PRINSLOO, PAULETTE

MOLECULAR AND CHROMOSOMAL PHYLOGENY OF THE HYRACOIDEA

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MOLECULAR AND CHROMOSOMAL PHYLOGENY OF THE HYRACOIDEA

by

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Molecular and chromosomal phylogeny of the Hyracoidea

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ABSTRACT

Chromosomal and molecular parameters were used to determine intraspecific and interspecific relationships of three species of the order Hyracoidea. A diploid chromosome number of 2n = 54 was found to be conserved in all three species and G-band homology was identified for most of the autosomes as well as the sex chromosomes within the *Procavia*, *Heterohyrax* and *Dendrohyrax* genomes. The relative amounts and distribution of constitutive heterochromatin was informative, particularly with regard to *D. arboreus*.

Restriction fragment length polymorphisms (RFLPs) in mitochondrial DNA (mtDNA) revealed 48 haplotypes among 141 hyrax specimens. *Procavia capensis capensis* clones showed strong geographic patterning and grouped into two geographically and genetically distinct clades. Marked genetic differentiation was also evident in *H. brucei* and *D. arboreus* which was thought to reflect the influences of habitat specificity, its fragmentation, and the effects of life history on mtDNA gene flow.

A high degree of congruence was found between RFLP and sequencing analysis in the determination of interspecific phylogenetic relationships. In addition, phenetic, cladistic and maximum likelihood analysis of mtDNA sequences yielded trees with similar topologies. *Dendrohyrax* was invariably placed as the most primitive and the northern *P. c. capensis* clade as the most recently derived of the taxa included in the study. The mtDNA data further suggest a close phylogenetic relationship between *Procavia* and *Heterohyrax* (which were estimated to have diverged approximately five million years B.P.), a relationship suggested by previous morphological studies as well as by similarities in ecological requirements. Sequencing results place the African elephant (*Loxodonta africana*) as a sistergroup to the Hyracoidea, while the grouping of the aardvark (*Orycteropus afer*) with the Paenungulate representatives was not supported by the cytochrome b sequences.

" Four things on earth are small
but they are exceedingly wise ...
The conies are a people not mighty,
yet they make their homes in the rocks. "

Proverbs 30: 24, 26

For my parents,
with love and gratitude

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Soli Deo Gloria

LIST OF CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

GENERAL INTRODUCTION

EVOLUTIONARY HISTORY OF THE HYRACOIDEA

The order Hyracoidea is thought to be African in origin with Oligocene fossil forms having been described from the Fayum depression in Egypt (Meyer 1978). At that time they are thought to have represented the most diversified but least specialized group of subungulates, with six genera ranging in size "from hare to horse" (Stahl 1974). Palaeontological data clearly suggest that the lineage has had a long independent history and, based on the large numbers of hyrax found in the Fayum deposits, probably constituted the most important medium-sized herbivores present at that time (Meyer 1978). Since the late Oligocene however, their numbers are thought to have declined due to competition from more advanced and specialized herbivores, as well as through immigration of large carnivores into Africa. This resulted in the larger forms becoming extinct with only a few small species surviving during the Miocene, Pliocene and Holocene (Stahl 1974).

The genus †*Sagatherium* is generally regarded as ancestral to the modern forms both because of its brachyodont molars (similar to the modern *Dendrohyrax*) and the small size of some of its species (Churcher 1954). During the Pliocene, extensive forests covered much of the African continent and *Dendrohyrax dorsalis* (having evolved from a †*Sagatherium* species in the west) ranged to the north and as far south as the eastern Cape Province of South Africa. Due to the effect of an ice age, the *D. dorsalis* distribution is thought to have decreased and more cold-adapted taxa, *D. validus* and *D. arboreus*, evolved. The retreat of the ice cap in the north was followed by increased aridity which, in turn, led to the establishment of an east-west savanna, conditions which are thought to have promoted the divergence of *Heterohyrax* from a *D. validus* stock (Bothma 1964).

The evolutionary relationship of *Procavia*, the other extant genus in this order, is more enigmatic. It has been postulated that *Procavia* was derived from *Dendrohyrax* via a *Heterohyrax*-like form (Brauer 1916 in Churcher 1954), while Hahn (1934) suggested that *Procavia* could have evolved from †*Sagatherium* through †*Prohyrax tertiarius*, an extinct species recorded from Namibia. The latter hypothesis is supportive of the idea that *Procavia* evolved in the south-western Cape and subsequently extended its range as far north as the Arabian region (Bothma 1964).

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† Extinct species.

TAXONOMIC POSITION

Biblical references to 'conies', an old English term for rabbits, represent the first documented account of the Hyracoidea (Romer 1968). On the basis of the possession of chisel-like incisors they have often been described as rodent-like mammals (Sclater 1900) and, as a result, were initially grouped with rodents, specifically with the guinea-pig, *Cavia*, by Storr in 1780. It was soon realized however, that the hyrax's external resemblance to the Rodentia was misleading, and that their body structure was, in fact, different from other mammalian groups (Romer 1968). In 1869 Huxley proposed the order Hyracoidea (Meyer 1978) but the evolutionary relationship of this enigmatic lineage continues to be disputed.

In addition to the suggested affinities to rodents, three other hypotheses concerning the evolutionary relationships of the Hyracoidea have been proposed. In 1897 Ameghino, using morphological criteria, suggested an association with the †Notoungulata, an extinct group of South American ungulates (Shoshani 1991). Cuvier, however, having studied their internal structure and dental characteristics, suggested the Perissodactyl ungulates to be a sister group to the Hyracoidea (Sclater 1900), an hypothesis supported by several other morphological studies (Osborn 1907; McKenna 1975; Fischer 1989).

Simpson (1945) combined the orders Proboscidea, Sirenia and Hyracoidea and two extinct fossil orders (†Pantodonta and †Embrithopoda) into the superorder Paenungulata. This grouping is supported by several morphological traits including dental characteristics, foot structure (Simpson 1945) and developmental patterns of foetal membranes (Novacek & Wyss 1986). Although the early Fayum fossils appear to have retained ancestral features common to all three lineages, it should be emphasized that the hyrax, proboscideans and sirenians were already distinct at their first appearance in the fossil record (Romer 1966).

Of the competing evolutionary scenarios outlined above, only the proposed hyracoidean, sirenian and proboscidean association enjoys support from a biochemical perspective. Immunodiffusion studies indicate a monophyletic origin for the Paenungulata (Shoshani 1986), while studies of haemoglobin (Kleinschmidt, Czelusniak, Goodman & Braunitzer 1986) and α-crystallin sequences (De Jong, Zweers & Goodman 1981) suggest a common origin for the orders Hyracoidea and Proboscidea; these investigations also clearly support the Paenungulata as one of the most basal branches of the Eutheria.

SYSTEMATIC OVERVIEW OF THE EXTANT HYRACOIDEA

Although the majority of studies on the Hyracoidea have dealt with their systematics (Hoeck 1982), the status of many taxa remains controversial. Thomas (1892) and Sclater (1900) proposed the Hyracoidea to be monotypic comprising the genus *Procavia* and, although other authors (Gray 1868, 1869, 1874; Blanford 1869; George 1874) recognized additional genera for the more divergent forms, Sclater defended his treatment on the grounds that all taxa within the order were morphologically very similar. Subsequently, two genera were recognized, *Procavia* and *Dendrohyrax,* with a third taxon, *Heterohyrax*, being regarded a subgenus of *Dendrohyrax* (Ellerman & Morrison-Scott 1951; Ellerman, Morrison-Scott & Hayman 1953; Roche 1972; Corbet 1978). Most recently Meester, Rautenbach, Dippenaar & Baker (1986), reflecting the consensus (Hahn 1934; Allen 1939; Roberts 1951; Swynnerton & Hayman 1951; Bothma 1971; Hoeck 1978; Swanepoel, Smithers & Rautenbach 1980; Allaerts, Van den Auenaerde & Van Neer 1982; Honacki, Kinman & Koeppl 1982), suggest the family Procaviidae to include three distinct genera - *Procavia*, *Heterohyrax* and *Dendrohyrax*.

Generic delineation has traditionally been based on differences in dentition and skull characteristics. In *Procavia* the molars are hypsodont, the first premolar is absent in adults, the length of the premolar toothrow is less than that of the molars, and the upper incisors are closer together than the width of an incisor. In contrast, *Heterohyrax* and *Dendrohyrax* have brachyodont molars, the first premolar is always present, the length of the premolar toothrow is equal to, or greater than, the length of the molar row, and the upper incisors are wider apart (Hahn 1934; Roberts 1951; Bothma 1971). Differences in skull features include the general shape of the skull, beading in the frontal region (which is well developed in *Dendrohyrax*) and the length of the muzzle (Roberts 1951). Additional morphological differences include coloration and hair structure (Roberts 1951), mammary formulae (Bothma 1971; Meester *et al.* 1986), the distance between the penis and anus (Bothma 1971), and penis structure (Hoeck 1978).

Bothma (1964, 1971) and Hoeck (1978) also incorporated ecological and behavioural features into their identification keys. *Procavia* and *Heterohyrax* are gregarious, diurnal inhabitants of rocky habitats, whereas *Dendrohyrax* is arboreal, nocturnal and occurs solitarily or in pairs. Furthermore, differences in the mating behaviour of *Procavia* and *Heterohyrax*, and distinctive male territorial calls in the three genera have been recorded (Hoeck 1978).

A multitude of species and especially subspecies has been described since Pallas' 1766 description of the rock hyrax from South Africa. Some authors regard *Procavia* as monotypic (Ellerman & Morrison-Scott 1951; Ellerman *et al.* 1953), while others recognize four (Allen 1939) or five different species within the genus (Hahn 1934; Bothma 1971; Roche 1972; Corbet 1978).

Most recently, Honacki *et al.* (1982) and Meester *et al.* (1986) recognize a single species, *P. capensis,* with five subspecies. The nominate, *P. c. capensis*, occurs throughout southern Africa but is replaced by *P. c. welwitchii* in the Namibian Kaokoveld and in the south-western reaches of Angola. *Procavia c. ruficeps* occurs in north-west Africa including Gambia, Senegal, Ghana, Nigeria, southern Algeria, Cameroon, Chad, Sudan and the Central African Republic, while *P. c. johnstoni* is found in Malawi, Zambia, northern Tanzania, Rwanda, Burundi, southern Uganda, north-eastern Congo and central Kenya. *Procavia c. syriaca* extends from Kenya north-eastwards to southern Egypt, Israel, Syria and south-eastern Arabia (Figure 1 a; Bothma 1971; Honacki *et al.* 1982; Smith 1985; Skinner & Smithers 1991).

Most authors recognize three species within both *Heterohyrax* and *Dendrohyrax* (Allen 1939; Roberts 1951; Bothma 1967, 1971; Roche 1972; Honacki *et al.* 1982; Meester *et al.* 1986). The most widely distributed *Heterohyrax* form is *H. brucei* which ranges from Egypt to western Somalia and as far south as northern South Africa and eastern Angola (Figure 1 b). The remaining congenerics have extremely restricted distributions with *H. antineae* limited to the Ahaggar mountains of southern Algeria and *H. chapini* to the Loadi Hill in Zaire.

Unlike the two rock dwelling genera *Procavia* and *Heterohyrax*, the arboreal *Dendrohyrax* is limited to natural forest (Figure 1 c); *D. arboreus* occurs in the Cape and Natal Provinces of South Africa and ranges north-eastwards through Mozambique, Zambia, Malawi, Zaire, Tanzania, Kenya and Sudan. In contrast, *D. dorsalis* occurs in western and central Africa including Gambia and extends through northern Angola, Bioko, Zaire and northern Uganda, while *D. validus* is distributed from eastern Tanzania to Zanzibar.

GENERAL BIOLOGY

In addition to the taxonomic studies briefly reviewed above, other aspects of hyracoid biology such as morphological adaptations, diet, thermoregulation, reproduction, social organization and ethology have received attention in the past. Most reports focus on *P. capensis* from eastern or southern Africa with some data available for *H. brucei*, a species that is often found in sympatry with *Procavia*. In marked contrast, little is known of the biology of the *Dendrohyrax* species, this being limited to reports on captive animals (Rudnai 1984 a, b).

Turner and Watson (1965) and Hoeck (1975) have argued that the kopje habitat, characteristic of both *Procavia* and *Heterohyrax*, represent islands of less extreme environmental conditions. Crevices offer a microclimate of constant temperature and humidity as well as providing shelter. Furthermore, within these areas the characteristic kopje vegetation provides protection from predators

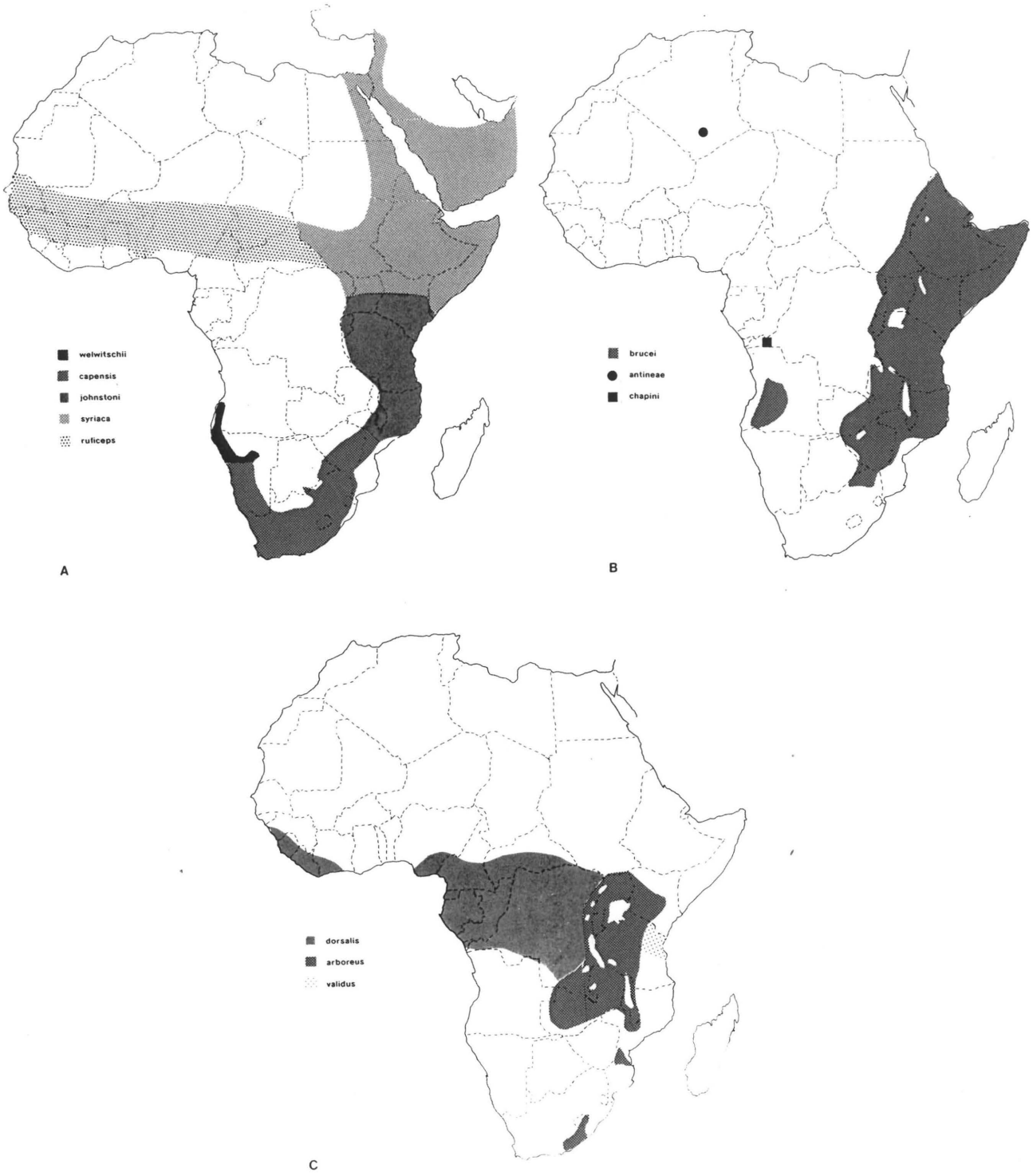


Figure 1 Distribution patterns of the seven recognized hyrax species in Africa and the Arabian region. (A) *P. capensis* subspecies, (B) *H. antineae*, *H. brucei* and *H. chapini*, and (C) *D. arboreus*, *D. dorsalis* and *D. validus* (drawn from Bothma 1971; Honacki *et al.* 1982; Smith 1985; Skinner & Smithers 1991).

in addition to serving as a food supply that, in some instances, is available throughout the year (Hoeck 1975).

The physiology of the rock hyrax species reflects adaptations to their habitat. Several authors have shown both *Procavia* and *Heterohyrax* to be thermolabile (Taylor & Sale 1969; Bartholomew & Rainey 1971; Louw, Louw & Retief 1972; McNairn & Fairall 1979) with low heat tolerance (Louw *et al.* 1972). Daily activity patterns are correlated with ambient temperature; the ability of the rock hyrax to live in a wide range of climatic conditions (from the waterless Sahara to the snowline of Mount Kenya) has been attributed to diurnal fluctuation in body temperature (Sale 1970) and their relative independence of drinking water (Louw *et al.* 1972).

The hyracoidean representatives are considered herbivorous and both *Procavia* and *Heterohyrax* have been classified as intermediate opportunistic feeders (Lensing 1983). Turner and Watson (1965) recorded no dietary overlap between the two taxa in an investigation that found *Procavia* to be predominantly a grazer and *Heterohyrax* a browser. This must be viewed with some caution, however, since Hoeck (1975) reported a degree of overlap during the dry season with *Procavia* switching from 78% grasses during the wet season to 57% browse during the dry season, while *Heterohyrax* predominantly utilizes browse throughout the year (81% during the wet season and 92% during the dry season). *Procavia* also tends towards a greater dietary variety (Hoeck 1975; Lensing 1983) and it seems likely that this plasticity has contributed to its wide distribution (Sale 1965).

Group size in the gregarious taxa (*Procavia* and *Heterohyrax*) appears to be determined by the availability of suitable shelter and space within rocky refuges (Fourie & Perrin 1987). The basic breeding unit in these species is a polygynous group with a dominant territorial male, several adult females and subadults and juveniles of both sexes (Sale 1965; Hoeck 1982; Hoeck, Klein & Hoeck 1982; Fourie & Perrin 1987).

Millar (1971) suggested that photoperiod is the proximate cue for regulating sexual activity in *Procavia* as there is a shift in the breeding season with decreasing latitude. This was confirmed and extended by Fourie (1983) who concluded that although this applied in temperate high latitudes, the proximate cue may, at lower latitudes, be influenced by rainfall and vegetation. Ultimate factors responsible for triggering the onset of the breeding season in this species include, in combination, rainfall, the need for more tolerable temperatures for the neonates and young, and adequate food for both lactating females and their offspring (Sale 1969; Fourie 1983; Stuart & Stuart 1984).

Litter size in this species varies from one to six (Sale 1969) and is likely to be determined by environmental factors; adverse nutritional conditions decrease the ovulation rate and increase prenatal mortality (Millar 1971). In all three genera, females give birth to precocious young after a relatively long gestation period of approximately 230 days (Sale 1965). Interestingly, given its small size,

Procavia, the only species for which data are available, is characterized by a prolonged developmental period with sexual maturity usually being attained at 16 to 17 months of age (Sale 1969; Millar 1971; Fourie & Perrin 1987). Fourie and Perrin (1987) have, however, found a developmental delay in males, while Millar (1971) recorded that females in certain areas reached precocious puberty at four to five months of age. This factor, together with their large litter sizes, low prenatal mortality under favourable environmental conditions, and the relative absence of infertility in females, contributes to the high reproductive capacity of *Procavia* (Millar 1971).

The juvenile sex ratio is in equilibrium, while that of adults has a female bias (Fourie & Perrin 1987). Dispersal is effected mostly by juvenile males. In contrast, juvenile females either stay with their natal groups or join adjacent breeding units, while emigration of adult females may occur when increased harem size leads to increased agonistic behaviour (Hoeck 1982). However, the dispersal is thought to be limited to distances of 2 - 20 km (Skinner & Smithers 1991), an observation that has not been rigorously assessed.

Fourie (1983) found juvenile female mortality to be the main factor regulating population size in South Africa's Mountain Zebra National Park, while predation appears to alter the amplitude of population fluctuations. In areas where the natural predators have been removed through agricultural development, enormous increases in hyrax numbers have been reported in periods of favourable climatic/vegetation conditions, with the population flush generally being followed by a crash in less favourable times (Kolbe 1967).

PHYLOGENETIC INFERENCE

The main objective of a phylogenetic study is to reconstruct the evolutionary history of a group of organisms (Hillis 1987). Comparative morphology concerns the relationship between form and function and, based on the evolutionary hypothesis of descent by modification from common ancestors, comparative studies involving structure, embryology and fossil evidence have traditionally shaped phylogenetic thinking (Patterson 1987). Different systematic philosophies have since been adopted to interpret these and more modern data (see recent reviews; Simpson 1975; Eldredge & Cracraft 1980; Ax 1987; Patterson 1987; Levinton 1988). While evolutionary systematics relies on both the genealogical position in a phylogenetic framework and the amount of morphological divergence from an ancestral lineage (Levinton 1988), phenetics (or numerical taxonomy) does not consider genealogy, and infers classifications strictly from overall similarity (Sokal & Sneath 1963). Phylogenetic systematic, or cladistic studies seek genealogically based relationships with no overall similarity criterion being employed (Hennig 1966).

Advances in the field of phylogenetic inference came about both through the inclusion of different kinds of characters as well as through the refinement of methods for handling larger numbers of these (Simpson 1975). However, with the understanding of an hereditary role for DNA it was realized that the evolutionary history of every organism is recorded in its macromolecules (Patterson 1987). This understanding led, progressively, to the development of more refined techniques that span a range from relatively crude serological methods to the analysis of proteins and the DNA itself (Simpson 1975).

Recently, the relative utility of morphological versus molecular data in phylogenetic studies has been a source of debate (Felsenstein 1985a; Hillis 1987; Patterson 1987; Lanyon 1988; Moritz & Hillis 1990; Dowling, Demarais, Minckley, Douglas & Marsh 1992; Wayne 1992). Despite conflicting opinions, the most important result has been the realization that these approaches are based on different assumptions, and that morphological change and molecular divergence respond independently to evolutionary stimuli. Critical to the effective use of the different analytical approaches therefore, is whether the strategy followed is appropriate given the questions posed for a particular investigation (Moritz & Hillis 1990). This can be further confounded by the species concept that is adopted and has led to suggestions that reproductive isolation is, in fact, the only direct measure of speciation under the biological species concept (Wayne 1992). Since this cannot be readily assayed however, Wayne (1992) has argued that speciation may be inferred from morphological or molecular divergence data.

Both approaches clearly have merit; morphological data can be expanded to include the wealth of information available from museum and fossil specimens and ontogenetic information, while the low cost of analysis is an additional advantage. Most molecular data, on the other hand, have a clear genetic basis, are less confounded by environmental influences, but are expensive to generate. Furthermore, while few morphological traits are shared between all major groups of organisms, molecules contain a phylogenetic record that extends to the origin of life. Since different portions of the genome evolve at different rates, a wide spectrum of comparisons, from populations to higher order relationships, is possible (Hillis 1987).

AIM AND OBJECTIVES

Traditionally, studies of the systematic relationships within the Hyracoidea have focused on morphological variation. However, individual variation in conventional features such as coloration, dentition and properties of the skull (Roberts 1951; Bothma 1964) often limited their usefulness. This clearly underscored the need to assess intraspecific and interspecific relationships within this order using a suite of new investigatory parameters (Hoeck 1978). Therefore, a variety of techniques, most of which have previously not been applied to the group, were used to determine the taxonomic status of the South African Hyracoidea, and to assess the phylogenetic relationships of taxa comprising this enigmatic mammalian order.

The main objectives of this study were:

1. To construct standardized G-and C-banded karyotypes for interspecific comparative purposes and to assess the extent of geographic chromosomal variation in *P. capensis*.

2. To isolate mitochondrial DNA from all study specimens and to screen the DNAs with a suite of restriction endonucleases. An analysis of restriction fragment length polymorphisms (RFLPs) and their use in determining geographic population genetic structure was to be undertaken.

3. To sequence portions of the mtDNA cytochrome b and 12S rRNA genes and to compare the results to appropriate outgroups using both phenetic and cladistic approaches. Additionally, attempts were to be made to assess the degree of concordance between the RFLP and sequence data sets used in phylogeny reconstruction.

CHAPTER 2

COMPARATIVE CYTOGENETICS

INTRODUCTION

Two aspects of cytogenetics are important with regard to cytosystematics, namely chromosome structure and chromosomal changes. The karyotype, or somatic chromosomal complement of a species (King & Stansfield 1985), forms the basis of its genetic system and chromosome number and morphology are important parameters for determining phylogenetic relationships, tracking speciation events (although whether chromosomal change is a cause or a consequence of speciation is moot - see Patterson 1978; Baker & Bickham 1986), and for the study of chromosomal organization (White 1973).

