus crassicaudatus, with the most restricted South African distribution, has as its range the easterly regions of the Eastern Cape, Kwazulu-Natal, eastern Lesotho, Swaziland, south eastern regions of Eastern Transvaal and southern Mozambique (Meester *et al* 1986; Figure 1.2). Of the currently recognized species *P. crassicaudatus* is physically the largest with stocky limbs and short, sparsely furred ears. The rufous-brown coat is somewhat coarse, with grey underfur. From the chin, a clearly discernible greyish-white band extends backwards to the nape patch. The remainder of the face is grey-brown (Skinner & Smithers 1990). The upper principal incisor is broad across the face (2.6 - 3.7mm per incisor); the bullae are small and this is reflected both proportionately (bulla breadth X 100/total length of skull: 5.5 - 7.8%) as well as in absolute terms (5.1 - 7.0mm). Diagnostically important ratios are maxillary premolar length x 100/frontal length (19.9 - 32.5%) and mesopterygoid space x 100/frontal length (11.7 - 19.9%) (Robinson 1982).

(ii) Pronolagus randensis Jameson 1907, Jameson's Red Rock Rabbit.

This species exists as two disjunct populations within the southern African subregion. One extends from Karibib, Namibia along the western parts of Namibia to the south western corner of Angola (Meester *et al* 1986; Figure 1.2). The other population's distribution extends from the northern parts of the Orange Free State, Gauteng, Western, North Western and Northern Transvaal, through south eastern Botswana, eastern Zimbabwe and western Mozambique (Meester *et al* 1986; Figure 1.2). In terms of overall size *P. randensis* is slightly smaller than *P. crassicaudatus*; it has a fine, silky coat, the fur is a grizzled, rufous-brown, although the rump and the back of the legs are somewhat lighter in colour. The underparts are a pinkish-light yellow. The cheeks, side of the neck and lower jaw are light grey and the chin white. The gular patch is a distinct rufous-brown. The ears may be tipped with black (Skinner & Smithers 1990). As with *P. crassicaudatus* the upper incisors are broad across the face and the bullae proportionately narrow (Robinson 1982). Diagnostically important ratios are the maxillary premolar length x 100/frontal length (16.9 - 22.9%) and the mesopterygoid space x 100/frontal length (9.4 - 14.7%) (Robinson 1982).

(iii) Pronolagus rupestris (Smith 1834) Smith's Red Rock Rabbit.

As with the previous species, *P. rupestris* occurs as two disjunct populations, one with a restricted distribution in north-east Africa and the other with a largely South African distribution. The latter encompasses the Western, Eastern and Northern Cape Provinces (except along the coastal regions) and extends northwards up to the Eastern Transvaal as well as into southern Namibia (Meester *et al* 1986; Figure 1.2). Smallest of the three *Pronolagus* species, *P. rupestris* has fur which is rufous-brown with distinct black grizzling; the rump and the back of the hindlimbs are more reddish. The cheeks are greyish-buff but not noticeably so, while the underparts of the body are pinkish-buff (Skinner & Smithers 1990). In contrast to *P. randensis* and *P. crassicaudatus*, the upper principal incisors are narrow across the face (2.2 - 2.8mm per incisor) and the bullae breadth x 100/total length of skull is 7.4 - 11.4%. The bullae are more robust than in the other two species, with absolute measurements ranging between 6.4 - 9.2mm (Robinson 1982). The bulla breadth x 100/mandibular height ratio (16.5 - 25.1%; Robinson 1982) is diagnostic for the species.

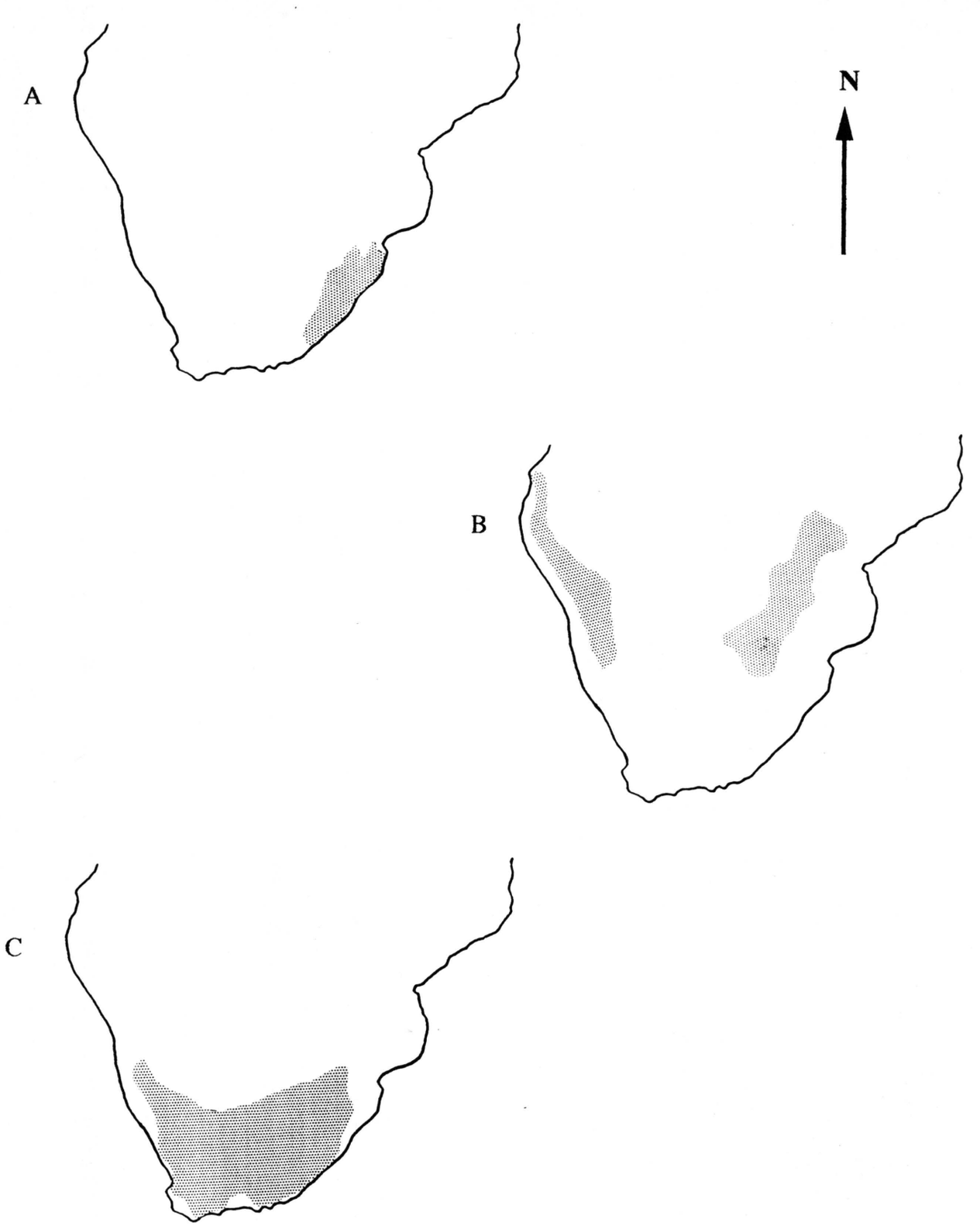

Figure 1.2 Distribution patterns of the three recognised *Pronolagus* species in southern Africa subregion. (A) *P. crassicaudatus*, (B) *P. randensis* and (C) *P. rupestris* (drawn from Skinner & Smithers 1990).

Molecular systematic studies

While the recognition of three conventionally accepted *Pronolagus* species was supported by morphometric studies (Robinson & Dippenaar 1983) most recently, however, an analysis of restriction fragment length polymorphisms (RFLPs) within *P. rupestris* revealed the presence of two well delineated clades (Figure 1.3). These comprise a south-eastern mitochondrial assemblage which is confined to the mountain ranges comprising the Great Escarpment of South Africa and its environs, and a north-western assemblage which is not so tightly constrained (Matthee & Robinson submitted). The sequence divergence separating the two clades (7.94%) approximates that detected between *P. rupestris* and *P. randensis* (9.30%; Matthee 1993; Matthee & Robinson submitted) and this, together with the fact that the clades are parapatric for part of their distribution and do not share mitochondrial DNA lineages, was interpreted as reflecting the presence of two undetected species in what has conventionally been regarded as a single taxon, *P. rupestris*.

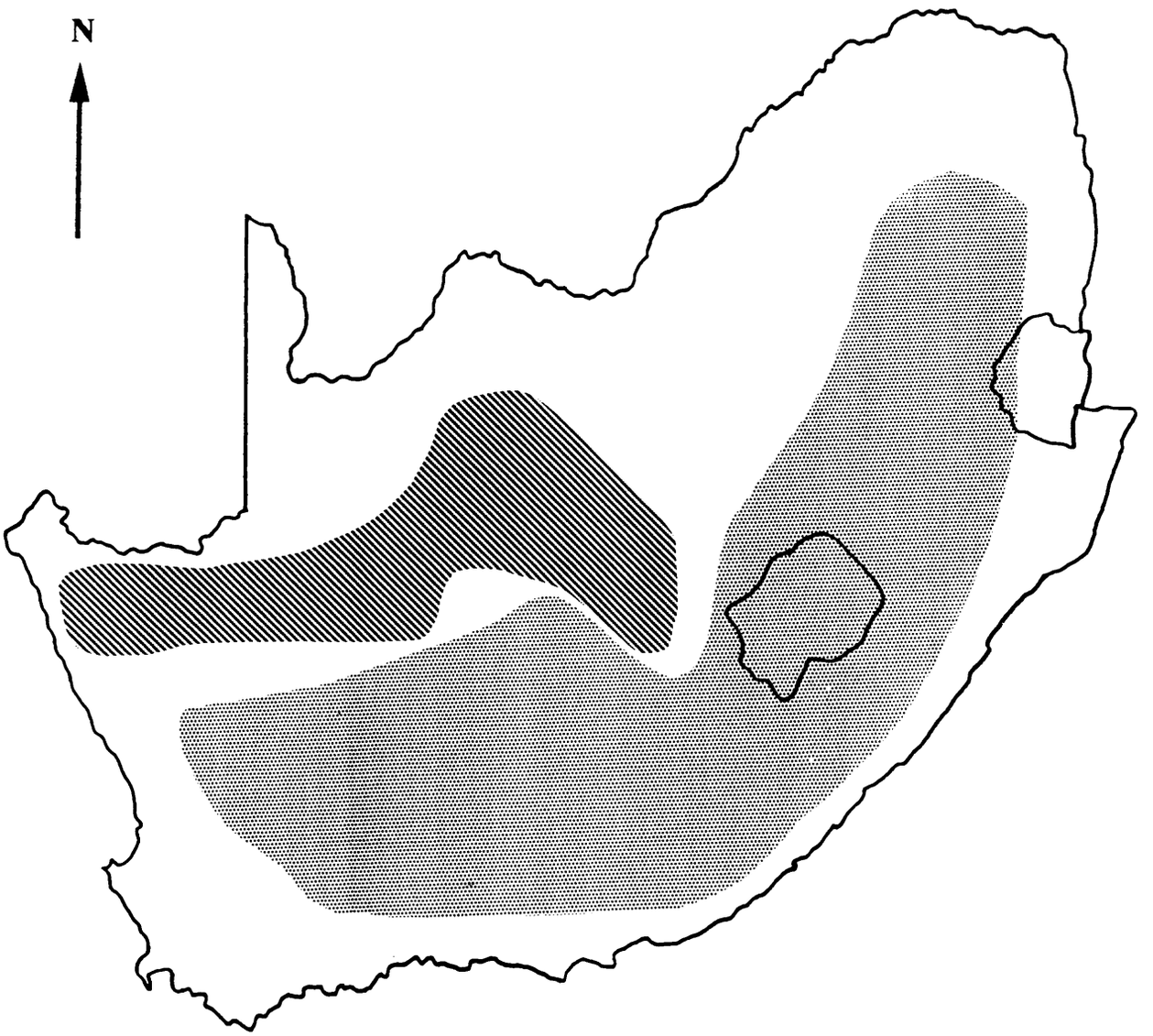

Figure 1.3 Geographic distribution of the two mitochondrial DNA clades delineated within *P. rupestris*, the (▨) north-western and (▩) south-eastern clade (drawn from Matthee 1993; Matthee & Robinson submitted).

AIMS AND OBJECTIVES

Given this background, it is clearly evident that a great deal of uncertainty exists with respect to the taxonomic status of taxa within the genus *Pronolagus* and, in a broader context, to the order's higher phylogenetic relationships. The aims of this study were therefore twofold:

(1) To assess the phylogenetic relationships and taxonomic status of the three morphometrically recognised species of *Pronolagus* and the two *P. rupestris* mitochondrial DNA clades using cytochrome b and 12S rRNA nucleotide sequence data. Two additional parameters, comparative cytogenetics and cranial morphometrics were utilized to evaluate the robustness of the *P. rupestris* mitochondrial DNA clades.

(2) To identify the most likely lagomorph sister taxon among the eutherian mammals using 12S rRNA sequence data.

CHAPTER 2

MOLECULAR ANALYSIS

PHYLOGENETIC INFERENCE USING MOLECULAR MARKERS

While morphology has been the traditional means by which the evolutionary path has been reconstructed, the recent application of molecular techniques to systematics has led to their extensive use in describing diversity and relationships in all forms of life (for example: Laerm *et al* 1982; Avise & Nelson 1989; Graves & Dizon 1989; Thomas *et al* 1989; Gilbert *et al* 1990; Thomas *et al* 1990; Bowen *et al* 1991; Disotell *et al* 1992; Janczewski *et al* 1992; Spears *et al* 1992; Horai *et al* 1993; Georgiadis *et al* 1994). Furthermore, the variation in evolutionary rates of different parts of DNA molecules allows resolution of the deepest to the most recent nodes of the evolutionary tree (Harrison 1991; Hillis & Dixon 1991).

Notwithstanding widespread support for molecular approaches in phylogenetic analyses, especially in areas that traditional methods consider troublesome, the lack of agreement between molecular based phylogenies is not dissimilar to that evidenced between morphological data sets (Patterson *et al* 1993). This results, paradoxically, from the variability of DNA and the ambiguities of species boundaries which often makes the estimation of specific status and other taxonomic relationships controversial (Harrison 1991; Baker 1994). Therefore, rather than being regarded as the panacea for systematics, molecular phylogenetics should occupy a place alongside the array of other taxonomic characters including morphology, physiology, behavioural, ecological and geographic parameters (Hillis & Dixon 1991).

The Database

In recent times DNA sequences have become the preferred data in the reconstruction of phylogenies. They present a vast array of characters with different structural/functional properties, mutational/selectional biases and evolutionary rates. Furthermore, the gathering of

sequencing data is becoming less expensive and technically less demanding (Cracraft & Helm-Bychowski 1991; Miyamoto & Cracraft 1991).

Region for sequencing

The phylogenetic value of a gene, or region of DNA, rests on the premise that the rate of evolution matches the divergence times of the taxa under consideration (Mindell & Honeycutt 1990; Larson 1991; Hillis & Huelsenbeck 1992; Graybeal 1994). A region that is too conserved will yield little historical information (or "signal"), and the more divergent sequences become, the greater the level of homoplasy or "noise" (parallelisms, convergences and reversals) which, in turn, lessens the robustness of a phylogeny (Mishler *et al* 1988; Smith 1989; Hillis & Dixon 1991). The rapid evolution of the extrachromosomal DNA found in animal mitochondria (Attardi 1985; Hixson & Brown 1986; Avise 1989; Harrison 1989; Brown *et al* 1993), together with varying rates of molecular evolution along the length of the molecule (Harrison 1989; Brown *et al* 1993), has resulted in its extensive use in phylogeny reconstruction.

The mitochondrial DNA molecule and its use in phylogeny reconstruction

The extrachromosomal DNA found in animal mitochondria is represented by a duplex, covalently closed circular molecule ranging from 15.7 to 19.5 kilobases in length in most organisms (Attardi 1985; Moritz *et al* 1987). The mammalian mitochondrial genome comprises 13 messenger RNAs (mRNA), 22 transfer RNAs (tRNA) and two ribosomal RNAs (rRNA) and is characterized by lack of intervening sequences within transcribed genes, no spacer sequences between genes, and no classes of repetitive DNA; the variable D-loop provides the only exception, in that it has no coding function (Anderson *et al* 1981; Avise 1986; Moritz *et al* 1987; Saccone *et al* 1991; Brown *et al* 1993).

The organization of the mitochondrial genome is conserved across animal orders (Kumazawa & Nishida 1993), ensuring that truly homologous loci can be compared for phylogenetic reconstruction. Other useful features include its clonal and almost invariably strict pattern of maternal inheritance (Avise 1989), its lack of recombination and ease of isolation and purification (Avise 1989; Harrison 1991; Kumazawa & Nishida 1993). In contrast, nuclear

DNA from any region of a diploid, sexually reproducing individual reflects two haplotypes and is subject to sequence rearrangements due to crossing over. The inherent weakness of mitochondrial DNA is, however, that it represents a single locus (Harrison 1989) reflecting only the female-to-female history of a population (Avise 1989; Avise 1991). In essence, a mitochondrial DNA phylogeny is synonymous with a gene tree and is in sharp contrast to a population or species tree which is a compilation of the genealogies for many independent genes (Avise 1989; Avise 1991). Clearly, mitochondrial DNA based phylogenies reflect only a single aspect of an organism's evolutionary history.

Two mitochondrial genes, cytochrome b and 12S rRNA, each with differing rates of evolution (with 12S evolving less rapidly than cytochrome b; Hixson & Brown 1986), have been widely used in phylogeny reconstruction (Miyamoto *et al* 1990; Allard & Miyamoto 1992; Geffen *et al* 1992; Thomas & Martin 1993). Both genes underpin two of the evolutionary pathways recognised by Graybeal (1994). Cytochrome b tracks the path of most protein genes in that there is a relatively rapid linear increase in sequence divergence at those sites free to change, followed by a levelling off of the curve where multiple substitutions predominate. The 12S rRNA undergoes a moderate but continuous rate of change, possibly having a greater proportion of sites free to change.

Cytochrome b

The structure/function relationship of cytochrome b is well known (Irwin *et al* 1991; Martin & Palumbi 1993). Cytochrome b is one of the proteins that comprise complex III (ubiquinol - cytochrome c oxireductase) of the mitochondrial oxidative phosphorylation system, and the only one found in the mitochondrial genome. This protein is thought to contain the two redox centres involved in the electron transfer. These redox centres coincide with highly conserved amino acid regions within the gene which, together with several other regions of 20 - 30 amino acid residues, are indicative of the heterogeneity in the molecular evolutionary rate of different regions along the length of the gene (Howell 1989; Irwin *et al* 1991; Ma *et al* 1993). While accurate alignment of a protein coding sequences is more easily obtainable than other sequences (for example rRNA; Irwin *et al* 1991; Mindell 1991; Miyamoto & Cracraft 1991; Kumazawa & Nishida 1993), the tendency to reach saturation fairly rapidly means that cytochrome b has limited resolving power and is usually only useful for investigating recent divergence events (Irwin *et al* 1991; Graybeal 1994).

12S rRNA

The rRNA genes code for ribosomal RNA which, together with a variety of smaller protein molecules, bond to form ribosomes for protein synthesis in the cell. Ribosomal RNA is therefore critical to an individual's survival. The vital functional role of rRNA not unexpectedly ensures that rRNA gene sequences are fairly resistant to change (Mindell & Honeycutt 1990). Animal rRNA genes are found both in the nuclear (5.8S, 18S and 28S) and mitochondrial (12S and 16S) genomes. Nuclear rRNA genes comprise repetitive sequence elements occurring in tandem arrays while in vertebrates the mitochondrial 12S and 16S rRNA genes are present as single copy loci (Baker *et al* 1989; Mindell & Honeycutt 1990). These two genes are adjacent to each other and are flanked by transfer RNAs. As with other mitochondrial genes, the mitochondrial rRNA genes have evolved at a faster rate than their two nuclear counterparts (Hixson & Brown 1986) both of which are characterized by approximately the same rate of change (suitable for estimating divergences of about 150 million years or less). Of the two, 12S rRNA has been used more extensively in constructing phylogenies, particularly for older divergences such as those inferring mammalian interordinal relationships (Ammerman & Hillis 1992; Springer & Kirsch 1993). As with cytochrome b, the 12s rRNA gene displays within-gene rate heterogeneity (Attardi 1985) with a 140 base pair region (corresponding to positions 1135 - 1275 of the human mitochondrial DNA sequence) evolving at a slower rate than the gene as a whole (Mindell & Honeycutt 1990).

MATERIALS

(1) Analysis of restriction fragment length polymorphisms (RFLPs)

Specimens that were collected for the cytogenetic and morphometric aspects of this investigation were initially screened by RFLP analysis to determine their mitochondrial clade affiliation (Matthee 1993). All sampled animals were shot at night with a shotgun. Kidneys, heart and liver were removed in the field as soon as possible after death and stored in liquid nitrogen until transfer to an ultra cold freezer in the laboratory. Standard body measurements (mass; total, tail, hind foot and ear length) and sex were recorded, and skulls and skins deposited in the mammal collection of the Transvaal Museum (Accession numbers listed in Appendix I).

Table 2.1 Collection localities of *P. rupestris* specimens used for mitochondrial DNA RFLP analysis (see Figure 4.1 p. 60 for map positions and Appendix V for coordinates).

Locality	Sample size
Bloemfontein	2
Boshof	3
Fauresmith	4
Volksrust	2

(2) Mitochondrial DNA sequencing

Although three *Pronolagus* species are currently recognized (Meester *et al* 1986), for the purposes of this study four sample groups were identified: *P. randensis*, *P. crassicaudatus*, *P.rupestris* (south-eastern clade) and *P. rupestris* (north-western clade). The *P. rupestris* specimens chosen for sequencing had been previously used in Matthee's (1993) study and their clade affiliation were known. Cytochrome b and the 12S rRNA genes were sequenced from two specimens representative of each of the above four groups (Table 2.2). As far as possible each group was represented by the taxon's most common and most divergent mitochondrial DNA haplotype (Matthee 1993). An ochotonid (*Ochotona princeps*) was specified as outgroup to the *Pronolagus* species in the phylogenetic analysis of the gene sequences.

In an attempt to identify the probable sister taxon to the lagomorphs, the 12S rRNA gene of an elephant shrew (*Elephantulus myurus*) and a tree shrew (*Tupaia glis*) were also sequenced. While 258 base pairs of sequence was available for the latter (Ammerman & Hillis 1992), this was supplemented by a further 442 base pairs generated in this study. In addition to these new sequences, data were also obtained from Genbank for *Brachyphyllum cavernarum* (bat), *Atelerix albiventris* (hedgehog), *Didelphis virginiana* (marsupial), *Homo sapiens* (human), *Mus musculus* (mouse), *Bos taurus* (cow), *Phoca vitulina* (seal) and *Balaenoptera physalus* (whale). Unfortunately the bat sequence was limiting since only 550 base pairs were available for this species.

Table 2.2 The taxonomic provenance, number of specimens sequenced and their geographic origins (see Figure 4.1 p. 60 for map positions and Appendix V for coordinates of southern African specimens).

Species/clade		Locality
P. randensis	1	Middelburg
	2	Louis Trichardt
P. rupestris (SE)	1	Donkerpoort
	2	Blyderivierspoort
P. rupestris (NW)	3	Springbok
	4	Kuruman
P. crassicaudatus	1	Hilton
	2	Waterval-onder
Ochotona princeps	1	Mono County, California
Elephantulus myurus	1	Bylderivierspoort
Tupaia glis	1	provided by L.K. Ammerman[1]

[1] Dr L.K. Ammerman, Department of Biology, Texas Wesleyan University, Fort Worth, TX 76105, USA.

METHODOLOGY

(1) Restriction fragment length polymorphisms

Mitochondrial DNA was extracted following standard procedures (Lansman *et al* 1981; Matthee 1993) and restriction site variation determined using the suite of 14 six-base restriction endonucleases employed by Matthee (1993): *Apa 1, Asp 718, Ava 1, Bam H1, Cla 1, Dra 1, Hind 111, Kpn 1, Pst 1, Sac 1, Sca 1, Stu 1, Sty 1* and *Xba1*. Mitochondrial DNA fragments were separated by gel electrophoresis. A molecular weight marker (*Hind 111* cleaved lambda-DNA; Promega and Boehringer Mannheim) was used for determining fragment sizes. Specimens of known mitochondrial DNA clade affiliation (Matthee 1993) were included as standards against which the fragment lengths of new specimens were compared. The methodology used followed that outlined in detail by Matthee (1993) and is therefore not repeated herein.

(2) Mitochondrial DNA sequencing

DNA Extraction

Total genomic DNA was extracted from frozen liver tissue (Amos & Hoelzel 1991) by digestion in 1M Tris pH 8.0, 1M NaCl, 0.5M NaEDTA, 10% SDS and proteinase K (10mg/ml) for several hours at 55°C followed by the addition of RNAse. The DNA was purified by phenol and chloroform-isoamyl alcohol (23:1) extractions, concentrated by ethanol precipitation and finally resuspended in Tris-EDTA. The optical density of the DNA was measured by spectrophotometry to determine both the purity and concentration of the sample (Maniatis *et al* 1982).

Polymerase Chain Reaction (PCR)

Amplification was performed in 50μl of a solution comprising distilled water, 25mM magnesium chloride ($MgCl_2$), 10 X *Taq* polymerase buffer, dNTP's, *Taq* polymerase (Promega), 50ng genomic DNA as well as 25pmol primer (synthesized by the Department of Biochemistry, University of Cape Town) for both strands of the targeted DNA (Figure 2.1 and 2.2; Table 2.3). Using a Hybaid Thermal Reactor, the reaction mixes were denatured for three minutes at 95°C. Amplification was obtained using a 35 cycle program with each cycle

having the following parameters: 94°C for 30 seconds (denaturing double stranded DNA), 50°C for 30 seconds (annealing of primer) and 72°C for 45 seconds (synthesis of new strand), and a final extension of 10 minutes at 72°C.

The newly generated fragments were electrophorised in 2% low melting point agarose gels (Nusieve, FMC Bioproducts). The fragments, visualized by ethidium bromide ultraviolet fluorescence, were excised from the gel and purified using either the Magic minipreps DNA purification system (Promega) or CLEANmix (Talent). The amplified DNA was finally suspended in Tris-EDTA.

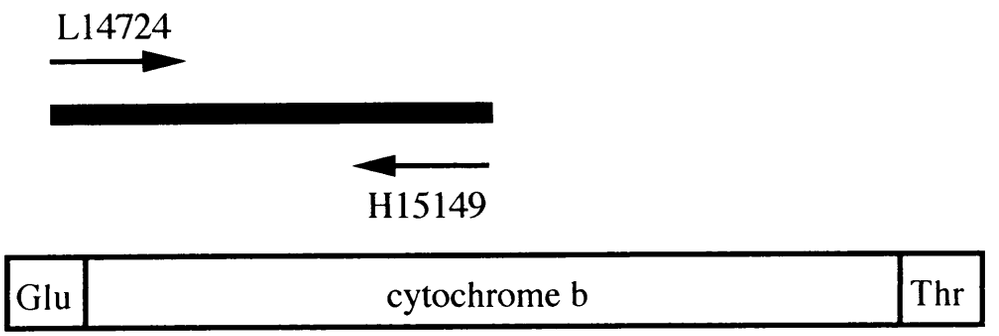

Figure 2.1 Primer position and direction of amplification for the generation of a 425 base pair segment of cytochrome b (adapted from Kocher *et al* 1989; Irwin *et al* 1991).

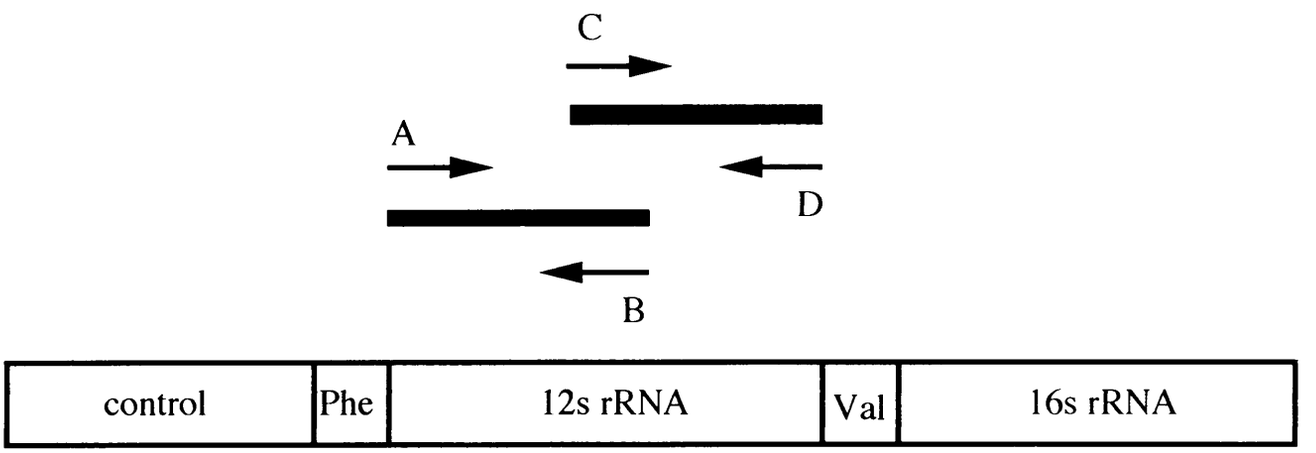

Figure 2.2 A - D indicate primer position and direction of amplification of the 12s rRNA gene (adapted from Kocher *et al* 1989; Allard & Honeycutt 1992). Solid lines show the region of overlap between the amplified segments.

Table 2.3 Primer sequences used for PCR and sequencing with the position of the 3' end of the primer according to the numbering system of the human sequence (Anderson *et al* 1981).

Primer	Sequence	3' position	Reference
Cytochrome b			
L14724	5' -CGAGATCTGAAAAACCATCGTTG- 3'	14724	1, 2
H15149	5' -AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA- 3'	15149	1, 2
12S rRNA			
A	5' -CATAGACACAGAGGTTTGGTCC- 3'	661	3
B	5' -TATCGATTATAGAACAGGCTCC- 3'	1200	3
C	5' -AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT- 3'	1091	1, 3
D	5' -TGACTGCAGAGGGTGACGGGCGGTGTGT- 3'	1487	1, 3

1. Irwin *et al* 1989.
2. Irwin *et al* 1991.
3. Allard & Honeycutt 1992.

Generation of single-stranded DNA and sequencing

The Sanger dideoxy, or chain termination method of sequencing was employed because of its simplicity and better band resolution (Hillis *et al* 1990). This technique requires as template, single stranded DNA. This was obtained either by the mechanical removal of one strand through the use of biotinylation and magnetic particle separation (Hultman *et al* 1989) or by heat denaturation. The single stranded DNA template was sequenced utilizing the PCR primers (Table 2.3), with a commercial kit (Sequenase Version 2.0; United States Biochemical) and sequencing reactions were run for all primer positions. Autoradiographic detection of the sequences was achieved through the incorporation of [α^{32}P] dATP (3000 Ci/mmol specific activity; Amersham) in the sequencing reaction.

Homology

Features in two or more taxa are described as homologous when they are derived from the same (or corresponding) feature in the nearest common ancestor (Mayr & Ashlock 1991). Clearly, the accuracy of sequence alignment will determine the reliability of the tree topology obtained (Thorne & Kishino 1992) and is therefore a critical aspect of any phylogenetic analysis. In this study, sequences were aligned manually and by computer (DAPSA version 2.4[2]; CLUSTAL version V - Higgins & Sharp 1989). Certain regions within the eutherian mammal data set presented alignment difficulties due to insertions or deletions and were therefore deleted prior to analysis (deleted regions are indicated in Appendix IV). This was considered justified since valid positional homology could not be established in these regions.

Data analysis

In constructing phylogenies, the most realistic evolutionary model would have the least amount of assumptions. In this analysis both distance (Neighbor-Joining; Saitou & Nei 1987) and parsimony methods (PAUP 3.1.2d5; Swofford 1993) were used to infer phylogenetic relationships.

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Distance methods calculate the evolutionary distance for all pairs of taxa (Nei 1991) and assume that this single coefficient of similarity, or dissimilarity, reflects the degree of evolutionary distance (Hillis *et al* 1993). This method does not, however, attempt to reconstruct evolutionary pathways. Maximum parsimony investigates the evolutionary relationships between various taxa and suggests a phylogeny that contains the minimum number of character transformations (Rodrigo 1992).