Mammalian diploid chromosome numbers (2n) range from 6-92, with 2n = 30-50 being most prevalent (Hsu 1979). Ohno, Becak & Becak (1964) measured the total length of the metaphase chromosomes of several mammalian species characterized by different diploid numbers and found little variation in this parameter. Therefore, while the basic amount of genetic material is similar between taxa, its packaging is responsible for the observed karyotypic variation found within evolutionary assemblages (Hsu 1979).

The degree of karyotypic conservatism reflected in both 2n and chromosome morphology is variable within mammalian infraclasses and orders (Wrigley & Graves 1988). There is evidence of substantial conservatism within both the Prototheria (order Monotremata; Wrigley & Graves 1988) and the Metatheria (Rofe & Hayman 1985). Within the Eutheria some groups show a high degree of chromosome or chromosome arm homology for example, the Bovidae (Evans, Buckland & Sumner 1973; Buckland & Evans 1978a; Gallagher & Womack 1992) and Felidae (Wurster-Hill & Gray 1973, 1975; Wurster-Hill & Centerwall 1982), while others, such as rodents (Jotterand-Bellomo 1988; review of South African species by Meester 1988) and canids (Wurster-Hill & Centerwall 1982; Wayne, Nash & O' Brien 1987), are often characterized by disruption of G-band sequences.

The variability of mammalian chromosome number in several orders may indicate that, since their origin, mammals seem to have been liable to acquire fusions, dissociations and pericentric inversions in their chromosomes. It is generally perceived that even the most closely related species of mammals frequently differ in karyotype to some extent, and occasionally differ greatly (White 1973), and therefore this seemed a highly appropriate investigatory parameter for inclusion in a phylogenetic study of the Hyracoidea.

Through appropriate staining, patterns of transverse bands can be demonstrated on the metaphase chromosomes of higher vertebrates. This not only enables the identification of individual chromosomes, but also small sections of chromosomes that have undergone rearrangement (Bickmore & Sumner 1989). G-banding involves the treatment of chromosomes with trypsin, a protease that causes collapse of chromosome structure. During subsequent staining, the G-band material is allowed to reconstitute and stain. In addition to G-band sequence homology, which often indicates genic homology (Qumsiyeh & Baker 1988), quantitative changes in constitutive heterochromatin have also been noted both within and between taxa (Arrighi & Hsu 1971).

Some authors are of the opinion that Giemsa-positive regions represent areas of reassociated, highly repetitious DNA (reviewed in Comings 1978), while others believe that it involves the removal of non-C-band DNA (Comings 1978). Resistance of heterochromatin to denaturation may be the result of a greater degree of condensation (Mac Gregor & Varley 1983), or the interaction with histone and non-histone proteins (Comings 1978). Variation in chromosome morphology can often be related to the presence or absence of heterochromatin, for example in the crab-eating fox (Wayne *et al.* 1987) and black rhino (Ryder, Houck & Kumamoto 1987) and, although C-bands are often strictly centromeric (e.g. in bovids; Buckland & Evans 1978b), some species such as lesser apes (Pellicciari, Formenti, Zuccotti, Stanyon & Romanini 1988), among others, show conspicuous terminal and/or interstitial heterochromatin.

It has been suggested (Hsu 1979) that the fixation of chromosomal rearrangements can be achieved rapidly, sometimes within a few generations, thus quickly propelling populations along divergent evolutionary pathways. Fixation of structural rearrangements, such as centric fusions that exhibit monobrachial homology, may result in genetically compatible populations becoming effectively reproductively isolated from one another (Baker & Bickham 1986). In general, chromosomal evolution in taxa whose ecology or social structure permits the formation of small demes or social groups has tended to be more rapid (Jotterand 1972) than those where social hierarchies are absent and that are generalists rather than habitat specialists (Arnason 1972).

Karyotypic data on the Hyracoidea is limited with the only published karyotype being that of the Kenyan *P. capensis* (Hungerford & Snyder 1969; Hsu & Benirschke 1971b). The species was reported to be characterized by $2n = 54$ with the karyotype comprising five pairs of metacentric and submetacentric chromosomes, and 21 pairs of acrocentric autosomes; both the X and Y chromosomes are submetacentric. Other Paenungulata which have been studied on a chromosomal level are the African (*Loxodonta africana*) and Indian (*Elephas maximus*) elephants (Hungerford, Snyder & Ulmer 1966; Hosli & Thurig 1970; Thurig 1970; Hsu & Benirschke 1971a; Wallace 1978) and the Amazonian manatee - *Trichechus inunguis* (Loughman, Frye & Herald 1970; Assis, Best, Barros &

Yonenaga-Yassuda 1988). The African elephant is characterized by 2n = 56 which is made up by three metacentric and submetacentric autosomal pairs and 21 pairs of acrocentric and subtelocentric chromosomes. In this species the X chromosome is submetacentric in morphology and the Y chromosome acrocentric. The Indian elephant differs slightly having six meta- and submetacentric autosomal pairs and 21 pairs of acrocentric chromosomes. Both the X and Y chromosomes are submetacentric (Hsu & Benirschke 1971a).

MATERIAL AND METHODS

Ear biopsies for tissue culture were collected from selected specimens (Table 1). The ear clippings (1 cm^2), taken in the field, were vigorously cleaned with 70% ethanol and cottonwool and placed in McCartney bottles containing tissue culture medium (McCoy's 5A or DMEM; Dulbecco's Modified Eagle's Medium; Highveld Biological) supplemented with 10% fetal calf serum (Highveld Biological). Where possible these were kept cool until processing in the laboratory.

Table 1 The species designation, collection localities and, where available, museum accession numbers of specimens used for cytogenetic analysis.

Species	Locality	Sex	Museum No
H. brucei	Blyde River	Male	-
D. arboreus	Pirie Forest	Male	KM 32 033
P. c. capensis	Karoo National Park	Male	-
P. c. capensis	Volksrust	Female	TM 42 437
P. c. capensis	Mica	Female	TM 42 481
L. africana	KNP	Female	-

Culture of fibroblast cells

Ear clippings were cleaned under sterile conditions and the excess hair removed. The tissue was subsequently incubated in fresh medium at 37°C for 24 - 48 hr to check for contamination. Primary cultures were initiated from uncontaminated ear clippings using routine procedures. The tissue was finely minced (0.5 - 1 mm in diameter) using a sterile scalpel, aseptically transferred to 25 cm^2 disposable culture flasks (Costar) and resuspended in 1 ml of McCoy's 5A medium or DMEM supplemented with 10 - 15% FCS. Cultures (incubated at 37°C with 5% CO_2) were checked on a regular basis and the depleted medium replaced. As soon as growth was noted, the volume of culture medium was increased to 5 ml. Subculturing was by trypsinization and cells from selected cultures were deep frozen in liquid nitrogen using FCS supplemented with 10% dimethylsulfoxid (DMSO).

Harvesting of metaphase chromosomes and slide preparation

Cell cultures in logarithmic growth were harvested. Three hr before harvest, the cultures were treated with colcemid (Gibco), a mitotic inhibitor which arrests cell division at metaphase. Cells were removed by trypsinization, transferred to 15 ml centrifuge tubes and pelleted at 2500 x g. Fibroblasts were subsequently resuspended in a hypotonic solution (0.075 M KCl) and incubated for 15 min following which the cells were pelleted and fixed in three changes of a 3:1 methanol:glacial acetic acid solution.

Before making drop preparations, slides were soaked in 70% ethanol and washed with a commercial soap solution (Sunlight Liquid) and distilled water. The cell solution was dropped onto wet slides that were allowed to dry in an upright position. Monitoring of the harvest (to check for cytoplasm) was done under a phase-contrast microscope or, following Giemsa staining, using a light microscope and a green filter.

Chromosome banding

Metaphase chromosomes were G-banded and C-banded using conventional methods (Wang & Fedoroff 1972; Sumner 1972). G-banding involved the treatment of chromosomes with 0.1% trypsin (pH 7.0; Enzar T; Armour Pharmaceutical Company) in phosphate buffered saline (PBS) for 30-50 sec, followed by staining in 2% buffered Giemsa solution (Sorensen's phosphate buffer pH 6.8;

Schwarzacher 1974) for 5 min. In the C-banding protocol, slides were immersed in 0.2 N HCl for 30 min prior to treatment with 5% $Ba(OH)_2$ (45-60 sec) at 60°C. Slides were subsequently incubated in 2 x SSC (0.3M NaCl, 0.03M trisodium citrate, pH 7.0) for 1 hr at 50°C and stained with 2% buffered Giemsa solution.

Preparation of standardized karyotypes

Slow emulsions with high contrast, fine grain and high resolving power are generally used to produce high quality photomicrographs of chromosomes. In the present study 50 ASA film, scaled down to 25 ASA using Perceptol developer (Ilford), was used. All photography was done with a 63 X oil immersion lens.

Diploid chromosome numbers were determined by counting 25 well spread metaphase plates from each specimen. Chromosome measurements, taken directly from photographs using a Quantimet 520 Image Analyzer (Cambridge Instruments, UK), were used to standardize the G-banded karyotypes of each specimen (Lee & Martin 1980). Chromosomes were grouped by chromosome arm ratios following a modification of the classification of Levan, Fredga and Sandberg (1964) as outlined in Table 2, and ordered in decreasing size.

Table 2 Chromosome classification based on chromosome arm ratio (modified from Levan *et al.* 1964).

Centromere position	Terminology	Arm ratio
Nearly median	Metacentric	< 1:1.1
Submedian	Submetacentric	1:1.1 - 1:1.9
Submedian	Subtelocentric	1:2 or >
Subterminal	Acrocentric	No visible, or extremely short second arm

Analyses

Interspecific comparisons included the assessment of 2n, chromosome morphology and banding patterns. The elephant was used as an outgroup in an attempt to determine primitive and derived karyotypic character states.

RESULTS

All three hyrax species included in this investigation were determined to have 2n = 54 which, in the case of *P. capensis*, confirms earlier reports (Hungerford & Snyder 1969; Hsu & Benirschke 1971b). The G-banded karyotypes of the respective species are shown in Figure 2 and the chromosomes numbered and standardized based on the percentage contribution of each to their respective genomes (Tables 3 - 5). Unfortunately, due to the low mitotic index in the elephant and the poor resolution of G-banded preparations, this species' karyotype could not be standardized.

The autosomal chromosome complement of *D. arboreus* (Figure 2 a) comprised 15 acrocentric pairs (1-15, one of which, pair 6, showed pronounced heteromorphism; see Figure 2a below), five pairs of subtelocentric autosomes (16-20), five pairs of submetacentric (21-25) and one pair of metacentric chromosomes (26). The X chromosome was determined to be the largest submetacentric element in the complement and contributes ± 5% to the female haploid complement; the submetacentric Y is intermediate in size between pairs 25-26 and constitutes approximately 2% of the female genome.

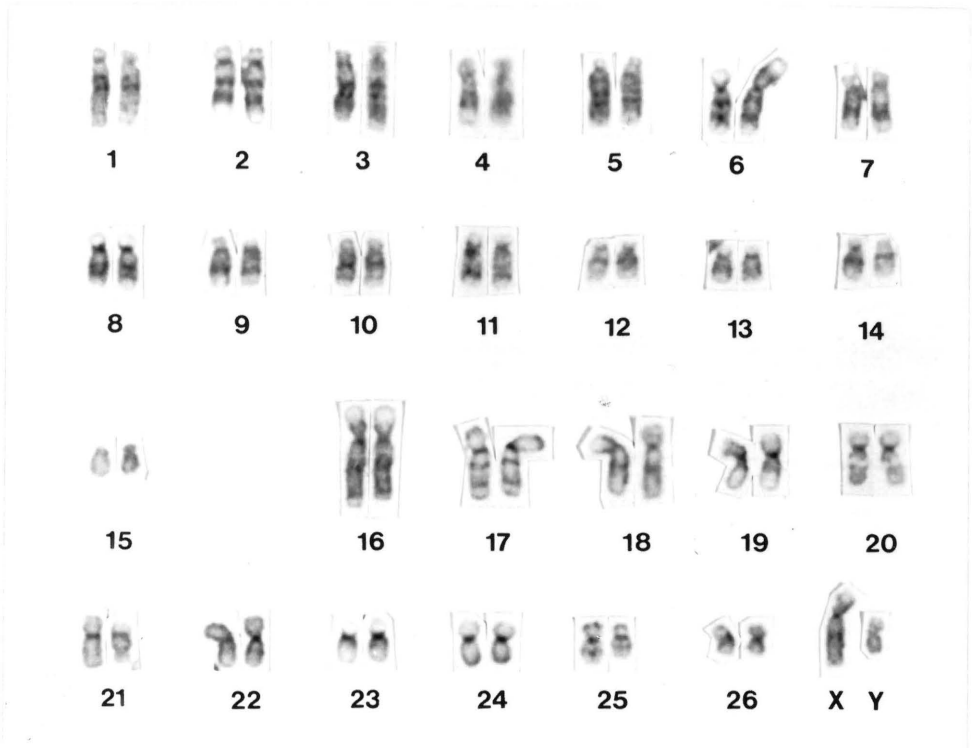
The *H. brucei* karyotype (Figure 2 b) has 20 acrocentric autosomal pairs (1-20), two subtelocentric pairs (21-22), two submetacentric pairs (23-24) and two metacentric autosomal pairs (25-26). The X chromosome is the largest submetacentric chromosome in the complement comprising 5.2% of the female genome while the acrocentric Y, due to its small size, is readily distinguishable and constitutes 1.4% of the haploid genome.

No variation was observed between the karyotypes of specimens from the three *P. c. capensis* localities (Table 1) and, as a result, a representative karyotype of the Karoo National Park specimen is shown (Figure 2 c). The chromosome complement of the South African species is similar to that reported for specimens from Kenya (Hungerford & Snyder 1969; Hsu & Benirschke 1971b) and comprises 42 acrocentric (pairs 1-21), six submetacentric (pairs 22-24) and four metacentric (pairs 25-26) autosomes. The X chromosome is the largest submetacentric chromosome constituting more than 5% of the female genome, while the Y chromosome is the smallest submetacentric.

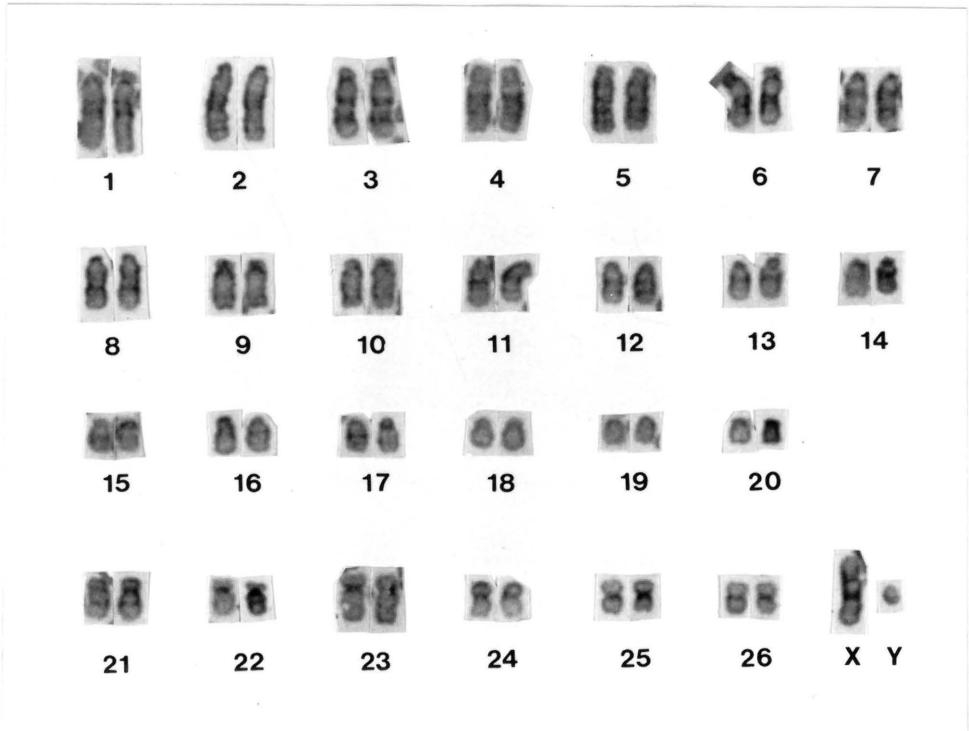
The G-banded karyotype of the African elephant presented in this study confirms the reported diploid number while the morphology of the respective chromosomes corresponds to those shown in standard stained karyotypes presented by earlier workers (Hungerford *et al.* 1966; Hosli & Thurig 1970; Thurig 1970; Hsu & Benirschke 1971a; Wallace 1978). The karyotype comprises 24 acrocentric autosomal pairs, two submetacentric autosomal pairs and a single metacentric pair. The X chromosome is the largest metacentric chromosome; no data are available on the Y chromosome. Although the karyotype could not be standardized, the chromosomes were arranged and conventionally numbered according to a decrease in length.

A comparison of the G-banded chromosomes of the different species is shown in Figure 3. Convincing G-band homology is evident for the euchromatic portions of the majority of the chromosomes of the three hyracoid genera. This includes the X chromosome that shows two dark bands in the middle of the long arm which are characteristic of most mammals (Pathak & Stock 1974). In several instances, homology could only be demonstrated for two of the three genera. *Procavia* autosomes 10, 15-18 and 21 could be identified in *Heterohyrax* (which correspond to chromosomes 9, 10, 15, 16, 17, 19 and 20) but not in *Dendrohyrax*, while chromosome 11 was considered to be homologous to chromosome 4 of *Dendrohyrax*. *Dendrohyrax* chromosomes 9, 17-19 and 21 were similarly unmatched in other hyrax species. Likewise, the weak resolution of many of the elephant chromosomes, especially the smaller ones, precluded the detection of G-band homology with hyrax chromosomes.

In determining homologies between the different species it is also informative to compare the relative amounts and distribution of constitutive heterochromatin present in their genomes (Figure 4). In all three genera, *Loxodonta*, *Heterohyrax* as well as *Procavia*, the distribution of C-band positive material is strictly centromeric (Figure 4 a-c). The paucity of C-bands in *Procavia* resulted in heterochromatin being detected in only the best preparations and not in all the chromosomes (Figure 4 a). In contrast, the *Dendrohyrax* karyotype is characterized by pronounced heterochromatic short arms in some of the autosomes, as well as by terminal bands of heterochromatin (Figure 4 d). This difference probably led to length discrepancies evident in several of the G-banded chromosomes of the various species (*Dendrohyrax* chromosome 16 *cf. Heterohyrax* 2, *Procavia* 2 and *Loxodonta* 2; *Dendrohyrax* 17 *cf. Loxodonta* 9; *Dendrohyrax* 22 *cf. Heterohyrax* 16 and *Procavia* 23). The length difference between the elephant's X chromosome and that of the Hyracoidea, may similarly be due to differing amounts of constitutive heterochromatin.

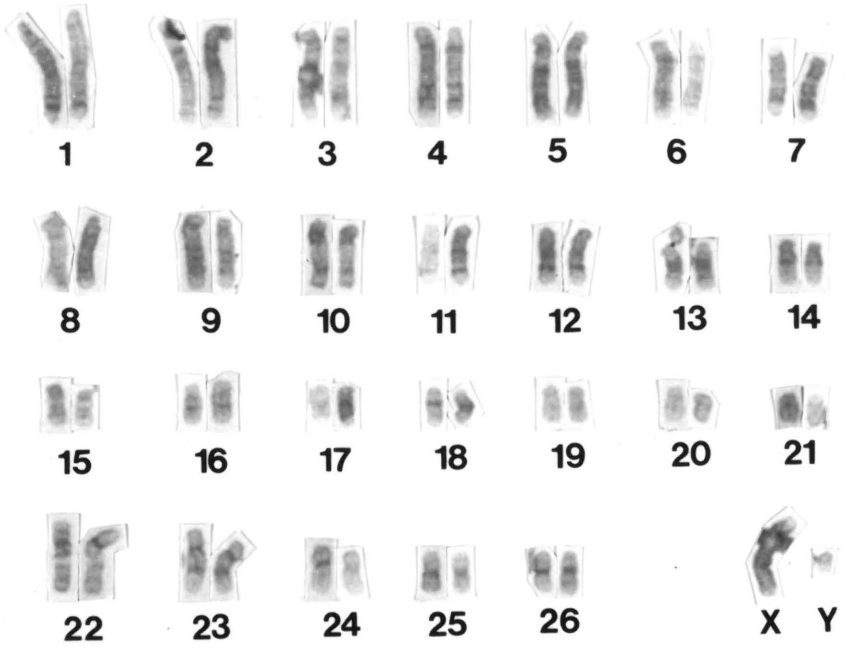


(a)

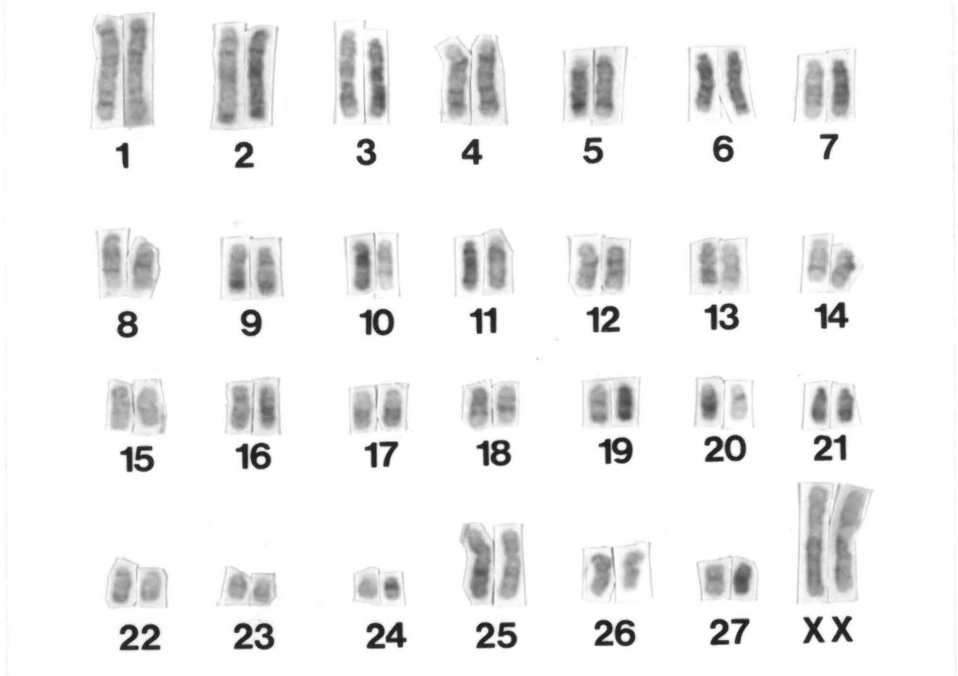


(b)

Figure 2 (a) G-banded karyotype of a male tree hyrax, *D. arboreus*, (2n = 54). The heteromorphism evident in the lengths of the short arms of the homologues constituting pair 6 is attributable to differences in heterochromatin. (b) G-banded karyotype of a male yellow spotted hyrax, *H. brucei*, (2n = 54).



(c)



(d)

Figure 2 (c) G-banded karyotype of *P. c. capensis* (Karoo National Park, 2n = 54). Specimens from the two other localities (Volksrust and Mica) showed identical karyotypes. (d) G-banded karyotype of a female African elephant, *L. africana*, (2n = 56).

Table 3 Relative chromosome lengths of *D. arboreus* expressed as a percentage of the haploid female genome (n = 4). SE = standard error.

Chromosome number	Relative length % of (A+X)		Chromosome number	Relative length % of (A+X)	
	Mean	SE		Mean	SE
1	5.212	0.076	15	2.056	0.069
2	5.038	0.112	16	6.417	0.154
3	4.944	0.127	17	4.896	0.129
4	4.424	0.171	18	4.598	0.059
5	4.292	0.159	19	3.658	0.042
6	3.238	0.057	20	3.541	0.071
7	3.587	0.114	21	3.475	0.086
8	3.572	0.212	22	3.345	0.098
9	3.428	0.037	23	2.712	0.030
10	3.332	0.091	24	2.705	0.065
11	3.322	0.092	25	2.249	0.100
12	2.822	0.049	26	1.985	0.054
13	2.529	0.163	X	4.938	0.145
14	2.351	0.045	Y	1.955	0.108

Table 4 Relative chromosome lengths of *H. brucei* expressed as a percentage of the haploid female genome (n = 4). SE = standard error.

Chromosome number	Relative length % of (A+X)		Chromosome number	Relative length % of (A+X)	
	Mean	SE		Mean	SE
1	6.588	0.166	15	2.770	0.151
2	6.010	0.200	16	2.560	0.073
3	5.732	0.159	17	2.449	0.083
4	5.360	0.279	18	2.145	0.104
5	5.226	0.120	19	2.119	0.038
6	4.748	0.157	20	1.972	0.091
7	4.400	0.115	21	3.353	0.085
8	4.257	0.066	22	2.796	0.058
9	3.916	0.108	23	4.027	0.047
10	3.865	0.097	24	2.713	0.052
11	3.774	0.067	25	2.548	0.061
12	3.240	0.030	26	2.327	0.084
13	3.034	0.041	X	5.199	0.130
14	2.884	0.054	Y	1.350	0.166

Table 5 Relative chromosome lengths of *P. c. capensis* expressed as a percentage of the haploid female genome (n = 4). SE = standard error.

Chromosome number	Relative length % of (A+X)		Chromosome number	Relative length % of (A+X)	
	Mean	SE		Mean	SE
1	6.139	0.168	15	2.561	0.096
2	6.002	0.136	16	2.530	0.073
3	6.098	0.370	17	2.526	0.096
4	5.798	0.124	18	2.494	0.068
5	5.607	0.118	19	2.297	0.050
6	4.996	0.111	20	1.916	0.070
7	4.188	0.091	21	1.620	0.122
8	4.153	0.113	22	4.280	0.227
9	4.032	0.169	23	3.574	0.141
10	3.947	0.085	24	2.635	0.050
11	3.746	0.118	25	2.347	0.048
12	3.353	0.057	26	2.305	0.070
13	2.935	0.101	X	5.779	0.473
14	2.608	0.061	Y	1.100	0.058

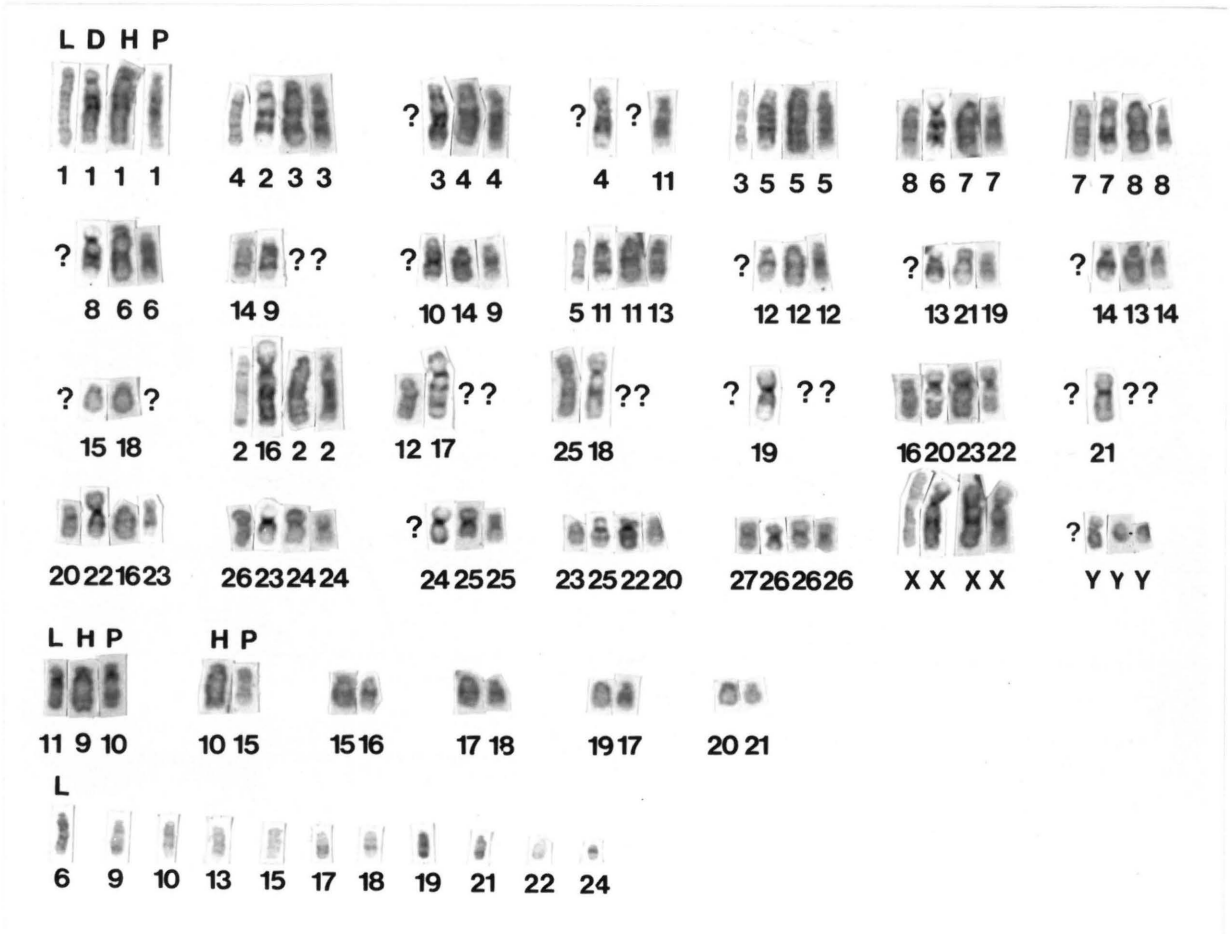


Figure 3 Comparison of the G-banded chromosomes of *D. arboreus* (D), *H. brucei* (H), *P. c. capensis* (P) and the African elephant, *L. africana* (L). The chromosomes were arranged according to the *Dendrohyrax* karyotype and unmatched chromosomes are indicated by (?). *Heterohyrax, Procavia* and *Loxodonta* chromosomes that could not be convincingly matched in the *Dendrohyrax* karyotype are arranged at the bottom of the figure.

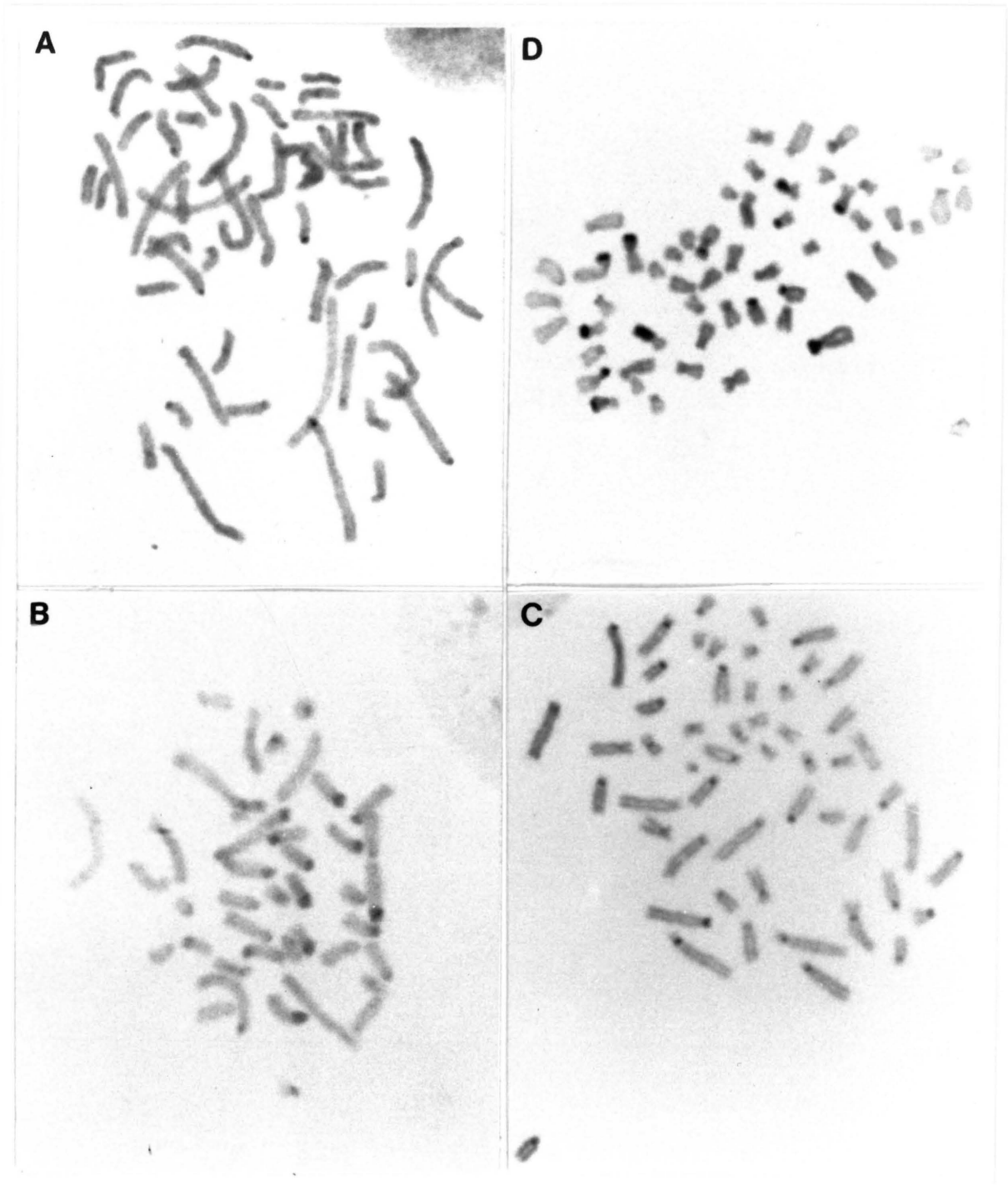


Figure 4 C-banded metaphase chromosomes of (a) *P. c. capensis*, (b) *L. africana*, (c) *H. brucei* and (d) *D. arboreus*.

DISCUSSION AND CONCLUSIONS

The conserved 2n = 54 in all the representatives of the Hyracoidea studied to date may indicate the ancestral chromosome number for the Hyracoidea although, clearly, commonality does not always imply the primitive condition (Qumsiyeh & Baker 1988). If this is a true reflection of the ancestral state and given the G-band homology for most chromosomes, karyotypic evolution in the Hyracoidea has not progressed through fusions or fissions of chromosomes or chromosome arms. Although homologues could not be identified for several of the autosomes in the G-band comparison of the three hyrax genera this most likely reflects weak G-band resolution and not G-band disruption through structural rearrangements such as inversions, reciprocal translocations or complex rearrangements. This does however, require confirmation.

The weak resolution of G-banded preparations, especially in the African elephant, unfortunately precluded more rigorous assessments of chromosomal homology and a subsequent cladistic analysis of the data set. Nonetheless, three of the *Dendrohyrax* chromosomes (9, 17 and 18), which could not be matched to elements in the other hyrax species, showed homology to elephant chromosomes, while chromosome 9 of *Heterohyrax* and chromosome 10 of *Procavia* matched chromosome 11 of the elephant.

C-banding proved more successful and marked differences in the relative amounts and distribution of constitutive heterochromatin were clearly evident between *Dendrohyrax* and the other taxa. As C-banding is thought to stain genetically inert blocks of DNA (John & Miklos 1979), it often shows intraspecific variability. However, in certain instances the distribution of C-band positive material may be useful in identifying phylogenetically informative changes (Van Tuinen & Ledbetter 1983; Van Tuinen & Valentine 1986), and in this respect, the unique presence of entirely heterochromatic short arms and terminal blocks of heterochromatin in the *D. arboreus* genome is likely to be a recently derived character.

In conclusion therefore, in spite of technical limitations, the present study demonstrated the conserved nature of the hyrax chromosome complement both with respect to chromosome number and to the G-band homology evident for most of the chromosomes. In addition, it revealed a lack of chromosomal differentiation between representatives of the two geographically and genetically distinct clades of *P. c. capensis* that, strikingly, are supported by RFLP and sequencing analysis of mitochondrial DNA (see Chapter 3).

CHAPTER 3

MOLECULAR ANALYSES

INTRODUCTION

Structure and unique features of mitochondrial DNA

Mitochondrial DNA (mtDNA) is a closed circular extranuclear molecule 94% of which encodes structural RNA. The mammalian mitochondrial genome contains 22 transfer RNA (tRNA) genes, genes for the 12S and 16S ribosomal RNAs (rRNAs) and 13 protein coding sequences including cytochrome b, cytochrome oxidase subunits I to III, ATPase subunit 6 and eight unidentified reading frames. An 879 base pair (bp) region surrounding the origin of the heavy (H) strand replication site and 32 bp near the origin of the light (L) strand replication site lacks coding function. The non-coding region also contains the displacement or D-loop (Avise & Lansman 1983).

In 1979, Barrell, Bankier and Drouin described codon assignments in human mtDNA that deviate from the universal genetic code. These included the use of UGA for tryptophan rather than a stop codon. Furthermore, the genetic code can vary within the same taxonomic group, a fact which suggests that the evolution of the genome has been subject to less stringent constraints (Attardi 1985). Another unique feature of mtDNA is that only 22 tRNAs are required to translate the genetic code, as opposed to 32 in the universal code; the significance of this is as yet unclear but is likely to be closer to the primitive condition of 15 to 16 anticodons from which the universal code is thought to have evolved (Avise 1991).

The size of the mitochondrial genome varies from 15.7 - 19.5 kilobase pairs (kb) (Brown 1983) yet the same 37 genes involved in cell metabolism are found in all animals. The genome contains few intergenic sequences, no introns and, except in the D-loop region, few insertions or duplications (Harrison 1989).

Evolution of mitochondrial DNA

In view of the functional constraints thought to apply to mtDNA (Brown 1983) the discovery of a high rate of mutation in this organelle was unexpected. The rate of 1-2% sequence divergence per lineage per million years is 5-10 times faster than that for single copy nuclear DNA (scnDNA),

a rate that was first described in primates (Brown, George & Wilson 1979). Subsequently, a similar mutation tempo has been reported for several taxa including the rhinoceros, rodents, equids, artiodactyls, gallinaceous birds, geese, *Xenopus*, salmonid fishes and Hawaiin *Drosophila* (reviewed in Wilson, Cann, Carr, George, Gyllensten, Helm-Bychowski, Higuchi, Palumbi, Prager, Sage & Stoneking 1985; Moritz, Dowling & Brown 1987 and Shields & Wilson 1987).

This high mutation rate (or high rate at which mutations become fixed) can be attributed to either a relaxation of functional constraint, or to inefficient repair mechanisms in mtDNA (Wilson *et al.* 1985). Gillespie (1986) further proposed that the association of nuclear DNA with histone could account for its more constrained evolution when compared to mtDNA.

The mutation rate can, however, vary across taxonomic groups as well as in the different regions of the genome. A rate similar to that of scnDNA has been reported in flies and sea urchins (reviewed in Harrison 1989), in turtles (Avise, Bowen, Lamb, Meylan & Bermingham 1992) and in the lemur (Hasegawa, Kishino, Hayasaka & Horai 1990). In view of these examples, and as illustrated by Wayne, Van Valkenburgh and O' Brien (1991), similar mutation rates of molecular evolution cannot simply be assumed for different taxonomic groups.

Of equal importance is the fact that the relationship between sequence divergence and divergence time is only linear up to 10-15 million years (Brown *et al.* 1979), at which point the rate slows down to reach a plateau at approximately 30 million years. In addition, only 25-30% of the mitochondrial genome appears to be affected by the high mutation rate. For example, tRNA genes exhibit the highest degree of homology followed by the rRNA genes and protein coding sequences. The D-loop, which displays relatively large size differences, is the most rapidly evolving portion of mtDNA (Attardi 1985). When considering the dynamics of mtDNA evolution it has been found that substitutions predominate over both deletions and additions and, that for lesser divergence times, transitions generally predominate over transversions. The transition-transversion ratio decreases with time, as multiple substitutions accumulate at many sites (Brown 1983).

Mode of inheritance

The maternal cytoplasmic inheritance of mtDNA has been demonstrated in several investigations (Dawid & Blackler 1972; Hutchison, Newbold, Potter & Edgell 1974; Hayashi, Yonekawa, Gotoh, Watanabe & Tagashira 1978; Giles, Blanc, Cann & Wallace 1980; Lansman, Avise & Huettel 1983b; Gyllensten, Wharton & Wilson 1985). To account for this, it was proposed that sperm mtDNA does not proliferate in the zygote either due to the failure of the midpiece of the sperm to penetrate the egg

or, alternatively, due to active alteration or degradation of sperm mtDNA following entry into the egg (Dawid & Blackler 1972). Gillham (1978) further suggested that maternal inheritance could be determined by interaction between nuclear and mitochondrial genes.

Until fairly recently, mtDNA sequences constituting less than 1-5% of the total mtDNA fraction would have gone undetected due to the limited sensitivity of available techniques (Avise & Lansman 1983) and the possibility of low level paternal leakage could therefore not be completely excluded. A recent study employing the polymerase chain reaction (PCR) showed paternally inherited molecules at a frequency of 10^{-4} compared to the maternal contribution (Gyllensten, Wharton, Josefsson & Wilson 1991) but since this was demonstrated using inbred interspecific crosses in mice it does not necessarily reflect a universal phenomenon in all vertebrates.

The maternal inheritance of mtDNA has profound implications for use in demographic studies. As mtDNA is clonally transmitted, the genome represents a set of completely linked markers which can be used to trace female genealogies (Harrison 1989). It is also considered to be a sensitive indicator of patterns of colonization and gene flow among insular populations (Ashley & Wills 1987), founder events (Moritz *et al.* 1987) and population bottlenecks (Wilson *et al.* 1985).

Mitochondrial DNA polymorphism

While individual organisms generally appear to be homoplasmic with regard to their mtDNA type, extensive mtDNA polymorphism can be detected between individuals, populations and also at higher hierarchical levels (Avise & Lansman 1983). Traditionally, the assay of sequence relatedness of mtDNAs has involved the use of variation in the number and size of fragments (restriction fragment length polymorphisms, RFLPs) generated by restriction endonuclease digestion of the DNA (Brown & Vinograd 1974; Wilson *et al.* 1985). Restriction endonucleases recognize particular DNA sequences, usually four to six bases long, and cleave the double-stranded DNA accordingly. As the majority of mtDNA variation is attributable to the gain or loss of specific restriction sites, without detectable alteration of genome size (Avise & Lansman 1983), sequence relatedness can be expressed as a function of the proportion of shared restriction fragments between mtDNAs (Upholt 1977; Nei & Li 1979). Mitochondrial genomes that differ by less than 0.05% can be resolved by using a suite of 10 enzymes, each recognizing four bases (Wilson *et al.* 1985).

Although the mapping of restriction sites may provide better resolution and improved accuracy of divergence estimates, the alignment of these maps is, however, subject to errors and restriction sites can offer a biased sample of nucleotide sequences (Wilson *et al.* 1985). Both restriction

techniques rely on the availability of at least milligram amounts of material, while another major limitation has been the fact that these methods do not provide insights on sequence evolution of mtDNA (Kocher, Thomas, Meyer, Edwards, Paäbo, Villablanca & Wilson 1989). The applicability of these techniques is also confounded by the non-linearity of the relationship between sequence divergence and divergence time beyond 10-15 million years (Brown *et al.* 1979) and can therefore only be applied to closely related species or genera.

In contrast, direct sequence comparisons provide higher resolution as the characters (nucleotides) are the fundamental units of information encoded in organisms (Hillis, Larson, Davis & Zimmer 1990). Through sequencing, "hot spots" for divergence have been identified within the mitochondrial genome and clues about sequence evolution provided (Wilson *et al.* 1985). In addition, the choice of a target sequence is determined by the evolutionary time scale involved, and comparisons at different levels of divergence are therefore possible (Hillis & Moritz 1990).

Early sequencing efforts were limited as the technique required cloning of the target DNA which is a time-consuming procedure. The discovery of PCR (Mullis & Faloona 1987), a fast and efficient amplification technique, thus had a major impact on the use of sequencing in molecular biology. PCR produces large amounts of specific DNA fragments from small amounts of complex template (Mullis & Faloona 1987; Saiki, Gelfand, Stoffel, Scharf, Higuchi, Horn, Mullis & Erlich 1988; White, Arnheim & Erlich 1989), permitting the isolation and characterization of specific DNA sequences. It can also be applied in forensic science and archaeology (Paäbo, Gifford & Wilson 1988) and in the study of preserved museum specimens (Appenzeller 1990), all sources that regularly contain degraded DNA that cannot be analysed using standard molecular techniques (Hillis *et al.* 1990).

A potential disadvantage of PCR is that it requires known sequences flanking the target fragment (Hillis *et al.* 1990). For mtDNA sequencing, this was largely overcome by the discovery of conserved areas in the published sequences of mammals, frog and fly (Kocher *et al.* 1989). The "universal primers" that were developed from these conserved regions, and modifications thereof, have since been used in various investigations involving groups as diverse as fish (Meyer, Kocher, Basasibwaki & Wilson 1990), lizards (Hedges, Bezy & Maxson 1991) and mammals (Irwin, Kocher & Wilson 1991).

The use of direct sequencing in combination with RFLP analysis can provide high-resolution information (Hillis & Moritz 1990), drawing from the advantages of both techniques. While RFLP analysis is more appropriate for the large scale investigation of geographic mtDNA variation within or between closely related species or genera, direct sequencing can estimate relatedness between more divergent groups and provide information about the sequence evolution of mtDNA.

Mitochondrial DNA phylogeography

The population genetic structure of a species is determined by its potential for gene flow and the environmental influence on that potential (Avise, Reeb & Saunders 1987). Given this relationship, different categories of genetic divergence patterns have been identified. The investigation of the relationships between mtDNA molecules and the geographic distribution of these phylogenetic groupings constitutes a discipline known as intraspecific phylogeography (Avise, Arnold, Ball, Bermingham, Lamb, Neigel, Reeb & Saunders 1987a).

Geographic structuring of lineages, defined by different branches in mtDNA phylogenies, has proved to be the most common pattern to emerge from comparative mitochondrial studies (Avise 1986). Most likely these discontinuous genetic divergence patterns have been shaped by long-term extrinsic barriers to gene flow, or alternatively, by the extinction of intermediate haplotypes in species with limited gene flow and dispersal capabilities (Avise *et al.* 1987a). Terrestrial and freshwater vertebrates typically reflect geographic patterning, for example, *Peromyscus* (Lansman, Avise, Aquadro, Shapira & Daniel 1983a), the desert tortoises (Lamb, Avise & Gibbons 1989) and sunfish species (reviewed in Avise 1992), while some continuously distributed marine species (the horseshoe crab - Saunders, Kessler & Avise 1986) show divergence patterns corresponding to recognized zoogeographic boundaries.

Generally, however, species occupying habitats free of significant impediments to gene flow (such as the marine environment), and more mobile species (for example birds and marine fish or mammals), tend to show limited phylogeographic patterning. Also, phylogenetic continuity may involve partial or complete spatial separation in the absence of geographic barriers in species exhibiting intermediate or limited gene flow. In these instances haplotypes are spatially separated, yet show limited differentiation involving few site changes (Avise *et al.* 1987a).

Phylogeographic studies have therefore highlighted the dominant role that historic demographic and zoogeographic factors play in the shaping of intraspecific gene phylogenies and can potentially allow the reconstruction of the historical events that shaped the evolution of contemporary populations (Avise 1986). Furthermore, by identifying concordant phylogeographic patterns between different species, the discipline offers the potential to assist in the identification of zoogeographic provinces that should form the focus of future conservation efforts (Avise *et al.* 1987a).

MATERIAL AND METHODS

Sample collection

The analysis of mtDNA RFLP variation was conducted on 141 individuals comprising 116 *P. c. capensis* specimens from 28 localities in southern Africa, two *P. c. syriaca* from Israel, 20 *H. brucei* from eight localities and three *D. arboreus* specimens from two localities. The collection localities of *Procavia* are shown in Figure 5 and those of *Heterohyrax* and *Dendrohyrax* in Figure 6. Map coordinates for all collection sites are listed in Appendix I. In most instances animals were shot with a large calibre rifle, or shotgun, and heart and liver samples removed and frozen in liquid nitrogen within 15 min after death. Standard body measurements were recorded and representative skulls and study skins were deposited in the mammal collections of the Transvaal Museum (Pretoria), Kaffrarian Museum (King Williams' Town) and the Bulawayo Natural History Museum (Zimbabwe). Museum accession numbers are presented in Appendix II. Specimen collection records are listed in Appendix III.

Although immediate freezing is preferable for preserving tissue for molecular analyses (Dessauer, Cole & Hafner 1990), this was not always possible. In the absence of freezer facilities, heart samples were finely sliced and preserved in a saturated salt solution supplemented with 20% dimethylsulphoxide (DMSO), a technique developed for the long term preservation of whale skin (Amos & Hoelzel 1991). Salt is a good preservative as it denatures most proteins, while DMSO enhances cellular permeability and, therefore, the rapid penetration of the preservative into the sample. Samples can be stored at 4°C, or at room temperature, for up to a year (Amos & Hoelzel 1991).

Selected specimens from each of the taxa analysed in the mtDNA RFLP analysis, together with representatives of appropriate outgroup species, were used for dideoxy-sequencing. The specimens, their mtDNA affinities, and the collection localities are presented in Table 6.