Sequence data from both genes were analyzed using all characters. However, the more distantly related taxa are, the more likely that sites become saturated through multiple substitutions. This usually skews the transition/transversion ratio towards 1:1 (Larson 1991). This saturation can be partially overcome by recoding the data set as purines (G and A) and pyrimidines (C and T), which would take only transversion events into account. In addition, cytochrome b sequences were analyzed both by excluding the third position of the codon and as protein sequence. Parsimony trees were constructed using PAUP 3.1.2d5 (Swofford 1993) from unordered character-state data unless the transition/transversion ratio differed from 1.0, in which case the empirical ratio was used to weight the data set.

In addition to parsimony analysis, a second phylogenetic-construction method was employed to determine whether similar topologies resulted from fundamentally different approaches. The Neighbor-Joining method was performed in PHYLIP (Felsenstein 1993) with distances being corrected for multiple hits by Kimura's (1980) two-parameter method.

Hypothesis testing

Since there are many hypotheses regarding the correct sister taxon to the lagomorphs (rodents and elephant shrews - Novacek & Wyss 1986; tree shrews - Dene *et al* 1982; primates - Goodman *et al* 1985; ungulates and insectivores - Novacek 1982) , the one-tailed binomial or winning-sites test (Prager & Wilson 1988; Hillis *et al* 1993) was used to compare various hypotheses with each other. The most parsimonious tree obtained was tested against all other hypotheses. In other words, an attempt was made to determine whether most parsimonious tree was significantly better than competing hypotheses. This was achieved by utilizing the number of parsimony sites supporting the trees being compared (the most parsimonious tree would therefore have the greatest number of parsimony sites).

The probability that the most parsimonious tree is either better, or worse, than the alternative is expressed as:

$$\left(\frac{1}{2}\right)^n \sum_{X=r}^{n} \binom{n}{X}$$

where n is the number of positions compared, r is the number of sites favouring one tree over another and

$$\binom{n}{X} = \frac{n!}{X!\,(n - X)!}$$

(Prager & Wilson 1988).

Confidence in resultant phylogeny

Once a phylogeny was obtained, it was necessary that some estimate be made regarding the reliability of the resulting topology. Of the variety of statistical procedures available (Hillis *et al* 1993), the bootstrap method and the g_1 statistic were selected for use in this study. Confidence values for internal nodes by bootstrapping were calculated using both the bootstrap option of PAUP or seqboot in PHYLIP.

The g_1 statistic (Hillis & Huelsenbeck 1992) examines the amount of phylogenetic signal present in sequence data. It measures the distribution of tree lengths:

$$g_1 = \frac{\sum (T_i - T)^3}{ns^3}$$

where n is the number of trees of length T, and s is the standard deviation of tree lengths.

When the distribution is significantly skewed from that which would be expected from random data (in other words the g_1 becomes more negative), the greater the phylogenetic signal present in the data. In this study, the g_1 value was obtained using the exhaustive search option of PAUP and the critical value for significance obtained from Hillis and Huelsenbeck (1992).

RESULTS

(1) Restriction fragment length polymorphisms

The mitochondrial DNA clade affiliations of the specimens collected specifically for the present investigation are indicated in Table 2.4. The specimens from Volksrust shared fragment profiles with the south-eastern mitochondrial DNA clade while the Bloemfontein, Boshof and Fauresmith specimens belong unequivocally to the north-western clade.

Table 2.4 Clade affiliation of specimens utilized in cytogenetic and morphometric analyses and their corresponding collection localities (see Chapters 3 and 4).

Locality	Clade affiliation
Bloemfontein	North-western
Boshof	North-western
Fauresmith	North-western
Volksrust	South-eastern

Whilst Matthee (1993) did not include specimens from Boshof and Fauresmith in the restriction enzyme analysis, it was anticipated *a priori* that they would align with the north-western clade, the prediction being based on geographic location. The clade affiliation is, however, inconsistent with the morphometric data, in that the two localities do not cluster within the north-western clade (see Chapter 4 for detailed discussion of the anomalous placement).

(2) Mitochondrial DNA sequencing

Phylogenetic relationships within Pronolagus

Phylogenetic trees were constructed using 745 and 333 aligned base pairs of 12S rRNA and cytochrome b respectively (Appendix II and III). Parsimony analysis of the 12S rRNA and cytochrome b sequences yielded, in each instance, a single most parsimonious tree. Significant g_1 statistics were obtained for both data sets (cytochrome b g_1 = -0.849; 12S rRNA g_1 = -1.025) which may be interpreted as reflecting that the data contained meaningful phylogenetic information (Hillis & Huelsenbeck 1992). Both parsimony and neighbor-joining trees were well supported by bootstrapping (Figure 2.3 and 2.4). The topologies based on cytochrome b and 12S rRNA sequences were identical (Figures 2.3 and 2.4) with the exception of the two *P. crassicaudatus* specimens which failed to cluster together in the cytochrome parsimony analysis. This is probably attributable to the high cytochrome b sequence divergences between these specimens (Table 2.5). Furthermore, tree topology remained unchanged irrespective of whether transversions only were used (data not shown). This stability was also reflected by the protein sequences as well as when third codon positions were excluded from the data set - although this was obviously only applied to the cytochrome b sequences (data not shown). These analyses revealed *P. crassicaudatus*, *P. randensis* and *P.rupestris* to be discrete taxa, with the four *P. rupestris* specimens clustering into two groups which mirrored the mitochondrial DNA results (Matthee 1993; Matthee & Robinson submitted). In all instances *P. crassicaudatus* was placed as the most basal of the *Pronolagus* taxa. Clearly, the large sequence divergence values obtained for the interspecific comparisons of the cytochrome b sequences (approaching 20%; Table 2.5) may not accurately reflect the relationships among taxonomic groups due to multiple substitutions (Mindell 1991) whereas the 12S rRNA values may be more informative.

Table 2.5 shows the average sequence divergence within and between the various taxa for the 12S rRNA gene. *Pronolagus crassicaudatus* and the southern clade of *P. rupestris* show greater within taxon variation (4.50% and 3.35% respectively) than do either the northern *P. rupestris* clade or *P. randensis* (1.65% and 1.64% respectively). Although the sequence divergence between the *P. rupestris* clades is lower than that between the recognised species,

it is nonetheless considerably higher than the variation within these two groups. Interestingly, the *P. rupestris* specimen from Blyderivierspoort in the Eastern Transvaal showed closer affinity to all of the other taxa, than any other pairwise comparison (barring, of course, the relationship with the other south eastern clade specimen from Donkerpoort; Table 2.6)

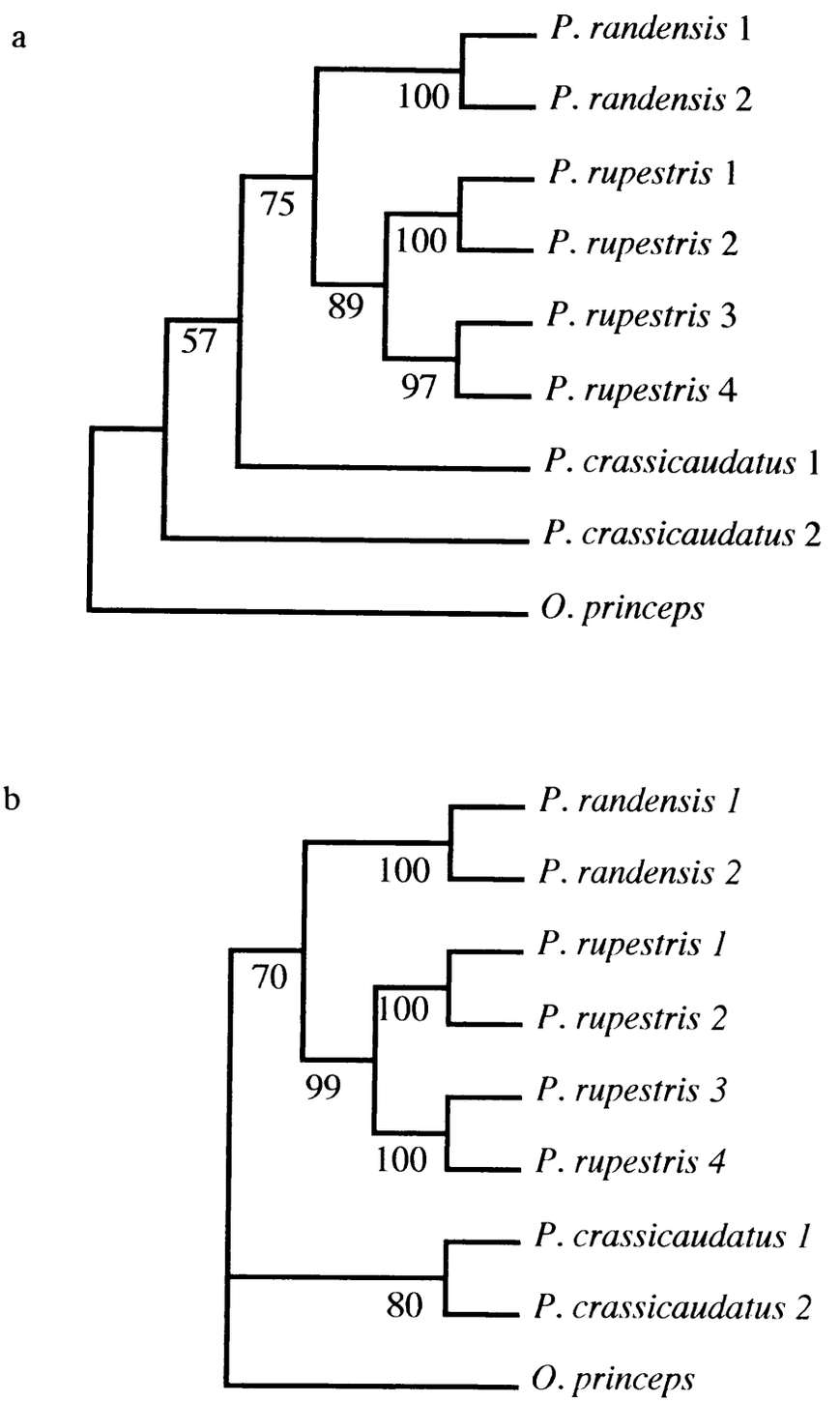


Figure 2.3 Phylogenetic analysis of 333 base pairs of cytochrome b (a) the single most parsimonious tree (Consistency index (CI) = 0.704; length = 305) and (b) an unrooted neighbor-joining tree using the *Ochotona* as outgroup to the *Pronolagus* taxa. Percentages of 1000 bootstrap trees in which a node is supported is given below each node.

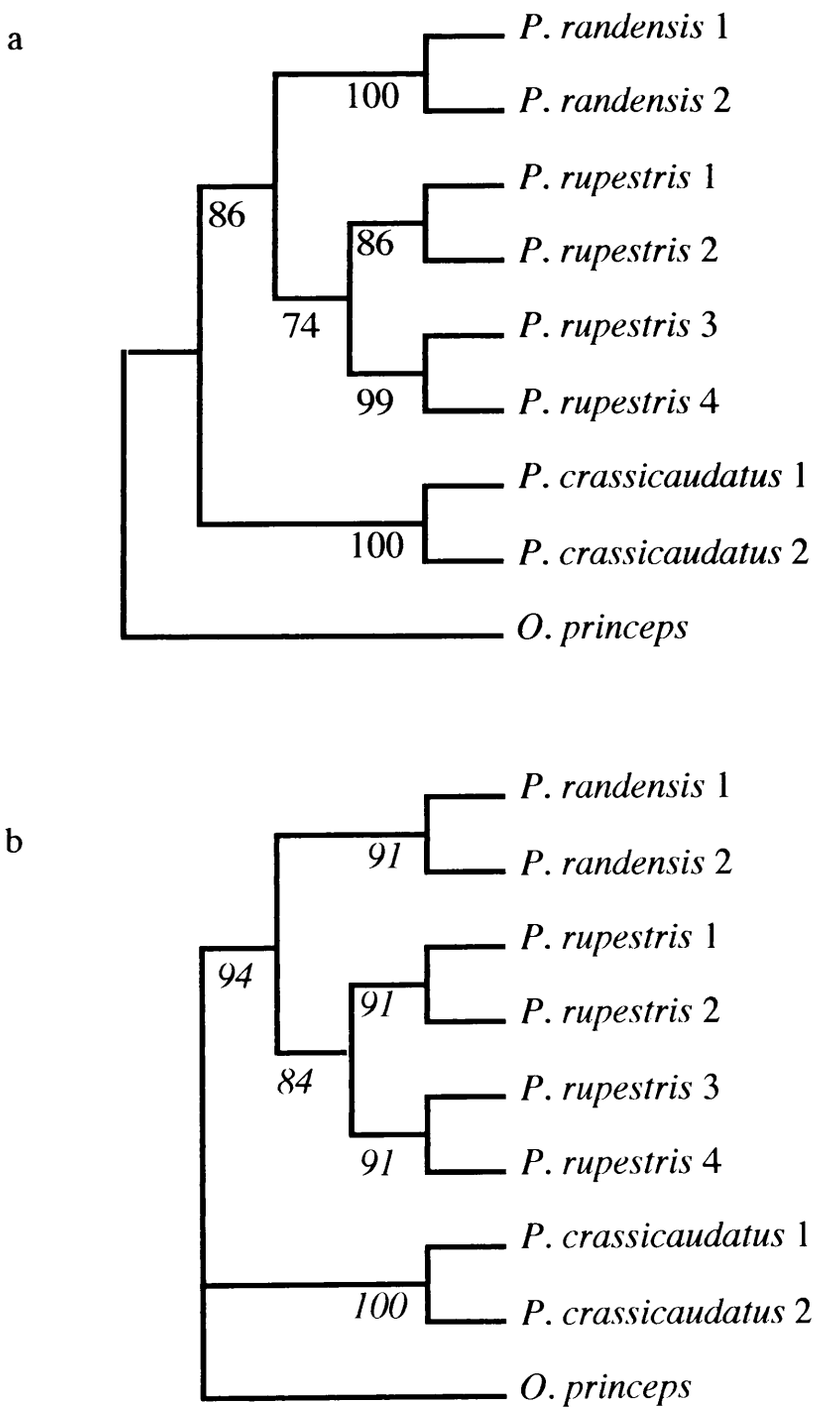


Figure 2.4 Phylogenetic analysis of 745 base pairs of 12S rRNA (a) the single most parsimonious tree (CI = 0.827; length = 455) and (b) an unrooted neighbor-joining tree using *Ochotona* as the outgroup to the *Pronolagus* taxa. Percentages of 1000 bootstrap trees in which a node is supported is given below each node.

Table 2.5 Mean sequence divergence values for the cytochrome b and 12S rRNA genes within the various taxa (a) and between the taxa (b).

	Sequence divergence (%)	
	Cytochrome b	**12S rRNA**
(a) Within:		
P. randensis	3.07	1.64
P. rupestris (N)	4.04	1.65
P. rupestris (S)	5.34	3.35
P. crassicaudatus	10.68	4.50
(b) Between		
P. randensis and *P. rupestris* (S)	19.92	7.23
P. randensis and *P. rupestris* (N)	18.85	6.39
P. randensis and *P. crassicaudatus*	18.09	9.11
P. rupestris (S) and *P. rupestris* (N)	14.53	5.09
P. rupestris (S) and *P. crassicaudatus*	22.64	9.73
P. rupestris (N) and *P. crassicaudatus*	18.58	9.09

Table 2.6 Mean sequence divergence values of pairwise comparisons between specimens based on the 12S rRNA gene

	Middelburg	Louis Trichardt	Donkerpoort	Blyderivierspoort	Springbok	Kuruman	Hilton	Waterval Onder
P. randensis 1 Middelburg	0.00	1.64	8.09	6.97	6.82	6.55	10.05	8.50
P. randensis 2 Louis Trichardt		0.00	7.18	6.67	6.23	5.96	9.41	8.50
P. rupestris 1 Donkerpoort			0.00	3.35	5.68	6.00	10.77	10.40
P. rupestris 2 Blyderivierspoort				0.00	3.90	4.78	8.80	8.95
P. rupestris 3 Springbok					0.00	1.65	8.80	8.50
P. rupestris 4 Kuruman						0.00	9.77	9.29
P. crassicaudatus 1 Hilton							0.00	4.50
P. crassicaudatus 2 Waterval Onder								0.00

The lagomorph relationships within the eutherian mammals

The removal of ambiguously aligned 12S rRNA sequences resulted in a truncated data set comprising 622 characters for this analysis (Appendix IV). The possible number of transitions and transversions in these data are 354 and 329 respectively, giving a transition - transversion ratio that approximates 1:1.

Rooted parsimony analysis (with the marsupial, *D. virginiana* specified as outgroup) yielded a single most parsimonious tree (Figure 2.5a). In this tree the lagomorphs cluster with the tree shrews. A strict consensus tree of all trees one step longer than the most parsimonious (seven trees found), showed support only for the bat-whale-hedgehog clade (Figure 2.5b). The g_1 statistic (-0.0079) was not significant at the 1% level (Hillis & Huelsenbeck 1992) showing absence of phylogenetic signal in the data set.

Transversion parsimony produced a single most parsimonious tree in which the elephant shrew and the tree shrew were placed as sister taxa to the lagomorphs (Figure 2.6a). Nine trees were found which were one step longer than the most parsimonious. As with the nucleotide character based topology, a strict consensus tree (Figure 2.6b) showed little support for any suggested relationships, although the rabbit-tree shrew-elephant shrew trichotomy is present. Bootstrap analysis (1000 replicates) revealed that no confidence could be placed in the suggested topology, since all nodes within the eutherian mammals collapsed at the 50% level. The bootstrap consensus tree utilizing transversions revealed 51% support for the rabbit-tree shrew-elephant shrew grouping (Figure 2.7). Distance analysis based on the neighbor-joining algorithm showed little concordance with the parsimony results, although the rabbit-tree shrew-elephant shrew clade is present (Figure 2.8).

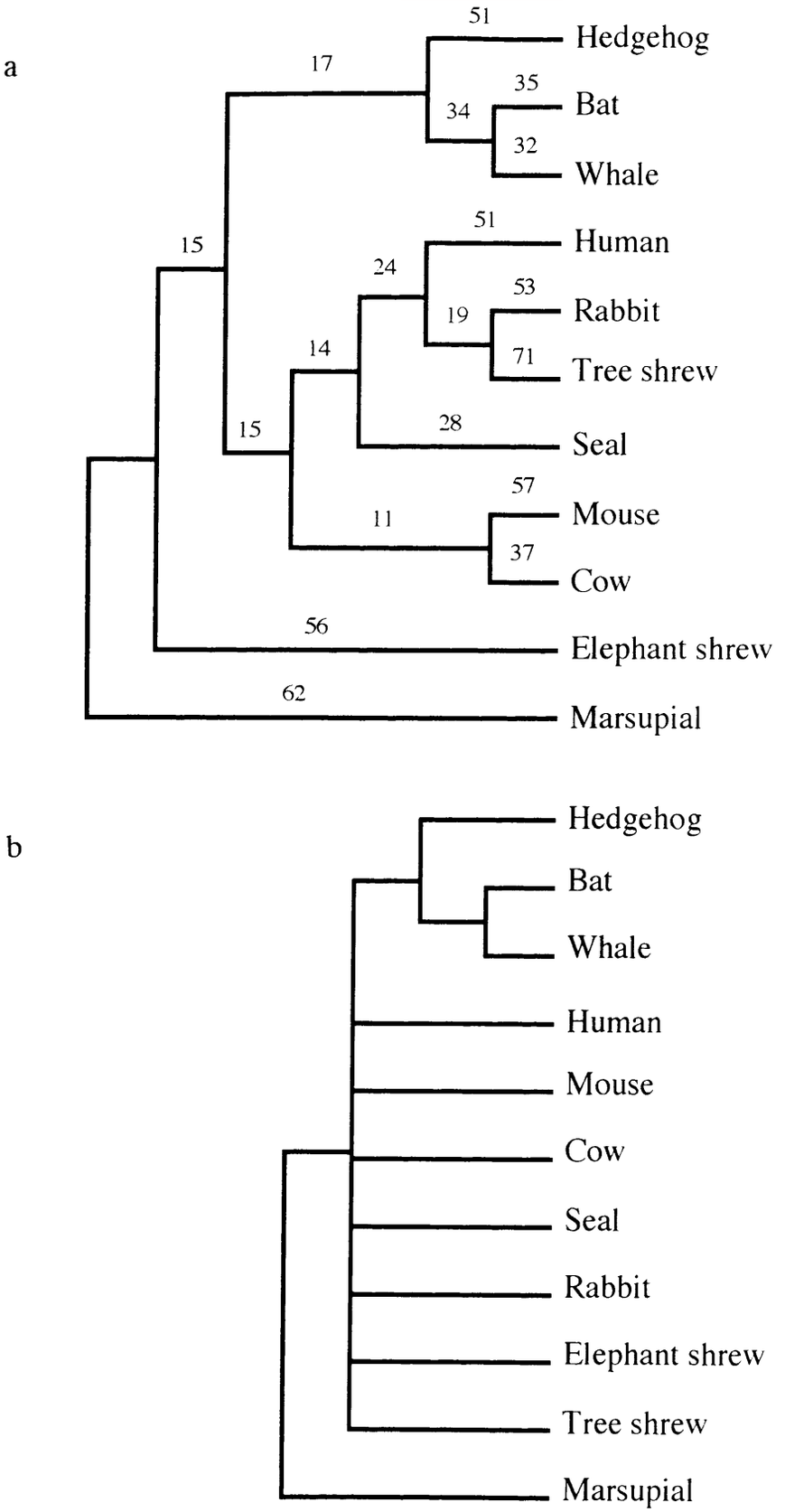


Figure 2.5 Phylogenetic analysis of 622 base pairs of the 12S rRNA gene showing (a) the single most parsimonious tree (CI = 0.614; length = 682) and (b) the strict consensus based on seven trees having ≤ 683 steps using the marsupial as specified outgroup. The number of changes along each branch are indicated in (a).

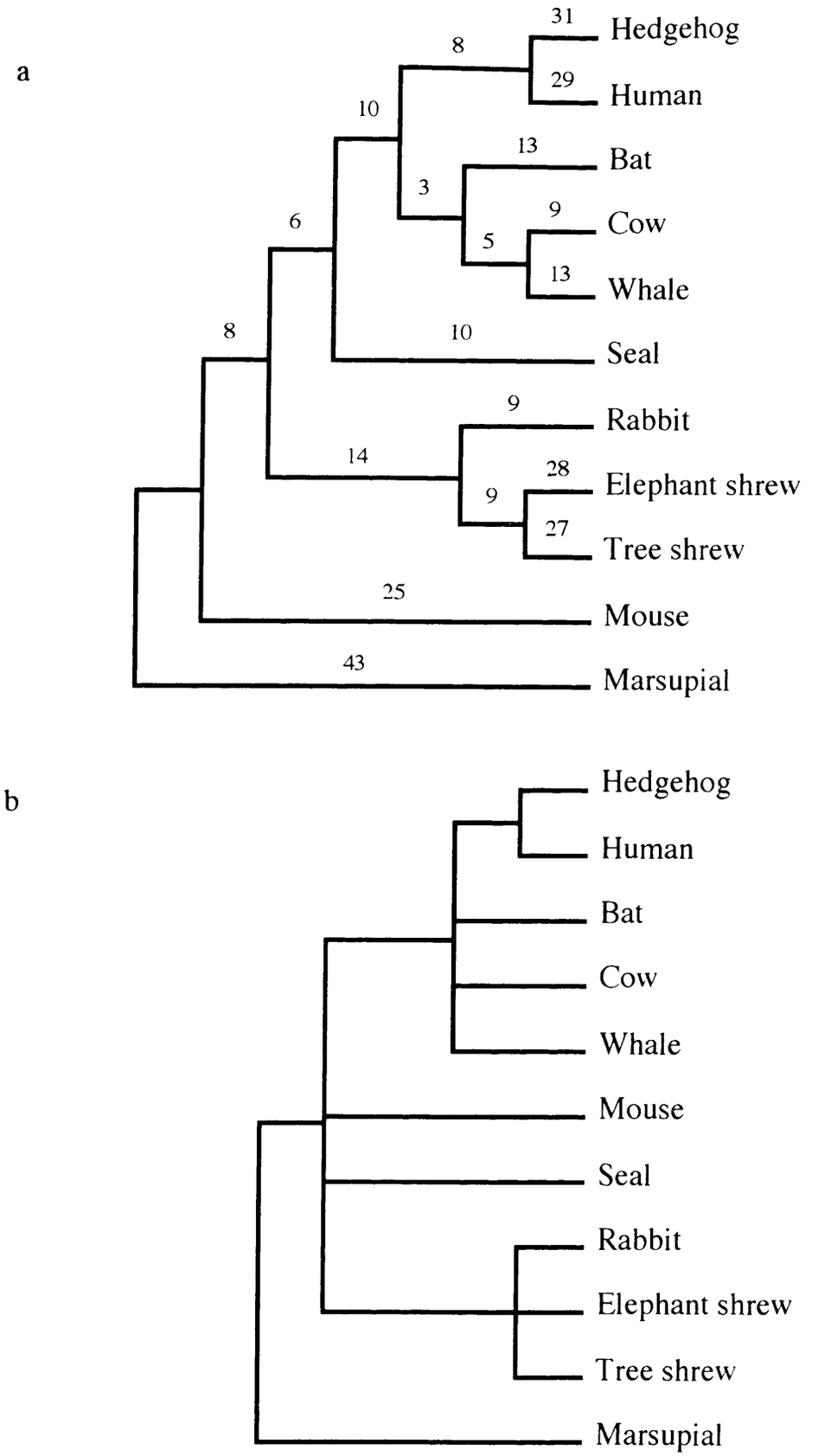


Figure 2.6 Phylogenetic analysis considering transversions only showing (a) the single most parsimonious tree (CI = 0.548; length = 292) and (b) strict consensus tree of nine trees of length ≤ 293 using the marsupial as specified outgroup. The number of changes along each branch length is shown in (a).

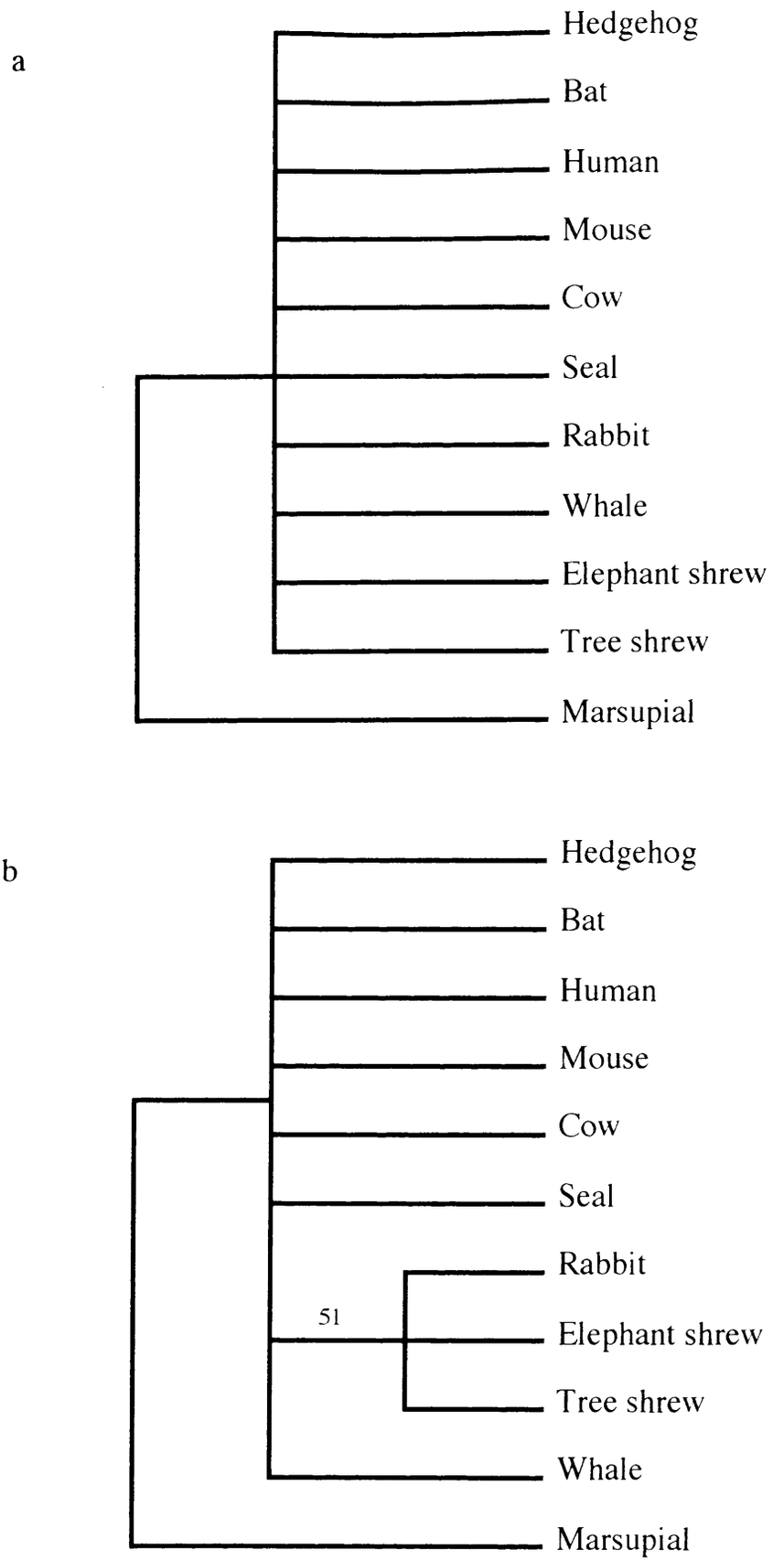


Figure 2.7 Bootstrap 50% majority rule consensus trees (1000 replicates) showing the percentages in which a node is supported utilizing (a) 622 base pairs of the 12S rRNA gene and (b) transversions events only. In both instances the marsupial was specified as outgroup.

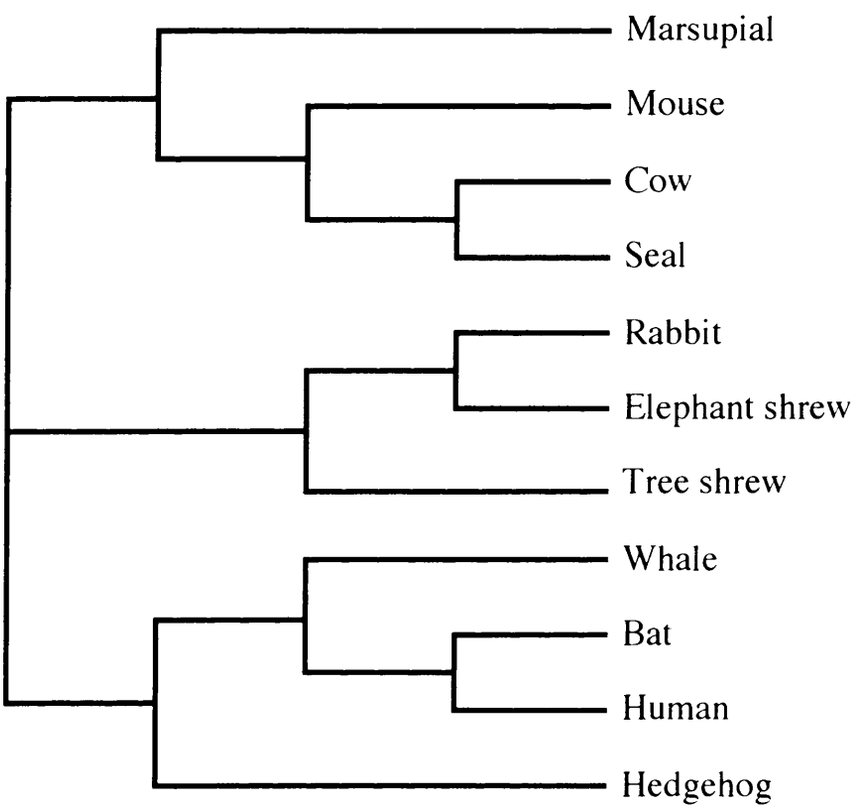


Figure 2.8 Branching order of the eutherian mammals with data weighted by Kimura's Two-parameter model as implemented in DNAdist of PHYLIP 3.4. The unrooted tree is based on 622 unweighted characters of the 12S rRNA gene.