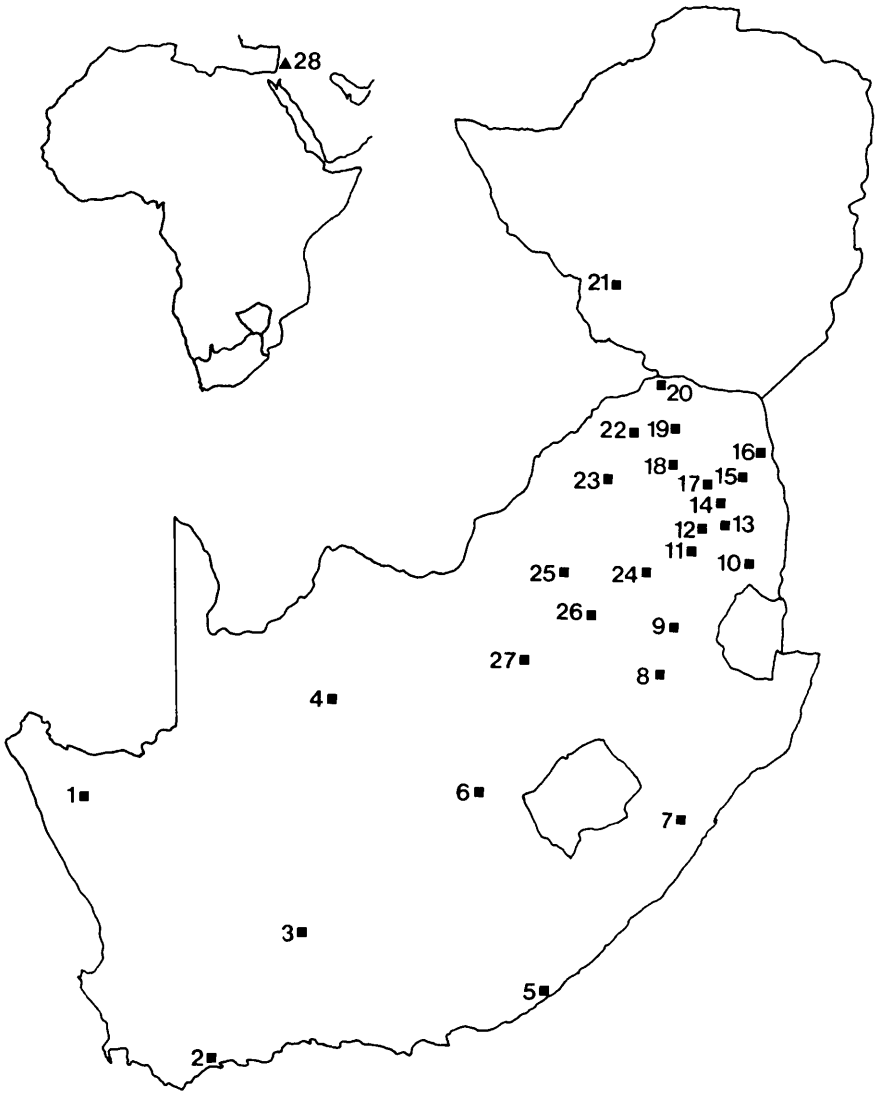


Figure 5 Collection localities of *P. c. capensis* in southern Africa. 1 - Springbok, 2 - De Hoop Nature Reserve, 3 - Karoo National Park, 4 - Kuruman Hills and Korannaberg, 5 - East London, 6 - Bloemfontein, 7 - Howick, 8 - Volksrust, 9 - Ermelo, 10 - Maqili (KNP), 11 - Lydenberg, 12 - Pilgrim's Rest, 13 - Blyde River Nature Reserve, 14 - Mica, 15 - Letaba (KNP), 16 - Shilowa (KNP Lebombo Mountains), 17 - Blackhills, 18 - Munnik, 19 - Buzzard Mountain Retreat (Soutpansberg), 20 - Vhembe Nature Reserve, 21 - Matopos (Zimbabwe), 22 - Blouberg, 23 - Potgietersrus, 24 - Middelburg, 25 - Rustenburg Nature Reserve, 26 - Suikerbosrand Nature Reserve, 27 - Vredefort. The insert shows the southern African localities in relation to that of *P. c. syriaca*.

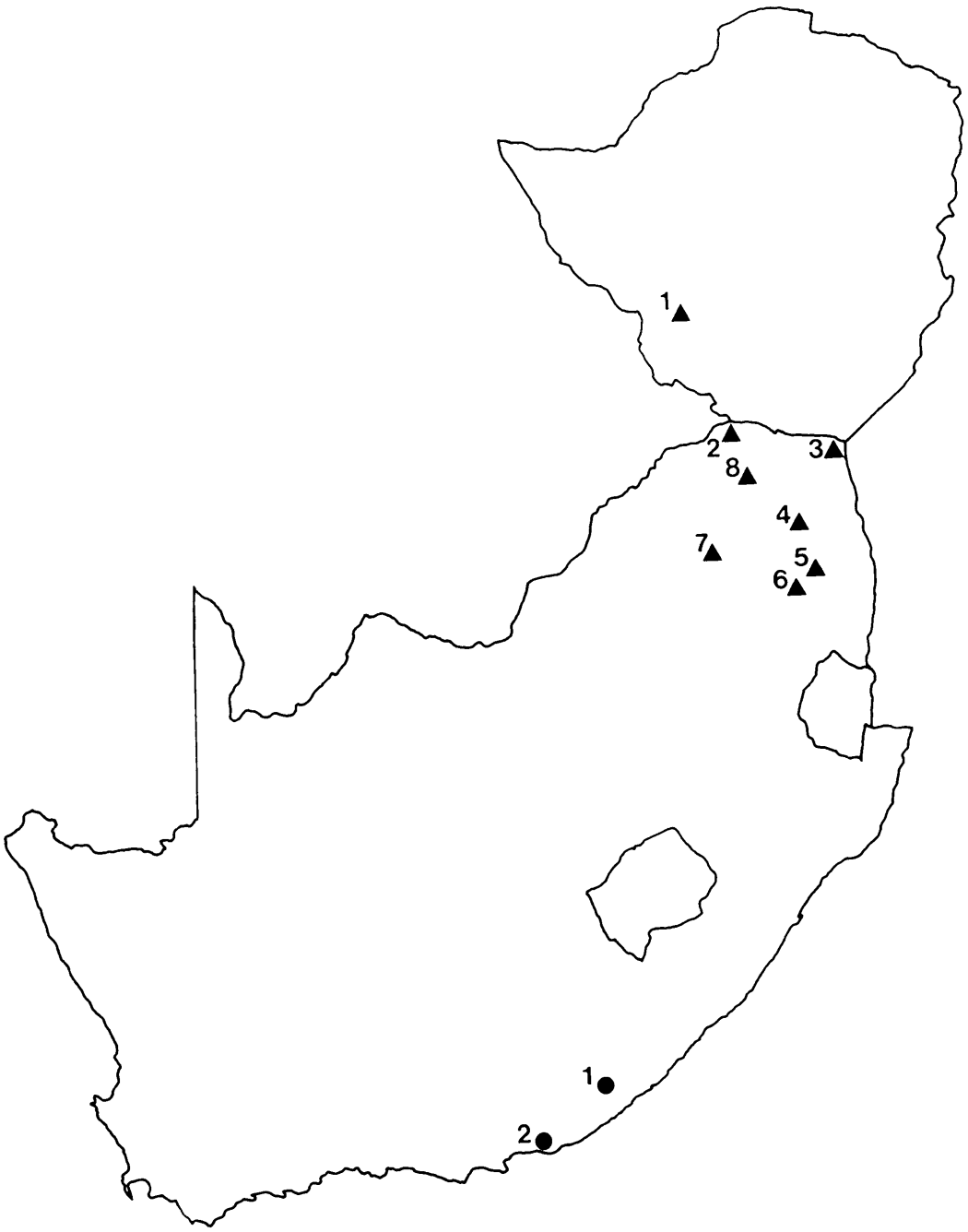


Figure 6 Collection localities of *H. brucei* (▲) and *D. arboreus* (●) in southern Africa. ▲: 1 - Matopos (Zimbabwe), 2 - Vhembe Nature Reserve, 3 - Pafuri (KNP), 4 - Munnik, 5 - Blyde River Nature Reserve, 6 - Ohrigstad, 7 - Buffelshoek Farm (Zebediela), 8 - Buzzard Mountain Retreat (Soutpansberg). ●: 1 - Pirie Forest (Ciskei), 2 - Alexandria State Forest (Grahamstown district).

Table 6 Representatives of the Hyracoidea and three outgroup taxa used in the sequencing analysis. The hyrax clones selected for interspecific comparisons are designated by asterisks. Numbers in parentheses correspond to the geographic localities presented in Figures 6 and 7.

Species	MtDNA clone	Locality
P. c. capensis	* 2	Beaufort West (3)
	11	Kuruman (4)
	19	Pilgrims' Rest (12)
	* 24	Vhembe (20)
	26	Shilowa (16)
P. c. syriaca	* 36	Haifa (28)
H. brucei	* 39	Vhembe (3)
	46	Soutpansberg (8)
D. arboreus	* 47	Pirie Forest (1)
	48	Alexandria (2)
L. africana	--	Kruger National Park
Orycteropus afer	--	Natal
Equus caballus	--	Pretoria

MtDNA extraction from soft tissue samples frozen in liquid nitrogen

Mitochondrial DNA was purified from heart or liver tissue using standard techniques (Lansman, Shade, Shapiro & Avise 1981). Eighty percent of extractions were done from heart tissue as it was found that this yielded purer mtDNA, and yields of up to 2.2 μg per gram of tissue were frequently obtained.

Thawed tissue samples (heart; mean = 6.06 g, or liver; mean = 46.38 g) were finely chopped in disposable petri dishes using sterile scalpel blades and resuspended in MSBCa^{2+} (0.21 M mannitol, 0.07 M sucrose, 0.05 M Tris-base, 3 mM $CaCl_2$, pH 7.5) prior to homogenizing the solution with a motor-driven glass teflon homogenizer. Ca-ions reduce nuclear breakage (Bogenhagen & Clayton 1974 in Lansman *et al.* 1981) while ethylenediamine-tetra-acetate (EDTA) was added to a final concentration of 10 mM to inhibit nuclease activity and prevent aggregation of mitochondria.

Cell debris was removed by centrifugation at 610 x g for 5 min before pelleting the mitochondria at 15 380 x g for 25 min. MtDNA pellets were washed using 20-30 ml of MSB-EDTA (MSB, 0.01 M EDTA, pH 7.5) and repelleted at 15 380 x g. Mitochondria were resuspended in STE-buffer (0.1 M NaCl, 0.05 M Tris-base, 0.01 M EDTA, pH 8) and the membranes lysed by the addition of 10% SDS (sodium dodecyl sulphate). Most of the protein fraction was removed by adding cesium chloride to 1 M and subsequent centrifugation at 15 000 x g for 15 min.

Cesium chloride - ethidium bromide gradients were prepared in Beckman thermoplastic ultracentrifuge tubes containing 9 ml of lysate, 8 g cesium chloride (density = 1.6 g/ml) and 600 μl ethidium bromide. Tubes were capped and the gradient overlaid with liquid paraffin to prevent tube collapse. Following 24 - 48 hr of ultracentrifugation (Beckman L7-55 Preparative Ultra-centrifuge) at 225 000 x g, the fractionized samples were visualized under UV-light (386 nm) and the mtDNA band (Figure 7) removed using a sterile syringe and 18 gauge needle. In a number of extractions no mtDNA band was visible and a layer 1 cm below the nuclear DNA band was removed.

Excess ethidium bromide was removed by four to five extractions with an equal volume of iso-amyl alcohol, while CsCl removal was by dialysis. This was done against 1 x TE (0.1 M Tris pH 8, 10 mM EDTA) for at least 48 hr when using dialysis tubing or, alternatively, for 30 min when using millipore filters in mini-dialysis. No ethanol precipitation was included in the procedure; mtDNA samples were subsequently stored at 4°C.

Restriction endonuclease digestion and end-labelling

Fifteen Type II restriction endonucleases were screened and 10 selected for the digestion of mtDNAs based on consistent digestion and their recognition of more than one site in the hyrax's mitochondrial genome. Purified mtDNA (10 - 20 ng) from each specimen was subsequently digested with the 10 restriction endonucleases following the manufacturers' specifications (Table 7). Digestions were routinely performed in 20 μl reaction volumes containing 10 - 20 units of enzyme, 10 X buffer and 2 mM spermidine; these were incubated at 37°C for 5 - 8 hr.

The resulting restriction fragments were end-labelled to high specificity with [α-^{32}P] dCTP (Deoxycytidine 5'- Triphosphate, tetra (Triethylammonium) salt, 3000 Ci/mmol specific activity; NEN products/Amersham) using the large Klenow fragment of *Escherichia coli* polymerase I (Brown 1980). This enzyme has the 5'-3' polymerase and the 3'-5' exonuclease activity of intact DNA polymerase I, but lacks the 5'-3' exonuclease activity. Consequently, slight overhangs are created by the exonuclease activity, while strong polymerase activity incorporates free nucleotides of the end-

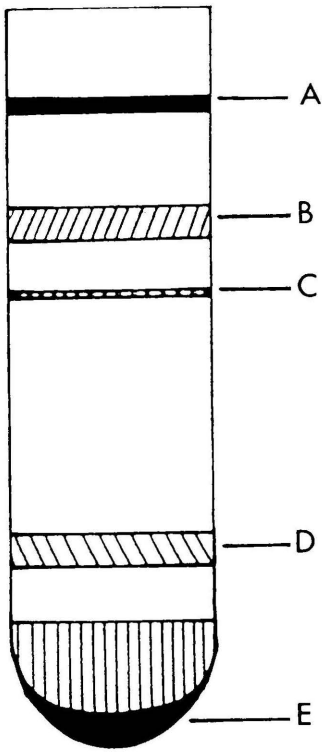


Figure 7 Cesium chloride-ethidium bromide density gradient visualized under UV-light following 24 - 48 hr of ultracentrifugation. A - protein, B - nuclear DNA, C - mitochondrial DNA, D - glycogen (when extracting from liver samples) and E - RNA (redrawn from Lansman *et al.* 1981).

Table 7 Ten restriction endonucleases used for the digestion of purified mitochondrial DNAs.

Enzyme	Recognition sequence*	r**	Spermidine	Buffer	Supplier(s)
*Ava*I	C/PyCGPuG	5.3	+	Medium	Promega
*Bam*HI	G/GATCC	6	+	High	Boehringer
*Dra*I	TTT/AAA	6	+	High	Boehringer
*Hinc*II	CTPy/PuAC	5.3	+	High	Promega/ Amersham
*Hind*III	A/AGCTT	6	+	High	Boehringer/ Amersham
*Pvu*II	CAG/CTG	6	-	Medium	Promega
*Sac*I	GAGCT/C	6	-	Low	Boehringer
*Sca*I	AGT/ACT	6	+	Medium	Promega
*Stu*I	AGG/CCT	6	+	Medium	Promega
*Xba*I	T/CTAGA	6	+	High	Boehringer

* - A - adenine, C - cytocine, G -guanine, T - thymine, Pu - any purine, Py - any pyrimidine

** - Enzyme class according to Nei (1987)

labelling buffer and radio-active label during incubation at 37°C for 15 min. The reaction was terminated by incubation at 65°C for 10 min followed by snap chilling.

Agarose gel electrophoresis and autoradiography

End-labelled restriction fragments were electrophoretically separated through 1% horizontal agarose gels (Biological Grade Agarose, Promega). Promega markers II (*Hind*III digested phage lambda DNA) and/or III (*Hind*III and *Eco*RI digested phage lambda DNA) were used as molecular weight standards. At this agarose concentration the efficient separation of linear fragments 9 - 0.5 kilobase (kb) in size was obtained. Tris-borate buffer (0.089 M Tris-base, 0.089 M boric acid, 0.02 M EDTA, pH 8.0), which provides good resolution of DNA fragments and has a high buffering capacity without needing recirculation, was used in electrophoresis. Since effective separation of fragments is inversely related to current (Maniatis, Fritsch & Sambrook 1982), gels were routinely run at low voltage (35 V) for 16 hr.

Nucleic acids trapped in the gel matrix cannot be efficiently detected by autoradiography as the radiation is quenched by the agarose. If transferred to a membrane however, it becomes sensitive to detection by autoradiography. In the present study, gels were dried onto 3MM Whatman chromatography paper by vacuum transfer (Slab Gel Dryer, Hoefer Scientific Instruments, San Francisco) at a temperature of 54°C and by applying vacuum for 1½ hr. Radio-actively labelled fragments were detected by autoradiography using Okomoto X-ray cassettes with one intensifying screen and Fuji RX Medical X-ray film. Subsequent to exposure at -70°C for 2-48 hr, autoradiographs were developed (Ilford Phenisol) and fixed (Ilford Hypam) using routine procedures.

Extraction of genomic DNA from soft tissue preserved in saturated salt solution

Genomic DNA was extracted from 100 mg of preserved heart tissue (p. 30) following Amos & Hoelzel (1991). The tissue sample was quick-frozen in liquid nitrogen and powdered using a pestle and mortar chilled in liquid nitrogen. The powder was transferred to a 1.5 ml microcentrifuge tube and resuspended in 500 µl digestion solution (50 mM Tris pH 8.0, 1% SDS, 10 mM EDTA, 100 mM NaCl). Proteinase K (10 mg/ml stock) was added to a final concentration of 1 mg/ml and the sample incubated at 65°C for 2 hr. The DNA was purified by phenol extractions (three repeats) and the phenol subsequently removed by two chloroform extractions. A 600 µl aliquot of the upper aqueous phase was retained in a fresh tube, an equal volume of 5 M LiCl added and the sample incubated at -20°C for 30 min. Proteins were removed by centrifugation at 12 000 x g for 5 min and the DNA finally precipitated by adding two volumes of 100% ethanol and overnight storage at -20°C. The

DNA pellets were subsequently washed with 70% ethanol and air dried before resuspending them in 25-50 μl 1 x TE.

Southern blot hybridization

Digestion profiles comprising very small fragments that were frequently obscured by background in the end-labelled preparations were subsequently confirmed by transfering the mtDNA fragments to hybridization membranes (Hybond N, Amersham) by southern blotting (Southern 1975). The membrane bound mtDNA fragments were screened using labelled hyrax mtDNA as probe. The same procedure was followed for the screening of high molecular weight DNA extracted from heart samples preserved in saturated salt solutions.

Enzymatically digested DNA samples were electrophoresed on 0.8 - 1% agarose gels containing ethidium bromide. After 16 hr of electrophoresis the gels were photographed on a UV transilluminator, rinsed in distilled water, agitated for 30 min in denaturation buffer (1.5 M NaCl, 0.5 M NaOH), and then neutralized (1.5 M NaCl, 0.001 M EDTA, 0.5 M Tris-base, pH 7.2). After rinsing in 10 x SSC (0.3 M NaCl, 0.03 M tri-sodium citrate pH 7.0) capillary blots were prepared using standard procedures (Maniatis *et al.* 1985) with 10 x SSC as the transfer buffer. Following 24 hr of transfer, membranes were rinsed in 2 x SSC to remove any adhering agarose, and baked at 80°C for 2 hr.

The mtDNA probe was oligolabelled following Feinberg and Vogelstein (1983). This involved the overnight incubation (at 37°C) of 100 ng of the denatured probe DNA in the presence of the Klenow fragment of DNA polymerase I, random hexanucleotide primers and dNTPs (including [α-^{32}P] dCTP). Subsequently, unincorporated nucleotides were removed by spermine precipitation. For this step spermine was added to a final concentration of 1.6 mM while 150 μg/ml carrier DNA (salmon sperm DNA) prevented loss of probe DNA during precipitation. The DNA was pelleted at 11 500 x g for 10 min at 4°C and the supernatant discarded (2 μl of the supernatant was retained for determination of total counts). The probe DNA was resuspended in 400 μl 0.5 M NaCl in TE^{-4} and chemically denatured by the addition of 4 N NaOH. Neutralization was effected by adding 2 M Tris; 2 μl of the precipitated probe was retained for counting. Incorporated counts per probe and the percentage incorporation were determined by scintillation counting (Packard 1500 tri-carb Liquid Scintillation Counter).

The pre-hybridization and hybridization steps were performed at 65°C in sealed plastic bags. Hybridization is dependent on the binding of complementary sequences and the rate of the reaction is determined by several factors including the concentration of the probe and filter-bound DNA, the second order rate constant, temperature, ionic strength, degree of mismatching and the pH (Anderson & Young 1985). Twenty ml of pre-hybridization solution (100 mM Denhardts, 20 x SSPE, 10% SDS)

was added to each blot and sealed bags incubated for 2 hr in a shaker waterbath. This step saturates binding sites on the membrane, and contributes to reducing non-specific background (Mason & Williams 1985).

Hybridization of the spermine precipitated mtDNA probe to the membrane bound fragments was allowed to progress for 24 hr. This was followed by two low stringency washes (2 x SSPE 0.1% SDS 24°C and 1 x SSPE 0.1% SDS 65°C) and one high stringency wash (0.1 x SSPE 0.1% SDS). The temperatures and salt concentrations of the washes effect hybrid stability allowing the removal of unincorporated nucleotides and mismatched hybrids (Anderson & Young 1985). To remove SDS, all membranes were rinsed in 0.1 x SSPE, resealed in plastic bags and subsequently exposed to autoradiography.

Estimation of restriction fragment size

Linear DNA molecules travel through gel matrices at rates that are inversely proportional to the $\log_{10}$ of their molecular weights (Helling, Goodman & Boyer 1974). The molecular weight markers run on each gel were used to construct a standard curve (molecular weight:distance migrated on the gel), and the molecular weights of individual restriction fragments read from the graph. Confirmation of shared fragments from different restriction profiles produced by the same enzyme was obtained by subsequently running these in adjacent lanes and possible co-migration clearly established.

Analyses of restriction fragment data

Subsequent to the identification of all the restriction fragment patterns, each enzyme was given a numeric designation (1-10) and each unique restriction fragment pattern produced by a specific enzyme assigned an alphabetical character (a, b, etc.). A 10 character code was thus formulated for each hyrax specimen - the haplotype. Specimens with identical haplotypes were considered to be representative of the same maternal lineage or clone.

Genotypic diversity was estimated as

$$G = n(1-\Sigma f_i^2)/(n-1)$$

where n is the number of specimens and f_i the frequency of the ith haplotype (Nei & Tajima 1981).

The Restsite computer program (version 1.1, Nei & Miller 1990) was used for calculations of sequence divergence. This was based on the proportion of shared fragments (F) between operational taxonomic units (OTUs) (equation 5.53 of Nei 1987)

$$F = 2M_{XY} / (M_X + M_Y)$$

where M_{XY} is the number of shared fragments between X and Y and M_X and M_Y the total number

of fragments for OTUs X and Y respectively.

The calculation of sequence divergence was by equations 5.54 and 5.55 of Nei (1987)

$$G2 = [F(3\text{-}2(G1))]^{0.25}$$

$$d = -2[\ln(G2)]/r.$$

G2 is calculated by iteration (equation 5.54) where F is the proportion of shared fragments and the first iteration value for $G1 = F^{0.25}$. In subsequent iteration cycles G2 replaces G1 until G2 = G1. Sequence divergence (d) is estimated by equation 5.55 where r represents the average number of base pairs recognized by all the restriction endonucleases used. Sequence divergence was estimated between clones, populations and species and the estimates between populations and species corrected for within group variation using equation 10.21 of Nei (1987)

$$d(\text{corrected}) = d_{XY} - (d_X + d_Y)/2$$

where d_X and d_Y represent variation within OTUs X and Y respectively, and d_{XY} the variation between them.

The different restriction enzyme classes (r) were first computed separately and then combined using equation 17 of Nei and Miller (1990)

$$d = \text{Sigma } \{m \times r \times d\} / \text{ Sigma } \{m \times r\} \text{ where}$$

$$m = (m(X) + m(Y))/2.$$

Correction for multiple hits was made following the Jukes and Cantor method incorporated in equation 5.3 of Nei (1987)

$$d = -3/4 \log_e(1\text{-}4/3p) \text{ and}$$

standard errors computed by bootstrapping with 200 replications and by random replacement. The resulting matrices were subsequently clustered using UPGMA (unweighted pair group method with arithmetic averages; Sneath & Sokal 1973) and the neighbor-joining algorithm (Saitou & Nei 1987).

In a more qualitative approach, the number of mutational steps between restriction fragment patterns was assessed for each of the 10 enzymes. All clones within each species were subsequently compared in a pairwise fashion to calculate the minimum number of mutational steps between them (summed over all 10 enzymes), and then connected in a minimum-path network invoking the least number of mutations between clones. The resulting parsimony networks where overlaid on geographic maps (Lansman *et al.* 1983a).

Geographical Information Systems (GIS) analysis was conducted to establish whether any correlation between contemporary environmental factors and geographic mtDNA structure could be detected. Elevation, rainfall and temperature data were obtained from the Water Board (Computer Centre for Water Research, Pietermaritzburg), while vegetation indices were digitized using Acocks (1988) as source.

Extraction of genomic DNA for sequencing analysis

Genomic DNA was extracted from frozen soft tissue samples following a modification of the protocol described for samples preserved in saturated salt (p. 37). The modification involved the treatment of the powdered sample in amniocyte buffer (1M Tris, 1M NaCl, 0.5M EDTA, 10% SDS) containing 750 μg proteinase K; the solution was incubated at 55°C for 6 hr. DNA was purified by phenol extraction, and the ethanol precipitation of the DNA was facilitated by the addition of 2M ammonium acetate.