Testing the various lagomorph sister-taxa hypotheses

Trees showing topologies of various lagomorph sister-taxa relationships were tested against the most parsimonious solution (suggesting a lagomorph and tree shrew relationship). The results of these analyses are summarized in Table 2.7. The lagomorph-primate and lagomorph-elephant shrew trees were not found to be significantly better than the lagomorph-tree shrew hypothesis. Table 2.7 shows that a lagomorph-primate and lagomorph-elephant shrew relationship required only one and two additional steps respectively to support their inclusion as sister taxa. This indicates that tree shrews, primates and elephant shrews are more likely sister taxa than the other tested lineages ($P < 0.05$). These results support parsimony analysis which revealed that the number of steps required to achieve sister-taxon affinity with rodents (17 steps), artiodactyls (22 steps) and insectivores (17 steps) is substantially more than required for the elephant shrew and primate solutions (Table 2.7).

The within-gene rate heterogeneity of 12S rRNA

Within-gene 12S rRNA rate heterogeneity was noted for the taxa in the present study and in this respect supports similar observations on other organisms (Attardi 1985; Mindell & Honeycutt 1990). In order to illustrate this, a region of 267 base pairs was selected on the basis that there were no deletions or insertions and that each site showed considerable conservatism across all orders. The interordinal divergence values were substantially lower (range 4.24 - 16.30%) than those calculated for the entire data set (range 15.38 - 28.10%) presumably indicating that these regions are evolving at a slower rate than the 12S rRNA gene as a whole.

Table 2.7 Results of a one-tailed binomial test. The most parsimonious tree is tested against alternate hypotheses and the number of sites supporting each topology, the level of significance and the number of steps required to obtain the proposed alternate topology is presented. An asterisk indicates those hypotheses that are significantly different from the most parsimonious solution.

Most parsimonious tree tested against suggested hypotheses	Number of sites supporting	Significance at 5% level	Additional number of steps
Rabbit - tree shrew	29	0.22*	17
Rabbit - rodent	2		
Rabbit - tree shrew	20	31.35	2
Rabbit - elephant shrew	18		
Rabbit - tree shrew	8	30.36	1
Rabbit - primate	7		
Rabbit - tree shrew	31	0.009	22
Rabbit - artiodactyl	19		
Rabbit - tree shrew	30	2.07	17
Rabbit - insectivore	13		

DISCUSSION

Phylogenetic relationships within Pronolagus

The concordance between the *Pronolagus* RFLP and sequence data sets reveals that phylogenetic analysis of the mitochondrial DNA molecule provides a robust indicator of the taxonomic status within this genus. The mitochondrial DNA sequence analysis supports both the recognition of the previously described *Pronolagus* morphospecies (Robinson & Dippenaar 1983) and the two assemblages apparent within *P. rupestris* (Matthee 1993; Matthee & Robinson submitted). Bootstrap values supporting these nodes were >80% (Figures 2.3 and 2.4).

Of the two genes sequenced however, cytochrome b provided much higher inter-taxon sequence divergence estimates (14.53% - 22.64%) than either those obtained from the RFLP data (7.94%- 9.30%) or the 12S rRNA gene (5.09% - 9.73%). This suggests a higher than average rate of sequence evolution for this gene, a result which is consistent with previously observed mammalian cytochrome b sequence variation (Irwin *et al* 1991; Geffen *et al* 1992). The inter-taxon cytochrome b sequence divergence values were, in most instances, larger than 15% (Table 2.5) and are therefore presumably subject to the increasing saturation of variable sites (Brown *et al* 1979; Mishler *et al* 1988). The inability to empirically observe these multiple substitutions leads to an underestimate of the evolutionary distance. While corrections can be made, it is not possible to accurately take into account rate-evolution among sites, nor to estimate the number of variable sites in the sequence (Nei 1991). The increase in multiple substitutions similarly leads to elevated rates of homoplasy which would be phylogenetically misleading. Therefore, patterns of relatedness inferred from sequence divergence values were based on the 12S rRNA gene.

The observed sequence divergence estimates for the 12S rRNA gene (5.09% - 9.73%; Table 2.5) within *Pronolagus* are, when compared to similar studies, nonetheless substantial. For example, values of 5.6 - 8.0% have been recorded for subfamily comparisons within the Cervidae (Miyamoto *et al* 1990) and an average inter-generic relationships of 16.8% within the family Bathyergidae has been reported (Allard and Honeycutt 1992). The relatively high sequence divergence values for the *Pronolagus* 12S rRNA gene mirror the restriction enzyme

divergence estimates (7.94% - 9.30%) which, on comparison to RFLP estimates for other taxa (Avise *et al* 1983; Cronin 1991), are similarly elevated (Matthee & Robinson submitted). These levels of divergence reflect either an elevated substitution rate for the *Pronolagus* 12S rRNA gene or an ancient origin for these particularly lineages. Elevated rates of molecular evolution have been postulated for small mammals with short generation times, including lagomorphs (Li *et al* 1990), while Grillitsch *et al* (1992) noted a similar trend based on allozyme evolution in lagomorphs. However, the second hypothesis cannot be discounted since Hibbard (1963) has suggested that *Pronolagus* represents an early leporine stock which now exists as evolutionary relic.

The accumulation of evolutionary change through mutation has led to the assumption that if mutation rate is constant over time, the number of nucleotide changes between two sequences (or lineages) will be proportional to time since the divergence of those two lineages (Harrison 1991). This assumption has been used to calculate the relative ages of various taxonomic groups (Avise & Nelson 1989; Thomas *et al* 1989; Hasegawa & Horai 1991; Saccone *et al* 1991; Avise 1992; Arnason & Gullberg 1993). Many objections have, however, been raised to the validity of a molecular clock. The variability of nucleotide substitution rates among genes, already noted for the two genes used in this study would, of necessity, mean that a rate of change would have to be calibrated separately for each gene investigated. There are however complications. First, the longer the portion of DNA being compared, even within a gene, the greater the possibility of finding heterogeneous substitutions rates (Takahata & Satta 1992; Saccone *et al* 1991; Irwin *et al* 1991; Mindell & Honeycutt 1990). Second, as the amount of divergence between two lineages increases, the probability of multiple substitutions occurring at the same nucleotide position similarly increases and, being undetected, divergence time will consequently be underestimated (Hasegawa *et al* 1993). This can be partially addressed by the incorporation of a correction factor. However, the plethora of corrections available in the literature (Brown *et al* 1982; Schöniger & von Haesler 1993; Tajima 1993), all of which may potentially generate different divergence estimates from the same sequence comparison, presents a dilemma as to which would be more appropriate under a given set of circumstances. Third, considerable rate heterogeneity exists between base substitutions that are synonymous (amino acid remains the same) or nonsynonymous (amino

acid replaced by another), as well as transversion and transition events. DNA sequences can thus be said to display several molecular clocks, each with a characteristic rate (Harrison 1991).

The universality of the molecular clock has further been criticised by the variation in nucleotide substitution rate among evolutionary lineages (Britten 1986; Avise *et al* 1988; Hasegawa *et al* 1990; Li *et al* 1990; Holmes 1991). Although many genes may display a fairly uniform substitution rate over 50-100 million years, it is feasible that the rate could be altered as a result of changing functional constraints within lineages (Wilson *et al* 1987). Given these considerations no attempt has been made to date the divergences in the present study. Instead the values are used merely as an indicator of variation within and between taxa.

The sequence divergences within the south-eastern *P. rupestris* clade and those within *P. crassicaudatus* are considerably higher than for the other two taxa (Table 2.6). In terms of *P. rupestris*, this supports the RFLP data (Matthee and Robinson submitted) which revealed the south-eastern clade to be characterized by deep divisions between populations, whereas those of the north-western lineages were separated by relatively few mutational steps. Although limited by small sample size, the high sequence divergence value observed for *P. crassicaudatus* (4.50%) may similarly reflect pronounced genetic structuring within this species. Interestingly, *P. crassicaudatus* is placed basally in all tree topologies (Figures 2.3 and 2.4) and is the most genetically distinct of the three recognised species on the sequence data. Clearly, whether this indicates *P. crassicaudatus* to be ancestral to other *Pronolagus* species or whether it has been subject to more intensive selective pressures, is moot.

The lagomorph relationships within the eutherian mammals

The parsimony and neighbor-joining tree topologies (Figures 2.5 - 2.8) suggest that the sister taxa of lagomorphs is to be found among the primates, tree shrews and elephant shrews, a result which is similarly reflected in the winning sites test. Although all three these sister taxa have not previously been included in a single analysis, these relationships have been suggested by previous molecular analyses (tree shrew - Dene *et al* 1982; primates and tree

shrew - Goodman *et al* 1985; elephant shrews - Novacek & Wyss 1986; primates - Easteal 1990; primates - Springer & Kirsch 1994). Contrary to many morphology based phylogenies however, the rodents are not well supported as a sister taxon by taxonomies based on molecular data (Chuan-Kuei *et al* 1987; Novacek 1992).

The lack of statistical support for the phylogenies presented herein, together with the obvious lack of concordance between them, must weaken any phylogenetic conclusions. Although the 12S rRNA gene is highly conserved in certain regions across all the compared orders (including Marsupialia; see Appendix IV), it is obvious that the nucleotide sites in the remaining regions have become randomized through multiple substitutions as evidenced by the transition/transversion ratio of 1:1. The high level of homoplasy present in the data set is further underscored by the weakness of nodes in trees one step longer than the most parsimonious tree and the absence of bootstrap support for them (Figures 2.5 - 2.7). The short internal branch lengths indicate the lack of informative sites between the orders, with most of the variation being specific to orders as evidenced by long terminal branch lengths (Figures 2.5 and 2.6). In fact, in light of the rapid radiation of the Mammalia (Benton 1990), it may not be possible to resolve branching orders using molecular data precisely because of short internal nodes and long terminal branches (Baker *et al* 1991). This loss of phylogenetic signal is further indicated by the nonsignificant g_1 statistic which shows that the data is not significantly skewed from that which may be expected from random sequences.

Given these results several generalizations may be made. First, the 12S rRNA gene does not appear to provide adequate resolution for delineating the interordinal relationships of mammals. The lack of support for eutherian mammal relationships in this study echoes that found in previous analyses (Ammerman & Hillis 1992; Springer & Kirsch 1993). However, even with the lack of significant bootstrap support for critical nodes in the trees generated by these studies, the authors' nonetheless conclude that 12S rRNA data retains significant signal to determine relationships among eutherian mammals. Bootstrapping is certainly not the only way in which the confidence in a phylogeny can be assessed, but the persistent collapsing of nodes in eutherian mammal phylogeny, based on varying lengths of the 12S rRNA gene, is clearly problematic. This suggests that, the phylogenies obtained from 12S rRNA data are, in essence, not sufficiently robust to allow for a rigorous assessment of mammalian evolutionary relationships.

CHAPTER 3

COMPARATIVE CYTOGENETICS

Diploid chromosome numbers vary considerably in mammals ranging from 2n = 6 in the Indian muntjac (*Muntiacus muntjak*; Hsu 1979) to 2n = 102 in a South American rodent, *Tympanoctomys barrerae* (Qumsiyeh 1994). The mammalian modal number has been calculated at 2n = 48 (Imai & Crozier 1980). The suggestion of an ancestral mammalian karyotype and the considerable range in diploid number has given rise to various hypotheses regarding chromosomal change: the fusion, fission and modal hypotheses set the ancestral diploid number at 2n ≈ 80, 2n = 6 - 14 or close to the present mode (Imai & Crozier 1980; Qumsiyeh & Baker 1988; Qumsiyeh 1994). However, it seems probable that both increases and decreases in diploid numbers have characterized mammalian chromosomal evolution, and that this occurred independently of the initial chromosome constitution of the lineage concerned (Qumsiyeh 1994).

The fission and fusion events which result in changes in chromosome number, together with varying arm lengths attributable to inversions have been used as crude indicators of the rate of chromosomal evolution, and hence the rate of organismal evolution (Wilson *et al* 1975; Bush *et al* 1977). The criticism levelled at this approach of estimating chromosomal evolutionary rates and associated speciation patterns, concerns its failure to take into account paracentric inversions, interstitial additions and deletions of chromatin which would not be observable by merely counting the number of chromosomes and chromosome arms (King 1993). For example, two populations of the creeping vole (*Microtus oregani*) were found to be identical in chromosome numbers, but differed in the banding patterns of the X chromosome (Libbus & Johnson 1988), the implication being that chromosome number alone would not have indicated the genetic variation between these populations. The use of chromosome banding patterns, on the other hand, allows individual chromosomes to be identified (establishing homology) and indicates domains which may have functional and structural significance (Bickmore & Sumner 1989). The variation in banding patterns between

organisms, or lack thereof, is of major importance in clarifying both chromosomal and organismal evolution, and can be used to compare individuals, populations, species, genera and even higher taxonomic categories (Qumsiyeh & Baker 1988; Qumsiyeh *et al* 1990).

The near-universal existence of karyotypic differences not only between the various mammalian orders, but also of related species poses a further dilemma: the role of chromosomal rearrangements in speciation. However, whether chromosomal rearrangements are causative agents or results of speciation remains controversial (Patterson 1978; Baker & Bickham 1986; Robinson & Roux 1986; King 1993). There does, nonetheless, appear to be a correlation between chromosome reorganization and species diversity (King 1987). Various authors (Arnason 1972; Wilson *et al* 1975; White 1973) have supported the idea that the extent of chromosomal variation in different groups may be a reflection of their life history patterns, for simplicity referred to here as r - and K - selected species. K - selected populations, usually of large size, low reproductive rate (late sexual maturity and small litter size), prolonged parental care, good mobility and a stable environment (Begon *et al* 1986) show karyotypic stability (Arnason 1972). On the other hand, r - selected populations which have opposing characteristics such as smaller size, restricted mobility, earlier sexual maturity, high mortality rates and occupation of unpredictable habitats (Begon *et al* 1986) tend to show extensive karyotypic variation (Arnason 1972; White 1973). The conditions under which a newly arisen chromosomal rearrangement becomes fixed in a popluation is limited, particularly if the population is K - selected. r - Selected populations by contrast, are more likely to form small isolated groups which have a higher probability of inbreeding which, in turn, could lead to fixation of chromosomal rearrangements (Wilson *et al* 1975; Qumsiyeh 1994).

The lagomorphs have been karyotypically intensively studied. Comparative cytogenetics supports the classic delineation of the ochotonids and leporids as distinct lineages within the Lagomorpha with centric fusions being the most important mechanism of karyotypic change in the order (Stock 1976). Within the Leporidae, considerable structural homology between karyotypes reveal a conserved organization of genetic material (Robinson 1979). In contrast, the amount of heterochromatin present varies extensively between the genera, from the least amount in *Oryctolagus*, to significant amounts in *Sylvilagus*, Ochotona and, to a lesser extent, *Pronolagus* (Stock 1976; Robinson *et al* 1981; Robinson *et al* 1983a).

The Leporidae are naturally divided into two groups: the hares (precocial young) and the rabbits (altricial young). Of these, rabbits (*Pronolagus*, *Romerolagus*, *Oryctolagus*, *Sylvilagus* and *Bunolagus*) show greater karyotypic diversity with diploid numbers ranging from 2n = 38 (*S. palustris*) to 2n= 52 (*S. transitionalis*; Robinson *et al* 1981; Robinson *et al* 1983a) while hares, genus *Lepus*, are remarkably uniform in chromosome complement with all species characterized by 2n = 48 (Robinson *et al* 1983). This variation in karyotype numbers in rabbits is thought to be attributable to differences in life history patterns. The speciose hares (29 species; Flux & Angermann 1990) are wide ranging with loose social structures whilst rabbits show greater social structure and may be restricted to certain habitats which would lead to population isolation and a greater possibility of chromosomal mutations becoming fixed within populations (Van der Loo *et al* 1979; Robinson *et al* 1983b). Of the rabbits, *Pronolagus* and *Sylvilagus* are the only two genera which are not monotypic, yet *Pronolagus* is characterized by a consistency in chromosome morphology and number (2n = 42; Robinson 1980) whilst *Sylvilagus* displays a range in diploid number (2n = 38 to 2n = 52; Ruedas *et al* 1989). Despite apparent karyotypic uniformity, both in terms of G - and C - banding patterns within *Pronolagus*, it was hoped that a closer examination of the chromosomal morphology of the two *P. rupestris* mitochondrial DNA clades may reveal the presence of subtle differences between them.

MATERIALS

Ear clippings were collected from specimens representing both the *P. rupestris* mitochondrial clades (see Table 2.1, p. 18). Both sexes were sampled at Bloemfontein, Boshof and Fauresmith representative of the north-western clade and at Volksrust, a south-eastern clade locality (coordinates for localities are given in Appendix V).

The portion of the ear to be biopsied was vigorously cleaned with 70% ethanol to remove sources of contamination in culture. Using sterile instruments, an approximately $1cm^2$ clipping was removed, the 70% ethanol wash repeated and the tissue placed in a McCartney bottle containing McCoy's medium (Highveld Biological) supplemented with 15% foetal calf serum (Highveld Biological). All sampled specimens were screened with restriction enzymes to ascertain their clade designation (see Chapter 2).

METHODOLOGY

Culture of Fibroblast Cells

All procedures follow standard protocols (Schwarzacher & Wolf 1974). To confirm absence of contamination, fresh medium was added to the samples which were subsequently incubated at 37°C for 24 to 48 hr. If free of contamination, the material was minced and placed into two 25cm^2 tissue culture flasks per specimen together with 1.5ml culture medium. Incubation was carried out at 37°C with 5% ambient CO_2.

Once substantial fibroblast growth was observed in the flask, the cells were washed with Phosphate Buffered Saline (PBS^-), followed by brief trypsinization to loosen cells which were then resuspended in 5ml medium and allowed to spread evenly in the flask thus encouraging growth. Routine subculturing proceeded once the cells were confluent (Schwarzacher & Wolf 1974).

Harvesting and slide preparation

Cell cultures in the logarithmic growth phase were harvested for cytogenetic analysis. Three hours before harvesting, colcimid (Gibco) was added (0.4 ug/ul) in order to arrest the cells in metaphase. After removal of cells by trypsinization, the cells were resuspended in a hypotonic solution (0.075M KCl) for 20 min which was followed by three changes of 1:3 acetic acid-methanol in order to fix the cells for microscopic examination.

Three to four drops of the acetic acid/methanol cell solution was spread onto cold slides covered by a thin film of water. To reduce the effects of cytoplasm slides were subsequently dried in a humid environment at 37°C. Once dried, the slides were boxed and incubated at 90°C for 20 minutes to harden the DNA for banding by trypsin.

G - banding

G - bands correspond to chromomeres (Comings 1978; Bickmore & Sumner 1989) with positive G - bands comprising regions that are A+T rich and, which condense early but

replicate late in the cell cycle. Negative G - bands are G+C rich on the other hand, condense late but replicate early in the cell cycle. The former category contains few genes but has long interspersed repetitive DNA sequences while the G - negative regions are thought to include both housekeeping and tissue-specific genes as well as short interspersed repetitive sequences (Bickmore & Sumner 1989). The protocol followed for enzymatic G - banding is that of Wang and Fedoroff (1972). Enzymatically treated chromosomes tend to be swollen and distorted, the bands, however, are crisp as they appear to be compressed by less stained, swollen areas (Schwarzacher & Wolf 1974).

C - banding

C - banding preferentially stains constitutive heterochromatin which comprises the late replicating regions of chromosomes and is usually associated with centromeres and telomeres in eukaryote chromosomes (Pardue & Hennig 1990). C - bands were visualized following Sumner (1972).

Silver staining

The nucleolar organizers are differentiated regions of DNA where ribosomal RNA synthesis and ribosome assembly occurs. These regions appear to be the sites where the specific binding of unfinished ribosome precursors occurs, consequently the more ribosomal RNA present, the darker the stain (Swanson *et al* 1981; Alberts *et al* 1983). Staining of active nucleolar organizers was achieved followed Goodpasture and Bloom (1975).

Preparation of karyotypes

Photomicrographs of high quality metaphase cells were taken using a 63 X oil immersion lens. In the case of G-banding, karyotypes were constructed from specimens representative of each of the mitochondrial DNA clades with chromosomes arranged according to the *Pronolagus* standard (Robinson 1980). A final composite karyotype, comprising a haploid set of chromosomes from each clade was constructed in order to detect possible subtle inter-band differences between the clades.

RESULTS

The diploid number, irrespective of clade affiliation, was 2n = 42 for all *Pronolagus rupestris* specimens analysed. No variation was apparent between the G - banded karyotypes of the specimens representing the mitochondrial DNA clades, a comparison of which is presented in Figure 3.1. Unfortunately, attempts to obtain fibroblast cell growth in several male specimens of the north-western clade proved unsuccessful, precluding comparisons of the Y - chromosome. The autosomal chromosome complement for specimens of both clades comprised 11 metacentric or submetacentric pairs (1 - 11; Figure 3.1) and nine subtelocentric or acrocentric autosomes (12 - 20; Figure 3.1). The X chromosome is submetacentric and the Y chromosome (in the case of the south-eastern clade), is telocentric. These results confirm earlier studies on representatives of the genus (Robinson 1980; Robinson 1982).

The amount and distribution of constitutive heterochromatin was invariant in the representiatives of the two *Pronolagus* mitochondrial DNA clades studied (Figure 3.2). As with the majority of mammals, the heterochromatin was centromeric in location. Counts of the number of NORs observed per cell varied quite considerably probably due to variation in the amount of ribosomal RNA present at these sites and suboptimal staining. Despite this variation, it was concluded that in *P. rupestris* five chromosome pairs bear nucleolar organizers which, in all instances, were terminally situated (Figure 3.3). No attempt was made to identify the NOR bearing chromosomes since Q - banding could not be performed and sequential G - banding/silver staining is unsuccessful.

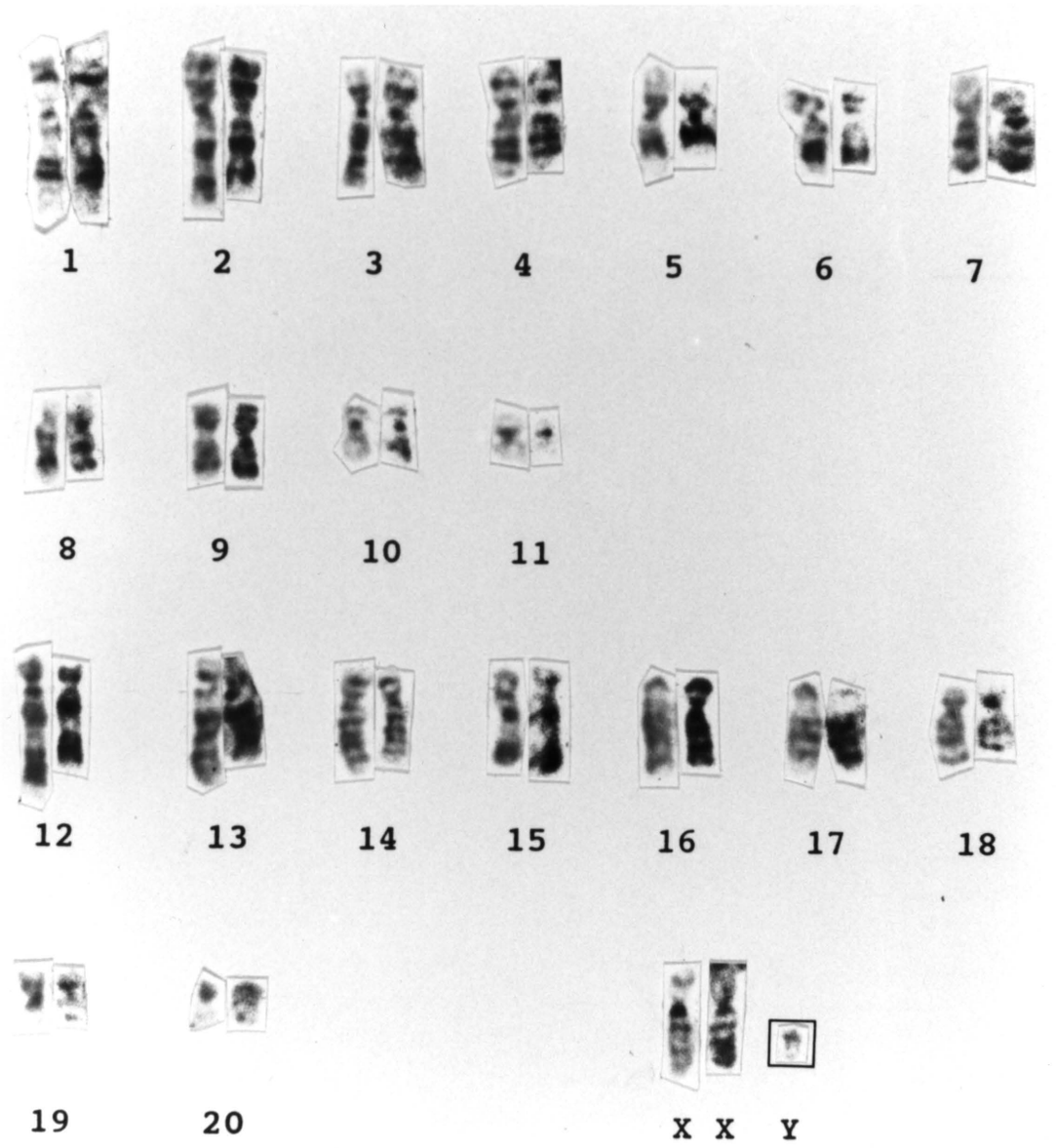


Figure 3.1 Comparison of the G - banded chromosomes of specimens of the *P. rupestris* south-eastern and north-western mitochondrial DNA clades. The first chromosome of each pair corresponds to the south-eastern clade and the second, to the north-western. The insert displays the Y - chromosome of a south-eastern clade specimen. The format of presentation follows the *P. rupestris* standard (Robinson 1979).

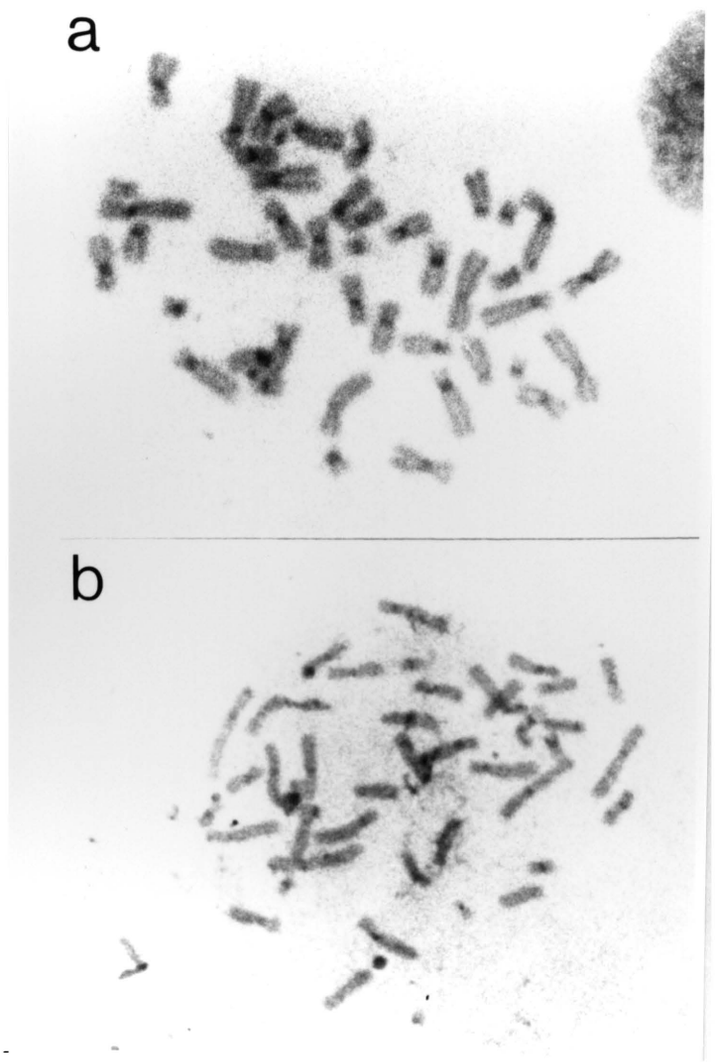


Figure 3.2 Representative C - banded metaphase chromosomes of (a) *P. rupestris* from the south-eastern clade and (b) *P. rupestris* from the north-western clade.

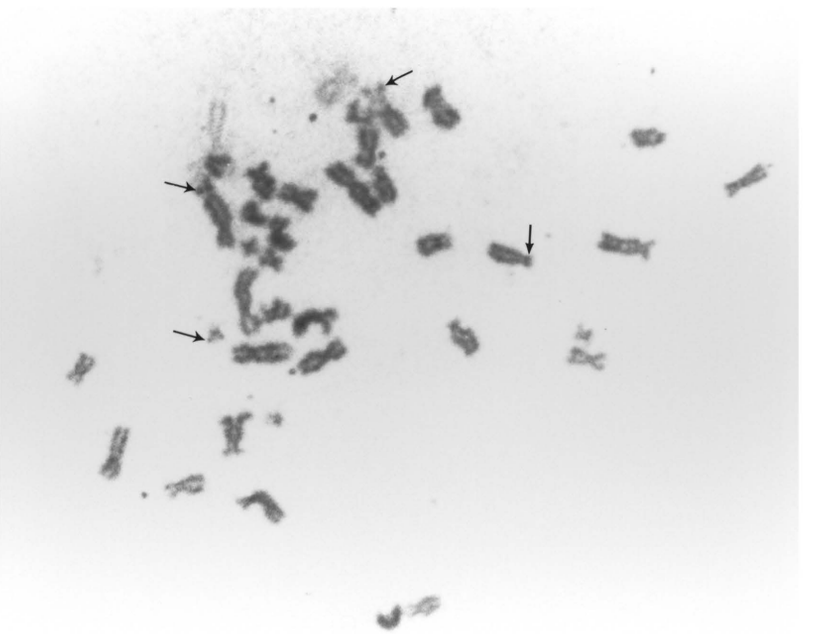


Figure 3.3: Silver stained chromosomes showing certain of the terminally located NOR regions (indicated by arrows).

DISCUSSION

From these results and published data (Robinson 1982) it is clear that *Pronolagus* shows no karyotypic variation either in terms of chromosomal number (consistently 2n = 42), G - or C - banding patterns. Additionally, the number of NORs (n = 10) in the genomes of the two mitochondrial DNA clades of *P. rupestris* was invariant. The taxa delimited within *P. rupestris*, whilst easily discernable at molecular (Matthee & Robinson 1994; Chapter 2) and morphological (Chapter 4) levels, do not appear to have undergone concurrent chromosomal evolution. The lack of variation within *P. rupestris* indicates the limitation of this parameter for the delineation of the taxa within this species.

CHAPTER 4

MORPHOMETRICS

Morphometric analysis is frequently used in association with other techniques such as karyotypic and allozymic data to determine the degree of concordance between data sets (Davis & Baker 1974; Schmidly *et al* 1988; Dowling *et al* 1989; Gallardo & Reise 1992; Hafner 1992). It is generally held that the level of congruence will, in turn, determine the degree of confidence that can be placed in the resultant taxonomy (Hillis *et al* 1993).

Morphometric analysis allows for the quantitative description, analysis, and interpretation of shape-related variation in organisms. The variation observed between taxa based on mensural characters are of importance to ecological, genetic, physiological and taxonomic studies, since this variation may be reflected in life history patterns (Somers 1986).

Unlike biochemical or karyotypic techniques, morphometric procedures allow the use of museum specimens which, apart from accessibility, are often geographically representative (Hillis 1987). Morphometrics also allows for the assessment of a reasonable portion of the phenotype. Cranial development and morphology for instance, results from the integrated expression of many nuclear genes, whereas individual phenotypic characters may reflect only one aspect of the evolutionary path of the organisms being considered. Morphometrics may therefore provide a more holistic view of evolutionary development (Taylor *et al* 1977; Cheverud 1982). Of additional importance, is that useful comparisons depend on the principle of homology. Whilst molecular techniques may appear more sophisticated, molecular homologies are no more secure than those based on morphology. Indeed, in cranial comparisons there is a fair degree of confidence that true homologues are, at least, being compared (Patterson 1987).

Morphometric analyses have proved to be particularly useful in distinguishing lagomorph species within the genera *Lepus* (Robinson & Dippenaar 1987; Palacios 1989) and *Pronolagus* (Robinson & Dippenaar 1983) as well as the identification and description of a new

Sylvilagus species, *S, obscurus* (Chapman *et al* 1992). At the infraspecific level, the technique has also been used successfully in the analysis of geographic variation within *Lepus arcticus* (Baker *et al* 1978) and *Oryctolagus cuniculus* (Taylor *et al* 1977).

MATERIALS

Most critical to the effective use of morphometrics is the need for sufficient specimens from each locality to allow for local variation as well as good geographic coverage of the area in question (Thorpe 1976). This aspect of the study is therefore based on 173 specimens of *P. rupestris* (29 of which were of known mitochondrial DNA affiliation) from 45 localities in South Africa (Figure 4.1; Appendix V). The samples provided excellent geographic coverage of the species in South Africa. For the purposes of assessing the phenetic integrity of *P. rupestris*, 25 additional specimens of *P. randensis* and 28 specimens of *P. crassicaudatus* were also examined.

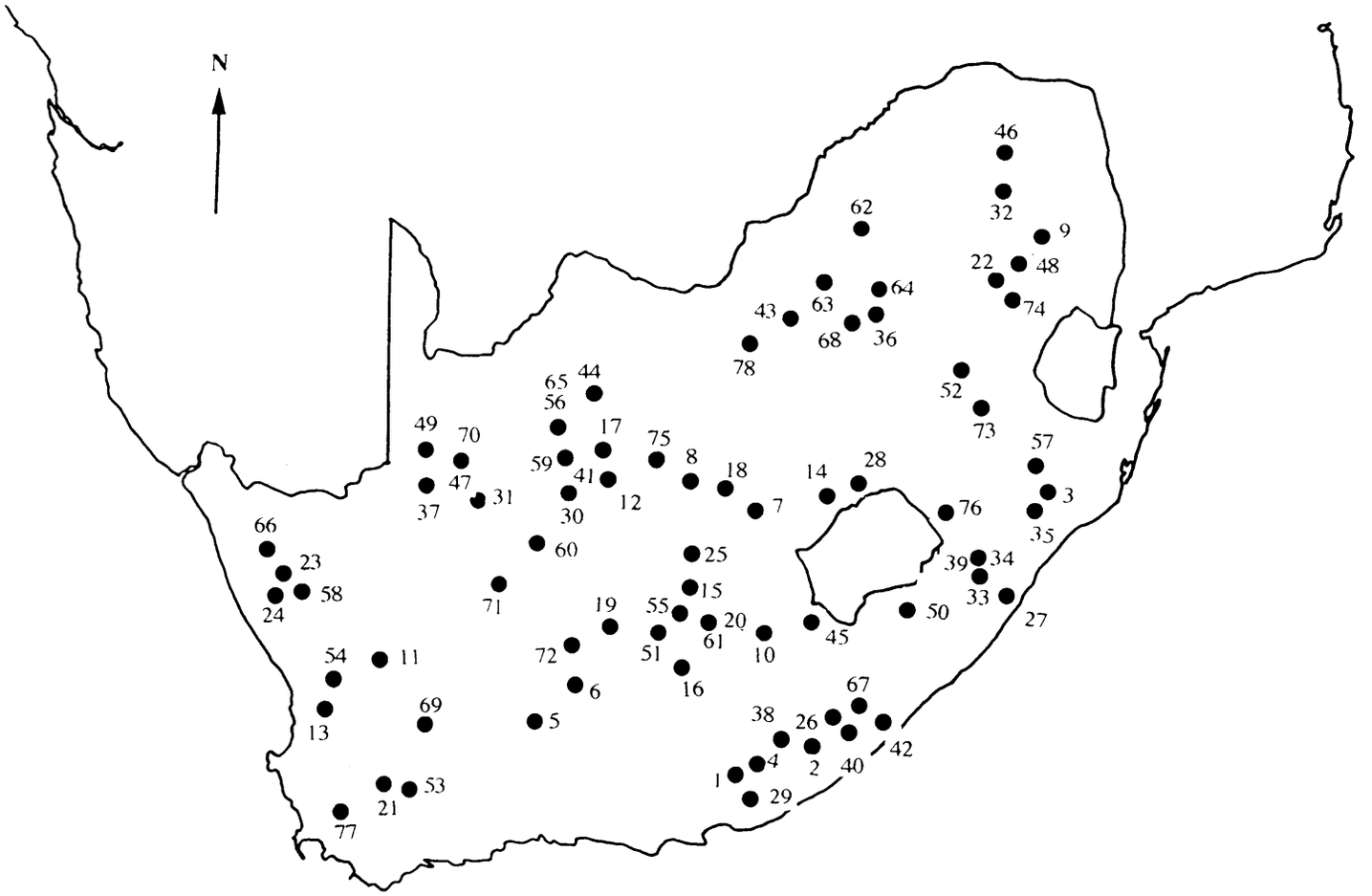


Figure 4.1 *Pronolagus* sampling localities. Locality codes are as follows: 1 - Albany district*; 2 - Amabele; 3 - Babanango; 4 - Bathurst; 5 - Beaufort West*; 6 - Biesiespoort*; 7 - Bloemfontein*; 8 - Boshof*; 9 - Bourke's Luck*; 10 - Burgersdorp; 11 - Calvinia*; 12 - Campbell*; 13 - Clanwilliam*; 14 - Clocolan*; 15 - Colesberg*; 16 - Cradock; 17 - Daniëlskuil*; 18 - Dealesville*; 19 - Deelfontein*; 20 - Donkerpoort*; 21 - Doornrivier*; 22 - Dullstroom; 23 - Eenriet*; 24 - Ezelfontein*; 25 - Fauresmith*; 26 - Fort Pato; 27 - Gillits; 28 - Golden Gate National Park*; 29 - Grahamstown*; 30 - Griekwastad*; 31 - Groblershoop; 32 - Haenertsburg; 33 - Hilton; 34 - Howick*; 35 - Itala; 36 - Johannesburg; 37 - Kakamas*; 38 - Kieskammahoek; 39 - Kilgobbin; 40 - King William's Town; 41 - Klipfontein; 42 - Komga; 43 - Koster; 44 - Kuruman*; 45 - Lady Grey*; 46 - Louis Trichardt; 47 - Louisvale*; 48 - Lydenburg*; 49 - Lutzputz*; 50 - Matatiele*; 51 - Middelburg, C.P.*; 52 - Middelburg, Tvl; 53 - Middlepost; 54 - Nieuwoudtville*; 55 - Noupoort*; 56 - Olifantshoek*; 57 - Pafuri; 58 - Platbakkies*; 59 - Postmasberg*; 60 - Prieska*; 61 - Rolfontein; 62 - Rooikrantz; 63 - Rustenburg; 64 - Silverton; 65 - Sishen*; 66 - Springbok*; 67 - Stutterheim; 68 - Suikerbosrand; 69 - Sutherland*; 70 - Upington*; 71 - Vanwyksvlei*; 72 - Victoria West*; 73 - Volksrust*; 74 - Waterval Onder*; 75 - Windsorton*; 76 - Winterton*; 77 - Wolesley; 78 - Zeerust. The asterisk indicates those localities used in the final multivariate analysis (Locality coordinates presented in Appendix V).

METHODOLOGY

Mensural characters

Twenty eight cranial measurements were recorded to the nearest 0.01mm using Mitutuyo digital callipers. These measurements were adopted from Robinson and Dippenaar (1983) and are defined and illustrated in Figure 4.2. To reduce the effect of age variation, measurements and analyses were based on adult specimens identified by the closure of the exoccipital and supraoccipital bones (Hoffmeister & Zimmerman 1967). Sexes were pooled due to the lack of sexual dimorphism within the genus (Robinson & Dippenaar 1983).

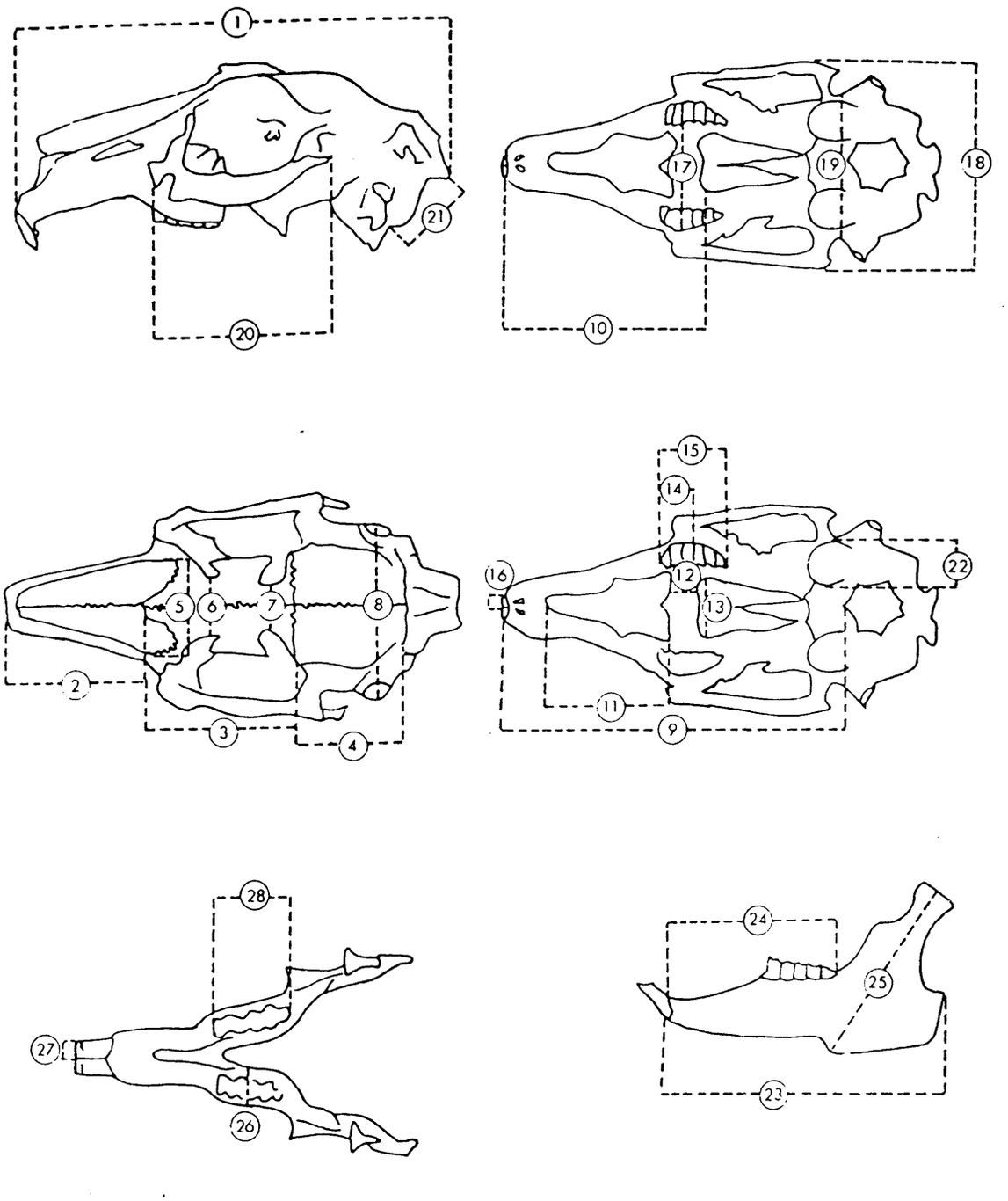


Figure 4.2 Reference points of cranial measurements: 1 = total length of skull; 2 = muzzle length; 3 = frontal length; 4 = parietal length; 5 = posterior muzzle breadth; 6 = anterior frontal breadth; 7 = posterior frontal breadth; 8 = interauditory breadth; 9 = basal cranial length; 10 = palate incisor length; 11 = palatal vacuity length; 12 = hard palate length; 13 = mesopterygoid space; 14 = maxillary premolar length; 15 = maxillary premolar-molar length; 16 = principal I^1 breadth; 17 = palatal breadth between p^2 and p^3; 18 = bizygomatic breadth; 19 = maximum cranial breadth; 20 = zygomatic arch length; 21 = posterior cranial height 22 = bulla breadth; 23 = maximum mandibular length; 24 = mandibular toothrow length; 25 = maximum mandibular height; 26 = mandibular body breadth; 27 = I_1 breadth; 28 = mandibular premolar-molar length (Robinson & Dippenaar 1983).

Analytical procedure

The morphological characters were specifically selected to examine cranial size and shape differences. Multivariate analyses provide information about objects where there is more than one variable (for example, size and shape of skulls). The relationship between skulls can therefore be determined by ordination which is the reduction of a matrix of distances, or similarities, among variables to a few dimensions (James & McCulloch 1990). This can be achieved by Principal Component Analysis (PCA) where eigen structure analysis of a correlation matrix, or a variance-covariance matrix among attributes, is performed.

PCA requires no formal assumptions about groups and is in this respect both a type of multidimensional scaling (James & McCulloch 1990) and a technique which ordinates ungrouped Operational Taxonomic Units (OTUs; Thorpe 1976). Since the advantage of PCA is that there is no prior assumption of groups, subsequently allowing for their discovery (Humphries *et al* 1981), PCA has been the main thrust of the multivariate analysis used in this revision of *Pronolagus* systematics.

Size may be defined as the coordinated increase or decrease of variables measured, or, alternatively, the magnitude of the variables (Somers 1986). Size variables can be seen as linear combinations which are positively and highly correlated. The most informative size variable can be obtained from the first principal component (Somers 1986; Zelditch *et al* 1989). The principal component axes independent of size can be regarded as reflecting shape variables which infer relationships between two or more characters (Somers 1986). Thus two categories exist when describing shape: (i) shape variation associated with general size and (ii) shape variation independent of general size.

When distinguishing between groups, certain authors (Thorpe 1976; Somers 1986) are of the opinion that size may be regarded as random noise and should be removed from the analysis. Consequently shape has traditionally been considered the more reliable parameter for delimiting groups (Sundberg 1989; Zelditch *et al* 1989). There are however dissenting views (Mosimann & James 1979; Humphries *et al* 1981; Somers 1986). It should be noted that size due to age and allometry does not fall into this category.

Cluster analysis, although viewed to be less efficient than PCA since it is more appropriate for categorical rather than continuous data (James & McCulloch 1990), provides an indication of similarity between OTUs. Discriminant analysis maximizes the existing variability, emphasizing characters that are important in discriminating between defined groups. PCA and the discriminant analysis, were computed using BIOΣTAT2 (Pimental & Smith 1986). Selected subroutines of the Numerical Taxonomy System of Multivariate Statistical Programmes (NT - SYS; Rohlf 1986) were employed for cluster analysis. Standard statistics, including the arithmetic mean, range, standard error, standard deviation and coefficient of variation were computed using UNIVAR (FORTRAN programme developed by Power (1970) and recompiled in PC version by N.J. Dippenaar).

Assessment of phenetic integrity of currently recognized species in South Africa

The taxonomic integrity of the three currently recognized species in South Africa, *P. rupestris*, *P. randensis* and *P. crassicaudatus* were first assessed by PCA and cluster analyses. This step allowed the verification of all museum specimens that have been identified as *P. rupestris*. Furthermore, this analysis was also deemed necessary to ensure that the measurements taken for this study were similarly able to distinguish the three morphospecies that have previously been identified by Robinson and Dippenaar (1983).

Selection of taxonomically useful characters

To produce a stable taxonomy the choice and number of characters should be carefully considered. The suite of characters used must be able to provide the most reliable information of the relationships between the organisms in question (Rohlf 1990). These characters must vary coherently between taxa and should not be ecophenotypic (Wiley 1981). It is therefore important to screen variables to avoid the non-heritable random noise components of variation.

Traditionally, as many characters as possible have been used in sampling the phenotype. This approach however, has not been proved to be empirically and theoretically justifiable (Sneath & Sokal 1973). Moreover, it has been shown that after screening for redundancy, large

character sets can be reduced significantly and still contain equivalent information (Chimimba & Dippenaar in press).

However, to reduce character redundancies, calculation of character correlations should be based on specimens from geographically contiguous localities. This reduces potential inter-locality geographic variation and simultaneously identifies highly correlated variables that may reflect a common epigenetic origin (Thorpe 1976). Such characters should be excluded where possible.

To select taxonomically useful non-redundant variables, a relatively large sample of *P. rupestris* specimens representing four geographically contiguous samples from four north-western Cape localities (Springbok, Eenriet, Platbakkies and Ezelfontein; Figure 4.1) was first subjected to R -mode PCA (James & McCulloch 1990) to test the homogeneity of the pooled samples. This was followed by a Q - mode PCA, cluster and univariate analyses to examine character associations. Selection of characters from within correlated subclusters of characters was based on the following criteria:

(i) A character's component loading on extracted PC axes of the three recognised species. High negative or positive loadings indicated the characters discriminating potential taxonomic groups.

(ii) A character's percentage variance contribution. Values are directly proportional to the amount of information provided by the character along that particular axis.

(iii) A character's coefficient of variation (CV) incorporating Haldane's (1955) correction for small samples. Values exceeding eight percent were considered to reflect substantial variation over the entire data set.

(iv) Measuring points associated with frequently damaged parts of the skull (with reference to analyses that require complete data sets).

(v) A character's previous diagnostic importance, particularly in original descriptions (Robinson & Dippenaar 1983; Chapman *et al* 1992).

Assessment of phenetic relationships within *P. rupestris*

(1) ***Analyses based on single specimens***

After the establishment of the phenetic integrity of the three currently recognised species in South Africa, the 108 specimens that were identified as *P. rupestris* and which were represented by full data sets were subjected to PCA. Clusters of specimens on the PCA scattergram was subsequently assessed in terms of geography.

(2) ***Analyses based on mean values***

To accommodate poorly represented samples and samples with incomplete data sets, localities such as Olifantshoek and Sishen, Upington and Louisvale (Figure 4.1) were pooled for the calculation of standard statistics. Pooling of geographically contiguous localities was undertaken with reference to phytogeographic zones (Acocks 1988). The only exception to the pooling procedure involved Burgersdorp and Rolfontein. None of the specimens from these two localities were represented by full data sets. These localities could not, however, be combined with adjacent localities since both fell within possible contact zones suggested by the exploratory PCA (data not shown). As a consequence, 43 geographic populations (represented by an asterisk in Figure 4.1) representing 173 specimens referable to as *P. rupestris* were subjected to PCA and form the basis of taxonomic conclusions in this study.

To assess characteristics of delineated phena, diagnostic differential cranial ratios (expressed as a percentage) were computed for combinations of characters with high component loadings in the PCA. Component loadings were derived from analyses based on the entire data suite and those based on pairwise comparisons between detected phena. The statistical integrity of the phenetic groups detected by the above procedure was tested by subjecting them to discriminant analysis and MANOVA (Willig *et al 1986*). In conjunction with the final morphometric analysis, Matthee's (1993) data pertaining to *P. rupestris* was reanalysed using both the neighbor-joining and UPGMA methods of tree construction. This step was included in order to elucidate mitochondrial DNA patterns within this species that may have been underemphasized in the original analysis by the inclusion of a second species *P. randensis* (presented in the discussion of this chapter).

(3) Analyses of specimens with known mitochondrial DNA affinity

A separate PCA using specimens of known mitochondrial DNA affinity was also undertaken. These also included specimens from either mitochondrial DNA sampled localities or adjacent localities. The need to reduce statistical bias and to comply with minimum sample size requirements ($n \geq 5$) for PCA led to the inclusion of specimens of inferred mitochondrial DNA affinity. This was however done with circumspection and implicated a single specimen from Postmasburg and three from Griekwastad. These localities, being geographically close to Prieska (Figure 4.1), were assumed to have mitochondrial DNA affinity with the Prieska specimens (the north-western clade; Matthee 1993).

(4) Status of Boshof and Fauresmith specimens

Extrapolation of Matthee's (1993) molecular data suggested that specimens from Boshof and Fauresmith would display a north-western restriction enzyme pattern. In contrast, preliminary morphometric analyses placed these localities within the south-Karoo clade. Lack of adequate material necessitated additional sampling from these areas. In doing so, seven additional specimens were screened to determine their mitochondrial DNA affiliations using standard procedures (see Chapter 2) and their cranial measurements recorded for subsequent morphometric analyses.

Geographic variation

The understanding of patterns and processes of diversification in a species is a necessity when attempting to distinguish between variation due to evolutionary relationships (heritable variation) within a species and variation resulting from environmental responses (non-heritable variation; Thorpe 1976; Smith & Patton 1984; Pankakoski *et al* 1987).

To assess geographic variation within phenetic groups obtained by multivariate analyses the sum of squares simultaneous test procedure (SS-STP), an *a posteriori* multiple comparison test was undertaken (UNIVAR: FORTRAN programme developed by Power (1970) and recompiled in PC version by N.J. Dippenaar).

RESULTS

Assessment of phenetic integrity of currently recognised species in South Africa

The measurements taken for this study allowed for the delineation of all three recognised *Pronolagus* species. Additionally, the species identification of all museum specimens was accurate with the single exception of a *P. crassicaudatus* specimen which was misclassified in the curated collection as *P. rupestris*. The preliminary PCA based on the three recognised species placed this specimen (from Bathurst) within the *P. rupestris* cluster but subsequent PCA analysis (*P. rupestris* specimens only) indicated it to be an outlier to the *P. rupestris* group (data not shown). Examination of the study skin and the characters separating this individual in the PCA (particularly bulla breadth which had a high negative loading in the factor matrix, a character diagnostically important for the delineation of *P. crassicaudatus*; Robinson & Dippenaar 1983) revealed that the Bathurst population in fact represented a southerly extension of *P. crassicaudatus*' conventionally accepted range.

Selection of taxonomically useful characters

Results of the PCA undertaken to assess the homogeneity of the four pooled samples used for character selection revealed no meaningful patterns between them (data not shown). This suggests that variation between populations is minimal and will not obscure interpopulational differences. This finding justified using the data from the pooled samples in screening characters for use in subsequent analysis.

A correlation phenogram (Figure 4.3) generated from the cluster analysis of the 28 mensural characters from 15 specimens comprising the four contiguous localities in the north-western Cape revealed six subclusters (distance 0.75 - 1.00). Component loadings and a character's percentage variance contribution gave an indication of those characters which were important in the discrimination between the three recognised species, and therefore, by inference may be important in the detection of phenetic differences within *P. rupestris*. These parameters were not obtained from the principal component analysis based on the fifteen specimens as variation between them was minimal.

Subcluster 1: Total length of skull, basal cranial length and mandibular toothrow length
Of the three characters in this subcluster basal cranial length and mandibular toothrow length were the most highly correlated. Both are characterized by high component loadings and percentage variance contribution along the first axis as well as low coefficient's of variations (Table 4.1). Basal cranial length was discarded on the grounds that this measurement was missing in a high percentage of specimens (N = 32). The remaining character of this subset, total length of skull, was retained on the grounds that it had been of diagnostic importance in previous studies (Robinson & Dippenaar 1983).

Subcluster 2: Muzzle length and palatal vacuity length
Palatal vacuity length was discarded due to a high coefficient of variation and a slightly lower component loading along the second axis (Table 4.1). Furthermore the decision to retain muzzle length was prompted by its importance in separating *P. randensis* and *P. crassicaudatus* (Robinson & Dippenaar 1983).

Subcluster 3: Zygomatic arch length and maximum mandibular length Although there is little difference in coefficient of variation and component loadings between the two characters (Table 4.1), maximum mandibular length was discarded in view of the large number of specimens lacking this measurement (N = 50).

Subcluster 4: Maxillary premolar-molar length and mandibular premolar-molar length
As with the previous subset of measurements there was little difference in the parameters used when comparing correlated characters (Table 4.1). However mandibular premolar-molar length had the greater number of missing measurements and was consequently eliminated (N = 9).

Subcluster 5: Posterior muzzle breadth and anterior frontal breadth
Anterior frontal breadth was rejected due to its exceptionally high coefficient of variation (CV = 11.392; Table 4.1).

Subcluster 6: Bizygomatic breadth and maximum cranial breadth
Both these characters were retained due to their diagnostic importance (Robinson & Dippenaar 1983).

Finally, although not showing high correlation with any of the other measurements used in this study, interauditory breadth was discarded in view of its high coefficient of variation and the large number of specimens having missing values for this measurement (N = 87).

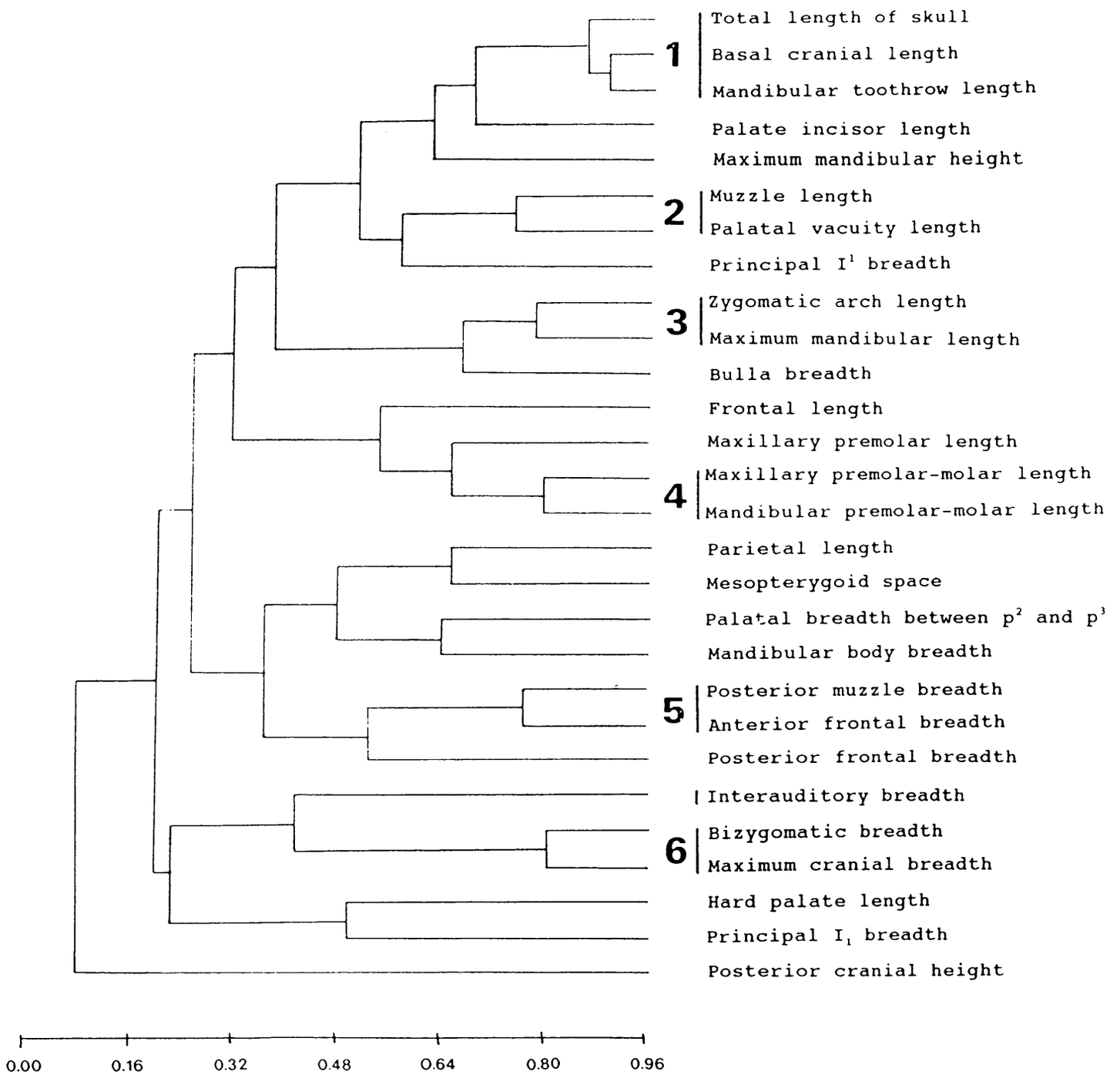


Figure 4.3 Correlation phenogram showing the correlation between various characters; the subclusters of characters all had correlation coefficients which varied between 0.75 - 1.00. The cophenetic correlation coefficient was 0.71.

Table 4.1 The first two principal component loadings of a PCA of the three recognized morphospecies (including percent variance contribution in parentheses) and the coefficients of variation (CV) corrected with Haldane's correction {(1 + 1/4n) CV} used to determine highly correlated characters.

Character	Principal Component axes		CV
	I	II	
Total length of skull	-0.967 (93.46)	-0.072 (0.52)	2.88
Muzzle length	-0.422 (17.82)	0.176 (3.09)	6.82
Frontal length	-0.661 (43.66)	0.202 (4.07)	6.21
Parietal length	-0.651 (42.44)	-0.233 (5.42)	8.45
Posterior muzzle breadth	-0.872 (75.99)	-0.060 (0.36)	6.07
Anterior frontal breadth	-0.768 (58.97)	0.203 (4.14)	11.39
Posterior frontal breadth	-0.755 (56.94)	0.271 (7.35)	7.21
Interauditory breadth	-0.334 (11.17)	0.473 (22.37)	9.03
Basal cranial length	-0.929 (86.38)	-0.088 (0.78)	3.40
Palate incisor length	-0.948 (89.87)	0.038 (0.14)	4.18
Palatal vacuity length	-0.860 (73.97)	-0.060 (0.36)	8.11
Hard palate length	-0.896 (80.23)	0.131 (1.73)	7.42
Mesopterygoid space	-0.158 (2.50)	-0.546 (29.80)	7.28
Maxillary premolar length	-0.808 (65.35)	-0.222 (4.90)	5.87
Maxillary premolar-molar length	-0.905 (81.98)	-0.178 (3.17)	4.32
Principal I^1 breadth	-0.915 (83.73)	-0.109 (1.19)	4.16
Palatal breadth between p^2 & p^3	-0.885 (78.40)	0.015 (0.02)	6.31
Bizygomatic breadth	-0.872 (75.99)	0.351 (12.35)	3.01
Maximum cranial breadth	-0.335 (11.21)	0.709 (50.27)	3.06
Zygomatic arch length	-0.934 (87.22)	-0.018 (0.03)	4.02
Posterior cranial height	-0.801 (64.11)	-0.088 (0.77)	3.64
Bulla breadth	0.485 (23.53)	0.197 (3.89)	5.67
Maximum mandibular length	-0.933 (86.99)	-0.095 (0.90)	3.67
Mandibular toothrow length	-0.960 (92.18)	-0.036 (0.13)	3.13
Maximum mandibular height	-0.952 (90.67)	-0.003 (0.00)	3.98
Mandibular body breadth	-0.734 (53.88)	-0.025 (0.06)	6.42
I_1 breadth	-0.853 (72.75)	-0.082 (0.68)	5.80
Mandibular premolar-molar length	-0.948 (89.78)	-0.113 (1.27)	3.63

Assessment of phenetic relationships within *P. rupestris*

(1) Analysis based on single specimens

The clustering of OTUs in the PCA scattergram based on 108 specimens (data not shown) formed three discrete phenetic assemblages corresponding to the following geographic regions: a northern Cape group (corresponding to the north-western mitochondrial DNA clade), a southern Cape-Karoo cluster and a group located along the eastern parts of the country (the latter two groups encompassing Matthee's (1993) south-eastern mitochondrial DNA clade; Figure 4.4).

However, the Boshof and Fauresmith OTUs plotted with the southern Cape-Karoo cluster rather than with the north-western group , an association which would have been predicted based on their mitochondrial affiliations (see Chapter 2). These specimens were therefore excluded from the final descriptive multivariate analyses and are dealt with separately (see section 4).