Horse DNA was extracted from venous blood collected directly into acid citrose dextrose (ACD) vacutainer tubes. Samples (10 ml volume) were lysed in 40 ml sucrose-triton X buffer and cells pelleted at 1000 x g at 4°C. The cells were subsequently washed, repelleted and treated overnight with 10% SDS and proteinase K at 50°C. Subsequently, DNA was recovered by the addition of saturated NaCl and by ethanol precipitation. Precipitated DNA was dissolved in 1 x TE.

Polymerase chain reaction (PCR)

The PCR reaction involves repeated cycles of heat denaturation (to make the DNA single stranded), hybridization of the primers to their complementary strands, and extension of the primers by DNA polymerase. This enzyme incorporates free nucleotides, resulting in amplification of the sequences defined by the 5' ends of the primers (White *et al.* 1989).

Taq DNA polymerase (Promega), isolated from the thermophilic eubacterium *Thermus aquaticus* which lacks *Taq* I restriction endonucleases as well as unspecific ends or exonucleases, was used in the reaction. *Taq* DNA polymerase is a highly processive 5'- 3' DNA polymerase, and the absence of exonuclease activity and ability to withstand high temperatures (75°C optimum temperature, but resistant to prolonged exposure to temperatures as high as 95°C) contribute to its almost universal use in PCR and sequencing.

Two sets of oligonucleotide primers, designed from conserved regions in mtDNA sequences of mammals, the frog and the fruit fly (Kocher *et al.* 1989) were used. The letters "L" and "H" refer to the light and heavy strands respectively, and the number refers to the 3' base of the primer in the human sequence (Anderson, Bankier, Barrell, De Bruijn, Coulson, Drouin, Eperon, Nierlich, Roe, Sanger, Schreier, Smith, Staden & Young 1981). The primers were:

cytochrome b - L14724 (Meyer & Wilson 1990)
- H15149 (Kocher *et al.* 1989)
12S rRNA - L1091 (Kocher *et al.* 1989)
- H1478 (Kocher *et al.* 1989).

The first primer pair amplifies a 425 bp fragment adjacent to the glutamine tRNA gene (Irwin *et al.* 1991) while the second amplifies a 386 bp fragment internal to the 12S rRNA gene at the 5' end of the valine tRNA coding region (Kocher *et al.* 1989). All primers used in this study were synthesized by the Department of Biochemistry, University of Cape Town, and supplied in concentrations of 40 to 70 OD units. The primers were stored in small aliquots (10 μl) at -70°C.

All preparative PCR reactions were done in a laminar flow hood to minimize contamination. Negative controls, comprising all components except DNA, were included for all reactions. The first negative control tests for contamination of stock solutions, while sterile water added to the second negative control subsequent to pipetting of DNA samples tests for aerosol contamination.

Double stranded DNA amplification was performed in 25 or 50 μl reaction volumes that contained: 10 x *Taq* polymerase buffer, 25 mM $MgCl_2$, 2 mM dNTPs, 12.5 - 25 pmol of both primers, 50 ng total genomic DNA and 1 unit of *Taq* polymerase. PCR was carried out in a Hybaid thermal reactor (Hybaid) and a 35 cycle program was used to generate double stranded products from the target species. Specifications were: denaturation for 30 sec at 93°C, annealing for 30 sec at 55°C and extension for 45 sec at 72°C. A final extension of 10 min at 72°C completed the reaction. After removal of the mineral oil layer, double stranded products were electrophoresed in 2-3% low melting point agarose gels (NuSieve, FMC Bioproducts) with ϕX174 (HaeIII digest) as molecular weight standard; at this point the negative controls were checked for possible contamination.

The amplified products were excised from the gels and purified using a commercial purification system (Magic Prep DNA purification system, Promega). The gel slices were transferred to 1.5 ml microcentrifuge tubes and incubated at 70°C to melt the agarose. A resin mix was added to bind the DNA and after vortexing for 1 min the resin/DNA solution was forced through a minicolumn to remove the agarose. The bound DNA was subsequently washed with Column Wash solution before dissolving the DNA in 10 - 25 μl of sterile 1 x TE which was followed by centrifugation for 20 sec at 12 000 x g.

Sequencing with chain terminating inhibitors

The method of Sanger, Nicklen and Coulson (1977), which is based on the inhibitory effects of dideoxy-triphosphates, was followed. Because ddTPs do not contain 3'-hydroxyl groups, termination of a synthesized strand occurs specifically at positions where they are incorporated. For example, if a primer and template are incubated with DNA polymerase and a mixture of ddTTP (dideoxythymidine triphosphate) and dTTP as well as the other three triphosphates (of which one is radio-actively labelled), fragments containing the same 5' end (complementary to the primer) and ddT residues at their 3' ends will be obtained. When these are separated on polyacrylamide gels the pattern of bands shows the distribution of dTs in the newly synthesized DNA sequence. By using the

appropriate terminator for each of the four nucleotides in separate incubations, a pattern of bands representing the whole sequence can be read from the gel.

Four μl of the purified double stranded PCR product was sequenced with a commercial kit (Sequenase, United States Biochemical Corporation). The Sequenase Version 2 enzyme is a genetic variant of bacteriophage T7 DNA polymerase that lacks 3'-5' exonuclease activity and is characterized by high processivity, speed and the efficient use of nucleotide analogues. The reaction consists of two steps: the annealing step extends the sequencing primer using limiting amounts of deoxynucleotide triphosphates while in the termination step, the concentrations of deoxynucleotide triphosphates are increased and dideoxynucleotide triphosphates added. CHASE solution containing dNTPs and sequencing buffer was routinely added to extend all fragments; the reaction was terminated by the addition of EDTA and formamide. DMSO was used at a final concentration of 10% in the annealing and labelling mixes to prevent the heat denatured DNA from reannealing. Labelled dATP in the form of [α-^{32}P]-dATP (3000 Ci/mmol specific activity, Amersham), or [α-^{35}S]-dATP (1000 Ci/mmol specific activity), was included for autoradiographic detection of the sequence.

Polyacrylamide gel electrophoresis

Six percent polyacrylamide gels were used for the electrophoretic separation of sequences. Acrylamide monomers polymerize into long chains that are covalently linked by a crosslinker, N, N'-methylene-bis-acrylamide, while the urea acts as a denaturing component. Chemical polymerization was accomplished with ammoniumpersulphate as initiator and N, N, N', N'-tetramethylene diamine (TEMED) as catalyst.

Polyacrylamide gel electrophoresis was performed using an adjustable vertical sequencing system (Hybaid) and a Consort E734 power supply. Gels were run at constant wattage (85 Watt), while a current of 50 mA and 1.5 kV was applied. At these settings, a gel temperature of 45° to 50°C was generated; TBE (pH 8.0) was used as buffer. Gels were pre-run for 1 hr before loading 2 - 3 μl of sample per lane in the conventional order: G A T C. Samples were heated to 80°C for 3 min immediately before loading. Smaller fragments were separated using 1½ - 2 hr of electrophoresis (until the bromophenol blue dye had migrated approximately 40 cm), while 4 - 6 hr were needed to separate the larger fragments (until the xylene cyanol dye front had migrated 40 cm). The gels were dried onto 3MM Whatman chromatography paper (Slab Gel Dryer, Hoefer) at 80°C for 2 hr under vacuum, and exposed to Fuji Medical X-ray film at -70°C for 2 - 8 days using Okomoto cassettes.

Analyses of sequencing data

Both strands of the two gene fragments were sequenced in order to score sequence close to the sequencing primers and to allow for the unambiguous scoring of bases in the regions of overlap. Most DNAs were amplified and sequenced more than once. Sequences were digitized with the aid of the GENEPRO computer program (Scientific Associates, California) and checked manually. Pairwise alignments were done using the GENEPRO program; final multiple alignments were performed manually and with CLUSTAL (version V), a computer programme based on the Higgins and Sharp (1989) alignment algorithm. This computer package also allows for the calculation of pairwise estimates of sequence divergence as well as rates of transition and transversion. Corrections for multiple hits were made using Kimura's (1980) two parameter model.

The corrected distances between sequences were used for clustering by the neigbor-joining algorithm (Saitou & Nei 1987) while the significance of internal branches was assessed using 1000 bootstrap replicates with random replacement (Felsenstein 1985b). Sequences of the two genes were analysed separately and then combined. In addition, cytochrome b sequences obtained from this study were translated and the amino acid sequences used both for clustering and for an assessment of the frequency of silent/replacement changes.

Parsimony analysis by PAUP (Phylogenetic Analysis Using Parsimony, version 2.4 Swofford 1985) and PHYLIP (version 3.3 Felsenstein 1990), was used for the construction of phylogenetic trees. Sites were treated as unordered characters with four possible character states (G, A, T or C). Invariant sites and symplesiomorphies were excluded from the data set for analysis by PAUP, and the remaining informative characters analysed using the Branch-and-Bound, Global Branch Swapping and Alltrees options. To account for the transition bias in mtDNA, sequences were recoded with only two character states; R (purine) and Y (pyrimidine), and only informative transversions considered for clustering using the same PAUP options.

Analysis by PHYLIP was done using the DNAPARS and DNABOOT (with 100 replicates and random replacement) subroutines which assume that each site and each lineage evolved independently. The programme also assumes that rate of change in the different lineages, as well as between different sites, does not vary significantly. In addition, the DNAML option (Felsenstein 1981), which considers all relevant sites (including invariant sites and symplesiomorphies), was used. This takes empirical base frequencies and transition bias into account.

RESULTS

RFLP analysis - Intraspecific variation within three species of the Hyracoidea

Figure 8 depicts representative digestion patterns obtained from the various taxa with the restriction enzyme *Ava*I. A total of 104 restriction fragment patterns was produced by the 10 restriction enzymes resulting in a mean of four fragments per pattern. The restriction fragment patterns are summarized in Appendix IV. The smallest fragment detected in the present study was 330 bp long. In a few instances, specifically after digestion of DNA with the restriction enzymes *Sca*I and *Stu*I, fragments of 100 to 130 bp could not be detected, but were calculated by subtraction from the total genome size. Four enzymes in particular (*Hinc*II, *Hind*III, *Sca*I, *Stu*I) were highly polymorphic, producing 15 - 16 different patterns.

Because measurements of large fragments are subject to error, the size of the hyrax mitochondrial genome ($\approx$ 16 626 $\pm$ 55 bp; N = 83) was estimated using appropriate restriction fragment patterns characterized by fragments ranging from 330 to 9 000 bp in size. A total of 28 - 45 restriction fragments was scored per individual resulting, on average, in a survey of 232.62 nucleotides or 1.4% of the hyrax mitochondrial genome.

Forty-eight haplotypes were detected in the study specimens (Table 8), all of which, with the exception of six (1, 2, 17, 23, 24 and 39), were restricted to specific geographic areas. This is supported by high estimates of genotypic diversity; 0.916 for the Cape rock hyrax and 0.837 for the yellow-spotted hyrax. The nucleotide diversities within sample populations of each species (excluding those populations that were represented by single individuals) are presented in Table 9.

Pairwise estimates of nucleotide sequence divergence among the 48 clones are presented in Table 10 and the relationships among them summarized in the neigbor-joining tree (Figure 9). The topology of a UPGMA tree generated from the same data set was, with minor branch swopping, identical to that derived from the neighbor-joining algorithm, and since the latter is thought to more accurately represent low values of sequence divergence (Jin & Nei 1991) only this is shown. Figure 10 depicts the clustering of populations within *P. capensis capensis* by the neighbor-joining algorithm.

Two major clusters are apparent within *P. c. capensis* (clones 1 - 35; Figure 9). The larger, and geographically more extensive, south-central cluster comprises populations from the south, central and north-eastern portions of the country (clones 1-20), while the second (northern) cluster (clones 21-35) is restricted to the extreme northern and north-western parts of South Africa and the south-western part of Zimbabwe. Nucleotide sequence divergence between these clades was estimated to be 3.32% ($\pm$ 0.73%) which, if a rate of sequence divergence of 2% per million years (Brown *et al.* 1979) is assumed, may be interpreted as indicating an evolutionary divergence time of approximately 1.7 million years. Diversity within the clades was low; 1.39% in the south-central clade and 1.18% in the northern clade. The two *P. c. syriaca* specimens from Haifa in Israel were identical and are

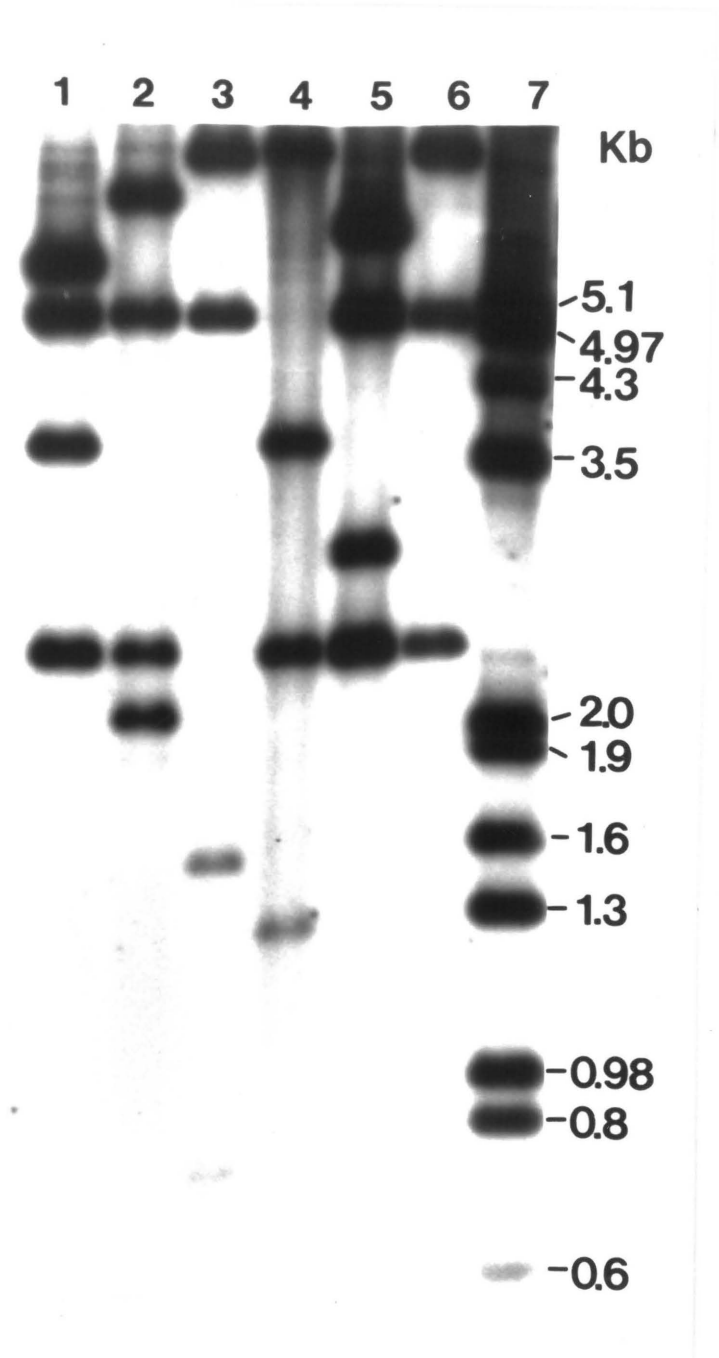


Figure 8 Restriction fragment patterns produced by digestion of mtDNAs with the enzyme *Ava*I. Lanes 1 - 6 represent six of the seven restriction fragment patterns obtained with this enzyme (Table 8; see Appendix IV for restriction fragment sizes). Patterns a - f are presented; taxa that share patterns are given in parentheses: lane 1 - f (*H. brucei*), 2 - c (*H. brucei*), 3 - d (*D. arboreus*), 4 - b (*P. c. capensis*), 5 - e (*P. c. capensis* and *P. c. syriaca*) and 6 - a (*P. c. capensis* and *H. brucei*). Lane 7 contains the molecular weight standard Lambda DNA digested with *Hind*III and *Eco*RI.

Table 8 Mitochondrial DNA haplotypes of 141 hyrax specimens based on RFLP analysis with 10 restriction endonucleases. Clones 1-35 are representative of *P. c. capensis*, clone 36 of *P. c. syriaca*, clones 37-46 of *H. brucei* and clones 47-48 of *D. arboreus*. Population designations of *Procavia* (P 1-28) correspond to Figure 5 and those of *Heterohyrax* (H 1-8) and *Dendrohyrax* (D 1-2) to Figure 6. * Numbers designate restriction endonucleases: 1 *Ava*I, 2 *Bam*HI, 3 *Dra*I, 4 *Hinc*II, 5 *Hind*III, 6 *Pvu*II, 7 *Sac*I, 8 *Sca*I, 9 *Stu*I, 10 *Xba*I; alphabetical characters correspond to restriction fragment patterns presented in Appendix IV.

No.	Haplotype*										N	Population
1	1b	2c	3a	4e	5d	6d	7a	8c	9c	10c	3	P 1,2
2	1a	2c	3a	4c	5d	6b	7a	8c	9c	10c	19	1,3,4,6,7,9
3	1b	2c	3a	4e	5d	6c	7a	8c	9a	10c	1	2
4	1b	2c	3a	4e	5d	6d	7a	8c	9a	10a	1	2
5	1b	2c	3a	4e	5d	6d	7a	8c	8h	10c	2	2
6	1e	2f	3b	4c	5h	6b	7a	8c	9f	10c	1	2
7	1e	2f	3b	4c	5h	6b	7a	8c	9c	10c	1	2
8	1a	2c	3c	4c	5d	6b	7a	8f	9c	10c	1	3
9	1a	2c	3a	4a	5d	6b	7a	8c	9c	10c	1	3
10	1a	2c	3a	4f	5d	6b	7a	8c	9c	10c	1	3
11	1e	2f	3a	4e	5i	6b	7a	8c	9g	10c	1	4
12	1a	2c	3a	4g	5f	6b	7a	8c	9c	10c	1	5
13	1a	2c	3a	4c	5f	6b	7a	8c	9c	10c	7	6
14	1a	2c	3a	4c	5d	6b	7c	8c	9c	10c	6	7
15	1a	2c	3b	4l	5i	6b	7a	8c	9c	10c	1	8
16	1a	2c	3a	4l	5d	6b	7a	8c	9c	10c	3	8
17	1a	2c	3a	4j	5d	6b	7a	8c	9g	10c	4	10,13
18	1a	2c	3a	4c	5d	6b	7a	8c	9g	10c	1	11
19	1a	2c	3a	4d	5d	6b	7a	8c	9a	10c	3	12
20	1a	2c	3a	4c	5d	6b	7c	8c	9a	10c	1	12
21	1a	2a	3b	4a	5a	6a	7a	8b	9a	10h	2	14

Continued/

No.	Haplotype*										N	Population
22	1a	2h	3b	4a	5c	6a	7e	8b	9i	10a	1	14
23	1a	2a	3b	4a	5c	6a	7a	8b	9a	10a	18	14,19,24,25, 26,27
24	1a	2a	3b	4a	5a	6a	7a	8b	9a	10a	19	14,15,18,20, 22,23
25	1e	2a	3b	4a	5k	6b	7a	8k	9l	10a	1	16
26	1e	2a	3b	4a	5k	6b	7a	8k	9m	10a	1	16
27	1a	2a	3f	4c	5a	6a	7a	8b	9a	10a	2	17
28	1a	2a	3f	4a	5a	6a	7a	8b	9a	10a	2	17
29	1e	2a	3b	4a	5k	6a	7a	8b	9a	10a	2	18
30	1a	2a	3b	4f	5j	6a	7a	8b	9a	10g	1	20
31	1a	2h	3b	4c	5a	6a	7a	8b	9a	10g	3	20
32	1a	2a	3b	4a	5j	6a	7a	8b	9m	10a	1	21
33	1a	2a	3b	4c	5n	6a	7a	8l	9l	10a	1	21
34	1a	2a	3b	4a	5c	6a	7a	8l	9l	10a	1	21
35	1a	2h	3b	4a	5a	6a	7e	8b	9a	10a	2	24
36	1e	2j	3h	4o	5e	6b	7a	8p	9o	10f	2	28
37	1a	2b	3b	4m	5b	6a	7e	8m	9k	10a	2	H 1
38	1a	2b	3b	4m	5b	6a	7e	8n	9k	10a	1	1
39	1a	2b	3b	4b	5b	6a	7b	8a	9b	10d	8	2,3
40	1a	2f	3b	4b	5b	6a	7b	8a	9b	10d	1	2
41	1a	2f	3b	4b	5l	6a	7b	8a	9b	10d	1	2
42	1c	2d	3d	4b	5e	6a	7a	8d	9d	10d	2	4
43	1c	2d	3d	4b	5e	6a	7a	8g	9j	10d	1	5
44	1a	2b	3a	4b	5i	6a	7a	8g	9e	10d	1	6
45	1a	2d	3a	4k	5m	6a	7a	8i	9e	10d	2	7
46	1f	2g	3a	4h	5e	6a	7e	8j	9e	10d	1	8
47	1d	2e	3e	4i	5g	6a	7d	8e	9p	10e	2	D 1
48	1g	2i	3g	4n	5o	6e	7f	8o	9n	10e	1	2

Table 9 Percent nucleotide sequence diversity (nucleotide substitutions per site) in sample populations represented by more than one individual. Species designations are: P - *P. c. capensis*, H - *H. brucei*, D - *D. arboreus*. Population designations correspond to those in Figure 5 (P 1-27) and Figure 6 (H 1-3, 5, 7 and D1).

Population	% D	Standard error	Population	% D	Standard error
P 1	1.137	0.725	17	0.238	0.231
2	1.850	0.516	18	0.549	0.439
3	0.439	0.282	20	0.769	0.395
4	1.723	0.881	21	0.820	0.327
6	0.222	0.231	23	0.000	0.000
7	0.222	0.250	24	0.703	0.382
8	0.453	0.387	25	0.000	0.000
9	0.000	0.000	26	0.000	0.000
10	0.000	0.000	27	0.000	0.000
12	1.137	1.130	H 1	0.000	0.210
13	0.000	0.000	2	0.000	0.331
14	0.675	0.229	3	0.000	0.000
15	0.000	0.000	5	0.000	0.000
16	0.222	0.186	7	0.000	0.000
			D 1	0.000	0.000

Table 10 Percent nucleotide sequence divergence (nucleotide substitutions per site) among the 48 haplotypes detected in the three hyracoid genera. Clones 1-35 are representative of *P. c. capensis*, clone 36 of *P. c. syriaca*, clones 37 - 46 of *H. brucei* and clones 47 - 48 of *D. arboreus*. Sequence divergences (d) are presented above the diagonal and standard errors derived via bootstrapping below the diagonal.

	1.14	0.93	0.88	0.46	3.13	2.64	1.58	1.00	1.27	1.64	1.36	1.36	1.36	1.76	1.27	1.53	1.39	2.29	2.06	5.26	5.97	4.51	4.96	6.74	6.66	5.13	5.04	5.38	5.87	6.33	5.44	6.20	5.52	6.25	8.61	9.55	9.44	11.70	11.58	11.58	8.39	9.63	8.00	6.92	9.35	13.18	10.07
0.75		2.13	2.12	1.67	1.46	1.05	0.45	0.26	0.25	1.72	0.81	0.22	0.22	0.71	0.25	0.95	0.23	1.56	0.88	4.62	5.31	3.88	4.33	5.34	5.25	4.11	4.42	4.46	4.92	5.27	4.81	5.20	4.90	5.60	6.71	8.92	8.81	11.00	10.88	10.88	7.22	8.43	7.28	6.38	9.23	11.14	9.95
0.60	1.00		0.52	0.96	3.97	3.85	2.67	2.01	2.25	2.67	2.39	2.39	2.39	2.87	2.25	2.38	2.26	1.88	1.65	4.02	5.26	3.45	3.80	5.61	5.54	3.98	3.88	4.23	4.53	4.85	4.18	4.82	4.25	4.76	9.46	9.76	9.65	11.93	11.81	11.81	8.54	9.80	8.21	7.12	9.60	14.86	11.76
0.57	0.83	0.37		0.91	4.04	3.91	2.68	2.00	2.25	2.68	2.39	2.39	2.39	2.89	2.25	2.38	2.25	1.88	1.63	3.79	4.62	2.96	3.30	4.69	4.62	3.48	3.37	3.75	4.00	4.28	3.65	4.27	3.73	4.18	8.53	8.61	8.51	11.58	11.47	11.47	8.31	9.55	7.89	6.81	9.23	14.56	11.35
0.42	0.77	0.64	0.62		3.22	3.35	2.19	1.54	1.81	1.90	1.93	1.93	1.93	2.38	1.81	1.74	1.61	2.35	2.12	5.51	5.79	4.70	5.18	6.57	6.99	5.35	5.26	5.61	6.15	6.65	5.69	6.03	5.35	6.58	9.11	9.34	9.23	11.47	11.35	11.35	8.24	9.46	8.57	7.37	10.24	13.00	9.82
1.35	0.66	1.74	1.29	1.42		0.30	1.83	1.84	1.77	1.25	2.38	1.54	1.75	1.39	1.77	2.39	1.38	3.29	2.20	4.37	4.57	4.02	4.11	4.32	4.63	4.13	4.54	3.86	4.61	4.42	4.54	4.78	4.63	4.84	5.90	7.19	7.10	8.53	7.74	9.22	6.65	8.35	9.49	9.01	11.56	11.71	9.57
1.28	0.71	1.85	1.28	1.41	0.27		1.38	1.41	1.35	1.22	1.94	1.12	1.30	1.01	1.35	2.32	1.34	3.19	2.13	4.19	4.75	3.86	3.94	4.51	4.42	3.98	4.36	3.70	4.41	4.22	4.36	4.96	4.80	4.63	5.59	7.37	7.28	8.72	7.93	9.43	7.36	8.55	8.80	8.34	10.42	11.93	9.83
0.64	0.30	1.04	0.78	0.69	0.74	0.75		0.71	0.69	2.25	1.27	0.69	0.69	1.02	0.69	1.41	0.71	2.06	1.42	4.42	5.04	3.72	4.15	5.10	5.02	3.93	4.23	4.27	4.69	5.01	4.60	4.96	4.68	5.33	6.38	9.15	9.04	11.25	11.13	11.13	8.02	9.43	9.23	8.06	12.08	11.37	10.20
0.65	0.27	1.02	0.74	0.71	0.80	0.84	0.44		0.35	1.59	0.48	0.48	0.48	0.82	0.35	0.59	0.49	1.43	1.15	4.41	5.09	3.66	4.11	5.04	4.95	4.42	4.21	4.14	5.09	5.60	4.60	5.51	4.69	5.40	7.11	8.88	8.76	10.97	10.85	10.85	7.10	8.31	7.20	6.31	9.18	11.74	10.06
0.84	0.26	1.20	0.91	0.90	0.71	0.71	0.36	0.45		1.86	0.93	0.46	0.46	1.01	0.56	1.07	0.47	1.70	1.12	4.67	5.35	3.94	4.38	5.41	5.32	4.31	4.47	4.54	4.61	5.47	4.85	5.38	4.94	5.64	7.32	8.98	8.87	11.04	10.92	10.92	7.36	8.55	7.37	6.45	9.30	11.93	14.00
1.06	0.91	1.35	1.17	1.21	0.99	0.91	0.84	0.78	0.85		1.79	1.79	1.98	1.86	1.86	1.62	1.47	2.84	2.62	5.79	6.10	5.33	5.42	4.80	5.15	5.60	5.51	4.70	6.46	6.47	5.51	5.86	5.60	6.39	6.05	9.12	9.01	11.23	9.96	11.11	7.55	8.91	7.57	7.91	11.38	12.81	9.55
0.75	0.89	1.07	0.91	0.81	1.16	1.14	0.75	0.35	1.01	1.13		0.58	1.04	1.24	0.93	0.71	1.06	1.24	1.74	4.72	5.41	3.97	4.42	5.44	5.34	4.80	4.51	4.54	5.50	5.99	4.90	5.88	4.99	5.70	6.81	9.04	8.92	11.13	11.00	11.00	7.32	8.53	7.40	6.17	8.95	11.25	13.94
0.78	0.25	1.08	0.81	0.80	0.64	0.73	0.39	0.35	0.39	0.97	0.79		0.45	0.78	0.46	1.18	0.46	1.81	1.15	4.72	5.41	3.97	4.42	5.44	5.34	4.20	4.51	4.54	5.01	5.37	4.90	5.29	4.99	5.70	6.81	9.04	8.92	11.13	11.00	11.00	7.32	8.53	7.40	6.49	9.35	11.25	10.07
0.86	0.27	1.12	0.92	0.88	0.92	0.84	0.41	0.37	0.33	1.07	0.93	0.38		0.94	0.46	1.18	0.46	1.81	0.61	5.14	5.41	4.33	4.81	5.90	5.80	4.59	4.90	4.93	5.47	5.88	5.33	5.76	5.42	5.70	7.39	9.04	8.92	11.13	11.00	11.00	7.93	9.33	8.18	7.16	9.35	11.25	10.07
1.17	0.47	1.14	1.16	1.08	0.52	0.67	0.58	0.58	0.77	0.87	1.16	0.60	0.72		0.45	1.57	0.97	2.25	1.69	3.94	4.90	3.61	3.70	4.62	4.54	3.96	4.11	3.86	4.41	4.60	4.11	4.58	4.56	4.76	6.41	7.46	7.36	8.93	8.82	8.82	7.36	8.55	7.37	7.11	10.42	11.19	9.83
0.92	0.25	0.92	0.89	0.85	0.84	0.83	0.36	0.43	0.73	0.90	1.01	0.38	0.35	0.43		1.07	0.47	1.70	1.12	4.67	5.35	3.94	4.38	5.41	5.32	4.31	4.47	4.54	5.19	5.47	4.85	5.38	4.94	5.64	6.41	8.98	8.87	11.04	10.92	10.92	7.36	8.55	7.37	6.45	9.30	11.19	9.83
0.96	0.95	1.10	1.02	0.91	1.20	1.26	1.02	0.38	1.02	1.06	0.38	0.87	0.88	1.23	1.08		0.73	1.18	1.70	4.99	5.25	4.20	4.67	4.91	5.23	5.09	4.76	4.82	5.84	6.36	4.76	5.34	4.47	6.05	7.23	8.87	8.76	10.92	10.80	10.80	7.27	8.45	8.02	6.64	9.84	11.08	13.92
0.79	0.21	1.19	0.89	0.76	0.77	0.72	0.30	0.32	0.28	0.85	0.79	0.30	0.31	0.45	0.31	0.99		1.67	0.99	4.95	5.21	4.14	4.62	4.84	5.16	4.40	4.72	4.75	5.27	5.67	4.72	4.68	4.42	6.01	6.62	8.81	8.70	10.88	10.75	10.75	7.13	8.33	7.94	6.93	10.24	11.02	9.82
1.30	1.16	1.41	1.10	1.18	1.57	1.60	0.99	0.98	1.20	1.36	0.69	1.15	1.30	1.20	1.22	0.77	1.14		1.14	3.79	5.00	3.22	3.57	4.69	4.62	3.75	3.65	4.02	4.31	4.61	3.94	4.59	4.02	4.51	7.21	8.92	8.81	11.00	10.88	10.88	7.22	8.43	7.28	6.38	9.23	12.57	15.33
0.79	0.61	0.90	0.83	0.91	1.00	0.92	0.58	0.58	0.59	1.07	0.95	0.68	0.56	0.80	0.60	1.13	0.73	1.12		3.64	4.53	3.04	3.41	4.27	4.19	3.20	3.50	3.56	3.87	4.11	3.81	4.11	3.89	4.06	7.39	9.04	8.92	11.13	11.00	11.00	7.93	9.33	8.18	7.16	9.35	12.69	11.49
0.94	0.96	1.56	1.30	0.91	0.84	0.83	0.91	1.08	1.01	1.14	0.99	1.10	0.88	0.91	1.00	0.99	1.02	1.63	1.67		1.42	0.46	0.22	2.03	1.96	0.69	0.45	0.80	1.13	0.94	0.78	1.78	1.21	0.70	7.20	5.24	5.15	6.19	6.65	6.65	6.64	7.10	8.11	6.42	9.30	11.86	10.19
0.88	0.88	1.20	1.45	1.03	0.92	1.03	1.10	0.98	0.97	1.26	0.96	0.88	0.88	1.44	0.90	1.03	0.86	1.24	1.36	0.48		0.83	1.09	2.60	2.78	1.61	1.37	1.38	1.74	1.34	1.37	2.27	1.45	0.55	8.27	4.26	4.16	5.50	5.90	5.90	7.61	7.51	9.92	8.56	9.05	11.63	11.35
0.62	0.66	1.21	1.06	0.59	0.77	0.73	0.61	0.64	0.65	0.98	0.67	0.60	0.62	0.87	0.60	0.81	0.65	1.24	1.04	0.33	0.44		0.22	1.61	1.53	0.69	0.45	0.48	0.80	0.94	0.45	1.40	0.68	0.70	7.20	4.82	4.73	6.19	6.65	6.65	6.15	6.55	7.31	5.83	8.35	11.86	11.62
0.93	0.95	1.50	1.41	1.01	0.80	0.76	0.92	1.01	1.01	1.12	1.09	1.00	1.19	0.90	0.93	0.99	1.18	1.37	1.75	0.23	0.42	0.24		1.69	1.61	0.45	0.22	0.55	0.87	0.69	0.52	1.47	0.93	0.45	7.29	4.52	4.44	5.79	6.19	6.19	6.23	6.64	7.43	5.94	8.47	11.97	10.32
1.58	1.68	1.95	2.02	1.58	1.69	2.15	1.20	1.64	1.47	2.20	1.58	1.50	1.57	1.59	1.42	1.66	1.68	2.17	1.97	0.74	0.76	0.57	0.65		0.22	2.28	1.97	1.07	2.45	2.66	1.56	1.89	1.44	2.34	6.44	6.28	6.19	8.00	8.61	7.90	7.32	7.83	9.73	7.59	11.94	16.64	13.38
1.45	1.51	2.62	1.92	1.68	1.76	1.56	1.07	1.78	1.71	1.94	1.46	1.73	1.50	1.46	1.51	1.52	1.68	1.95	1.56	0.78	0.93	0.65	0.61	0.18		2.21	1.89	0.99	2.38	2.58	1.30	2.22	1.76	2.26	6.34	6.19	6.10	7.90	8.51	7.81	7.22	8.43	8.68	6.81	10.37	19.45	16.02
1.04	0.98	1.66	1.81	0.94	0.84	0.71	1.05	1.02	0.91	1.21	1.20	0.93	0.92	0.83	0.98	1.23	1.07	1.46	1.36	0.45	0.60	0.42	0.37	0.65	0.76		0.24	1.10	0.99	0.67	0.99	1.48	1.40	0.92	6.99	5.09	5.00	6.47	6.93	6.93	6.45	6.86	7.62	6.11	8.64	11.49	10.33
1.10	1.01	1.42	1.36	1.09	0.66	0.64	1.04	1.08	1.14	1.04	1.36	1.01	0.92	0.85	0.96	1.12	1.08	1.44	1.46	0.33	0.50	0.32	3.22	0.73	0.67	0.27		0.76	1.10	0.92	0.76	1.73	1.18	0.68	7.37	5.00	4.91	6.39	6.85	6.85	6.32	6.73	7.54	6.04	8.58	12.06	10.44
0.99	0.88	1.58	1.50	1.11	1.01	0.97	0.85	0.93	0.76	1.02	1.04	0.90	0.82	0.76	0.91	0.81	0.84	1.38	1.53	0.51	0.54	0.36	0.37	0.58	0.64	0.57	0.46		1.24	1.37	0.78	1.64	1.19	1.04	6.92	5.73	5.65	7.28	7.81	7.19	6.68	7.13	7.89	6.22	9.23	13.09	11.35
1.24	1.02	1.55	1.61	1.16	0.88	0.96	1.03	1.06	1.03	1.11	1.26	1.04	1.17	1.06	1.19	1.54	1.10	1.90	1.67	0.79	0.57	0.44	0.56	0.74	0.67	0.45	0.52	0.61		0.76	0.78	1.37	1.52	1.38	7.40	5.88	5.78	6.37	6.82	6.82	6.42	6.82	7.48	5.98	8.49	12.03	15.56
1.13	1.20	2.21	1.78	1.40	1.11	1.09	1.25	1.22	1.24	1.07	1.22	1.30	1.31	1.28	1.27	1.30	1.39	1.87	1.88	0.63	0.53	0.48	0.40	0.61	0.63	0.41	0.48	0.51	0.34		1.26	1.80	1.71	0.69	6.90	5.42	5.33	5.88	6.28	6.28	6.86	7.32	7.51	6.60	9.48	11.37	10.20
1.04	1.09	1.22	1.28	1.31	1.02	0.98	1.07	1.10	1.03	1.04	1.16	1.16	1.08	1.23	1.12	1.08	0.96	1.16	1.26	0.38	0.59	0.26	0.31	0.61	0.71	0.42	0.38	0.36	0.49	0.44		1.03	0.83	1.03	7.37	5.00	4.91	6.39	6.85	6.85	6.32	7.28	7.54	6.04	8.58	13.56	13.83
1.36	1.40	1.70	1.56	1.37	1.50	1.38	1.18	1.26	1.25	1.19	1.35	1.42	1.26	1.28	1.20	1.34	1.31	1.60	1.42	0.64	0.61	0.56	0.53	0.97	1.04	0.62	0.65	0.47	0.38	0.56	0.37		0.64	2.06	8.55	5.35	5.26	7.31	7.85	7.22	7.20	7.67	9.76	7.03	11.13	11.82	12.15
0.94	1.00	1.25	1.15	0.85	0.99	1.04	0.83	1.07	0.94	1.07	0.92	1.06	0.86	1.22	0.98	0.80	0.80	1.21	1.17	0.35	0.47	0.39	0.36	1.03	0.92	0.41	0.40	0.44	0.48	0.36	0.35	0.55		1.48	8.07	4.70	4.61	6.48	6.95	6.95	6.40	6.82	8.47	6.76	9.67	12.18	12.02
1.78	1.25	1.99	1.97	1.40	0.95	0.89	1.15	1.38	1.48	1.12	1.65	1.69	1.63	1.23	1.53	1.36	1.46	1.77	1.95	0.50	0.30	0.49	0.34	0.82	0.73	0.59	0.42	0.56	0.64	0.52	0.46	0.65	0.46		8.57	4.16	4.08	5.79	6.19	6.19	7.90	8.51	9.18	8.01	8.47	11.97	10.32
1.31	1.33	1.76	1.10	1.39	1.50	1.40	1.32	1.48	1.51	1.78	1.39	1.32	1.25	1.29	1.34	1.62	1.36	1.69	1.30	1.19	1.17	1.39	1.24	1.64	1.68	1.08	1.20	1.38	1.45	0.96	1.29	1.50	1.48	1.23		9.58	9.48	9.61	9.52	10.41	8.18	9.47	12.13	9.54	11.50	16.72	20.32
2.74	2.75	2.89	3.24	3.02	2.38	2.45	3.13	2.58	3.04	2.99	3.17	3.24	3.17	2.82	3.26	2.89	2.90	2.76	2.91	2.06	1.64	1.98	1.88	2.19	2.08	1.89	1.89	2.13	1.82	1.86	2.05	1.64	1.61	1.75	1.90		0.00	1.99	2.43	2.43	4.82	6.99	6.14	7.82	7.66	12.14	14.90
2.75	2.64	2.74	2.79	2.98	2.56	2.44	2.67	3.06	2.86	2.55	2.68	2.72	3.11	2.91	3.10	3.23	3.00	2.99	2.70	1.98	1.66	1.86	1.83	2.16	1.87	1.82	1.64	2.01	1.85	1.86	1.93	1.73	1.55	1.60	1.88	0.96	0.88		1.66	2.07	2.07	4.29	6.20	6.01	6.94	7.55	12.01	14.80
2.53	2.96	2.68	2.43	2.70	2.60	2.65	2.66	2.53	2.62	2.54	2.57	2.51	3.00	2.72	2.51	2.70	2.86	2.49	2.32	2.33	1.87	1.95	2.13	2.22	1.98	1.76	1.59	1.95	2.18	2.29	1.91	2.04	1.84	1.80	1.88	0.96	0.88			0.00	0.00	3.09	3.72	3.87	5.81	6.42	10.16	16.89
2.72	2.80	2.34	2.47	2.83	2.58	2.46	2.57	2.93	2.42	2.91	2.49	2.71	2.77	2.89	2.54	2.85	2.82	2.78	2.78	2.52	1.94	1.90	1.67	2.24	2.39	2.08	1.94	2.13	2.01	1.96	1.98	2.12	2.11	1.88	1.99	1.18	0.92	0.28		0.17	3.34	4.02	4.80	6.27	6.33	10.03	16.78
2.58	2.77	3.03	2.46	2.59	2.65	2.73	2.66	2.50	2.38	2.46	2.85	2.67	2.51	3.13	2.55	2.64	2.60	2.69	2.80	2.23	2.19	1.84	1.88	2.27	2.12	1.79	2.00	1.90	2.09	2.00	2.06	2.00	1.89	1.89	1.66	1.07	0.83	0.24	0.46		3.34	4.02	3.74	5.71	6.91	10.03	16.78
1.44	1.40	1.53	1.69	1.67	1.26	1.27	1.71	1.32	1.63	1.18	1.54	1.50	1.32	1.55	1.54	1.39	1.54	1.44	1.23	1.89	1.50	1.61	1.39	1.58	2.21	1.60	1.56	1.56	1.62	1.45	1.60	1.75	1.66	1.51	2.74	1.35	0.91	1.04	0.99	1.19		1.09	3.06	4.40	4.63	11.84	16.81
2.05	1.76	1.98	1.93	1.79	1.45	1.54	2.36	1.93	1.74	1.64	1.87	1.75	1.57	1.74	1.80	1.77	1.75	1.59	1.54	2.15	1.70	1.68	1.77	2.00	2.56	1.76	1.83	1.75	1.74	1.64	2.12	2.06	1.83	1.71	3.30	1.76	1.42	1.00	0.99	1.10	1.00		2.59	4.32	4.21	10.36	14.81
1.89	1.87	1.85	1.68	2.28	2.26	1.83	2.41	1.90	1.92	2.12	1.95	1.88	1.62	1.74	1.79	2.39	2.72	1.90	1.80	2.67	2.77	2.07	2.28	2.95	2.22	2.38	2.50	2.28	2.14	2.12	2.25	2.83	3.01	2.44	3.28	2.19	1.72	1.82	2.20	1.58	1.23	1.22		2.79	4.02	9.23	14.26
1.86	1.78	1.82	1.59	2.20	2.23	1.56	2.71	1.74	1.78	2.06	1.67	1.81	1.75	1.83	1.81	2.23	2.36	2.00	1.72	2.50	2.59	1.99	2.11	2.42	1.71	1.79	1.77	1.57	1.64	1.73	1.81	2.46	2.23	1.93	2.41	1.90	1.61	1.90	1.88	1.71	1.88	1.86	1.53		2.79	13.07	19.12
2.27	1.95	2.04	1.94	2.45	2.61	1.33	2.73	1.94	1.65	2.89	2.01	1.87	1.87	1.31	1.95	2.75	2.74	2.02	2.18	2.07	3.19	1.81	2.03	2.65	1.79	1.88	1.84	1.75	1.68	2.43	1.80	2.94	2.78	2.10	3.71	1.82	1.66	2.01	1.86	1.91	1.81	1.71	1.83	0.74		12.17	18.24

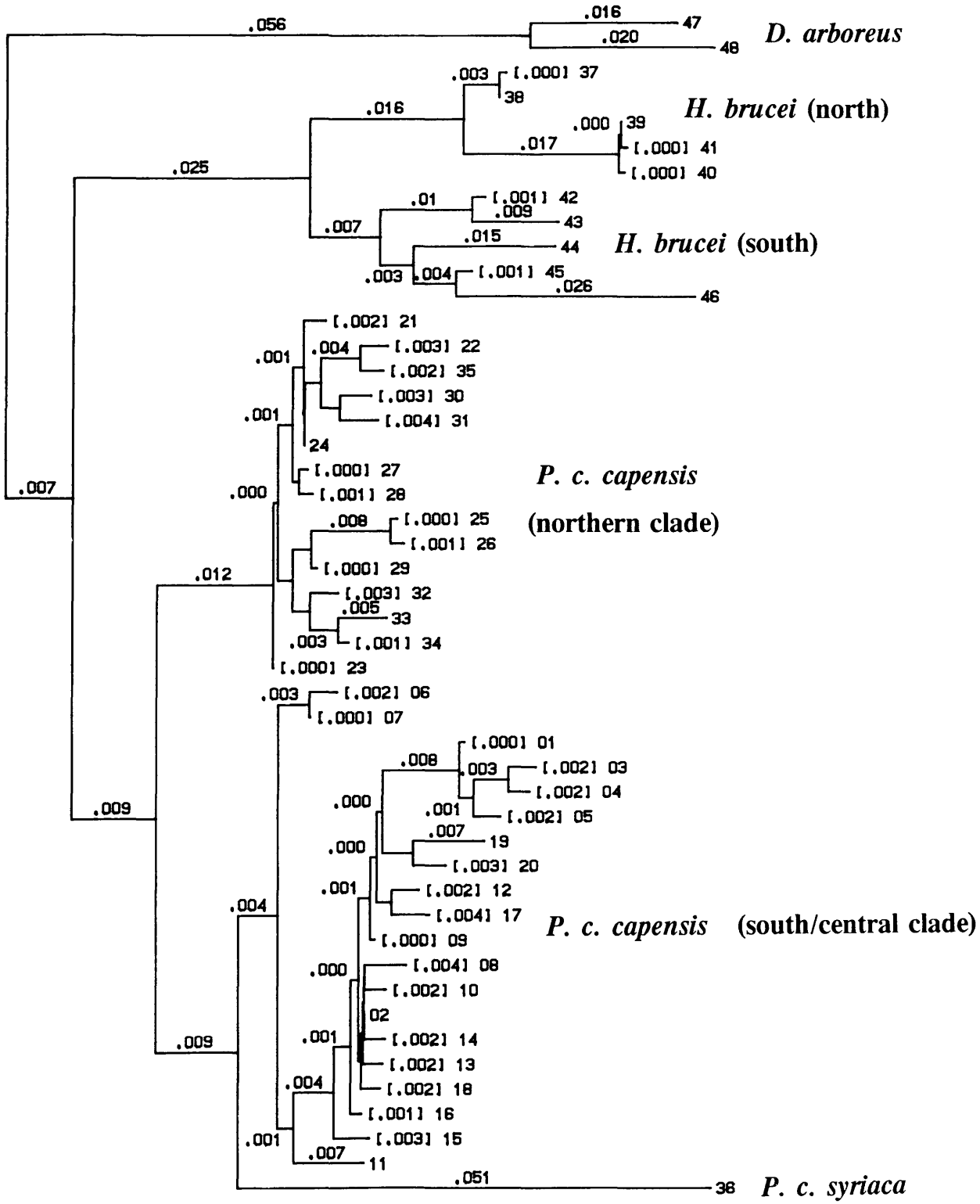


Figure 9 Neighbor-joining tree based on nucleotide sequence divergence (nucleotide substitutions per site) between 48 clones of three hyrax species. Clone numbers are as in Table 8. The values over each branch represent sequence divergence.

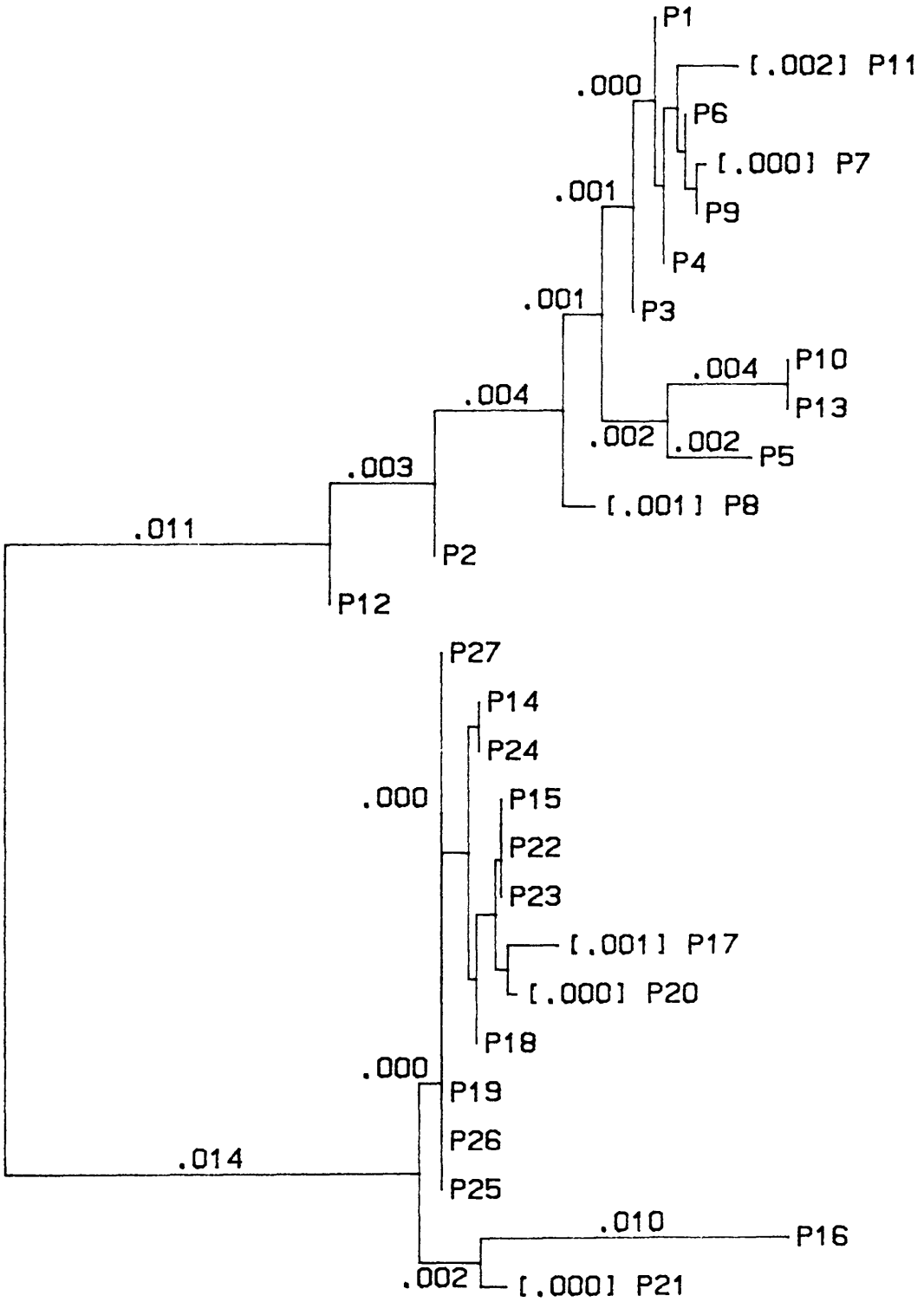


Figure 10 Neighbor-joining tree based on nucleotide sequence divergence (nucleotide substitutions per site) between 27 *P. capensis capensis* populations. Population designations correspond to Figure 5. The values over each branch represent sequence divergence.

represented by clone 36.