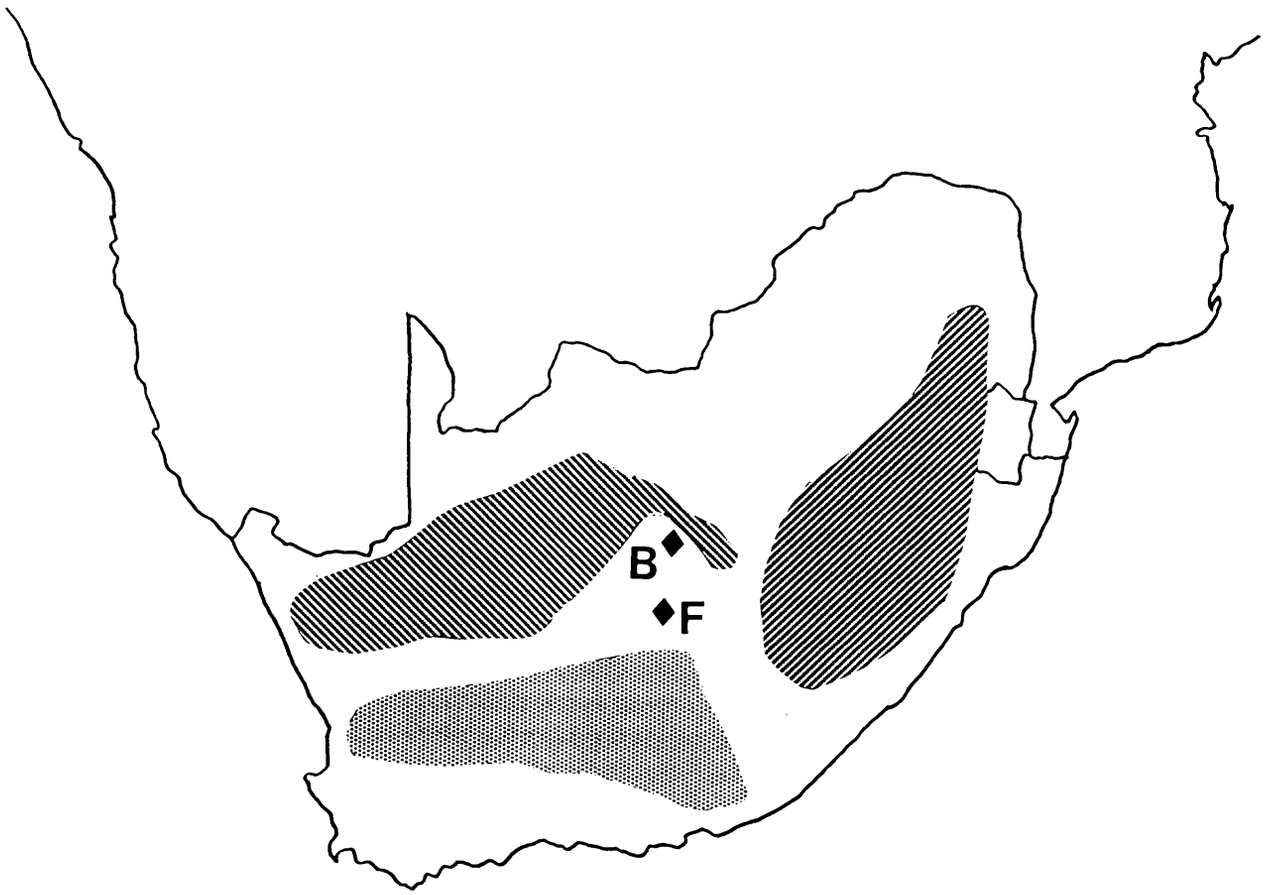


Figure 4.4 Map showing the geographic extent of the *P. rupestris* north-western (▨), south-Karoo (⬚) and eastern (▨) phenetic assemblages as well as the localities of (B) Boshof and (F) Fauresmith. The south-Karoo and eastern groups jointly comprise the south-eastern mitochondrial DNA clade referred to in the text.

(2) Analyses based on mean values

The following multivariate statistics represent the descriptive analyses of *P. rupestris*. Component loadings along the first axis of the PCA were all negative and high with the exception of maximum cranial breadth which had a comparatively low negative component loading and bulla breadth which was positive (Table 4.2). The first principal component, which accounts for 54.24% of the variation, separates the previously defined mitochondrial DNA south-eastern clade (Matthee & Robinson submitted) into two distinct assemblages, one limited to the southern-Karoo and the other to the eastern escarpment (Figure 4.5). The second component, which accounts for an additional 8.95% of the trace, delineates a north-western and a south-eastern clade corresponding to the north-western and south-eastern mitochondrial clades (Figure 4.6). Interestingly, the two most important measurements segregating OTUs along the second axis are posterior frontal breadth and, in particular, bulla breadth (Table 4.2). Examination of the third principal component provided no additional information.

The minimum spanning tree (Figure 4.5) joined an eastern clade OTU (OTU 76: Winterton) and a south-Karoo OTU (OTU 20: Donkerpoort) to the north-western assemblage. The Winterton skulls were found to be smaller than the average for the eastern clade, but were not incorrectly classified in either the correlation or distance phenograms (Figures 4.6 and 4.7). Donkerpoort, together with other south-Karoo localities, OTU 54 (Nieuwoudtville), OTU 55 (Noupoort), OTU 21 (Doornrivier) and OTU 51 (Middelburg) were incorrectly classified in the distance phenogram (Figure 4.6) due to large skulls. The misplaced OTUs in the correlation phenogram (Beaufort West, Victoria West, Colesburg, Upington/Louisvale, Clanwiliam and Middelburg) were found to have broader skulls than the average for their respective clades. None of these OTUs with the exception of Middelburg (already mentioned) were misclassified in any of the other analyses. Generally though, cluster analyses, both distance (Figure 4.6) and correlation (Figure 4.7) algorithms, displayed the same trends shown by the PCA.

PCA based on pairwise comparisons of the identified three groups were also carried out (data not shown). In all instances, the delineation between the clusters was distinct, bulla breadth proving to be the most important character responsible for the separation. Standard statistics for the three groups are given in Appendix VI.

Table 4.2 The first two principal component loadings and percentage variance contribution (in parentheses) of characters based on mean values for *P. rupestris* localities, excluding the Boshof and Fauresmith specimens.

Character	Principal Component axes	
	I	II
Total length of skull	-0.933 (87.041)	0.111 (1.223)
Muzzle length	-0.623 (38.820)	0.436 (18.967)
Fronatl length	-0.709 (50.206)	-0.408 (16.679)
Parietal length	-0.539 (29.024)	-0.364 (13.258)
Posterior muzzle breadth	-0.713 (50.861)	-0.168 (2.825)
Posterior frontal breadth	-0.533 (28.397)	-0.517 (26.688)
Palate incisor length	-0.889 (79.003)	-0.228 (5.217)
Hard palate length	-0.819 (67.042)	0.013 (0.017)
Mesopterygoid space	-0.589 (34.648)	-0.189 (3.578)
Maxillary premolar length	-0.687 (47.138)	0.171 (2.913)
Maxillary premolar-molar length	-0.868 (75.319)	0.293 (8.565)
Principal I^1 breadth	-0.855 (73.091)	0.147 (2.174)
Palatal breadth between p^2 & p^3	-0.851 (72.438)	-0.219 (4.788)
Bizygomatic breadth	-0.874 (76.366)	-0.012 (0.014)
Maximum cranial breadth	-0.356 (12.645)	-0.116 (1.353)
Zygomatic arch length	-0.660 (43.617)	0.135 (1.811)
Posterior cranial height	-0.733 (53.789)	-0.119 (1.417)
Bulla breadth	0.099 (0.983)	0.857 (73.432)
Mandibular toothrow length	-0.926 (85.811)	0.161 (2.601)
Maximum mandibular height	-0.892 (79.485)	0.113 (1.266)
Mandibular body breadth	-0.716 (51.291)	-0.221 (4.890)
I_2 breadth	-0.750 (56.220)	0.182 (3.308)

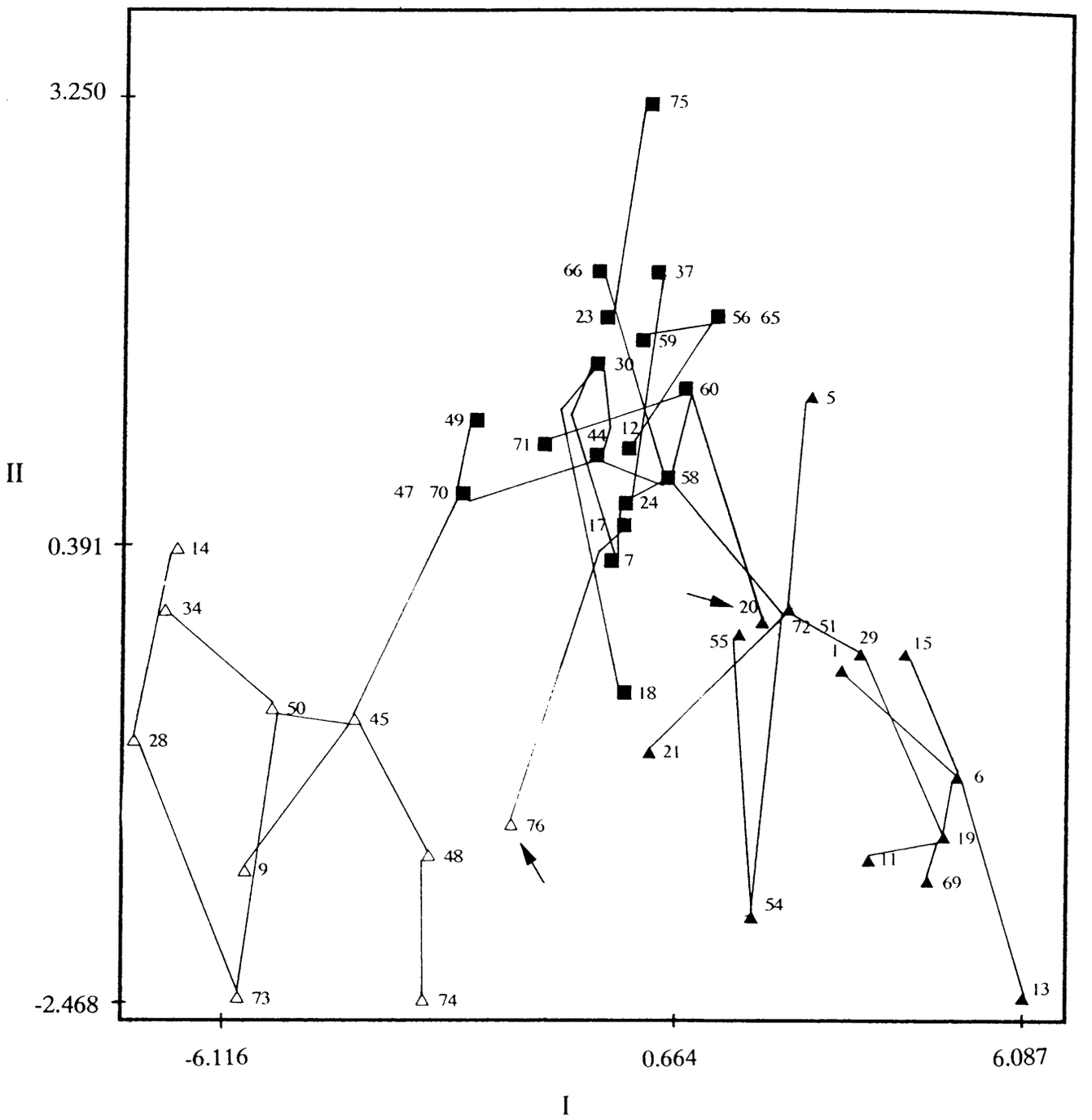


Figure 4.5 Scattergram of components I and II of a principal component analysis where OTUs are population means of the constituent specimens. The clade affiliation is designated as follows: north-western (■), southern-Karoo (▲) and eastern groups (△). The superimposed MST indicates single connections between the three phenetic groups, with the two incorrectly classified OTUs indicated by arrows (see text for details). Numbering of OTUs corresponds to Figure 4.1.

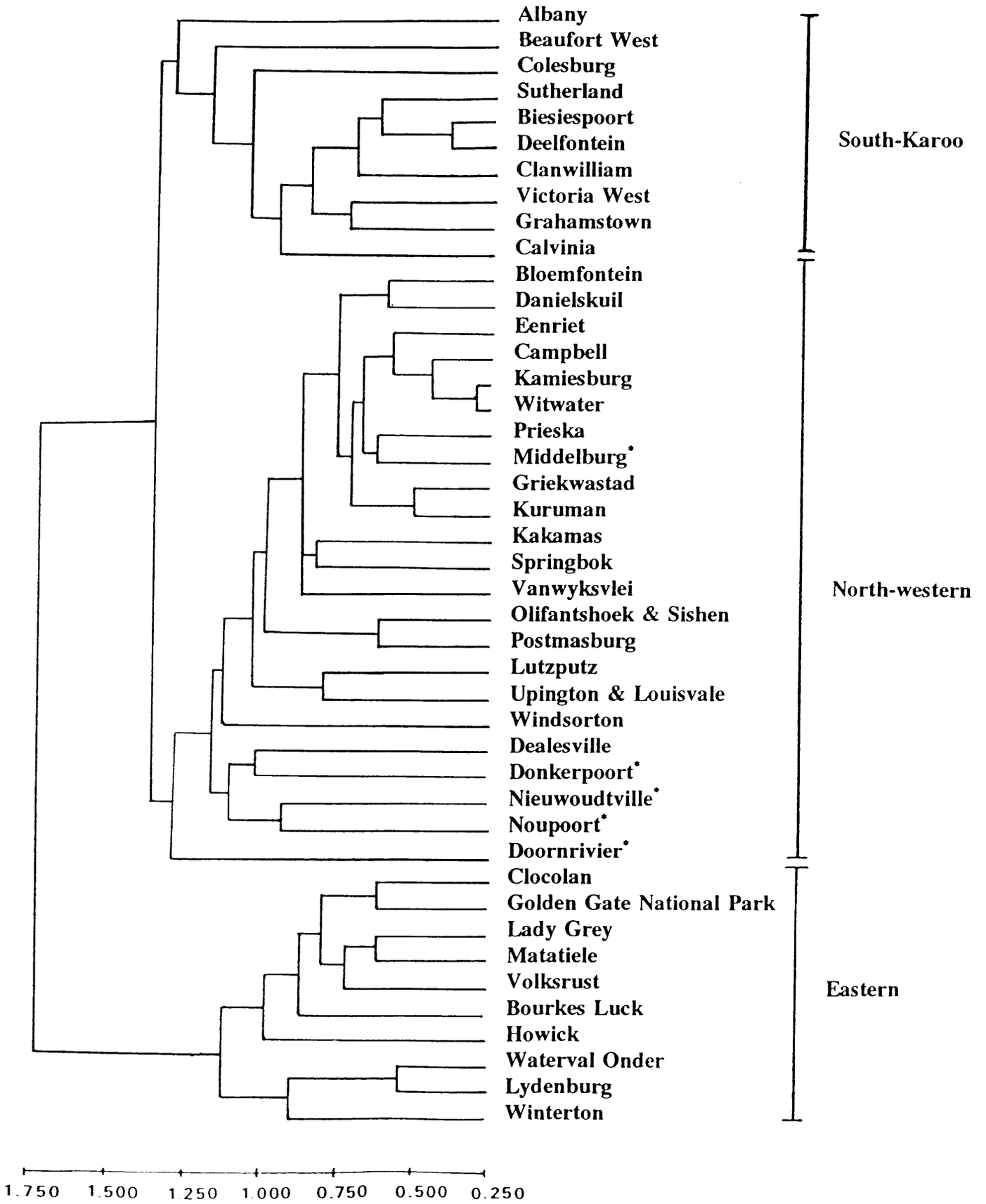


Figure 4.6 Phenogram based on average taxonomic distances for *P. rupestris*, indicating three phenetic groups. The five south-Karoo specimens that cluster with the north-western clade are marked by asterisks (see text for details). Cophenetic correlation coefficient = 0.72.

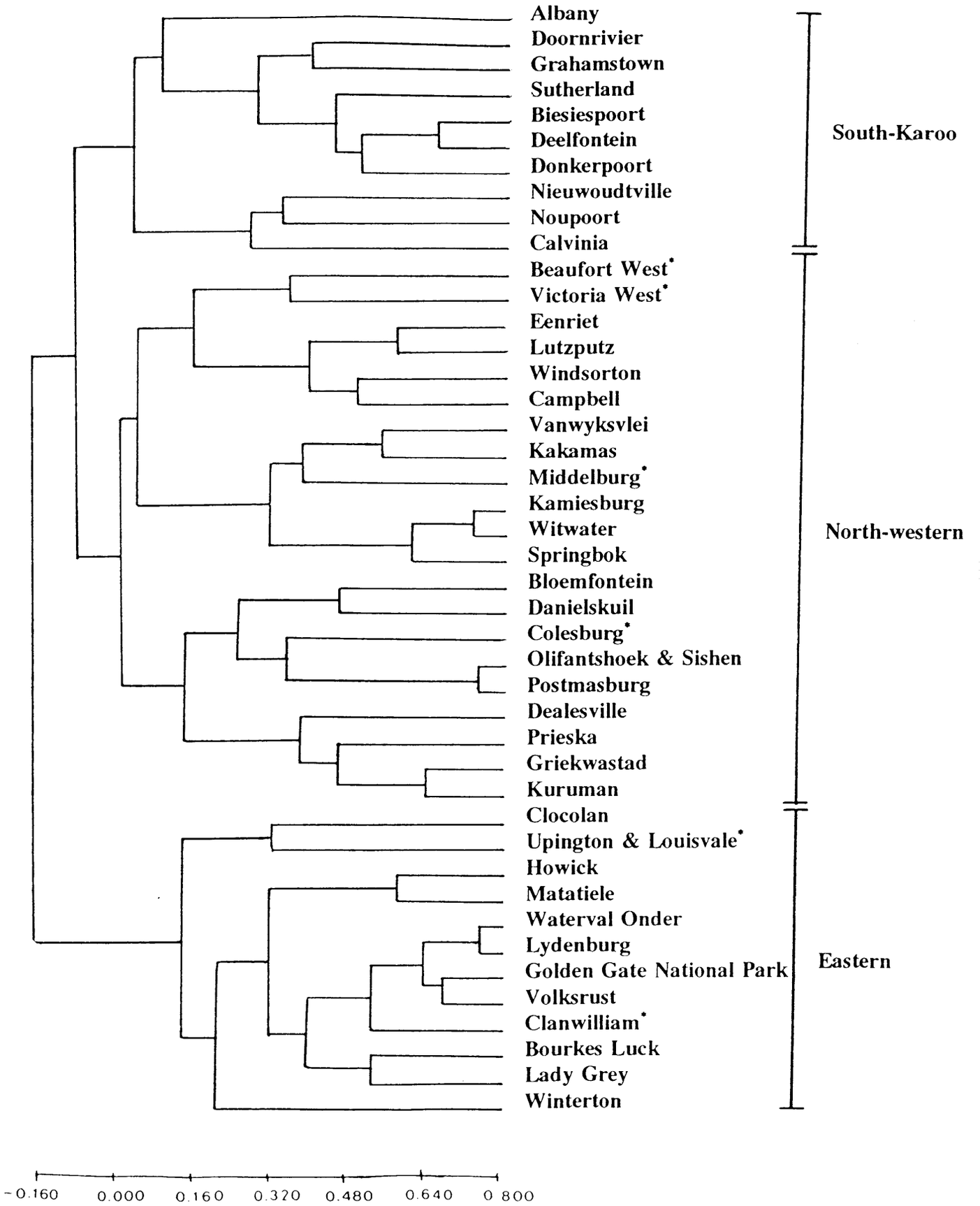


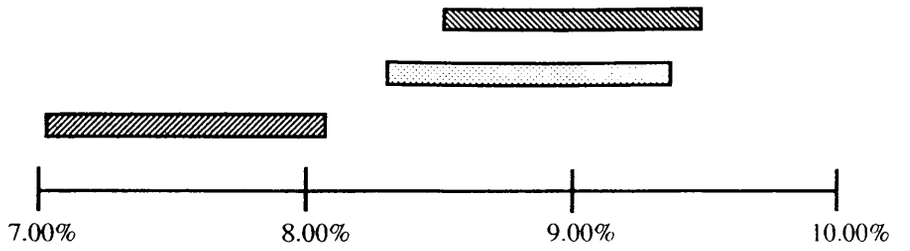
Figure 4.7 Correlation phenogram based on mean values calculated for each locality of *P. rupestris*. Misplaced OTUs are marked by asterisks (see text for details). Cophenetic correlation coefficient = 0.63.

Cranial ratios (Table 4.3) were calculated by contrasting high and low component loadings along the first and second components. The ratio of bulla breadth to total length of skull, and to mandibular toothrow length (component I) and frontal length (component II) illustrated that the eastern assemblage has significantly narrower bullae relative to either the north-western or south-Karoo clades, and that these measurements can be used to distinguish specimens of the eastern clade from the those of other two assemblages (Table 4.3; Figure 4.8). The ratio of frontal length and muzzle length enables the distinction between representatives of the north-western and south-Karoo clades (Table 4.3; Figure 4.8).

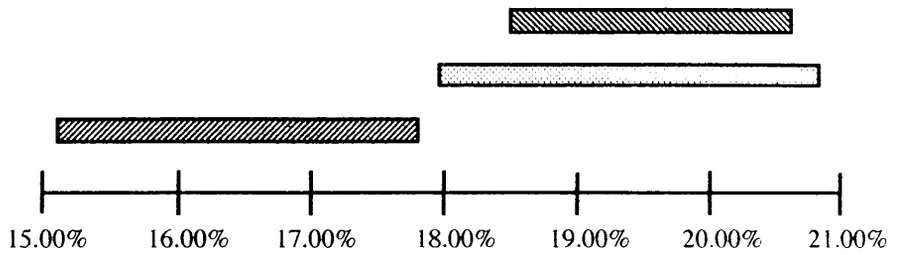
The discriminant analysis of 43 *a priori* grouped OTUs resulted in all OTUs being correctly classified. The south-Karoo and eastern clades plotted apart along the first canonical variate axis (data not shown; 85.91% of the total variance) with bulla breadth proving to be a significant factor in this separation. The mitochondrial DNA clades separated along the second canonical variate axis (4.09% of the total variance). Characters that showed high loadings along the second canonical variate axis were palate incisor length, maxillary premolar-molar length, principal I^1 breadth, bulla breadth, mandibular toothrow length and maximum mandibular height. Significant differences were indicated between the group centroids (respectively for the first and second cmponents: north-western clade - 3.062, 2.441; south-Karoo - 4.567, -2.519; eastern - -6.181, -0.308 by MANOVA ($F = 13.82$; $P < 0.01$).

Table 4.3 Variation in ratios calculated by contrasting cranial measurements which have high and low loadings in component I and II in a factor matrix. X = Arithmetic mean (expressed as a percentage; SD = Standard deviation; Observed ranges in parentheses).

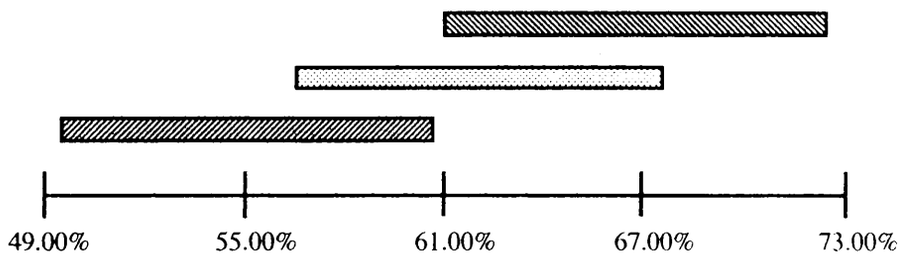
Ratio	*P. rupestris*		
	North-western	South-Karoo	Eastern
		Component I	
Bulla breadth X 100/ Total length of skull	X = 8.99% SD = 0.46 (8.53% - 9.45%)	X = 8.83% SD = 0.56 (8.27% - 9.39%)	X = 7.57% SD = 0.52 (7.05% - 8.09%)
Bulla breadth X 100/ Mandibular toothrow length	X = 19.54% SD = 1.05 (18.49% - 20.59%)	X = 19.43% SD = 1.45 (17.98% - 20.88%)	X = 16.43% SD = 1.31 (15.12% - 17.74%)
		Component II	
Bulla breadth X 100/ Posterior frontal breadth	X = 66.64% SD = 5.79 (60.85% - 72.43%)	X = 62.20% SD = 5.61 (56.59% - 67.81%)	X = 54.88% SD = 5.51 (49.37% - 60.39%)
Frontal length X 100/ Muzzle length	X = 99.96% SD = 4.47 (95.49% - 104.42%)	X = 88.85 SD = 0.22 (88.63% - 89.06%)	X = 100.02 SD = 4.45 (95.57% - 104.47)



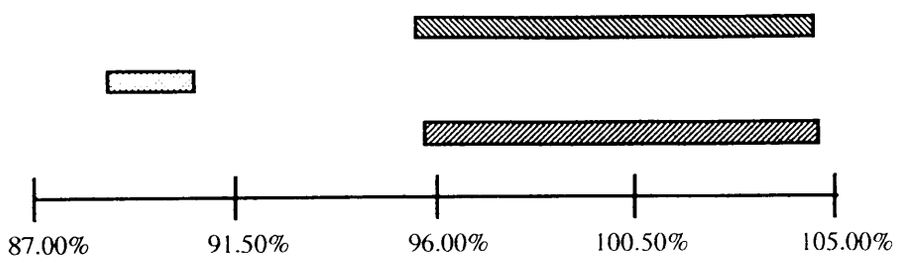
Bulla breadth X 100/ Total length of skull



Bulla breadth X 100/ Mandibular toothrow length



Bulla breadth X 100/ Posterior frontal breadth



Frontal length X 100/ muzzle length

Figure 4.8 Graphic representation of diagnostic ratios presented in Table 4.4: north-western ( ), south-Karoo ( ) and eastern ( ).

(3) Analyses of specimens with known mitochondrial DNA affinity

Principal component analysis performed on specimens with known mitochondrial clade affiliation underscores the previous analyses in that the three morphotypes were observed (data not shown). This clearly emphasizes the congruency between the morphometric and mitochondrial DNA data sets.

(4) Status of Fauresmith and Boshof specimens

The Boshof and Fauresmith localities did not cluster as expected with the north-western clade (Figure 4.10), despite analysis of their mitochondrial DNA (see Chapter 2) confirming that they fall within the mitochondrial DNA north-western clade. These are therefore the only two localities in the morphometric analyses which failed to show congruence with the mitochondrial DNA data (Matthee 1993).

Discriminant analysis was performed on the Fauresmith and Boshof specimens to obtain some indication of which of the three groups they were most closely affiliated to. Discriminant analysis probabilities (Table 4.4) show that the Boshof and Fauresmith individuals could not confidently be assigned to any of the designated groups. The values presented in Table 4.5 can be contrasted to the probabilities of 0.9 - 1.0 obtained for the rest of the OTUs (based on mean values) being correctly assigned to the *a priori* defined groups. A second discriminant analysis, using individual specimens as OTUs rather than population mean values similarly show that the discriminant probabilities for the Boshof and Fauresmith specimens are low and inconclusive.

Table 4.4 Discriminant analysis probability of the Fauresmith and Boshof specimens being assigned to each of the three morphometric clades based on the discrminant analysis of the entire *P. rupestris* sample: NW - north-western; SK - south-Karoo and E - eastern groups.

Locality	NW	SK	E
Fauresmith 1	0.46	0.41	0.13
Fauresmith 2	0.50	0.32	0.18
Fauresmith 3	0.35	0.46	0.19
Fauresmith 4	0.48	0.24	0.28
Boshof	0.40	0.21	0.39

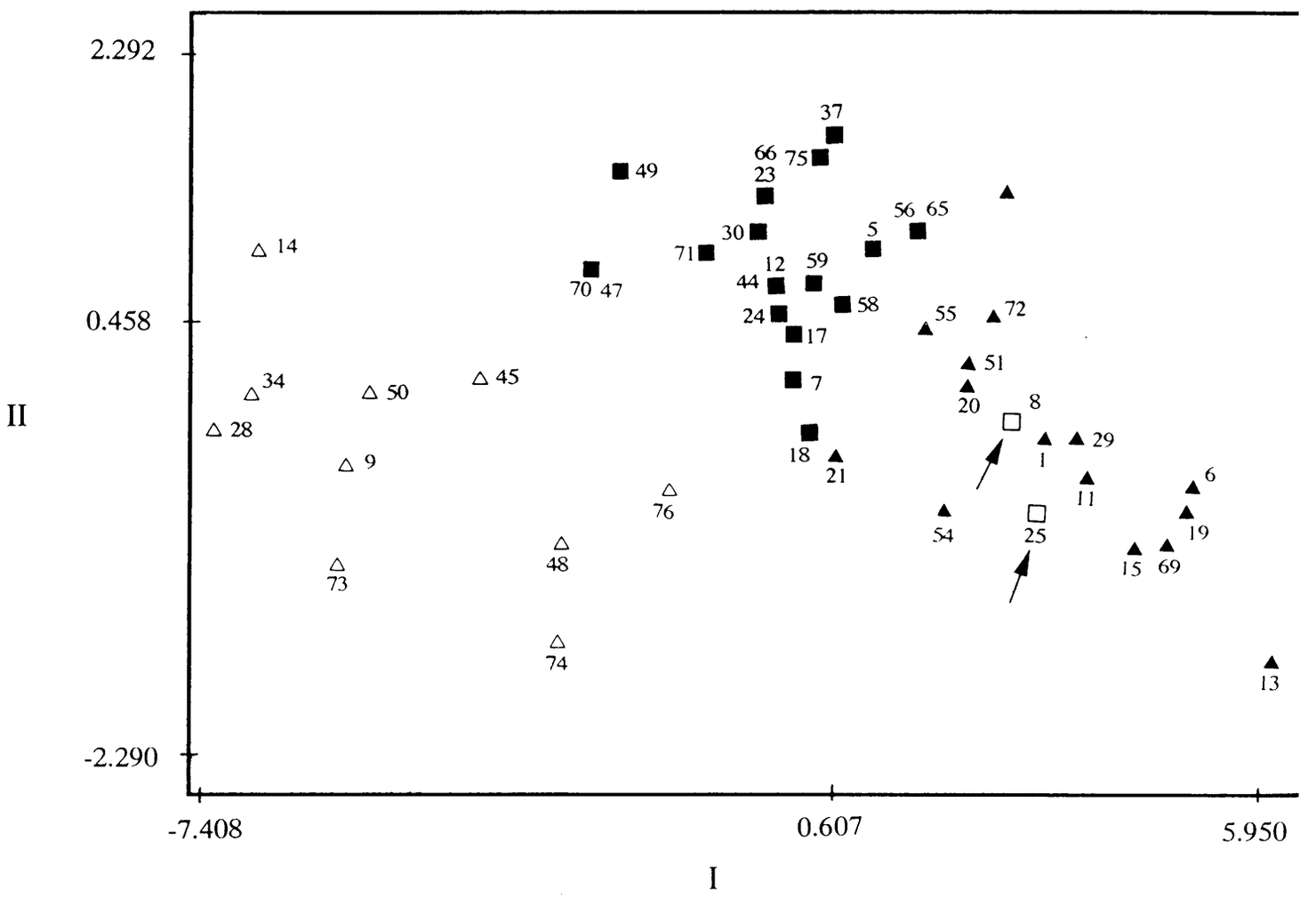


Figure 4.9 Scattergram of components I and II of a principal component analysis where OTUs are represented by population mean values. The Boshof and Fauresmith OTUs arrowed and the morphometric groups are designated as follows: north-western (■), southern-Karoo (▲) and eastern assemblages (△). Numbering of OTUs corresponds to Figure 4.1.

Geographic variation

The two assemblages within the south-eastern mitochondrial DNA clade are differentiated mostly in terms of size (component I; Figure 4.6). Analysis of geographic variation (SS-STP) was performed on 22 cranial measurements for 91 individuals representing 13 localities. No statistically significant differences were observed among samples. SS-STP diagrams are presented for all 22 cranial measurements (Table 4.5).

Table 4.5 Results of the SS - STP analysis of geographic variation in 22 cranial characters in the south-eastern mitochondrial DNA clade of *P. rupestris*. Vertical lines include ranked means falling in nonsignificant (P > 0.95) subsets. The locality codes correspond to those in Figure 4.1. The asterisk denotes OTUs referable to the morphometric eastern clade, the remainder belonging to the south-Karoo clade.