The 10 clones (37 - 46) within *H. brucei* grouped into two clusters (Figure 9). The northern group includes the Matopos (clones 37 and 38), Vhembe (clones 39-41) and Pafuri (clone 39) populations, while the southern cluster (clones 42-46) comprised two sub-clusters, Blyde River (clone 42) and Ohrigstad (clone 43) in the east, and Munnik (clone 44), Zebediela (clone 45) and Soutpansberg (clone 46) in the central Transvaal area. The two *D. arboreus* populations are represented by two distinct lineages (clones 47 - 48; Figure 9) which are separated by 3.63% sequence divergence.

Appendix V lists the minimum number of mutational steps between the restriction fragment patterns produced for the three South African hyrax species studied in this investigation. The numbers of mutational steps between clones within *P. c. capensis* are presented in Table 11 and, in the case of *H. brucei*, in Table 12. The two *D. arboreus* clones are separated by a minimum of 19 mutational steps.

Figure 11 shows geographic overlays of the phylogenetic networks involving the least number of mutational steps between clones detected in the two *P. c. capensis* clades. These networks mirror the topology of the neighbor-joining trees based on sequence divergence between clones (Figure 9) and between populations (Figure 10). Thirteen mutational steps separate the two clades (Table 11). The minimum-path network for the *H. brucei* clones is illustrated in Figure 12 and reflects the geographic relationships evidenced between sub-clusters in the neighbor-joining tree (Figure 9).

RFLP analysis - interspecific variation between three hyrax species

Seven major groupings were identified in the mtDNA RFLP analysis of intraspecific variation in the three hyrax species (Figure 9). *P. c. capensis* south-central (clones 1 - 20, Table 8 and Figure 9), *P. c. capensis* north (clones 21 - 35), *P. c. syriaca* (clone 36), *H. brucei* north (clones 37 - 41), *H. brucei* south (clones 42 - 46), *D. arboreus* clone 47 and *D. arboreus* clone 48.

Sequence divergence estimates between the seven clusters are presented in Table 13 and the relationships between them summarized in an UPGMA tree (Figure 13). This unrooted tree depicts *Dendrohyrax* as the most distantly related taxon, while *P. c. capensis* and *H. brucei* are very closely related. *Procavia c. syriaca* is more distant to the latter grouping. The minimum number of mutational steps between the different species could not be accurately estimated from the restriction fragment analysis.

Table 11 Pairwise estimates of the minimum number of mutational steps between the 35 *P. c. capensis* clones detected in this investigation. Clone numbers correspond to those in Table 8.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
2	5																																	
3	10	15																																
4	9	14	3																															
5	4	9	10	9																														
6	14	9	20	19	14																													
7	12	7	22	21	16	2																												
8	7	2	17	16	11	11	9																											
9	4	1	14	13	8	10	8	3																										
10	6	1	16	15	10	10	8	3	2																									
11	8	9	16	15	10	6	6	11	8	10																								
12	6	3	16	15	10	12	10	5	2	4	10																							
13	6	1	16	15	10	10	8	3	2	2	10	2																						
14	6	1	16	15	10	10	8	3	2	2	10	4	2																					
15	8	3	18	17	12	8	6	5	4	4	10	6	4	4																				
16	6	1	16	15	10	10	8	3	2	2	10	4	2	2	2																			
17	7	4	15	14	9	11	11	6	3	5	9	3	5	5	7	5																		
18	6	1	14	13	8	8	8	3	2	2	8	4	2	2	4	2	3																	
19	15	12	9	8	15	17	19	14	11	13	17	11	13	13	15	13	10	11																
20	14	9	8	7	14	14	16	11	10	10	16	12	10	8	12	10	11	8	5															
21	25	22	19	16	25	21	23	24	21	23	23	23	23	23	23	23	22	21	16	15														
22	23	20	21	18	23	19	21	22	19	21	21	21	21	21	21	21	20	19	18	17	6													
23	23	20	17	14	23	19	21	22	19	21	21	21	21	21	21	21	20	19	14	13	2	4												
24	24	21	18	15	24	20	22	23	20	22	22	22	22	22	22	22	21	20	15	14	1	5	1											
25	28	25	22	19	28	22	24	27	24	26	24	26	26	26	26	26	25	24	19	18	7	9	5	6										
26	28	25	22	19	28	22	24	27	24	26	24	26	26	26	26	26	25	24	19	18	9	11	7	8	2									
27	26	21	20	17	26	20	22	23	22	22	24	24	22	22	22	22	23	20	17	14	3	7	3	2	8	10								
28	25	22	19	16	25	21	23	24	21	23	23	23	23	23	23	23	22	21	16	15	2	6	2	1	7	9	1							
29	25	22	19	16	25	19	21	24	21	23	21	23	23	23	23	23	22	21	16	15	4	6	2	3	3	5	5	4						
30	27	22	21	18	27	21	23	24	23	21	25	25	23	23	23	23	24	21	18	15	6	8	4	5	9	11	5	6	6					
31	27	22	21	18	27	21	23	24	23	23	25	25	23	23	23	23	24	21	18	15	4	6	4	3	9	11	3	4	6	4				
32	26	23	20	17	26	22	24	25	22	24	24	24	24	24	24	24	23	22	17	16	5	7	3	4	6	6	6	5	5	5	7			
33	30	25	24	21	30	24	26	27	26	26	28	28	26	26	26	26	27	24	21	18	9	11	7	8	10	12	8	9	9	7	9	6		
34	27	24	21	18	27	23	25	26	23	25	25	25	25	25	25	25	24	23	18	17	6	8	4	5	7	9	7	6	6	8	8	5	3	
35	26	23	20	17	26	22	24	25	22	24	24	24	24	24	24	24	23	22	17	16	3	3	3	2	8	10	4	3	5	7	3	6	10	7

Table 12 Pairwise estimates of the minimum number of mutational steps between 10 *H. brucei* clones (Clone numbers correspond to those in Table 8).

Clone no.	37	38	39	40	41	42	43	44	45
38	1								
39	11	10							
40	12	11	1						
41	15	14	4	3					
42	26	25	17	18	19				
43	22	21	13	14	15	10			
44	25	24	16	17	16	19	9		
45	31	30	24	25	24	25	15	10	
46	29	28	22	23	24	25	15	12	8

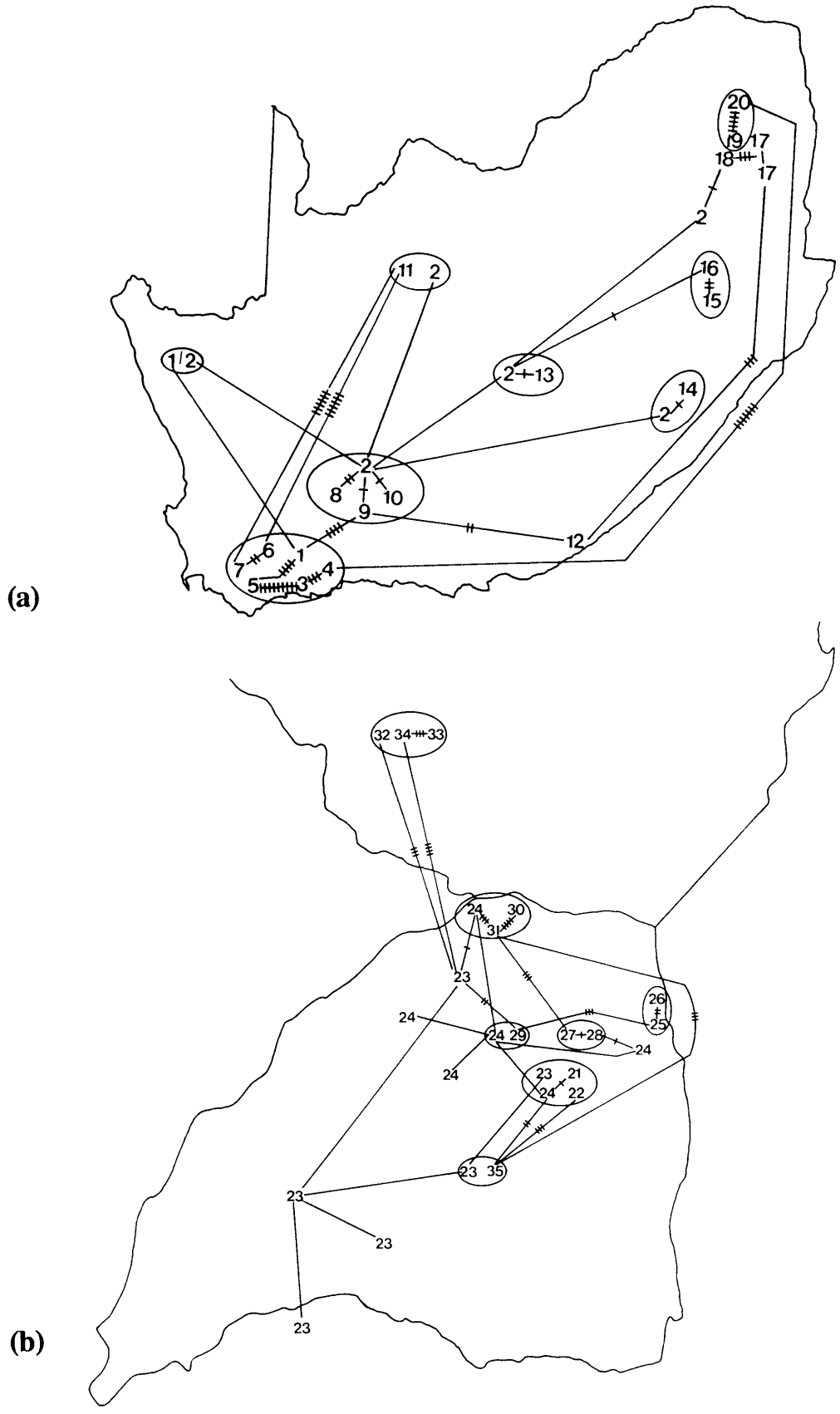


Figure 11 Geographic overlays of the minimum number of mutational steps between *P. c. capensis* clones of the south-central (a) and northern (b) clades. Cross-hatching along connecting branches indicates the least number of mutational steps between haplotypes. Thirteen mutational steps separate the two mtDNA clades; these connect clone 20 of the south-central clade to clone 23 of the northern clade.

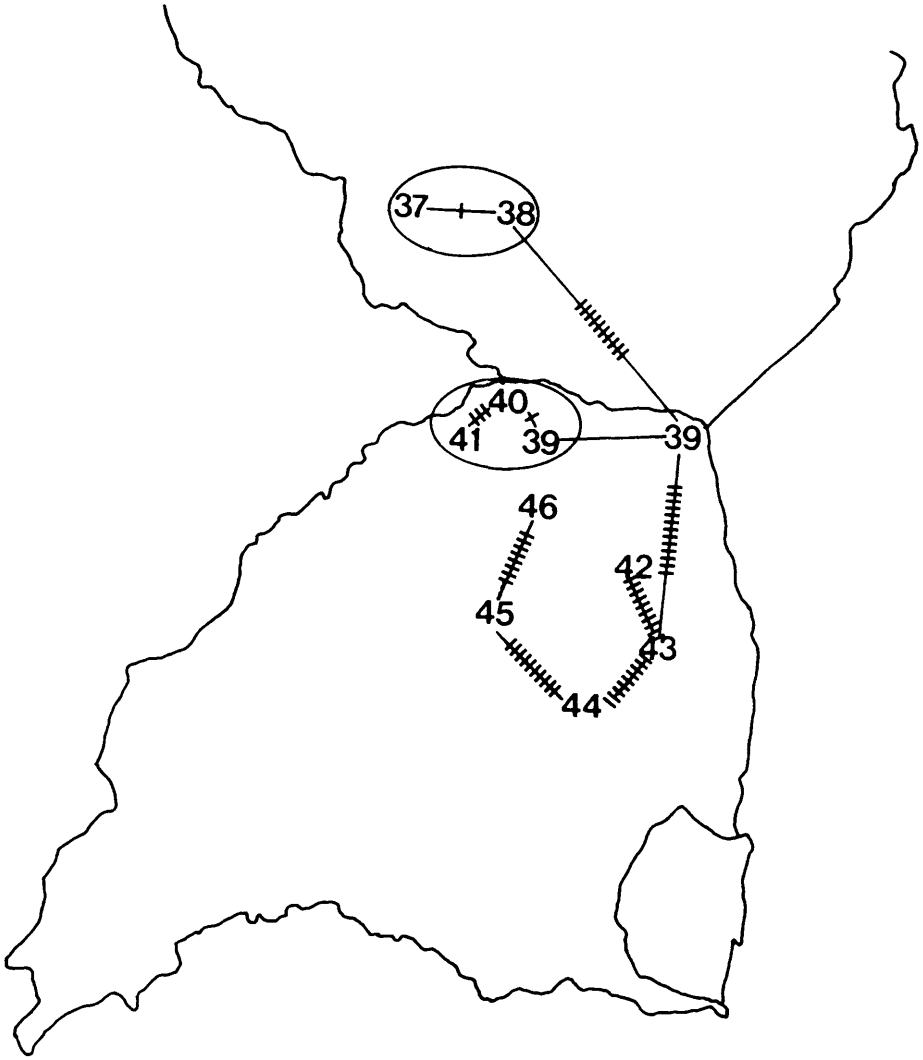


Figure 12 Minimum number of mutational steps separating closely related *H. brucei* lineages. Cross-hatching along connecting branches indicates the least number of mutational steps between these haplotypes.

Table 13 Percent sequence divergence (nucleotide substitutions per site) between seven groups within the three hyrax species based on restriction enzyme analysis of mitochondrial DNA. 1 - south-central *P. c. capensis;* 2 - northern *P. c. capensis;* 3 - *P. c. syriaca*; 4 - northern *H. brucei*; 5 - southern *H. brucei*; 6 - *D. arboreus* clone 47; 8 - *D. arboreus* clone 48. Sequence divergences (d) are presented below the diagonal and standard errors derived via bootstrapping above the diagonal. All values were Jukes-Cantor corrected.

Group no.	1	2	3	4	5	6	7
1		0.73	1.19	2.78	1.45	1.92	3.36
2	3.32		1.10	1.75	1.61	1.93	3.62
3	6.26	6.68		1.46	2.73	2.87	2.30
4	8.38	4.83	9.13		0.79	2.38	2.82
5	5.61	5.17	8.06	3.02		2.15	4.32
6	11.07	11.84	16.72	10.22	9.45		1.44
7	16.68	20.13	20.32	15.33	14.21	3.63	

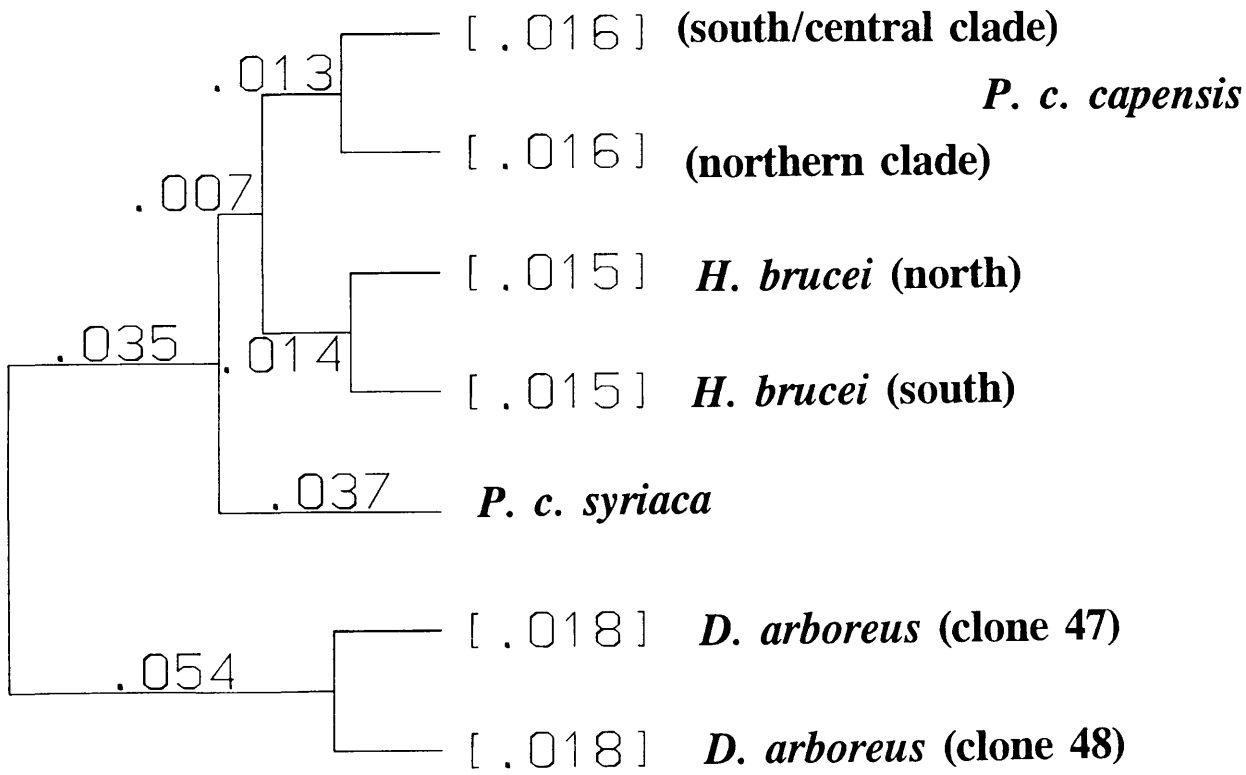


Figure 13 UPGMA tree based on sequence divergence estimates between the seven major mtDNA groupings detected in the three hyrax species. Values over branches and in parentheses represent sequence divergence.

PCR and sequencing

Figure 14 shows the amplified fragments of two mtDNA genes after 35 cycles of the polymerase chain reaction. Primers designed for amplification of the 12S rRNA gene routinely gave stronger amplification, while the formation of primer dimers (lanes 2 - 7) and non-specific amplification (lane 5) is evident in the case of the cytochrome b amplification. Contamination of negative controls was experienced on several occasions and in most instances involved aerosols created during pipetting. Recovery of PCR products from agarose gels was highly successful and only a small fraction of the purified product was required for use as template in the sequencing reactions.

The use of ^{35}S in sequencing experiments gave inconsistent results and required excessively long exposure times. Diluted ^{32}P was subsequently used as a substitute. This necessitated using only 0.02 μl per reaction and significantly shortened the exposure times that varied from 24 - 96 hr. Although the ^{32}P was aliquoted and stored under N_2 at -70°C (Amersham, *in litt.*), it nonetheless tended to lose activity after three weeks. Figure 15 depicts representative cytochrome b and 12S rRNA sequences using ^{35}S and ^{32}P respectively. As is apparent, the quality of sequences obtained with the two radio-nucleotides is comparable and, in spite of its short half-life, ^{32}P was found to be more cost-effective and generally gave greater consistency and clearer sequencing results.

In total 690 bp of mtDNA sequence was obtained from the specimens included in this analysis (this excludes intraspecific comparisons). In the case of cytochrome b, 362 nucleotides of the 425 bp fragment were sequenced, while 331 nucleotides of the 386 bp 12S rRNA fragment were similarly analysed. Sequences close to the respective primers were lost, although they may have been resolved through further experimentation with higher dilutions of the labelling mix, or by reducing the length of the labelling step.

The mtDNA gene sequences from five representatives of the Hyracoidea and the five outgroup species are given in Figure 16. The cytochrome b sequences (Figure 16 a) of the hyrax, aardvark and horse were aligned to the published sequences of the rhino and elephant (Irwin *et al.* 1991), and the human (Anderson *et al.* 1981). Likewise, the 12S RNA sequences (Figure 16 b) of the hyrax, aardvark and elephant were aligned to the human sequence (Anderson *et al.* 1981). The amino acid sequences derived from the cytochrome b data are shown in Appendix VI.

To justify the use of single hyracoid representatives for the interspecific comparisons, a within species comparison involving approximately 180 nucleotides of the cytochrome b fragment (H-strand) was done (Appendix VII). Although limited variation was evident in the *P. c. capensis* clades, the two *D. arboreus* lineages (sequences not shown) were identical. In sharp contrast however, the two *H. brucei* specimens differed markedly from each other. However, the Vhembe sequence (clone 39) was chosen for interspecific comparisons since its taxonomic status is undisputed and it represented the most common mtDNA type in *H. b. ruddi*.

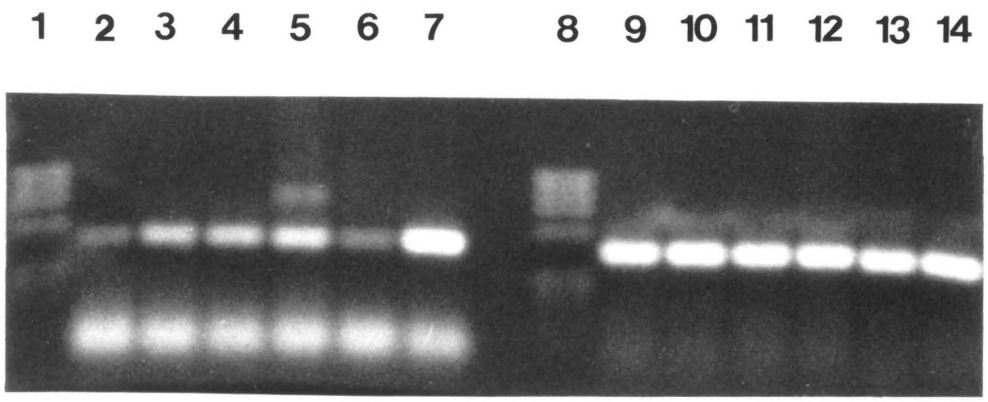


Figure 14 PCR amplification of portions of two mtDNA genes. Lanes 1 and 8 contain the size standard ϕX174 while lanes 2-7 reflect amplification of the cytochrome b fragment and 9-14 that of the 12S rRNA fragment. The same loading order was retained for the two genes: *D. arboreus*, *H. brucei*, *P. c. capensis* (south-central clade), *P. c. capensis* (northern clade), *P. c. syriaca* and *L. africana*.