Locality Code	Sample Size	Arithmetic Mean	Locality Code	Sample Size	Arithmetic Mean
Total length of skull			**Muzzle Length**		
28*	6	88.50	28*	6	39.84
73*	3	85.13	50*	9	38.01
9*	5	85.00	73*	4	37.60
50*	7	84.31	45*	8	37.54
48*	6	83.57	20	4	37.48
45*	7	83.43	51	11	35.05
20*	3	78.58	29	7	34.97
51	10	77.44	48*	7	34.85
11	4	76.78	9*	5	34.56
29	7	76.64	19	7	33.42
6	5	76.11	6	7	33.34
19	7	74.88	13	6	31.15
13	6	73.47	11	4	29.22
Frontal length			**Parietal length**		
9*	5	39.16	73*	3	18.97
73*	4	36.81	20	4	18.54
48*	7	36.33	28*	6	18.37
28*	6	35.93	50*	10	18.27
45*	8	35.57	45*	6	18.25
50*	9	35.44	9*	5	18.19
11	4	34.31	29	8	17.97
51	12	31.26	48*	6	17.75
13	6	31.01	51	12	17.40
6	7	30.98	19	7	17.35
19	7	30.56	6	7	17.29
29	7	30.38	13	6	16.95
20	4	29.83	11	4	16.33

Table 4.5 (continued)

Locality Code	Sample Size	Arithmetic Mean	Locality Code	Sample Size	Arithmetic Mean
Posterior muzzle length			**Posterior frontal breadth**		
28*	6	17.63	73*	4	12.40
9*	5	17.24	28*	6	12.40
48*	7	16.37	50*	10	11.62
73*	4	16.24	48*	7	11.62
45*	10	15.95	45*	8	11.44
50*	10	15.81	9*	5	11.42
11	4	15.37	13	6	11.20
29	8	15.05	19	7	11.14
20	4	14.56	20	4	11.01
6	7	14.50	51	12	10.81
19	7	14.44	11	4	10.80
51	12	14.37	29	8	10.76
13	6	14.37	6	6	10.72
Palate incisor breadth			**Hard palate length**		
28*	6	38.05	28*	6	8.11
9*	5	37.20	73*	4	7.79
73*	4	37.05	48*	7	7.66
50*	9	36.97	9*	5	7.62
45*	8	36.28	50*	10	7.51
48*	7	36.00	45*	10	7.50
29	8	32.34	51	12	6.51
51	11	32.24	20	4	6.34
6	7	32.21	29	8	6.23
11	4	32.05	13	6	6.21
20	4	31.78	19	7	6.16
19	7	31.16	6	7	6.10
13	6	30.58	11	4	5.87

Table 4.5 (continued)

Locality Code	Sample Size	Arithmetic Mean	Locality Code	Sample Size	Arithmetic Mean
Mesopterygoid space			**Maxillary Premolar length**		
9*	5	5.96	28*	6	8.11
73*	4	5.94	45*	10	8.02
28*	6	5.85	50*	10	8.00
50*	10	5.82	73*	4	7.99
20	4	5.75	9*	5	7.83
11	4	5.72	48*	7	7.82
45*	9	5.46	29	8	7.66
29	8	5.23	51	12	7.64
19	7	5.23	20	4	7.33
48*	6	5.20	13	6	7.16
51	12	5.18	19	7	7.13
6	7	5.06	11	4	7.12
13	6	4.67	6	7	7.03
Maxillary premolar-molar length			**Principal I^1 breadth**		
9*	5	15.27	9*	5	2.87
28*	6	15.17	73*	4	2.81
45*	10	15.15	28*	6	2.79
73*	4	14.89	45*	8	2.74
50*	10	14.68	48*	7	2.65
48*	7	14.24	50*	9	2.61
51	12	14.07	29	8	2.38
20	4	13.73	51	11	2.33
29	8	13.63	20	4	2.33
11	4	13.46	13	6	2.30
6	7	13.38	11	4	2.30
19	7	13.26	19	7	2.29
13	6	13.04	6	7	2.21

Table 4.5 (continued)

Locality Code	Sample Size	Arithmetic Mean	Locality Code	Sample Size	Arithmetic Mean
Palatal breadth between P^2 and P^3			**Bizygomatic breadth**		
28*	6	15.13	28*	6	39.11
73*	4	15.05	45*	6	38.54
9*	5	14.33	73*	3	38.31
50*	10	14.04	9*	5	38.07
48*	7	14.03	50*	10	38.01
45*	10	13.42	48*	5	37.34
20	4	13.35	51	9	36.53
11	4	12.75	20	4	36.37
51	12	12.42	11	4	35.90
29	8	12.34	29	8	34.85
19	7	12.33	19	5	34.85
13	6	12.00	6	5	34.66
6	7	11.68	13	5	34.24
Maximum cranial breadth			**Zygomatic arch length**		
28*	5	27.90	9*	5	34.83
73*	10	27.43	28*	6	34.83
45*	8	27.09	45*	6	34.03
20	4	26.74	50*	10	33.72
48*	7	26.60	48*	6	33.54
50*	3	26.43	73*	3	33.01
19	7	26.36	20	4	31.49
51	12	26.34	51	11	30.90
6	6	26.31	6	6	30.67
9*	5	26.30	11	4	30.09
11	4	26.27	29	6	29.84
13	6	25.82	19	7	29.00
29	8	25.48	13	5	28.10

Table 4.5 (continued)

Locality Code	Sample Size	Arithmetic Mean	Locality Code	Sample Size	Arithmetic Mean
Posterior cranial height			**Bulla breadth**		
28*	6	19.86	20	3	7.31
45*	7	19.49	51	11	6.90
50*	8	19.49	11	4	6.79
73*	2	19.32	29	7	6.78
48*	6	19.28	28*	6	6.73
9*	5	18.83	19	7	6.69
20	5	18.15	45*	7	6.59
51	11	18.15	13	6	6.52
11	4	17.94	50*	8	6.46
6	5	17.86	6	5	6.44
19	7	17.85	9*	5	6.38
13	6	17.73	48*	7	6.32
29	7	17.35	73*	11	5.97
Mandibular toothrow length			**Maximum mandibular height**		
28*	6	41.06	28*	6	40.30
9*	5	40.44	9*	5	39.73
73*	3	40.40	48*	6	38.73
50*	10	39.05	50*	10	38.60
45*	7	37.94	73*	4	38.52
48*	6	37.90	45*	6	36.94
20	3	36.59	51	12	35.80
51	11	35.77	20	3	34.78
29	7	34.98	29	8	34.70
11	4	34.85	11	4	34.60
6	5	34.23	13	7	33.81
13	7	33.56	6	6	33.62
19	7	33.29	19	7	33.48

Table 4.5 (continued)

Locality Code	Sample Size	Arithmetic Mean	Locality Code	Sample Size	Arithmetic Mean
Mandibular body breadth			**Principal I_1 breadth**		
9*	5	5.39	73*	3	2.91
45*	7	5.25	28*	6	2.88
28*	6	5.19	45*	7	2.80
73*	4	5.13	9*	5	2.80
48*	7	5.01	48*	7	2.76
51	12	4.94	50*	10	2.67
50*	10	4.92	51	11	2.61
19	7	4.78	29	7	2.54
29	8	4.73	11	4	2.51
11	4	4.71	13	7	2.50
20	3	4.62	20	3	2.46
6	6	4.61	6	5	2.46
13	7	4.59	19	7	2.43

DISCUSSION

The most significant result of this investigation is the degree of concordance between two unrelated data sets. The phena delineated by the skull morphometric analyses corresponds closely to the mitochondrial DNA clades previously detected within *P. rupestris* by Matthee (1993). At a finer level, however, equally striking was the presence of a third phenotypically distinct assemblage confined to the north eastern escarpment of South Africa.

The two independent data sets (skull morphology and mitochondrial DNA) indicate that *P. rupestris* does indeed show extensive genetic structuring which corresponds to geographically discrete regions. The division of the previously described south-eastern mitochondrial DNA clade into two phenotypically distinct assemblages was however not previously observed at a molecular level (Matthee 1993; Chapter 2).

A reassessment of the data from the south-eastern mitochondrial DNA clade was clearly called for. Matthee's (1993) analysis of the mitochondrial DNA RFLP data was based on both mitochondrial DNA clades of *P. rupestris* as well as a second species *P. randensis*. The inclusion of the *P. randensis* specimens tends to over emphasize the differences between the groups, and downplay subtle intra-group variation. Reanalysis (both UPGMA and neighbor-joining) of the Matthee's south-eastern mitochondrial DNA data in isolation, yielded two clusters which correspond well to the phenetically defined eastern and south-Karoo groups (Figure 4.6). The misclassified lineages (indicated with an asterisk in Figure 4.6) originate from the geographic contact zones between the two morphologically defined clades (Figure 4.7), and may represent relict lineages from the time when gene flow occurred unhindered along the Great Escarpment. Recent introgression of mitochondrial DNA between the south-Karoo and eastern clades seems unlikely as there are no intermediate skull types between the clades. Thus, the beginnings of mitochondrial DNA differentiation within the south-eastern clade can be observed.

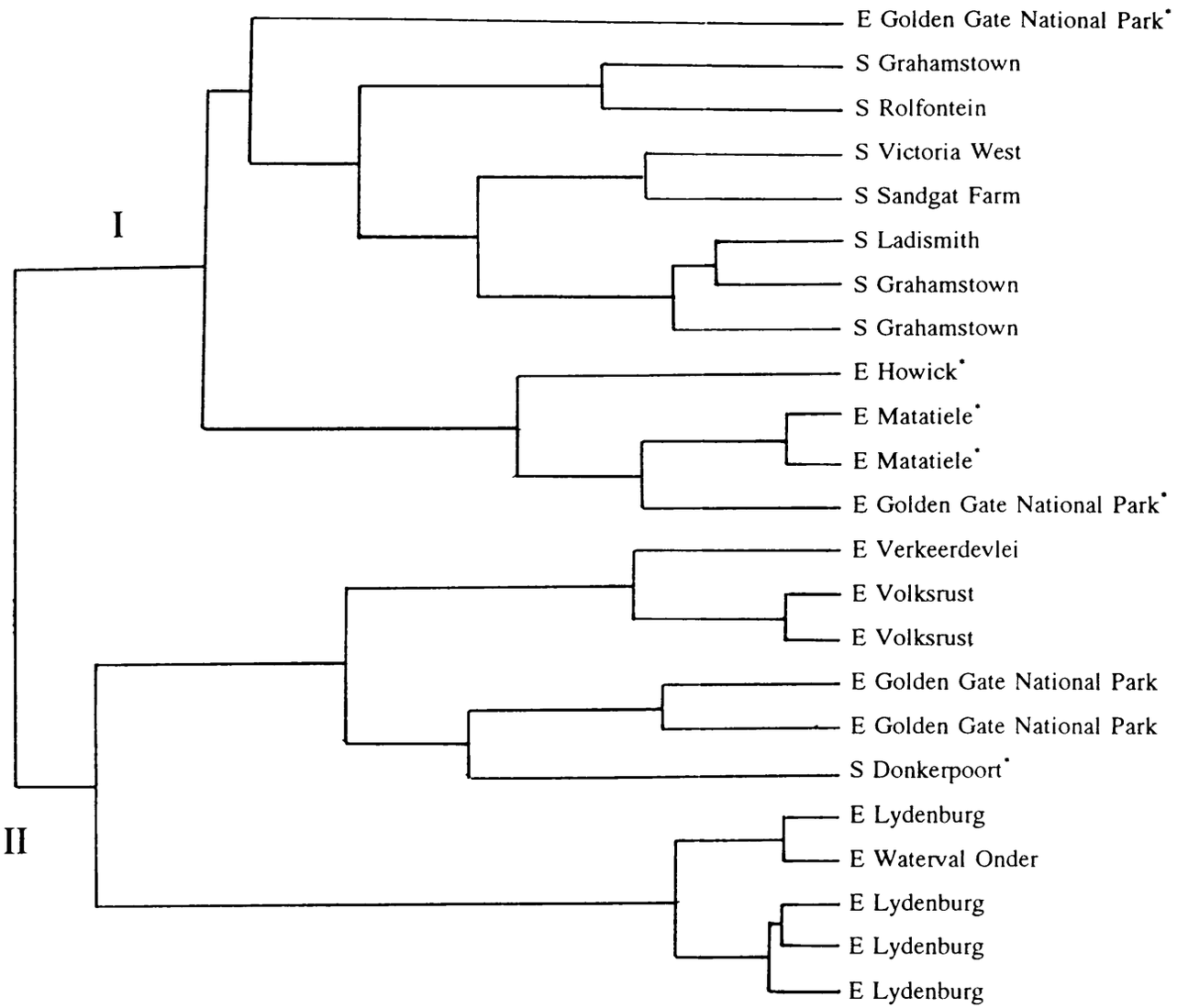


Figure 4.10 UPGMA tree based on mitochondrial DNA sequence divergence values between haplotypes of the south-eastern mitochondrial DNA clade. Haplotypes occurring geographically in either the eastern or South-Karoo morphometric groups are respectively indicated by an E or an S. The two major clusters (indicated by I and II) correspond to the geographic regions in Figure 4.11, while the misplaced OTUs are indicated with an asterisk and their geographic location shown in Figure 4.11.

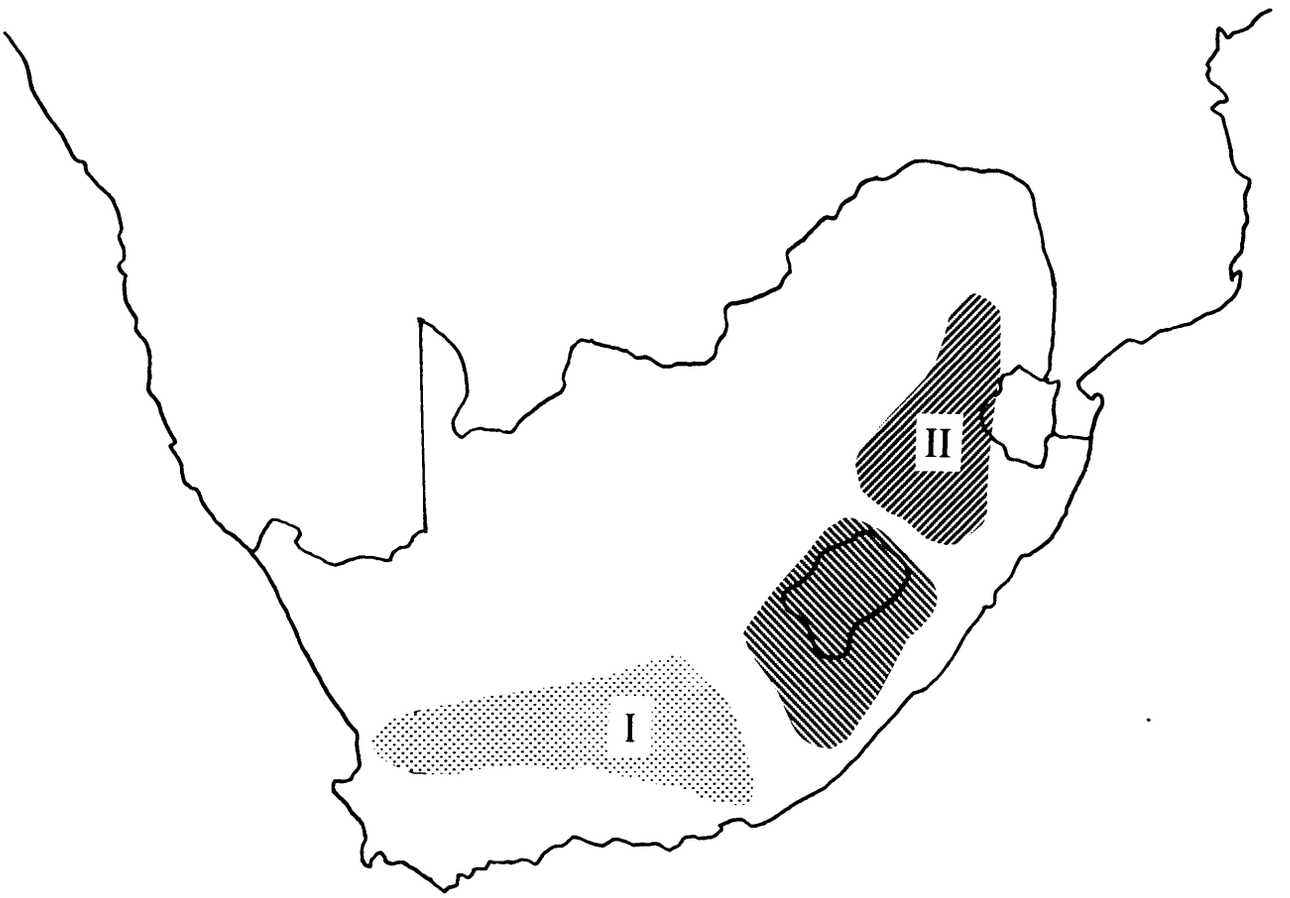


Figure 4.11 Geographic location of cluster I (south-Karoo ▒) and cluster II (eastern ▨) and the misclassified OTUs (▩) which fall within a zone of contact between the two major phenetic assemblages.

Status of Boshof and Fauresmith specimens

The Boshof and Fauresmith specimens showed no clear cranial affinity to any of the observed groups. Based on the data presented here the most likely explanation for this result would be that regional hybridization may have occurred between representatives of the two clades at some time in the recent or evolutionary past. Two possible explanations may be advanced to account for the origin of these postulated hybrids. First, hybrids may have resulted from unidirectional introgression which may have occurred by two means. Males from the south-Karoo group may have interbred with a population of north-western clade individuals. The contribution of south-Karoo nuclear genes could be reflected by cranial morphology but the absence of south-Karoo females from the populations would preclude the presence of maternally inherited mitochondrial DNA. Consequently, mitochondrial DNA analyses would be unable to reflect this hybridization event. This theory, however, assumes that within *Pronolagus* there is male biased dispersal, a life history characteristic which has not been recorded for this species. In fact, as is evidenced by other leporids, dispersal generally seems to involve juveniles of both sexes (Gibb 1990). Alternatively, the observed patterns could result from the asymmetrical outcome of the two reciprocal hybrid crosses: offspring from a cross between south-Karoo males and northern females are viable and fertile but the reciprocal cross of south-Karoo females and northern males produce inviable or infertile hybrids. This would explain the loss of south-Karoo mitochondrial DNA from these specimens and mirror the results for pocket gophers (Baker *et al* 1989). A second explanation may be, that males and females from both clades were present in the region and interbred but that the south-Karoo mitochondrial DNA lineages was subsequently lost by stochastic lineage extinction (Avise *et al* 1984). This would imply a historic hybridization event.

The possibility that these specimens are hybrids, is however clearly speculative given the poor sampling of this region. Furthermore, these localities fall within a described subspecies of *P. rupestris*, namely *P. r. curryi* (Meester *et al* 1986) with the distribution given as the western Orange free State from Fauresmith and Boshof to Vryburg. Extensive sampling of this region may reveal that this subspecies may be morphometrically distinct from the assemblages described in this chapter. If this is shown to be so, the north-western mitochondrial DNA clade could well be divided into two distinct morphotypes as is the case with the south-eastern mitochondrial DNA clade.

CHAPTER 5

GENERAL CONCLUSIONS AND IDENTIFICATION KEY TO THE SOUTHERN AFRICAN *P. RUPESTRIS*

The taxonomic revision of the genus *Pronolagus* has shown the genus to be more diverse than recognized in the previous descriptions by Robinson and Dippenaar (1983) and Meester *et al* (1986). Although *P. crassicaudatus*, *P. randensis* and *P. rupestris* remain valid, most of the taxonomic characters utilized in the present study revealed further subdivision within *P. rupestris*. The mitochondrial DNA data (both RFLP and sequence) define two discreet geographic clades, referred herein as the north-western and south-eastern clades (Chapter 2). Cranial morphology on the other hand delineated three phena, a north-western, south-Karoo and eastern assemblage (Chapter 4). To rigorously assess the relationships within *Pronolagus*, and to establish phylogenetic reliability, congruence between independent data sets (molecular and non-molecular, in this instance morphological) is clearly desirable (Hillis *et al* 1993). Congruence would suggest that the underlying historical pattern has been identified, whereas conflict could suggest theoretical and/or procedural problems (Hillis 1987).

Comparison of the molecular and morphological data sets used in this study revealed striking correspondence between assemblages defined by each parameter. A north-western assemblage is supported by both molecular and morphological data, while the phenotypic south-Karoo and eastern groups coincide with the mitochondrial DNA south-eastern clade (Matthee 1993). Although the molecular data did not unequivocally reveal the presence the two morphometric phena within the south-eastern clade, the high sequence divergence values within this clade (3.35% *cf* the north-western clade's 1.65%; see Table 2.5 p. 33) supports further subdivisioning within this taxon.

Proposed model for speciation patterns within *Pronolagus*

The unusually high levels of variation within the *Pronolagus* genus may be attributable to habitat specificity. Rock rabbits are restricted to discontinuous rocky outcrops and dispersal would require suitable intervening habitat. In its absence, animals inhabiting these outcrops would be isolated and may, through time, accumulate genetic change. This mode of speciation invokes geographical barriers as the likely cause of divergence between isolates.

Pronolagus fossil remains have been recorded at Langebaanweg (south western Cape) and Makapansgat (Northern Transvaal) as early as five million years before present (Klein 1994), indicating the presence of *Pronolagus* in areas at almost opposite ends of the current distribution (the absence of fossils in the intervening areas may be a sampling artefact). Unfortunately, fossiliferous localities between 10 and 5.2 mya in southern Africa are unknown, thus there is no indication of *Pronolagus* distribution prior to 5 mya. The widespread southern African dispersal of *Pronolagus* may have been initiated by the Terminal Miocene Event when the declining global temperatures culminated in the spread of grasslands and savannah (6.5 to 5 mya). Previous forests and woodlands, occurring during warm humid periods, may have restricted the dispersal of these leporids. In contrast, the opening up of the intervening areas through the spread of grasslands may have allowed migration through previously impenetrable vegetation.

The continual decline in temperatures toward the present was characterised by climatic fluctuations, alternating between warm humid periods and those of extreme aridity (Butzer 1983). It seems likely therefore that a cycle of aridity, following the initial widespread dispersal of *Pronolagus*, may have caused the extinction of many subpopulations. Arid intervening regions are likely to have reduced or at least impeded the movement (and gene flow) of animals between remaining relict populations. If populations were geographically and genetically isolated for a sufficient period of time, the accumulation of genetic change within these populations may have led to the initial division of *Pronolagus* into genetically and morphologically distinct groups which eventually gave rise to *P. crassicaudatus*, *P. randensis* and *P. rupestris*. Similar environmental perturbations may have shaped the more recent cladogenic events within *P. rupestris*. *Pronolagus rupestris*' distribution, particularly

of the north-western and south-Karoo clades, coincides with the semi-arid regions of South Africa and where climatic fluctuations are more dramatic than that of the subhumid interior (Butzer 1983). It seems possible therefore that *P. rupestris* was subject to more frequent population fragmentation than the other two species, which, in turn, may have contributed to the substantial variability observed between populations within this species.

Taxonomic status of assemblages defined within *P. rupestris*

While *P. crassicaudatus* and *P. randensis* are clearly good species, the status of the three phena described within *P. rupestris* must be determined and more specifically, whether these warrant species status. Views that species should be morphologically distinct or exhibit a minimum level of genetic divergence, are simplistic. Due to differing evolutionary pressures, patterns of morphological and molecular variation may not necessarily be similar, or divergence of taxa may not be apparent at either of these levels. Differences indicative of discrete units within taxa may be caused by simple changes not affecting morphology or measures of genetic divergence (Hillis 1987; Hillis *et al* 1993).

Attempts to quantify distinct taxa can be summarized by the biological and evolutionary species concepts (and their derivatives; King 1993). While the first emphasizes interbreeding natural populations that are reproductively isolated from each other, the latter accentuates lineages with their own evolutionary tendencies and historical fates (Ax 1984). While both have avid opponents, the biological species concept is the least practical. The criterion of reproductive isolation is particularly difficult to test (Wayne 1992) and the correspondence between morphological, genetic, ecological and reproductive discontinuities are often simply assumed (Mishler & Donaghue 1982).

Since reproductive isolation can only be inferred, no inferences will be made regarding biological species. Instead the observed molecular and morphological variation detected in this study have been interpreted from a taxonomic species perspective. Taxonomic species are those that are identified by a set of characters subjectively perceived by man (Lovtrop 1979; Pattterson 1981; Lambert *et al* 1987). Within the framework of this dissertation therefore, inferred groupings and species are to be regarded as taxonomic rather than biological, and relationships between these groupings are based on observable differences or

similarities. Using these criteria it seems justified that the three morphometric taxa described in Chapter 3 should be given species status. In describing these I have followed convention by elevating the first described subspecies occurring within the geographic boundaries of the morphometric assemblages to species status. In sum therefore, I recognise *P. rupestris* (north-western), and suggest two species (the names of which are available) for the eastern (*P. barretti*) and south-Karoo (*P. saundersiae*) morphometrically delineated assemblages.

Identification key:

This key is to be used in conjunction with diagnostic ratios and information generated from multivariate statistics. Standard statistics are presented in Appendix VI. For diagnosis of *P. crassicaudatus* and *P. randensis* refer to Robinson and Dippenaar (1983).

1. Skull robust (Total length of skull: 71.71 - 91.86 mm) with small bullae both in absolute measurements (Bulla breadth: 5.58 - 7.87 mm) and proportionately (Bulla breadth X 100/ total length of skull = 7.05 - 8.09%; Bulla breadth X 100/ mandibular toothrow length = 15.12 - 17.74%; Bulla breadth X 100/ posterior frontal breadth = 49.37 - 60.39%; Figure 5.1). Tail consistently a light sandy colour with usually sandy undertone to fur. Geographic range: occurring along the eastern escarpment from the vicinity of Lady Grey and extending into the eastern Transvaal (Figure 4.4).....*P. barretti.*

2. Skull gracile (Total length of skull: 70.60 - 85.27mm) with robust inflated bulla both in absolute measurements (Bulla breadth: 5.61 - 8.53mm) and proportionately (Bulla breadth X 100/ total length of skull = 8.27 - 9.45%; Bulla breadth X 100/ mandibular toothrow length = 17.98 -20.88%; Bulla breadth X 100/ posterior frontal breadth = 56.59 - 72.43%; Figure 5.1). Tail ranging in colour from dark red to black. Geographic range: Northern Cape Province, eastern Orange Free State, Western and Eastern Cape Provinces (Figure 4.4).....3

3[1]. Tail completely black or partially black/dark red (not consistent in eastern parts of range). Fur frequently characterised by reddish undertone. Total length of skull: 75.09 -85.27mm. Frontal and muzzle lengths almost equal: frontal length X 100/ muzzle length = 95.49 - 104.42% (Figure 5.2). Geographic range: Northern Cape Province extending eastward into the western Orange Free State (Figure 4.4).....*P. rupestris.*

3[2]. Tail usually deep red colour. Small skull (Total length of skull: 70.60 - 82.11mm). Frontal length shorter than muzzle length: Frontal length X 100/ muzzle length = 88.63 - 89.06% (Figure 5.2). Geographic range: Western and Eastern Cape provinces (Figure 4.4).....*P. saundersiae.*

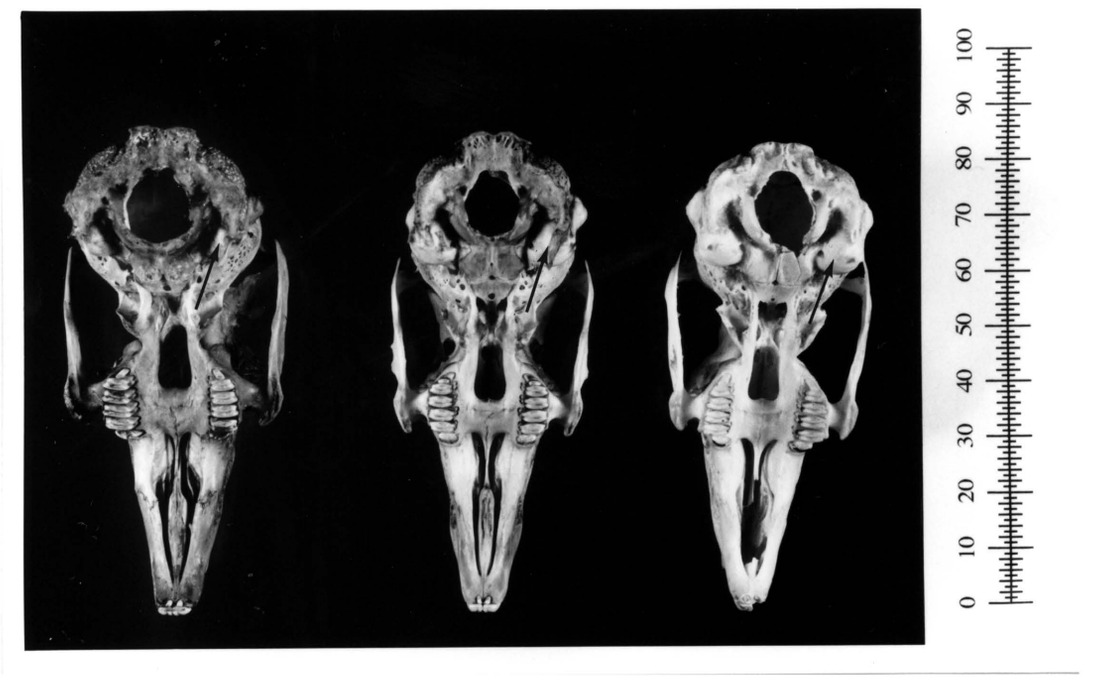


Figure 5.1 Photograph of the ventral aspect of the skulls. Bullae are indicated by an arrow and species from left to right are *P. barretti*, *P. saudersiae* and *P. rupestris*.

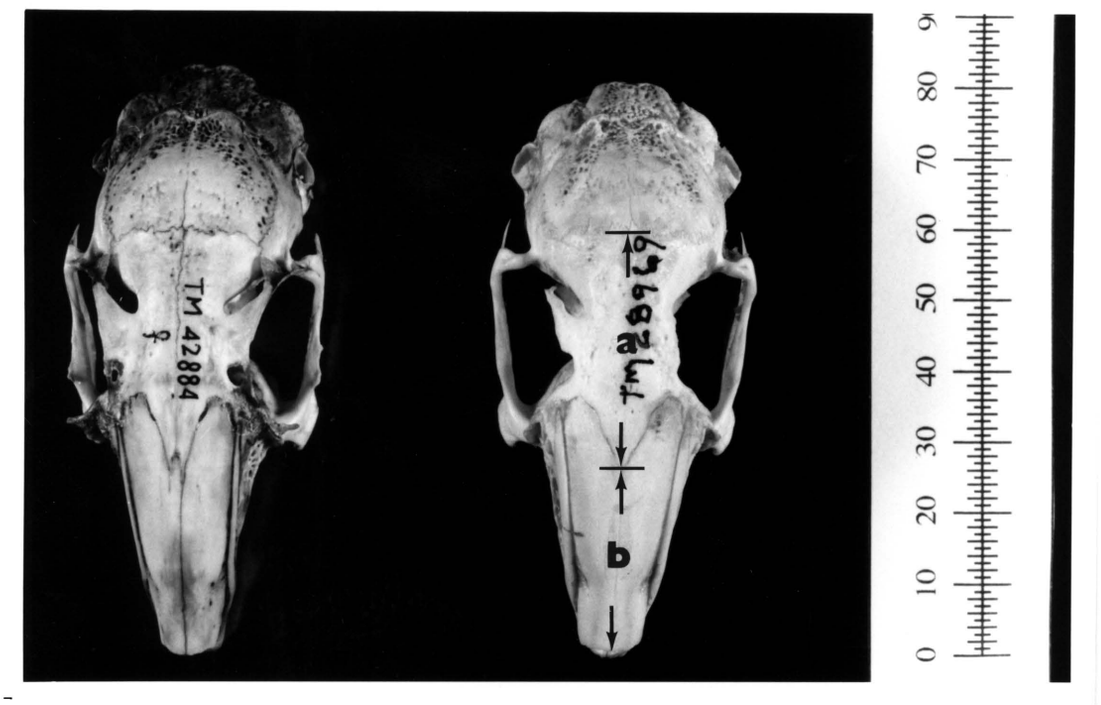


Figure 5.2 Photograph showing dorsal aspect of the skull. Character A = frontal length; Character B = muzzle length. Species from left to right are *P. saundersiae* and *P. rupestris*.

SPECIES DESCRIPTION

Genus *Pronolagus* Lyon, 1904

Pronolagus Lyon, 1904: Smithsonian Misc. Col. 45: 321, 416.

Pronolagus rupestris (Smith, 1834)

Lepus rupestris Smith, 1834: South African Quarterly Journal Ser. 2,2: 174.
Lepus melanurus Rüppell, 1842: Museum Senckenbergianum, Frankfurt a. M. 3: 137.
Oryctolagus crassicaudatus curryi Thomas, 1902. Annals and Magazine of Natural History 10: 245.

Holotype: BM (NH) 45.7.3.8; sex unknown; no type locality, see Meester *et al* 1986 for discussion.

Distribution: Northern Cape province extending eastwards into the Orange Free State.

Diagnosis: Medium-sized (Total length of skull: 75.09 - 85.27mm). The bullae are robust. Frontal and muzzle lengths almost equal. The tail is usually black or black/dark red although this is not consistent in the eastern parts of the distribution. The fur is frequently characterized by a reddish undertone.

General remarks: Characterized by distinctive mitochondrial DNA haplotypes which are not shared by the other species in the genus. The subspecies in the eastern region of its distribution, *P. r. curryi* may represent either hybrids between this species and *P. saundersiae*, or be phenetically distinct from the other specimens in this species.

Pronolagus saundersiae Hewitt, 1927

Pronolagus crassicaudatus saundersiae Hewitt, 1927: Records of the Albany Museum 3: 434.
Pronolagus crassicaudatus australis Roberts, 1933: Annals of the Transvaal Museum 15: 270.
Pronolagus crassicaudatus mulleri Roberts, 1938: Annals of the Transvaal Museum 19: 243.

Holotype: Type specimen in Kaffrarian Museum, number not allocated; sex unknown; Albany district (pers. commumnication Lloyd Wingate, Kaffrarian Museum, King William's Town).

Distribution: Western and Eastern Cape Provinces.

Diagnosis: Small skull (Total length of skull: 70.09 - 85 - 82.11mm). The bullae are robust. Frontal length shorter than muzzle length. The tail usually a deep red colour.

General Comments: Cannot be distinguished unequivocally from *P. barretti* using molecular characters. Roberts (1951) did not record museum number; possibly lost or not allocated.

Pronolagus barretti Roberts, 1949

Pronolagus barretti Roberts, 1949: Annals of the Transvaal museum 21: 179.

Holotype: T.M. 10014; sex unknown; Matiwane, Ladysmith, Natal.

Distribution: Occurring along the eastern escarpment from the vicinity of Lady grey and extending into the Eastern Transvaal.

Diagnosis: Skull robust (Total length of skull: 71.71 - 91.86mm). Bullae are considerably smaller than for the previous two species. The tail is a light sandy colour with a usually sandy undertone to fur.

General remarks: Cannot be distinguished from *P. saundersiae* using molecular characters.

SUMMARY

A taxonomic revision of the genus *Pronolagus* utilizing sequence data from two mitochondrial genes, cytochrome b and the 12S rRNA, indicated that the currently accepted morphometrically defined species within the leporid genus *Pronolagus* (*P. crassicaudatus*, *P. randensis* and *P. rupestris*) are similarly distinguishable at a molecular level. More specifically, the mitochondrial DNA clades identified within *P. rupestris* (Matthee 1993) were well supported by the sequence data of the present study. Cladistic and phenetic approaches based on the sequence data yielded similar tree topologies which clearly reveal *P. rupestris* to be characterized by two distinct genetic clades. These two mitochondrial assemblages occur in geographically discrete regions of South Africa; the first occurring throughout the Northern Cape Province and the eastern parts of the Orange Free State while the second ranges from the Western and Eastern Cape Provinces along the Great Escarpment into the Eastern Transvaal. Attempts at clarifying two mitochondrial DNA clades using a variety of chromosomal staining techniques (G-banding, C-banding and silver staining) showed all representatives of the genus to be karyotypically invariant.

The third parameter utilized in this study, cranial morphology, delineated three distinct phena: a north-western group, the geographic localities of which corresponded to the mitochondrial DNA north-western clade and two assemblages which jointly comprise the mitochondrial DNA south-eastern clade, and are designated herein as the south-Karoo and eastern phenetic groups. These morphologically delineated taxa are distinguishable from each other based on diagnostic cranial ratios. Only two localities, Boshof and Fauresmith, which are situated in the eastern parts of the north-western clade's distribution, and characterized by corresponding mitochondrial DNA haplotypes, could not be assigned to any of the morphometric groups with any degree of confidence. It is hypothesized that specimens from these areas represent either hybrids between the north-western and south-Karoo morphometric clades or, alternatively, may represent a distinctive but not clearly resolved sub-assemblage within the north-western mitochondrial DNA clade.

The marked degree of congruence between the molecular and morphological data sets is indicative of the robust nature of inferred taxonomic groups within *P. rupestris*. Taxonomic recognition at the species level of the three assemblages within *P. rupestris* by their elevation to species status is strongly recommended. The convention of elevating the first described subspecies within the geographic confines of the morphometric clades to species status was followed. Therefore, north-western clade retains *P. rupestris* as species designation and two new species names are suggested: *P. saundersiae* (Hewitt 1927) and *P. barretti* (Roberts 1949) respectively for the south-Karoo and eastern morphometrically delineated assemblages.

In a secondary aspect of this study an attempt was made to identify the probable sister taxon of the Lagomorpha among eutherian mammals utilizing 12S rRNA sequence data. The phylogenies constructed from these data were unable to provide conclusive resolution of the interordinal relationships within the eutherian mammals. It is, however, suggested that the orders Scandentia (tree shrews), Primates or Macroscelidea (elephant shrews) are the strongest contenders for lagomorph sister taxon status.

OPSOMMING

'n Taksonomiese hersiening van die genus *Pronolagus*, gebaseer op nukleotiedopeenvolgingsdata van twee mitochondriale gene, sitochroom b en 12S rRNA, dui daarop dat die huidig aanvaarde morfometries gedefinieerde spesies binne die Leporidae genus *Pronolagus* (*P. crassicaudatus, P. randensis en P. rupestris*) ook op die molekulêre vlak van mekaar onderskei kan word. Meer spesifiek, die mitochondriale DNA groepe geïdentifiseer binne *P. rupestris* (Matthee 1993), word ondersteun deur die nukleotiedopeenvolgingsdata verkry in die huidige studie. Kladistiese en fenetiese benaderinge, gebaseer op die nukleotieddata, het soortgelyke boom topologieë tot gevolg wat onomwondelik aantoon dat *P. rupestris* gekarakteriseer word deur twee duidelike genetiese groepe. Hierdie twee mitochondriale groeperings kom voor in geografies diskrete gebiede van Suid-Afrika: die eerste kom voor regdeur die Noord-Kaapprovinsie asook in die oostelike dele van die Oranje-Vrystaat terwyl die tweede groep strek vanaf die Wes- en Oos-Kaapprovinsies al langs die Groot Platorand tot in die Oos-Transvaal. Pogings om hierdie twee mitochondriale groepe van mekaar te onderskei op grond van verskeie chromosoom bandkleuringstegnieke (G-band, C-band en silwer kleuring) het aangetoon dat verteenwoordigers van die groepe kariotipies identies is.

Die derde parameter wat gebruik is in die huidige studie, skedelmorfometrie, het drie diskrete fena aangedui: 'n noord-westelike groep waarvan die geografiese lokaliteite ooreenstem met die mitochondriale noord-westelike groep, asook twee groeperings wat gesamentlik die mitochondriale suidelike groep vorm en word onderskeidelik aangedui word as die suid-Karoo en die oostelike fenetiese groepe. Hierdie morfologies gekarakteriseerde taksa is onderskeibaar op grond van diagnostiese skedel verhoudings. Slegs twee lokaliteite, Boshof en Fauresmith, wat geleë is in die oostelike dele van die noord-westelike groep se geografiese verspreiding, en gekarakteriseer word deur gepaardgaande mitochondriale DNA haplotipes, kon nie oortuigend toegewys word aan enige van die morfologiese groepe nie. Daar word gehipotiseer dat eksemplare van hierdie areas verteenwoordigend is van basters tusse diere van die noord-westelike en suid-Karoo morfologiese groepe of verteenwoordigend kan wees van 'n bepaalde, maar nie duidelik gedefinieerde, sub-groepering binne die noord-westelike mitochondriale groep.

Die duidelike graad van ooreenstemming tussen die molekulêre en morfologiese datastelle is 'n aanduiding van die robuustheid van die voorgestelde taksonomiese groepe binne *P. rupestris*. Taksonomiese herkenning op die spesies vlak van die drie groepe binne *P. rupestris* word ten sterkste aanbeveel. Die konvensie om die subspesie wat eerste beskryf is binne die grense van die morfologiese groepe se status te verhoog tot dié van 'n spesie is nagevolg. Dus behou die noord-westelike groep *P. rupestris* terwyl twee nuwe spesie name voorgestel word naamlik *P. saundersiae* vir die suid-Karoo groep en *P. barretti* vir die oostelike morfologies gedefinieerde groep.

As sekondêre aspek van die studie is gepoog om 'n moontlike sister-takson vir die Lagomorpha te identifiseer binne die eutherian soogdiere, deur gebruik te maak van 12S rRNA nukleotiedopeenvolgingsdata. Die filogenetiese verwantskappe, gekonstrueer vanaf hierdie data, kon geen defnitiewe resolusie binne die interordinale verwantskappe vir die eutherian soogdiergroep aandui nie. Daar word egter voorgestel dat die ordes Scandentia (skeerbekkies), Primate of die Macroscelidea (klaasneuse) die sterkste aanspraakmakers is as die sister-takson van die Lagomorpha.

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APPENDIX I

Museum accession numbers of *Pronolagus rupestris* specimens collected for the present study.

LOCALITY	MUSEUM ACCESSION NUMBERS
Fauresmith	TM 44547 - 44550
Bloemfontein	TM 44482 - 44483
Boshof	TM 44545 - 44546

APPENDIX II

Aligned sequences (a) of a 333 bp region of the mitochondrial DNA cytochrome b gene with the corresponding amino acid sequences (b) for eight representatives of *Pronolagus* and the single outgroup species *Ochotona princeps*. If a nucleotide could not be scored with certainty it is indicated by N.

(a)

```
                                                                      50
P. randensis 1          ACCCACCCCATTATTAAAATTGTGAACCACTCCCTGATCGACCTCCCCGC
P. randensis 2          ACCCACCCCATTATTAAAATTGTGAACCACTCCCTGATCAGCCTCCCCGC
P. rupestris 1          ACCCACCCCCTACTTAAAATCGTGAATCATTCCCTAATTGACCTCCCCGC
P. rupestris 2          ACCCACCCCCTACTTAAAATCGTGAACCATTCCCTAATTGACCTCCCAGC
P. rupestris 3          ACCCACCCCCTACTTAAAATCGTGAACCATTCCCTAATTGACCTCCCGGC
P. rupestris 4          ACCCACCCCCTACTTAAAATCGTGAACCATTCCCTAATTGACCTCCCGGC
P. crassicaudatus 1     ACCCACCCCCTTATTAAAATCTTGAACCACTCCCTAATTGACCTCCCAAC
P. crassicaudatus 2     ACCCACCCCTTTATTAAAATTTTAAATCACTCCCTAATTGACCTTCCAAC
O. princeps             NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN

                                                                     100
P. randensis 1          CCCATCAAACATCTCTGCCTGATGGAACTTTGGCTCTCTCCTAGGCCTTT
P. randensis 2          CCCATCAAACATCTCTGCCTGATGGAACTTTGGCTCTCTCCTAGGCCTTT
P. rupestris 1          CCCATCAAATATTTCCGCTTGATGGAACTTTGGGTCTCTCCTGGGCCTTT
P. rupestris 2          CCCATCAAATATTTCCGCTTGATGGAACTTTGGGTCTCTCCTGGGCCTTT
P. rupestris 3          CCCATCGAATATCTCTGCTTGATGAAATTTTGGCTCCCTTCTGGGCCTCT
P. rupestris 4          CCCATCGAATATCTCTGCTTGATGAAATTTTGGCTCCCTTCTGGGCCTCT
P. crassicaudatus 1     CCCATCAAATATCTCTGCTTGATGAAACTTTGGAACTCTCCTAGGCTTAT
P. crassicaudatus 2     CCCATCAAACATCTCTGCCTGATGAAATTTTGGGTCTCTCCTAGGCTTAT
O. princeps             NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGGTCTCTCCTANNNNNNN

                                                                     150
P. randensis 1          GCCTAGTTATCCAAATTTTCACCGGGCTTTTCCTGGCCATGCACTATACA
P. randensis 2          GCCTAGTTATCCAAATTTTCACCGGGCTCTTCCTGGCCATGCACTATACA
P. rupestris 1          GCCTAATCCTGCAAATCCTAACAGGACTATTCCTAGCAATACACTATACA
P. rupestris 2          GCCTAATCCTGCAAATCCTAACAGGACTATTCCTAGCAATACACTATACA
P. rupestris 3          GCCTAATCATCCAAATCCTAACCGGACTTTTTCTAGCCATACACTACACA
P. rupestris 4          GCCTAATCATCCAAATCCTAACCGGACTTTTTCTAGCCATACACTACACA
P. crassicaudatus 1     GCCTAGCCATCCAAATTTTTACCGCGCTCTTCCTAGCCATACATTACACA
P. crassicaudatus 2     GCCTAGCCATCCAAATTTTTACCGGGCTCTTCCTAGCCATACACTACACA
O. princeps             NGCTAGGTATCCAAATCATCACCGGCTTATTCCTGGCCATACACTACACG

                                                                     200
P. randensis 1          TCAGATACAATGACAGCATTCTCATCCGCTACCCACATTTGCCGAGACGT
P. randensis 2          TCTGATACAATGACAGCATTCTCATCCGTCACCCACATTTGCCCAGACGT
P. rupestris 1          TCTGACACAATAACAGCATTTTCCTCTGTCACCCACATTTGCCGAGACGT
P. rupestris 2          TCTGACACAATAACAGCATTTTCCTCTGTCACCCACATTTGCCGAGACTT
P. rupestris 3          TCAGACACAACAACAGCATTTTCATCTGTTACCCACATTTGTCGAGACGT
P. rupestris 4          TCAGACACAACAACAGCATTTTCCTCTGTTACCCACATTTGTCGTGATGT
P. crassicaudatus 1     TCAGATACAATAACAGCATTTTCATCTGTCACTCATATTTGTCGAGATGT
P. crassicaudatus 2     TCAGACACAATAACAGCATTTTCATCCGTCACTCACATTTGCCGAGATGT
O. princeps             TCAGACACACTAACAGCATTCTCATCAGTCACTCACATCTGCCGAGATGT
```

	250
P. randensis 1	CAACCACGGCTGACTCATTCGCTACCTCCACGCCAATGGAGCTTCCATAT
P. randensis 2	CAACCACGGCTGACTCATTCGCTACCTCCACGCCAATGGAGCTTCCATAT
P. rupestris 1	CAACCATGGCTGAATCATTCGTTACCTCCATGCCAACCGGAGCTCTATAT
P. rupestris 2	TAATTATGGCTGAATCATCCGTTACATCCATGCCAACCGGACGTTCATAT
P. rupestris 3	CAATCACGGCTGGCTCATTCGCTACCTCCACGCCAATGGGGCCTCTATAT
P. rupestris 4	TAATCACGGCTGGCTCATTCGCTACCTCCACGCCAATGGGGCCTCTATAT
P. crassicaudatus 1	TAATTATGGTTGACTAATTCGCTACCTCCACGCCAACGGGAGCCCTATGT
P. crassicaudatus 2	CAATTACGGCTGGCTTATTCGCTACCTCCACGCCAATGGAGCCTCTATAT
O. princeps	AAACTACGGCTGAATTATCCGCTACCTACACGCCAACGGCGCATCCATAT

	300
P. randensis 1	TTTTTATTTGCCTCTATATACACGTAGGCCGAGGAATTTACTATGGCTCA
P. randensis 2	TTTTTATCTGCCTCTATATACACGTAGGCCGAGGAATTTACTATGGCTCA
P. rupestris 1	TTTTTATTTGCCTCTACATGCACGTAGGCCGAGGAATTTACTATGGCTCA
P. rupestris 2	TTTTTATTTGCCTCTACATGCACGTGGGCCGAGGGATTTACTATGGTTCT
P. rupestris 3	TTTTTATTTGTCTATACATGCACGTAGGTCGAGGGATTTACTATGGTTCT
P. rupestris 4	TTTTTATTTGTCTCTACATGCACGTAGGTCGAGGAATTTACTATGGCTCA
P. crassicaudatus 1	TCTTTATCTGCCTTTACTTGCACGTAGGTCGAGGAATTTACTACGGCTCA
P. crassicaudatus 2	TCTTTATTTGCCTTTACATGCACGTGGGTCGAGGAATCTACTACGGCTCA
O. princeps	TCTTCATCTGCCTCTTCTTACACGTAGGTCGAGGTATCTATTACGGCTCC

	333
P. randensis 1	TATACATATTCAGAAACCTGAAATATTGGCATC
P. randensis 2	TATGCATATTTAGAAACCTGAAATATTGGCATC
P. rupestris 1	TATACATATACAGAAACCTGAAATATCGGCATC
P. rupestris 2	TATACATATACAGAAACCTGAAACATCGGCATC
P. rupestris 3	TATACATATACAGAAACCTGAAACATCGGCATC
P. rupestris 4	TATACATACATAGAGACCTGAAATATCGGTATC
P. crassicaudatus 1	TACACATACTCAGAAACCTGAAACATTGGCATT
P. crassicaudatus 2	TACACATACTTAGAAACCTGAAATATTGGCATT
O. princeps	TTCACCTACTCAGAGACATGAAACATCGGAATC

(b)

```
                                                                      50
P. randensis 1          THPIIKIVNHSLIDLPAPSNISAWWNFGSLLGLCLVIQIFTGLFLAMHYT
P. randensis 2          THPIIKIVNHSLISLPAPSNISAWWNFGSLLGLCLVIQIFTGLFLAMHYT
P. rupestris 1          THPLLKIVNHSLIDLPAPSNISAWWNFGSLLGLCLILQILTGLFLAMHYT
P. rupestris 2          THPLLKIVNHSLIDLPAPSNISAWWNFGSLLGLCLILQILTGLFLAMHYT
P. rupestris 3          THPLLKIVNHSLIDLPAPSNISAWWNFGSLLGLCLIIQILTGLFLAMHYT
P. rupestris 4          THPLLKIVNHSLIDLPAPSNISAWWNFGSLLGLCLIIQILTGLFLAMHYT
P. crassicaudatus 1     THPLIKILNHSLIDLPTPSNISAWWNFGTLLGLCLAIQIFTALFLAMHYT
P. crassicaudatus 2     THPFIKILNHSLIDLPTPSNISAWWNFGSLLGLCLAIQIFTGLFLAMHYT
O. princeps             NNNNNNNNNNNNNNNNNNNNNNNNNNGSLLNNNLGIQIITGLFLAMHYT

                                                                     100
P. randensis 1          SDTMTAFSSATHICRDVNHGWLIRYLHANGASMFFICLYMHVGRGIYYGS
P. randensis 2          SDTMTAFSSVTHICPDVNHGWLIRYLHANGASMFFICLYMHVGRGIYYGS
P. rupestris 1          SDTMTAFSSVTHICRDVNHGWIIRYLHANRSSMFFICLYMHVGRGIYYGS
P. rupestris 2          SDTMTAFSSVTHICRDFNYGWIIRYIHANRTFMFFICLYMHVGRGIYYGS
P. rupestris 3          SDTTTAFSSVTHICRDVNHGWLIRYLHANGASMFFICLYMHVGRGIYYGS
P. rupestris 4          SDTTTAFSSVTHICRDVNHGWLIRYLHANGASMFFICLYMHVGRGIYYGS
P. crassicaudatus 1     SDTMTAFSSVTHICRDVNYGWLIRYLHANGSPMFFICLYLHVGRGIYYGS
P. crassicaudatus 2     SDTMTAFSSVTHICRDVNYGWLIRYLHANGASMFFICLYMHVGRGIYYGS
O. princeps             SDTLTAFSSVTHICLDVNYGWIIRYLHANGASMFFICLFLHVGRGIYYGS

                                        119
P. randensis 1          YTYSETWNIGILLLFTVMA
P. randensis 2          YAYLETWNIGILLLFTVMA
P. rupestris 1          YTYTETWNIGILLLFTVMA
P. rupestris 2          YTYTETWNIGILLLFMVMA
P. rupestris 3          YTYTETWNIGILLLFMVMA
P. rupestris 4          YTYLETWNIGILLLFTVMA
P. crassicaudatus 1     YTYSETWNIGILLLFAVMA
P. crassicaudatus 2     YTYLETWNIGILLLFATMA
O. princeps             FTYSETWNIGILLLFAVMR
```

The single letter codes for amino acids according to Anderson *et al* (1981) were used: A - Ala; C - Cys; D - Asp; E - Glu; F - Phe; G - Gly; H - His; I - Ile; K - Lys; L - Leu; M - Met; N - Asn; P - Pro; Q - Gln; R - Arg; S - Ser; T - Thr; V - Val; W - Trp; Y - Tyr.

APPENDIX III

Aligned sequences of a 745 bp region of the mitochondrial 12S rRNA gene for eight representatives of *Pronolagus* and the single outgroup *Ochotona princeps*.

```
                                                                      50
P. randensis 1          ATTACACATGCAAGACTCCCCGCGCCAGTGAGAATGCCCTTAACATCAGC
P. randensis 2          ATTACACATGCAAGACTCCCCGCGCCAGTGAGAATGCCCTTAACATCAGC
P. rupestris 1          ATTACACATGCAAGATTCCCCGCACCAGTGAGAATGCCCTTAACATCAAT
P. rupestris 2          ATTACACATGCAAGATTCCCCGCACCAGTGAGAATGCCCTTAACATCAAT
P. rupestris 3          ATTACACATGCAAGACTCCCCATACCAGTGAGAATGCCCTTAACATCAAA
P. rupestris 4          ATTACACATGCAAGACTCCCCTAACCAGTGAGAATGCCCTTAACATCAGA
P. crassicaudatus 1     ATTACACATGCAAGATTCCCCATGCCAGTGAGAATGCCCTTAACATCAAC
P. crassicaudatus 2     ATTACACATGCAAGATTCCCCATGCCAGTGAGAATGCCCTTAACATCAAC
O. princeps             ATTACACATGTAAGCATCCCCGAACCGGTGAAAATGCCCTCCAACTCACC

                                                                     100
P. randensis 1          --CCAGATCAAGAGGAGCAGGCATTAAGCACACCAACTGGTAGCTCACAA
P. randensis 2          --CCAGATCAAGAGGAGCAGGCATTAAGCACACCAACTGGTAGCTCACAA
P. rupestris 1          --CTAGATCAAGAGGAGCAGGCATTAAGCACACCAATTGGTAGCTCACAA
P. rupestris 2          --CTAGATCAAGAGGAGCAGGCATTAAGCACACCAACTGGTAGCTCACAA
P. rupestris 3          --CTAGATCAAGAGGAGCAGGCATCAAGCACACCAACTGGTAGCTCACAA
P. rupestris 4          --CTAGATCAAGAGGAGCAGGCATTAAGCACACCCACTGGTAGCTCACAA
P. crassicaudatus 1     --CCAGATCAAGAGGAGTAGGCATTAAGCACACCAGCCGGTAGCTCACGA
P. crassicaudatus 2     --CCAGATCGAGAGGAGCAGGCATTAAGCGCACCAACTGGTAGCTCACGA
O. princeps             CACC-GATTAAGAGGGGCGGGTATTAAG--CACACACTAGTAGCTCAAAA

                                                                     150
P. randensis 1          CGCCTTGCTTACCCACGCCCCCAAGGGACACAGCAGTGATAAGAATTCAG
P. randensis 2          CGCCTTGCTTACCCACGCCCCCAAGGGACACAGCAGTGATAAGAATTCAG
P. rupestris 1          CGCCTTGCTTACCCACGCCCCCAAGGGACACAGCAGTGATAAGAATTTAG
P. rupestris 2          CGCCTTGCTTACCCACGCCCCCAAGGGACACAGCAGTGATAAGAATTTAG
P. rupestris 3          CGCCTTGCTTACCCACACCCCCAAGGGACACAGCAGTGATAAAAATTCAG
P. rupestris 4          CGCCTTGCTTACCCACACCCCCAAGGGACACAGCAGTGATAAAAATTCTG
P. crassicaudatus 1     CGCCTTGCTCACCCACACCCCCAAGGGATACAGCAGTGAATTAAACTTAG
P. crassicaudatus 2     CGCCTTGCTTACCCACACCCCCAAGGGATACAGCAGTGATTAGAATTCAG
O. princeps             TACCTTGCTTAACCACACCCCCAAGGGACACAGCAGTGATAAATCTTAAG

                                                                     200
P. randensis 1          CAATGAACGTAAGTTCGACTGAGCTATGCTACTCTAGGGTTGGTAAATTT
P. randensis 2          CAATGAACGAAAGTTCGACTGAGCTATGCTACTCTAGGGTTGGTAAATTT
P. rupestris 1          CAATGAACGAAAGTTCGACTGAGCTATGCTATCCTAGGGTTGGTAAATTT
P. rupestris 2          CAATGAACGAAAGTTCGACTAAGCTATGCTATCCTAGGGTTGGTAAATTT
P. rupestris 3          CAATGAACGAAAGTTCGACTGAGCTATGCTATCCTAGGGTTGGTAAATTT
P. rupestris 4          CAATGAACGAAAGTTCGACTGAGCTATGCTATCCTAGGGTTGGTAAATTT
P. crassicaudatus 1     CAATGAACGAAAGTTCGACTGAGTTATGCTACTTAAGGGTTGGTAAATTT
P. crassicaudatus 2     CAATGAACGAAAGTTCGACTGAGTTATGCTACTTAAGGGTTGGTAAATTT
O. princeps             CAATAAACGAAAGTTTGACTAAGTTAAACTACATCAGAGTTGGTAAATTT
```

```
                                                                                                                  250
P. randensis 1          CGTGCCAGCCACCGCGGTCATACGATTAACCCAAACTAATAAAACTCCGG
P. randensis 2          CGTGCTAGCCACCGCGGTCATACGATTAACCCGAACTAATAAAACTCCGG
P. rupestris 1          CGTGCCAGCCACCGCGGTCATACGACTAACCCAAACTAATAAAACCTCGG
P. rupestris 2          CGTGCCAGCCACCGCGGTCATACGATTAACCCAAACTAATAAACTCTCGG
P. rupestris 3          CGTGCCAGCCACCGCGGTCATACGATTGACCCAAACTAATAAACTCCCGG
P. rupestris 4          CGTGCCAGCCACCGCGGTCATACGATTGACCCAAACTAATAAAACTCCGG
P. crassicaudatus 1     CGTGCCAGCCACCGCGGTCATACGATTAACCCAAACTAATAAATACCCGG
P. crassicaudatus 2     CGTGCCAGCCACCGCGGTCATACGATTAACCCAAACTAATAAAATGCCGG
O. princeps             CGTGCCAGCCACCGCGGTCATACGATTAACCCAAATTAACAAAACCCCGG

                                                                                                                  300
P. randensis 1          CGTAAAGCGTGATTAGAAGCAAACAAAAAATAAAATCAAATAGCGTCTGA
P. randensis 2          CGTAAAGCGTGATTAGAAGCAAACAAAAAATAAAATCAAATAGCGTCTGA
P. rupestris 1          CGTAAAGCGTGTTTAGAATAGAACAAAAAATAAAATCAAATACCGTCTAA
P. rupestris 2          CGTAAAGCGTGATTAGAAATGAACAAAAAATAAAATCAAATACCGTCTAA
P. rupestris 3          CGTAAAGCGTGATTAGAAGCGAACAAAAAATAAAATCAAATACCGTCTAA
P. rupestris 4          CGTAAAGCGTGATTAGAAGCGAACAAAAAATAAAATCAAATGCCGTCTAA
P. crassicaudatus 1     CGTAAAGTGTGATTAGAAGTAAATGAAAAATAAAATCAAATAGCGTTTAA
P. crassicaudatus 2     CCTAAAGTCTGATTAGAAGTAAATGAAAAATAAAATCAAATAGCGTCCAA
O. princeps             CGTAAAGCGTGTTTAAGACTACAACT-CAATAAAGTTAAATAATACCCAA

                                                                                                                  350
P. randensis 1          ACTGTAAAAAGTACAAGCCGCGAACAAAAATAGT-CAACGAAAGTGATCT
P. randensis 2          ACTGTAAAAAGTACAAGCCGCGAACAAAAATAGT-CAACGAAAGTGATCT
P. rupestris 1          GCTGTAAAAAGTAATAGACACGAATAAAAATAAA-CAACGAAAGTGATTT
P. rupestris 2          GCTGTAAAAAGTAATAGACACGAACAAAAATAAC-CAACGAAAGTGATTT
P. rupestris 3          GCTGTAAAAAGTACTAGCCACGAACAAAAATAAT-CAACGAAAGTGATTT
P. rupestris 4          GCTGTAAAAAGTACTAGCCACGAACAAAAATAAT-CAACGAAAGTGATTT
P. crassicaudatus 1     GCTGTAAAAAGTACTAACCACGAACAAAAATAAT-CAACGAAAGTGATCT
P. crassicaudatus 2     GCTGTAAAAAGTACTAGCCACGAACAAAAATAAT-CAACGAAAGTGATCT
O. princeps             GCTGTAAAAAGCTATAGCTATAATTAAGCCCCT--CAACGAAAGTGACTT

                                                                                                                  400
P. randensis 1          TACAATTTTCGAACTCACGATAGCTAAGACCCAAACTGGGATTAGATACC
P. randensis 2          TACAATTTTCGAACTCACGATAGCTAAGACCCAAACTGGGATTAGATACC
P. rupestris 1          TTATATTTTCGAACTCACGATAGCTAAGACCCAAACTGGGATTAGATACC
P. rupestris 2          TACAATTTTCGAACTCACGATAGCTAAGATCCAAACTGGGATTAGATACC
P. rupestris 3          TATAATTTTCGAACTCACGATAGCTAAGATCCAAACTGGGATTAGATACC
P. rupestris 4          TATAATTTTCGAACTCACGATAGCTAAGATCCAAACTGGGATTAGATACC
P. crassicaudatus 1     TATAATTTTCGAACTCACGAGAGCTAAGACCCAAACTGGGATTAGATACC
P. crassicaudatus 2     TCTAATTTTCGAACTCACGAGAGCTAAGACCCAAACTGGGATTAGATACC
O. princeps             TATCACCCTTGAATACACGAAAGCTAGGACCCAAACTGGGATTAGATACC

                                                                                                                  450
P. randensis 1          CCACTATGCCTAGCCCTAAACTTCAATAATTTCATAACAAAATTACTCGC
P. randensis 2          CCACTATGCCTAGCCCTAAACTTCAATAATTTCATAACAAAATTATTCGC
P. rupestris 1          CCACTATGCTTAGCCCTAAACTTTAATAATTCCATAACAAAACTATTAGC
P. rupestris 2          CCACTATGCTTAGCCCTAAACTTTAATAATTCCATAACAAAACTATTCGC
P. rupestris 3          CCACTATGCTTAGCCCTAAACTTTAATAATTATATAACAAAATTATTCGC
P. rupestris 4          CCACTATGCTTAGCCCTAAACTTTAATAATTATATAACAAAATTATTCGC
P. crassicaudatus 1     CCACTATGCTTAGCCCTAAACCTTAATAGTTTCATAACAAAACTATTCGC
P. crassicaudatus 2     CCACTATGCTTAGCCCTAAACCTCGATAATTCCATAACAAAATTACTCGC
O. princeps             CCACTATGCCTAGCCACAAACCTAAATAGTTAAACAACAAAACTATTCGC
```

```
                                                                      500
P. randensis 1          CAGAGAACTACAAGCCAGGGCTTGAAACTCAAAGGACTTGGCGGTGCTTC
P. randensis 2          CAGAGAACTACAAGCCAGGGCTTGAAACTCAAAGGACCTGGCGGTGCTTC
P. rupestris 1          CAGAGAACTACAAGCCAGGGCTTGAAACTCAAAGGACCTGGCGGTGCTTC
P. rupestris 2          CAGAGAACTACAAGCCAGGGCTTGAAACTCAAAGGACTTGGCGGTGCTTC
P. rupestris 3          CAGAGAACTACAAGCCAGGGCTTGAAACTCAAAGGACTTGGCGGTGCTTC
P. rupestris 4          CAGAGAACTACAAGCCAGGGCTTGAAACTCAAAGGACTTGGCGGTGCTTC
P. crassicaudatus 1     CAGAGAACTACAAGCCAGAGCTTAAAACTCAAAGGACTTGGCGGTGCTTT
P. crassicaudatus 2     CAGAGAACTACAAGCCAGAGCTTAAAACTCAAAGGACTTGGCGGTGCTTT
O. princeps             CAGAGAACTACTAGCAACAGCTTAAAACTCAAAGGACTTGGCGGTGCTTT

                                                                      550
P. randensis 1          ACACCCACCTAGAGGAGCCTGTTCTATAACCGACAATCCCCGATAAACCC
P. randensis 2          ACACCCACCTAGAGGAGCCTGTTCTATAATCGACAATCCCCGATAAACCC
P. rupestris 1          ACACCCTCCTAGAGGAGCCTGTTCTATAATCGATAATCCCCGATAGACCT
P. rupestris 2          ACACCCACCTAGAGGAGCCTGTTCTATAATCGATAATCCCCGATAAACCC
P. rupestris 3          ACACCCATCTAGAGGAGCCTGTTCTGTAATCGATAATCCCCGATAAACCC
P. rupestris 4          ACACCCATCTAGAGGAGCCTGTTCTATAATCGATAATCCCCGATAAACCC
P. crassicaudatus 1     ATACCCGCCTAGAGGAGCCTGTTCTATAATCGATAATCCCCGATAAACCC
P. crassicaudatus 2     ATACCCGCCTAGAGGAGCCTGTTCTGTAATCGATAATCCCCGATAAACCC
O. princeps             ATACCCACTTAGAGGAGCCTGTTCTATAATCGATAAACCCCGCTCCACCT

                                                                      600
P. randensis 1          CACCACTCTTTGCCAACTCAGCCTATATACCGCCATCTCCAGCAAACCCT
P. randensis 2          TACCACTCTTTGCCAACTCAGCCTATATACCGCCATCTCCAGCAAACCCT
P. rupestris 1          TACCACTCTTTGCCAACTCAGCCTATATACCGCCATCTCCAGCGAACCCT
P. rupestris 2          TACCACTCTTTGCCAACTCAGCCTATATACCGCCATCTCCAGCGAACCCT
P. rupestris 3          TGCCACTCTTTGCCAACTCAGCCTATATACCGCCATCTCCAGCAAACCCT
P. rupestris 4          TGCCACTCTTTGCCAACTCAGCCTATATACCGCCATCTCCAGCAAACCCT
P. crassicaudatus 1     TACCTCTCTTTGCCAACTCAGCCTATATACCGCCATCTCCAGCGAACCCT
P. crassicaudatus 2     TACCACTCTTTGCCAACTCAGCCTATATACCGCCATCTCCAGCGAACCCT
O. princeps             CACCACCCTTTGCTAACTCAGCCTGTATACCGCCATCTCCAGCAAACCCT

                                                                      650
P. randensis 1          AAAAAGGAATAAAAGTGAGCTCAATTACCCCATAAAAACGTTAGGTCAAG
P. randensis 2          AAAAAGGAATAAAAGTGAGCTCAATTACCCCATAAAAACGTTAGGTCAAG
P. rupestris 1          AAAAAGGAATAAAAGTGAGCTCAATCACCCCATAAAGACGTTAGGTCAAG
P. rupestris 2          AAAAAGGAACAAAAGTGAGCTCAATCACCTCATAAAAACGTTAGGTCAAG
P. rupestris 3          AAAAAGGAACAAAAGTGAGCTCAATCACCCCATAAAAACGTTAGGTCAAG
P. rupestris 4          AAAAAGGAACAAAAGTGAGCTTAATCACCCCATAAAAACGTTAGGTCAAG
P. crassicaudatus 1     GAAAAGGAGCAAAAGTGAGCTCAATCACCCCATAAAAACGTTAGGTCAAG
P. crassicaudatus 2     GAAAAGGAGTAAAAGTGAGCTTAATTACCCCATAAAAACGTTAGGTCAAG
O. princeps             AAAAAGGTATCATAGTAAGCCCAATAACCCCATAAAAACGTTAGGTCAAG

                                                                      700
P. randensis 1          GTGTAGCCCATAGAGTGGAAAGTAATGGGCTACATTTTCTACCCTAGAAT
P. randensis 2          GTGTAGCCCATAGAGTGGAAAGTAATGGGCTACATTTTCTACCCTAGAAC
P. rupestris 1          GTGTAGCCTATAGAGTGGAAAGTAATGGGCTACATTTTCTATCATAGAAC
P. rupestris 2          GTGTAGCCTATAGAGTGGAAAGCAATGGGCTACATTTTCTATCATAGAAT
P. rupestris 3          GTGTAGCCTATAGAGTGGAAAGTAATGGGCTACATTTTCTATCATAGAAC
P. rupestris 4          GTGTAGCCTATAGAGTGGAAAGTAATGGGCTACATTTTCTATCATAGAAC
P. crassicaudatus 1     GTGTAGCCTATGGAGTGGAAAGCAATGGGCTACATTTTCTACTATAGAAT
P. crassicaudatus 2     GTGTAGCCTATGGAGTGGAAAGCAATGGGCTACATTTTCTACTATAGAAT
O. princeps             GTGTAGCCCATAGGGTGGGAAGCAATGGGCTACATTTTCTTATATAGAAC

                                                                    745
P. randensis 1          ATA--CGGAAGCCCCTATGAAAGC-TAGGGGCCGAAGGAGGATTT
P. randensis 2          ATA--CGGAACTCCCTATGAAAGC-TAGGGACCAAAGGAGGATTT
P. rupestris 1          ATA--CGGAAGTCCCTATGAAATC-TAGAGACCAAAGGAGGATTT
P. rupestris 2          ATA--CGGAAGTCCCTATGAAATC-TAGAGACCAAAGGAGGATTT
P. rupestris 3          ATA--CGGAAGTCCCTATGAAACC-TAGAGACCAAAGGAGGATTT
P. rupestris 4          ATA--CGGAAGTCCCTATGAAACC-TAGAGACCAAAGGAGGATTT
P. crassicaudatus 1     ACA--CGGAACTCCCTGTGAAACC-TAGGGACCAAAGGAGGATTT
P. crassicaudatus 2     ACA--CGGAAGTCCCTGTGAAACC-TAGGGACCAAAGGAGGATTT
O. princeps             ACATACGAAAGCCCATGTGAAACCATAGG--CCGAAGGCGGATTT
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APPENDIX IV

Aligned sequences of a 700 bp region of the mitochondrial 12S rRNA gene with single representatives of various mammalian orders. Nucleotides that could not be scored with certainty are indicated by a N

```
                                                                                                  50
Hedgehog          ATTACACATGCAAGTATCAGCTAACCAGTGAGAATGCCCTCTAACCCTAT
Bat               NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Marsupial         CCTACACATGCAAGTTTCCGCTACCCAGTGAGAATGCCCTTTAAGTCTTA
Human             ATTACACATGCAAGCATCCCCGTTCCAGTGAGTTCACCCTCTAAATCAC-
Mouse             ATTACACATGCAAACCTCCATAGACCGGTGTAAAATCCCTTAAA--CATT
Cow               CTTACACATGCAAGCATCTACACCCCAGTGAGAATGCCCTCTAGGTTATT
Seal              ATTATACATGCAAGCCTCCGCGCCCCGGTGAAAATGCCCTCCAAATCCCA
Rabbit            ATTACACATGCAAGACTCCCCGCGCCAGTGAGAATGCCCTTAACATCAG-
Whale             CTTACACATGCAAGTATCCACATCGCAGTGAGAACGCGGTCTAAATCATA
Elephant shrew    ATTATACATGCAAGTATCCGCTAACCAGTGGAAATGCCCTTTTACTCG--
Tree shrew        ATTACACATGTAAGAATCCCCGATCCAGTGAGAATGCCCTCTAAGTCC--

                                                                                                 100
Hedgehog          TATATAGGTATAAAGGAGCAGGTATCAAGTACACAA-TAAATGTTGCTTA
Bat               NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Marsupial         TAAATTAAGCAAAAGGAGCTGGTATCAGGCACACAA---AATGTAGCCGA
Human             --CAC-GATCAAAAGGAACAAGCATCAAGCACGC-AGC-AATGCAGCTCA
Mouse             TACTTAAAATTTAAGGAGAGGGTATCAAGCACATTA---AAA-TAGCTTA
Cow               AAAAC----TAAGAGGAGCTGGCATCAAGCACAC-A-CCC-TGTAGCTCA
Seal              ATAACCGATTAAAAGGAGCAGGTATCAAGCACACTAA-ACAAGTAGCTTA
Rabbit            --CCCAGATCAAGAGGAGCAGGCATTAAGCACACCA--ACTGGTAGCTCA
Whale             AA----GATTAAAAGGAGCGGGTATCAAGCACGCTAGCACTAGCAGCTCA
Elephant shrew    --ACCCGATCCCAAGGAGCCGGCATCAAGCACACGA-CA---GTAGCTCA
Treeshrew         -AA-CCGACTAAAAGGAGCTGACATCAAGCACACTAAA-CAG-TAGCTCA

                                                                                                 150
Hedgehog          TAACATCTTGCTTAACCACACCCCCACGGGAAACAGCAGTGATAAATCTT
Bat               NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Marsupial         TAACACCTTGCTTTACCACACCCCCACGGGAGACAGCAGTGATTAAAATT
Human             AAACGCTTAGCCTAGCCACACCCCCACGGGAAACAGCAGTGATTAACCTT
Mouse             AGACACCTTGCCTAGCCACACCCCCACGGGACTCAGCAGTGATAAATATT
Cow               CGACGCCTTGCTTAACCACACCCC-ACGGGAAACAGCAGTGACAAAAATT
Seal              CAACGCCTTGCTCAACCACACCCCCACGGGATACAGCAGTAATAAAAATT
Rabbit            CAACGCCTTGCTTACCCACGCCCCCAAGGGACACAGCAGTGATAAGAATT
Whale             GAACGCCTCGCTTAGCCACGCCCCCACGGGACACAGCAGTGATAAAAATT
Elephant shrew    AAACGCCTTGCTTAGCCACACCCCCACGGGATACAGCAGTAATAAAAATT
Tree shrew        CAACGTTTTGCTCAACCACACCCCCACGGGTTACAGCAGTGATAAAAATT

                                                                                                 200
Hedgehog          GAGCTATAAACGAAAGTTTGACTAAGTCATACTAATAC---AGGGTTGGT
Bat               NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Marsupial         AAGCAATAAACGAAAGTTTGACTAAGTCATAATTTACATT-AGGGTTGGT
Human             TAGCAATAAACGAAAGTTTAACTAAGCTATACTAA-CC-CCAGGGTTGGT
Mouse             AAGCAATAAACGAAAGTTTGACTAAGTTATACC--TC-TT-AGGGTTGGT
Cow               AAGCCATAAACGAAAGTTTGACTAAGTTATATTAA---TT-AGGGTTGGT
Seal              AAGCTATGAACGAAAGTTTGACTAAGCTATATTAAACCTCCAGGGTTGGT
Rabbit            CAGCAATGAACGTAAGTTCGACTGAGCTATGCTA---CTCTAGGGTTGGT
Whale             AAGCTATAAACGAAAGTTCGACTAAGTCATGTTAAT--TTAAGGGTTGGT
Elephant shrew    AAGCAATAAACGAAAGTTTGACTAAGCCATACTGATA---GAGGGTTGGT
Tree shrew        AAGCAATGAACGAAAGTTTGACTAGGTTATACTACC----TAGGATTGGT
```

```
                                                                 250
Hedgehog       AAATCTCGTGCCAGCCACCGCGGTCATACGATTAACCCATGTTAATAGGA
Bat            NNNNNNNNNNNNNNNNNNNGCGGTCATACGATT-ACCCTAATTAATAGAT
Marsupial      CAATTTCGTGCCAGCCACCGCGGTCATACGATTAACCCAAATTAATAA-A
Human          CAATTTCGTGCCAGCCACCGCGGTCACACGATTAACCCAAGTCAATAGAA
Mouse          AAATTTCGTGCCAGCCACCGCGGTCATACGATTAACCCAAACTAATTA-T
Cow            AAATCTCGTGCCAGCCACCGCGGTCATACGATTAACCCAAGCTAACAGGA
Seal           AAATTTCGTGCCAGCCACCGCGGTCATACGATTAACCCAAACTAATAGGC
Rabbit         AAATTTCGTGCCAGCCACCGCGGTCATACGATTAACCCAAACTAATAAAA
Whale          AAACTTCGTGCCAGCCACCGCGGTCATACGATCGACCCAAATTAATAGAA
Elephant shrew CAATCTCGTGCCAGCCACCGCGGTCATACGATTAACCCAAATTAATAACA
Tree shrew     AAATTTGGGGCCAGCCACCGCGGTCATACGATTAACCCAAATTAATAGCT

                                                                 300
Hedgehog       ATTCGGCGTAAAGAGTGTTTTAGATATTATTAA---ACTGAAATTAAAAT
Bat            ATACGGCGTAAAGCGTGTTTAGGATCCCTAAAA---CAATAAAGCCAAAC
Marsupial      TAACGGCGTAAAGAGTGTTTAAGTTATATACAA---AAATAAAGTTAATA
Human          -TCCGGCGTAAAGAGTGTTTTAGATCACCCCCTCCCCAATAAAGCTAAAA
Mouse          CTTCGGCGTAAAACGTGTCAACTATAAATAAAT---AAATAGAATTAAAA
Cow            GTACGGCGTAAAACGTGTTAAAGCACCATACC----AAATAGGGTTAAAT
Seal           CCTCGGCGTAAAGCGTGTTAAAGATCAACCCA----CACTAAAGCTAAAA
Rabbit         CTCCGGCGTAAAGCGTGATTAGAAGCAAACAAA---AAATAAAATCAAAT
Whale          GCACGGCGTAAAGAGTGTTAAGGAGCCACATG----AAATAAAGTCAAAC
Elephant shrew AACCGGCGTAAAGTGTGGTTAAAAATTCGACAT---AAATAAAGTTAAAT
Tree shrew     CCTCGGCGTAAAGTGTGTTTATGAGAAACTGCG----AATAAAGTCAAAT

                                                                 350
Hedgehog       ATGA-TTCAGATGTAAAAAGCTTAAATTATTTATTAAAATTCAACTACAA
Bat            TTTAGCTACGCTGTAAAAAGCCACAGCTACTG--TAAGC-CCCATTACGA
Marsupial      ATTAACTAAACTGTAGCACGTTCTAGTTAATA-TTAAAA-TACATAATAA
Human          CTCACCTGAGTTGTAAAAAACTCCAGTTGACA--CAAAA-TAGACTACGA
Mouse          TCCAACTTATATGTGAAAATTCATTGTTAGGACCTAAAC-TCAATAACGA
Cow            TCTAACTAAGCTGTAAAAAGCCATGATTAAAA-TAAAAA-TAAATGACGA
Seal           CCTAACCAAGCCGTAAAAAGCTACCGTTAACA--TAAAA-TAGACCACGA
Rabbit         AGCGTCTGAACTGTAAAAAGTACAAGCCGCGAACAAAAA-TAGTCAACGA
Whale          CTTAATTAAGCTGTAAAAAGCCCTAATTAAAA-TTAAGC-CAAACTACGA
Elephant shrew TAGCTCTCTGCTGTAAAAAGCTAAAGACCTAAGAAAATAGACTACAACGA
Tree shrew     ACAGCCCAAGCTGTAAAATGCCACAGGCCCAC--TAAGA-ACAATGACGA

                                                                 400
Hedgehog       AAGTGA-TTTCATAAAATCTTATTACACGTGAGCTAAGATCCAAACTAGG
Bat            AAGTAACTTTAA-ATAATCCGAATACACGATAGCTAAGGCCCAAACTGGG
Marsupial      AAATGACTTTAATATCA-CCGACTACACGAAAACTAAGACACAAACTGGG
Human          AAGTGGCTTTAACAT-ATCTGAACACAGAATAGCTAAGACCCAAACTGGG
Mouse          AAGTAATTCTAGTCATTTAT-AATACACGACAGCTAAGACCCAAACTGGG
Cow            AAGTGACCCTACAAT-AGCCGAC-GCACTATAGCTAAGACCCAAACTGGG
Seal           AAGTGACTTTACTAA-TTCTGACTGCACGATAGCTAAGATCCAAACTGGG
Rabbit         AAGTGATCTTACAATTTTCGAACT-CACGATAGCTAAGACCCAAACTGGG
Whale          AAGTGACTTTAATATAATCTGATCACACGACAGCTAAGATCCAAACTGGG
Elephant shrew AAGTGACTTTACCCAGATATGACC-CACGATAGCTAAGATACAAACTGGG
Tree shrew     AAGTAACTTTATAAACTCAGACTA-CACGACAATTAAGACCCAAACTGGG

                                                                 450
Hedgehog       ATTAGATACCCTATTATGCTTAGCCCTAAACTTAGACAGTT--ACTTAAC
Bat            ATTAGATACCCCACTATGCTTAGCCCTAAACTTAAAGAGTC-CCCATAAC
Marsupial      ATTAGATACCCCACTATGCTTAGTAATAAACTAAAATAATTTAACA-AAC
Human          ATTAGATACCCCACTATGCTTAGCCCTAAACCTCAACAGTTAAA-TCAAC
Mouse          ATTAGATACCCCACTATGCTTAGCCATAAACCTAAATAATTAAATTTAAC
Cow            ATTAGATACCCCACTATGCTTAGCCCTAAACACAGATAATT-ACATAAAC
Seal           ATTAGATACCCCACTATGCTTAGCCCTAAACATAAATAATT-CACGTAAC
Rabbit         ATTAGATACCCCACTATGCCTAGCCCTAAACTTCAATAATTTCA-T-AAC
Whale          ATTAGATACCCCACTATGCTTAGTCGTAAACCCCAATAGT--CACAAAAC
Elephant shrew ATTAGATACCCCACTATGCTTAGCCTTAAACCTTGATAATTTA--GTAAC
Tree shrew     ATTAGATACCCCACTATGCTTAATTGTAAACCCAGGTAATTAAA--TAAC
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                                                                      500
Hedgehog          AAAACTGTACGCCAGAGAACTACGAGCCACAGCTTAAAACTCAAAGGACT
Bat               AAGACCCTTCGCCAGAGTACTACTAGCCAAAGCTTAAAACTCAAAGGACT
Marsupial         AAAATTATTCGCCAGAGAACTACTAGCAATTGCTTAAAACTCAAAGGACT
Human             AAAACTGCTCGCCAGAACACTACGAGCCACAGCTTAAAACTCAAAGGACC
Mouse             AAAACTATTTGCCAGAGAACTACTAGCCATAGCTTAAAACTCAAAGGACT
Cow               AAAATTATTCGCCAGAGTACTACTAGCAACAGCTTAAAACTCAAAGGACT
Seal              AAAATTATTCGCCAGAGAACTACTAGCAACAGCTTAAAACTCAAAGGACT
Rabbit            AAAATTACTCGCCAGAGAACTACAAGCCAGGGCTTGAAACTCAAAGGACT
Whale             AAGACTATTCGCCAGAGTACTACTAGCAACAGCCTAAAACTCAAAGGACT
Elephant shrew    AAAAATATCCGCCAGAGAACTACAAGCAACCGCTTAAAAC-CAAAGGACT
Tree shrew        AAAATTACTCGCCAGAGAACTACGTGCCACAGCCCAAAACTCAAAGGACT

                                                                      550
Hedgehog          TGGCGGTGCTTTATACCC-CCTAGAGGAGCCTGTTCTATAATCGATAAAC
Bat               TGGCGGTGCTTTATATCCTCCTAGAGGAGCCTGTTCTATAATCGATAAAC
Marsupial         TGGCGGTGCCCTAAACCCACCTAGAGGAGCCTGTTCTATAATCGATAAAC
Human             TGGCGGTGCTTCATATCCCTCTAGAGGAGCCTGTTCTGTAATCGATAAAC
Mouse             TGGCGGTACTTTATATCCATCTAGAGGAGCCTGTTCTATAATCGATAAAC
Cow               TGGCGGTGCTTTATATCCTTCTAGAGGAGCCTGTTCTATAATCGATAAAC
Seal              TGGCGGTGCTTCACACCCCTCTAGAGGAGCCTGTTCTGTAATCGATAAAC
Rabbit            TGGCGGTGCTTCACACCCACCTAGAGGAGCCTGTTCTATAACCGACAATC
Whale             TGGCGGTGCCTCATACCCATCTAGAGGAGCCTGTTCTGTAACCGATAAAC
Elephant shrew    TGGCGGTGCTTCACACCCA-CTAGAGGAGCCTGTTACATAATCGATAAAC
Tree shrew        TGGCGGTGCTTTATATCCATCTAGAGGAGCCTGTCCGATAATCGATAAAC

                                                                      600
Hedgehog          CCCGATCAACCTCACCAATCTTTGCTAATTCAGCCTTTATACCGCCATCT
Bat               CCCGATAAACCTCACCAACCCTTGCCAACTCAGCCTATATACCGCCATCT
Marsupial         CCCGATAAACCAGACCTTATCTTGCCAATACAGCCTATATACCGCCATCG
Human             CCCGATCAACCTCACCACCTCTTGC----TCAGCCTATATACCGCCATCT
Mouse             CCCGCTCTACCTCACCATCTCTTGCTAATTCAGCCTATATACCGCCATCT
Cow               CCCGATAAACCTCACCAATTCTTGCTAATACAGTCTATATACCGCCATCT
Seal              CCCGATAAACCTCACCATTCCTTGCTAATACAGTCTATATACCGCCATCT
Rabbit            CCCGATAAACCCCACCACTCTTTGCCAACTCAGCCTATATACCGCCATCT
Whale             CCCGATCAACCTCACCAACCCTTGCTACTTCAGTCTATATACCGCCATCT
Elephant shrew    CCCGATAAACCCTACCATCACTTGCTAAATCAGTCTATATACCGCCATCT
Tree shrew        CCCGATACACCTCACCATCTCCTGCTATTCCAGCCTATATACCGCCATCT

                                                                      650
Hedgehog          TCAGCAAACCCTAAA-AAGGAGGAAAAGTAAGCACAATAATCCGCCATCT
Bat               TCAGCAAACCCTAAA-AAGGAGACACAGTAAGCTCAACTA-CCGCCATCT
Marsupial         TCAGCTAACCTTTAA-AAAGAATTACAGTAAGCAAAATCATCCGCCATCG
Human             TCAGCAAACCCTGATGAAGGCTACAAAGTAAGCGCAAGTACCCGCCATCT
Mouse             TCAGCAAACCCTAAA-AAGGTATTAAAGTAAGCAAAAGAATCCGCCATCT
Cow               TCAGCAAACCCTAAA-AAGGAAAAAAAGTAAGCGTAATTATCCGCCATCT
Seal              TCAGCAAACCCTTAA-AAGGAACAAAAGTAAGCACAATAATCCGCCATCT
Rabbit            CCAGCAAACCCTAAA-AAGGAATAAAAGTGAGCTCAATTACCCGCCATCT
Whale             TCAGCAAACCCT--A-AAGGGAGAAAAGTAAGCATAACCATCCGCCATCT
Elephant shrew    TCAGCAAACCCTTAA-AAGGAACTACAGTAAGCTCAAACGTCCGCCATCT
Tree shrew        TCAGCAAACCC-AAA-AAGGACGACAAGTAAGCTTAATCATCCTACATA-

                                                                      700
Hedgehog          TAAAAAGTTAGGTCAAGGTGCAGCCAATAGATTGGGATGAAATGGGCTAC
Bat               TAAAACGTTAGGTCAAGGTGTAGCTTATGGGTTGGAAAGAAATGGGCTAC
Marsupial         TAAAACGTTAGGTCAAGGTGTAGCATATGATAAGGAAAGTAATGGGCTAC
Human             TAAGACGTTAGGTCAAGGTGTAGCCCATGAGGTGGCAAGAAATGGGCTAC
Mouse             TAAAACGTTAGGTCAAGGTGTAGCCAATGAAATGGGAAGAAATGGGCTAC
Cow               TAAAACGTTAGGTCAAGGTGTAACCTATGAAATGGGAAGAAATGGGCTAC
Seal              TAAAAAGTTAGGTCAAGGTGTAACCTATGGAATGGGAAGAAATGGGCTAC
Rabbit            CAAAACGTTAGGTCAAGGTGTAGCCCATAGAGTGGAAAGTAATGGGCTAC
Whale             TAAAACGTTAGGTCAAGGTGTAACCCATGGGTTGGGAAGTAATGGGCTAC
Elephant shrew    TAAAACGTTAGGTCAAGGTGTAACCTATGTGATGGAGCATAATGGGCTAC
Tree shrew        -AAAAAGTTAGGTCAAGGTGTAGCCCATGGGATGGGAAGTGATGGGCTAC
```

APPENDIX V - GAZETEER

Map coordinates of localities, taken in most instances with reference to the nearest town, were derived from Skead's Zoo Historical Gazetteer (Skead 1973) and the Annals of the Cape Provincial Museums vol. 12 (Leistner & Morris 1976). Where place names were not listed in either of the above sources, use was made of the 1:250 000 topocadastrial map series.

Albany district	33°14'S	26°33'E
Amabele	32°39'S	27°32'E
Babanango	28°22'S	31° 4'E
Bathurst	33°30'S	26°50'E
Beaufort West	32°20'S	22°35'E
Biesiespoort	31°43'S	23°10'E
Bloemfontein	29° 7'S	26°13'E
Boshof	28°33'S	24°14'E
Burgersdorp	31° 0'S	26°20'E
Bourkes Luck	24°35'S	30°48'E
Calvinia	31°28'S	19°47'E
Campbell	28°41'S	23°44'E
Clanwilliam	32°11'S	18°53'E
Clocolan	28°55'S	27°34'E
Colesberg	30°42'S	25° 6'E
Cradock	32°10'S	25°38'E
Danielskuil	28°11'S	23°33'E
Dealesville	28°38'S	25°46'E
Deelfontein	30°58'S	23°48'E
Donkerpoort	30°40'S	25°30'E
Doornrivier	33° S	20° E
Dullstroom	25°26'S	30° 4'E
Eenriet	30° 2'S	18°13'E
Eselfontein, Kamiesberg	30°23'S	18° 6'E
Fauresmith	29°45'S	25°20'E
Fort Pato	32°54'S	27°23'E
Gillits	29°48'S	30°48'E
Golden Gate National Park	28°28'S	28°43'E
Grahamstown	33°14'S	26°33'E

Griekwastad	28°52′S	23° 1′E
Groblershoop	28°56′S	21°59′E
Haenertsburg	23°55′S	29°57′E
Hilton	29°33′S	30°17′E
Howick	29°28′S	30°12′E
Itala	28°31′S	31° 2′E
Johannesburg	26° 0′S	28° 0′E
Kakamas	28°46′S	20°38′E
Kieskammahoek	32°43′S	27° 8′E
Kilgobbin	29°28′S	30° 6′E
King William's Town	32°53′S	27°25′E
Klipfontein	28°10′S	23° 8′E
Komga	32°34′S	27°54′E
Koster	25°51′S	26°54′E
Kuruman	27°28′S	23°25′E
Lady Grey	30°43′S	27°14′E
Louis Trichardt	23° 3′S	29°53′E
Louisvale	28°35′S	21°12′E
Lutzputs	28°22′S	20°41′E
Lydenburg	25° 3′S	30°28′E
Matatiele	30°21′S	28°43′E
Middelburg, C.P.	31°27′S	24° 1′E
Middelburg, Transvaal	25°47′S	29°28′E
Middlepost	31°52′S	20°13′E
Nieuwoudtville	31°22′S	19° 8′E
Noupoort	31° 9′S	24°56′E
Olifantshoek	27°57′S	22°44′E
Postmasburg	28°19′S	23° 4′E
Platbakkies, Witwater	30°16′S	18°29′E
Prieska	29°40′S	22°45′E
Rolfontein	30° 0′S	24°43′E
Rooikrantz	24°53′S	27°48′E
Rustenburg	25°40′S	27°15′E
Silverton	25°44′S	28°18′E
Sishen	27°49′S	22°59′E
Springbok	29°40′S	17°53′E
Stutterheim	32°34′S	27°25′E
Suikersbosrand	26°30′S	28°13′E

Sutherland	32°24′S	20°40′E
Upington	28°27′S	21°15′E
Vanwyksvlei	30°21′S	21°49′E
Victoria West	31°24′S	23° 7′E
Volksrust	27°22′S	29°53′E
Waterval Onder	25°39′S	30°20′E
Windsorton	28°20′S	24°43′E
Winterton	28°48′S	29°32′E
Wolesley	33°25′S	19°12′E
Zeerust	25°33′S	26° 1′E

APPENDIX VI

Standard statistics for *P. rupestris*: NW = north-western clade; SK = south-Karoo; E = eastern; Char = characters; N = number of individuals; SD = standard deviation; CV = coefficient of variation.

Char	N	Mean	SD	Range	CV
Total length of skull					
NW	67	80.738	2.597	75.09-85.27	3.217
SK	42	76.709	3.006	70.60-82.11	3.919
E	67	83.961	4.977	71.71-91.86	5.928
Muzzle length					
NW	76	36.023	2.803	27.13-40.81	7.781
SK	47	34.263	2.904	27.98-41.33	8.476
E	50	37.181	3.191	29.80-42.54	8.582
Frontal length					
NW	79	32.540	2.114	28.33-38.97	6.497
SK	50	31.021	2.458	24.92-36.63	7.924
E	52	35.402	3.200	28.48-44.44	9.039
Parietal length					
NW	77	17.198	1.211	14.14-20.77	7.042
SK	51	17.502	1.211	14.30-20.10	6.919
E	52	18.257	1.220	14.92-21.13	6.682

Posterior muzzle length					
NW	79	14.895	0.946	12.97-16.97	6.351
SK	51	14.605	0.940	13.05-16.75	6.436
E	56	16.225	1.473	13.38-22.87	9.079
Posterior frontal breadth					
NW	78	10.969	0.875	8.97-12.66	7.977
SK	50	10.982	0.680	9.52-12.60	6.192
E	53	11.634	0.880	9.83-13.78	7.564
Palate incisor breadth					
NW	78	34.691	1.610	30.52-37.26	4.641
SK	47	32.009	1.908	27.27-35.26	5.961
E	51	36.361	2.594	30.21-40.86	7.134
Hard palate length					
NW	81	6.868	0.587	5.41- 8.55	8.547
SK	50	6.275	0.588	4.99- 8.75	9.371
E	56	7.457	0.748	5.48- 8.75	10.031
Mesopterygoid space					
NW	81	5.293	0.446	4.30- 6.38	8.426
SK	50	5.176	0.438	4.08- 6.09	8.462
E	55	5.642	0.631	4.52- 8.49	11.840

Maxillary premolar length					
NW	81	7.690	0.506	6.60-10.04	6.580
SK	50	7.336	0.567	6.18- 8.54	7.729
E	56	7.910	0.494	6.86- 9.49	6.245
Maxillary premolar-molar length					
NW	81	14.465	0.695	12.33-16.48	4.805
SK	50	13.621	0.787	11.99-15.33	5.778
E	56	14.706	0.871	12.58-16.88	5.923
Principal I^1 breadth					
NW	78	2.530	0.187	2.11- 2.97	7.391
SK	48	2.297	0.179	2.00- 2.71	7.793
E	51	2.682	0.222	2.21- 3.30	8.277
Palatal breadth between P^2 and P^3					
NW	81	12.762	0.819	11.00-14.66	6.418
SK	50	12.280	0.947	9.96-14.62	7.712
E	56	13.934	1.207	11.24-16.60	8.662
Bizygomatic breadth					
NW	67	36.683	1.067	34.52-39.41	2.909
SK	40	35.596	1.188	32.73-38.52	3.338
E	49	37.643	1.758	33.26-41.38	4.670

Maximum cranial breadth					
NW	75	26.376	1.058	23.35-29.03	4.011
SK	50	26.325	0.859	24.56-28.20	3.263
E	52	26.679	1.098	23.79-28.68	4.116
Zygomatic arch length					
NW	71	31.803	1.377	28.40-34.45	4.330
SK	45	30.394	2.347	27.72-39.21	7.722
E	48	33.425	2.044	28.27-36.19	6.115
Posterior cranial height					
NW	72	18.438	0.656	16.85-20.16	3.558
SK	43	18.102	0.654	16.49-19.32	3.613
E	45	19.087	1.099	15.58-21.31	5.758
Bulla Breadth					
NW	72	7.233	0.452	6.03- 8.53	6.249
SK	45	6.766	0.478	5.61- 8.10	7.065
E	48	6.491	0.482	5.58- 7.87	7.426
Mandibular toothrow length					
NW	79	37.050	1.561	32.72-40.04	4.213
SK	46	34.861	2.065	29.90-40.24	5.924
E	49	38.786	2.594	32.91-42.55	6.688

Maximum mandibular height					
NW	75	36.301	1.650	31.05-39.50	4.545
SK	49	34.485	1.876	31.33-40.79	5.440
E	49	38.335	2.562	31.92-42.74	6.683
Mandibular body breadth					
NW	81	4.851	0.303	3.99- 5.92	6.246
SK	50	4.772	0.324	4.08- 5.62	6.790
E	52	5.079	0.333	4.40- 5.87	6.556
Principal I_1 breadth					
NW	82	2.664	0.209	2.16- 3.12	7.845
SK	48	2.543	0.207	2.06- 3.25	8.140
E	50	2.756	0.202	2.30- 3.17	7.330