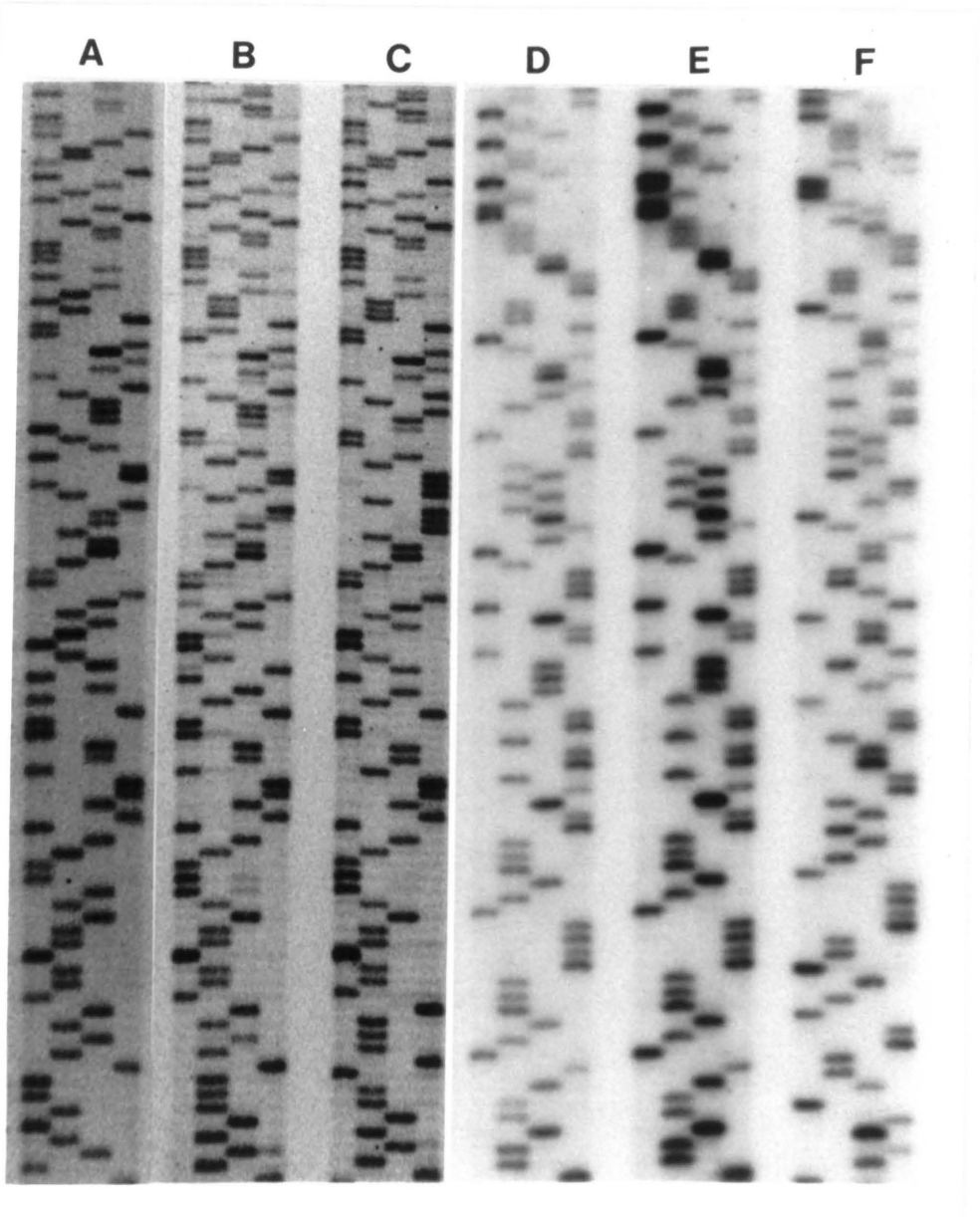


Figure 15 Autoradiograph illustrating sequence from the cytochrome b (lanes A-C) and 12S rRNA (lanes D-F) mtDNA genes. Sequences A-C were obtained using ^{35}S, while ^{32}P was used for sequencing D-F. Sequences of the following species are shown: A - *H. brucei*; B - *P. c. capensis* (northern clade); C,D - *P. c. capensis* (southern clade); E - *P. c. syriaca* and F - *L. africana*. Note the shift in alignment between the hyracoid 12S rRNA sequences and those of the elephant.

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HOMO          AACCCCCTAATAAAATTAATTAACCACTCATTCATCGACCTCCCCACCCC
DICEROS       CACCCACTAATCAAAATTATCAATCACTCATTCATCGACCTACCCACCCC
LOXODONTA     CACCCCTTACTTAAAATCATCAATAAATCCTTCATTGATCTACCTACCCC
ORYCTEROP     CACCCTCTTATCAAAATTATTAATCACTCATTTATTGACCTTCCAACTCC
EQUUS         CACCCACTAATTAAAATCATCAATCACTCTTTTATTGACCTACCTACTCC
PROCSOUTH     CACCCACTACTTAAAACCATCAATGACGCCTTCATTGACCTCCCAACGCC
PROCNORTH     TACCCACTGCTCAGAACCATCAATGACGCCTTCATTGACCTTCCAACGCC
PROCSYR       CACCCACTGTTTAGAACCATCAATGAAGTGTTCTTTGACCTCCCAACGAT
HETEROHYR     CACCCACTAATAAAAACAATCAACGATGCCAACATTGACCTACCAACACC
DENDROHYR     CTCCCACTATTCAAAGTGATGAACAATGTCTTCATTGATCTCCCAACACC
                ***  *  * * *   ** **  *        * ** ** ** **

HOMO          ATCCAACATCTCCGCATGATGAAACTTCGGCTCACTCCTTGGCGCCTGCC
DICEROS       ATCAAACATTTCAGCCTGATGAAATTTTGGCTCTCTACTAGGAATCTGCC
LOXODONTA     ATCCAACATATCAACATGATGAAATTTCGGCTCACTACTAGGAGCATGCC
ORYCTEROP     TTCTAACATCTCAGCATGATGAAACTTTGGATCCCTCCTTGGAATCTGCC
EQUUS         CTCAAACATTTCATCATGATGAAACTTCGGCTCCCTCCTAGGAATCTGCC
PROCSOUTH     ATCCAACATCTCAACATGATGAAACTTTGGATCCCTGCTAGGAGCCTGCC
PROCNORTH     ATCCAACATCTCAGCATGATGAAACTTTGGATCACTCCTAGGAGCCTGCC
PROCSYR       ATCTAAGATCTAAGCATGATGAATGTTGGGATCCCTACTAGGAGCCTGCC
HETEROHYR     CCCCAATATCTCAACATGATGAAACTTCGGATCCCTTCTAGGAGCCTGCC
DENDROHYR     CTCCAATATCTCAGCATGATGAAATTTGGGATCCCTATTAGGAGCCTGCC
                * ** ** *   * *******  ** ** ** **  * **    ****

HOMO          TGATCCTCCAAATCACCACAGGACTATTCCTAGCCATGCACTACTCACCA
DICEROS       TAATCCTACAAATCCTAACCGGACTATTTCTTGCTATACATTATACACCA
LOXODONTA     TAATTACACAAATCCTAACAGGATTATTCCTAGCCATACATTATACACCC
ORYCTEROP     TCATCATCCAAATTGTCACAGGCCTATTTTTAGCTATACACTACACATCA
EQUUS         TAATCCTCCAAATCTTAACAGGCCTATTCCTAGCCATCCTACCTAGCCGC
PROCSOUTH     TAATCATCCAAATTCTAACAGGCCTATTCCTAGCCATACATTACACATCA
PROCNORTH     TAATCATCCAAATTCTAACAGGCCTATTCCTAGCCATACATTACACATCA
PROCSYR       TAATAATCCAAATTCTAACAGGCCTATTCCTAGCCTTACATTACACACCA
HETEROHYR     TAATCATCCAAATTCTAACAGGCCTATTTTTAGCCATACATTACACATCA
DENDROHYR     TAATTATCCAAATCCTAACAGGACTATTTCTCGCTTTACACTACACACCA
              * **    *****    ** **  ****  * **  * *

HOMO          GACGCCTCAACCGCCTTTTCATCAATCGCCCACATCTCACGAGACGTAAA
DICEROS       GACACAACAACTGCCTTCTCATCCGTTGCCCACATCTGTCGAGAGGTAAA
LOXODONTA     GACACAATAACTGCATTTTCATCTATATCCCATATTTGCCGAGATGTAAA
ORYCTEROP     GATACCTCAACCGCATTCTCATCTGTAACCCATATTTGCCGAGATGTAAA
EQUUS         GCTGCCACAACTGCCTTCTCATCCGTTACCCACATCTGCCGAGACGTTAA
PROCSOUTH     GATACAATAACCGCATTCGCATCAGTAACCCACATTTGCCGAGACGTGAA
PROCNORTH     GATACAATAACCGCATTCGCATCAGTAACCCACATTTGTCGAGACGTAAA
PROCSYR       GATACAATAAGCGCATTTGCGTCAGTAACCCACGTTTGCCGAGAAGTAAA
HETEROHYR     GACACAACAACCGCATTCGCATCAGTAACCCACATCTGCCGAGACGTAAA
DENDROHYR     GTCACTACAGCTGCGTTCTCGTCAGTCACTCGCGTTTGCCGAGCTGTGAA
              *   *   *   ** **  * **  *  * *   * *  ****  ** **
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Figure 16 Sequences for the (a) 362 bp portion of cytochrome b and the (b) 331 bp fragment of the mitochondrial 12S rRNA gene. The sequences of the L-strand are shown. Alignment is according to the previously published human sequence (Anderson *et al.* 1981). The cytochrome b fragment corresponds to positions 14 767 - 15 129 of the published human sequence and the 12S rRNA sequence to positions 1 117 - 1 444. (*) = invariant nucleotides detected for all taxa.

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HOMO          TTATGGCTGAATCATCCGCTACCTTCACGCCAATGGCGCCTCAATATTCT
DICEROS       CTACGGCTGAATTATCCGCTACCTACATGCAAACGGAGCATCCATATTTT
LOXODONTA     CTACGGCTGAATTATTCGACAACTACACTCAAACGGAGCATCCATTTTCT
ORYCTEROP     CTACGGCTGACTAATCCGCTATCTTCACGCAAACGGAGCATCCATATTCT
EQUUS         CTACGGATGAATTATTGGCTACCTCCATGCCAACGGAGCATCAATATTTT
PROCSOUTH     CCATGGGTGGGTAATCCGATACCTACATGCCAATGGAGCATCCCTATTCT
PROCNORTH     CCATGGATGGATAATCCGATACCTGCATGCCAACGGAGCATCCCTATTCT
PROCSYR       CCATGGCTGAATAATTTGATACCTCCATGCTAATGGAGCATCCCTATTCT
HETEROHYR     CTACGGCTGAATAATCCGATATCATCACGCCATCGGAGCATCCATATTCT
DENDROHYR     CTACGGCTGAGCTGTTTGCTGTCTCCACGCTAACGGAGCATCAGTATTCT
                * ** **     *  *    *  **  * *  ** ** **  * ** *

HOMO          TTATCTGCCTCTTCCTACACATCGGGCGAGGCCTATATTACGGATCATTT
DICEROS       TTATCTGCCTATTCATCCACATAGGACGCGGCCTCTATTACGGATCCTAC
LOXODONTA     TCCTCTGCCTATACACACACATTGGACGAAACATCTACTATGGGTCCTAC
ORYCTEROP     TCATCTGCTTATTTATCCACATTGGACGAGGGATTTATTACGGATCATAC
EQUUS         TTATCTGCCTCTTTATCCACGTAGGACGCGGCCTCTACTACGGCTCCTAC
PROCSOUTH     TCGTTTGCTTATATGCCCACATCGGACGAGGTATTTACTACGGATCCTAT
PROCNORTH     TCATATGTTTATATGCCCACATCGGACGAGGTATTTACTACGGATCCTAC
PROCSYR       TCGTATGCCTATGTGCCCACATTGGACGAGGCATTTACTATGGCTCCTAC
HETEROHYR     TCATATGCCTCTACGCTCATATTGGACGAGGTATCTACTACGGATCTTAC
DENDROHYR     TCATTTGCTTATACGCCCGCAATGGACGACGCATTCACTATGACTCATAC
              *  * **  * *     *     ** **     *  * ** *  ** *

HOMO          CTCTACTCAGAAACCTGAAACATCGGCATTATCCTCCTGCTTGCAACTAT
DICEROS       ACCTTCCTAAAAACCTGAAACATCGGAGTTATTCTACTACTCACAGTAAT
LOXODONTA     CTATACTCGGAAACTTGAAATACCGGCATTATATTACTACTAATCACCAT
ORYCTEROP     CTATACTCTGAAACCTGGAACATCGGAATTATCCTCCTATTCACCACAAT
EQUUS         ACATTCCTAGAGACATGAAACATTGGAATCATCCTACTTTTCACAGTAAT
PROCSOUTH     CTGTATTCAGAAACTTGAAACATCGGAGTTTTACTCTTACTAACAACAAT
PROCNORTH     CTGTACTCAGAAACTTGAAACATCGGAGTTTTACTCTTACTAACAACAAT
PROCSYR       CTGTACCCAGAAACCTGAAACAGTGGAGTTTTACTCTTACTAACAACAAT
HETEROHYR     CTATACTCAGAAACCTGAAACATCGGAGTTTTACTCCTACTAACAACTAT
DENDROHYR     CTATATCTAGAAACCTGAAACAGTGGAATCCTCCTAGTATTAGCAACCAT
                 *      * ** ** ** *  **  *  *  *  *  *       **

HOMO          AGCAACAGCCTT
DICEROS       AGCCACAGCATT
LOXODONTA     AGCCACCGCCTT
ORYCTEROP     AGCTACTGCTTT
EQUUS         AGCCACAGCATT
PROCSOUTH     AGCAACAGCATT
PROCNORTH     AGCAACAGCATT
PROCSYR       AGCAACAGCATT
HETEROHYR     AGCAACAGCATT
DENDROHYR     AGCAACAGCATT
              *** ** ** **
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(a)

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HOMO 12S        AATCAACAAAACTGCTCGCCAGAACACTACGAGCCACAGCTTAAAACTCA
LOXODONTA       CCTTTACAAAGCTATCCGCCAGAGAACTACTAGCCAGAGCTTAAAACTTA
ORYCTEROP       TA---ACAAAATTATTCGCCAGAGAACTACAAGCAACAGCTTAAAACTCA
PROCSOUTH       TATTAACGAAGCTGTTCGCCAGAGAACTACCAGCAAAAGCTAAAAACTCA
PROCNORTH       TATTAACGAAGCTGTTCGCCAGAGAACTACCAGCAAAAGCTGAAAACTCA
PROCSYR         TATTAACGAAGCTGTTCGCCAGAGAACTACCAGCAAAAGCTAAAAACTCA
HETEROHYR       TATTAACAAAGCTGTTCGCCAGAGAACTACTAGCTAAAGCTAAAAACTCA
DENDROHYR       TATTAACTAAACTGTTCGCCAGAGAACTACTAGCAATAGCTAAAAACTCA
                     ** **  *   *******  ***** *** * **** ****** *

HOMO 12S        AAGGACCTGGCGGTGCTTCATATCCCTCTAGAGGAGCCTGTTCTGTAATC
LOXODONTA       AAGGACTTGGCGGTGCTTTATATCCACCTAGGGGAGCCTGTCTCGTAACC
ORYCTEROP       AAGGACTTGGCGGTGCTTTATATCCATCTAGAGGAGCCTGTTATGTAATC
PROCSOUTH       AAGGACTTGGCGGTGCTTCACACCCACCTAGAGGAGCCTGTCCAATAATC
PROCNORTH       AAGGACTTGGCGGTGCTTCACACCCACCTAGAGGAGCCTGTCCAATAATC
PROCSYR         AAGGACTTGGCGGTGCTTCACACCCACCTAGAGGAGCCTGTCCAATAATC
HETEROHYR       AAGGACTTGGCGGTGCTTCACACCCACCTAGAGGAGCCTGTCCCATAACC
DENDROHYR       AAGGACTTGGCGGTGCTTCACACCCACCTAGAGGAGCCTGTCCCATAATC
                ****** ********** * * **  **** *********    *** *

HOMO 12S        GATAAACCCCGATCAACCTCACCACCTCTTGCT----CAGCCTATATACC
LOXODONTA       GATGAACCCCGATATACCTTACCGTCACTTGCTAATTCAGTCCATATACC
ORYCTEROP       GATAAACCCCGATATACCTCACCATCACTTGCCAATACAGCCTATATACC
PROCSOUTH       GATAAACCCCGATAAACCTCACCACCATTTGCCTGCCCAGTCTATATACC
PROCNORTH       GATAAACCCCGATAAACCTCACCACCATTTGCCTGCCCAGTCTATATACC
PROCSYR         GATAAACCCCGATAAACCTCACCACCATTTGCCTGCCCAGTCTATATACC
HETEROHYR       GATAAACCCCGATAAACCTCACCACCATTTGCCTGCCCAGTCTATATACC
DENDROHYR       GATAAACCCCGATAAACCTCACCACCATTTGCCTGCTCAGTCTATATACC
                *** *********  **** ***  *  ****     *** * *******

HOMO 12S        GCCATCTTCAGCAAACCCTGATGAAGGCTACAAAGTAAGCGCAAGTACCC
LOXODONTA       ACCATCTTCAGCAAACCCCTATAGGGCACAAA-AGTGAGCTTAATCATAC
ORYCTEROP       GCCATCTTCAGCAAACCCTTACAAGGGAATAATAGTAAGCCAAATTATTA
PROCSOUTH       GCCATCTTCAGCAAACCCTTAAAAGGAAGTAA-AGTAAGCACAACAATAC
PROCNORTH       GCCATCTTCAGCAAACCCTTAAAAGGAAGTAA-AGTAAGCACAATAATGC
PROCSYR         GCCATCTTCAGCAAACCCTTAAAAGGAGGTAA-AGTAAGCACAACAATAC
HETEROHYR       GCCATCTTCAGCAAACCCTTAAAAGGAAGTAA-AGTAAGCTCAACAACAC
DENDROHYR       GCCATCTTCAGCAAACCCTTAAAAGGAAATAA-AGTAAGCGCAATAATGC
                 ******************  *    *     * *** ***  **  *

HOMO 12S        ACGTAAAGACGTTAGGTCAAGGTGTAGCCCATGAGGTGGCAAGAAATGGG
LOXODONTA       CCATGAAAAAGTTAGGCCGAGGTGTAGCTTACGTGACGGTCAAAGATGGG
ORYCTEROP       CCATAAAAACGTTAGGTCAAGGTGTAGC-AATGTGATCGGAATAAACGGG
PROCSOUTH       CCATAAAAAAGTTAGGTCAAGGTGTAGCTAATATGGTGGACTTAGATGGG
PROCNORTH       CCATAAAAAAGTTAGGTCAAGGTGTAGCTAATATGGTGGACTTAGATGGG
PROCSYR         CCATAAAAAAGTTAGGTCAAGGTGTAGCTAATATGGTGGACTCAGATGGG
HETEROHYR       CCATAAAAAAGTTAGGTCAAGGTGTAGCTAATATGGTGGACTCAGATGGG
DENDROHYR       CCATAAAAAAGTTAGGTCAAGGTGTAGCTAATATGGTGGACTAAGATGGG
                 * * ** * ****** * *********  *   *   *    * * ***
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HOMO 12S        CTACATTTTCTACCCCAGAAAAC----TACGATAGCCCTTATGAAACTTA
LOXODONTA       CTACATTCTCTATTATAGAACA-GACAAACGGATATCACTCTGAAA-TGG
ORYCTEROP       CTACATTTTCTATTTTAGAACATATCCTACGAAACCTAA-ATGAAA-CCA
PROCSOUTH       CTACATTCTTTAC-ACATAA-A-GCAATACGAATGTTACCATGAAA-TTA
PROCNORTH       CTACATTCTTTAC-ACATAA-A-GCAATACGAACGTTACTATGAAA-TTA
PROCSYR         CTACATTCTTTAC-ACATAA-A-GCAATACGAATGTCGCTATGAAA-TTA
HETEROHYR       CTACATTCTTTAC-ACACAA-A-GTAATACGAATGTCACTATGAAA-CTA
DENDROHYR       CTACATTCTTTGC-ACAAAA-A-GCAATACGAATGTCACTATGAAA-TTA
                ******* * *     * ** *      ***          *****

HOMO 12S        AGGGTCGAAGGTGGATTTAGCAGTAAACTAAGAGTA
LOXODONTA       GTGGTTGAAGGCGGATTTAGTAGTAAACCAACATTA
ORYCTEROP       AAGGCTAAAGGAGGATTTAGTAGTAAATTAAGAATA
PROCSOUTH       GTGACTGAAGGAGGATTTAGCAGTAAATTAAGAATA
PROCNORTH       GTCACTGAAGGAGGATTTAGCAGTAAATTAAGAATA
PROCSYR         GCCACTGAAGGGGGATTTAGCAGTAAATTAAGAATA
HETEROHYR       GTCACTGAAGGAGGATTTAGTAGTAAATTAAGAATA
DENDROHYR       GTAACTGAAGGAGGATTTAGCAGTAAATTAAGAATA
                     **** ******** ******  ** * **
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(b)

The pairwise estimates of sequence divergence (uncorrected estimates and values corrected by Kimura's two parameter method; see p. 44) and the rates of transitions and transversions are presented in Table 14. The relationship between the transition/transversion ratio (TS/TV) and sequence divergence estimates for the two genes are graphically presented in Figure 17. An analysis of the base pairs comprising the regions sequenced in cytochrome b and 12S rRNA, together with the empirical base pair frequencies (Felsenstein 1990), are presented in Figure 18. The neighbor-joining trees generated from the cytochrome b and 12 S rRNA data, and from the combined data sets are shown (Figure 19). The neighbor-joining tree based on the amino acid sequences for cytochrome b is also presented.

A total of 140 cytochrome b sites was phylogenetically informative and an analysis of the distribution of these revealed 35 at first codon positions (25%), 13 at second positions (9%) and 92 at third positions (66%). These data, together with 67 informative 12S rRNA sites, were used for subsequent cluster analysis. Cladograms using unweighted values for the cytochrome b and 12S rRNA data, and for the two data sets combined, are shown in Figure 20. In each instance, a single most parsimonious tree resulted from the analysis. The combined matrix of 217 characters similarly yielded a single tree (CI = 0.67). A re-analysis of the cytochrome b dataset with only the elephant as outgroup (71 informative characters) yielded a tree of 174 steps and CI of 0.67; the branching order remained unaltered.

In a further analysis only informative transversions were used; this included 112 for the complete cytochrome b dataset and 32 for the 12S rRNA dataset. The analysis of transversions in cytochrome b yielded a tree (not shown; 154 steps, CI 58%) of similar topology to that based on the full cytochrome b data set (Figure 20 a), while seven equally parsimonious trees (not shown) were produced in the analysis of the 12S rRNA transversions. In comparison to the tree based on the full data set (Figure 20 c), the strict consensus tree (not shown; Rolf's CI 0.714, Mickevich's consensus 0.5) revealed the same branching order for the outgroups but the relationships within the Hyracoidea were unresolved. In all instances the branch-and-bound, global branch swapping and alltrees options yielded the same results and, consequently, only those for the branch-and-bound option are shown.

Bootstrap analysis using PHYLIP (Figure 21) mirrored the PAUP topologies. Most of the cytochrome b relationships were not well supported, while those based on 12S rRNA sequences were well supported, but not significantly so. Since the percentage base composition of the two genes analysed for the different taxa were not found to deviate significantly from the empirical base frequencies (Figure 18), the aligned sequences were subjected to analysis by the Maximum Likelihood method (DNAML option of PHYLIP). The topologies of the trees generated for the two genes were similar to each other, as well as to those derived from the other analytical procedures adopted in this study.

Table 14 Pairwise estimates of sequence divergence (p) and values corrected for multiple hits (d). Also shown are the transition (TS) and transversion (TV) rates for portions of two mtDNA genes derived from the five hyrax representatives and the five outgroups. **(a) Cytochrome b** - Comparisons correspond to the order of sequences in Figure 16 a: 1 - *H. sapiens*, 2 - *D. bicornis*, 3 - *L. africana*, 4 - *O. afer*, 5 - *E. caballus*, 6 - *P. c. capensis* (south-central), 7 - *P. c. capensis* (northern), 8 - *P. c. syriaca*, 9 - *H. brucei*, 10 - *D. arboreus*.

Comparison	% p	% d	TS rate	TV rate
1 vs. 2	22.93	27.49	0.1050	0.1243
1 vs. 3	27.62	34.71	0.1298	0.1464
1 vs. 4	22.65	27.21	0.1160	0.1105
1 vs. 5	26.80	33.31	0.1188	0.1492
1 vs. 6	25.41	31.15	0.1105	0.1436
1 vs. 7	25.14	30.74	0.1105	0.1409
1 vs. 8	30.11	38.61	0.1243	0.1768
1 vs. 9	23.48	28.22	0.0967	0.1381
1 vs. 10	31.49	41.76	0.1713	0.1436
2 vs. 3	23.20	28.14	0.1271	0.1050
2 vs. 4	20.99	24.78	0.1022	0.1077
2 vs. 5	16.30	18.44	0.0773	0.0856
2 vs. 6	23.76	29.05	0.1354	0.1022
2 vs. 7	22.65	27.21	0.1160	0.1105
2 vs. 8	26.80	33.48	0.1326	0.1354
2 vs. 9	22.93	27.49	0.1050	0.1243
2 vs. 10	28.45	36.56	0.1602	0.1243
3 vs. 4	25.14	31.10	0.1381	0.1133
3 vs. 5	27.90	35.11	0.1271	0.1519
3 vs. 6	22.65	27.49	0.1354	0.0912
3 vs. 7	23.20	28.32	0.1381	0.0939
3 vs. 8	24.86	30.41	0.1188	0.1298

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Comparison	% p	% d	TS rate	TV rate
3 vs. 9	22.65	27.18	0.1133	0.1133
3 vs. 10	27.62	34.98	0.1464	0.1298
4 vs. 5	24.03	29.09	0.1077	0.1326
4 vs. 6	19.89	23.31	0.1050	0.0939
4 vs. 7	18.78	21.73	0.0912	0.0967
4 vs. 8	24.59	30.01	0.1188	0.1271
4 vs. 9	19.06	22.06	0.0856	0.1050
4 vs. 10	26.52	33.25	0.1436	0.1215
5 vs. 6	26.24	32.60	0.1298	0.1326
5 vs. 7	26.24	32.57	0.1271	0.1354
5 vs. 8	28.73	36.30	0.1160	0.1713
5 vs. 9	26.24	32.53	0.1243	0.1381
5 vs. 1	30.11	39.27	0.1630	0.1381
6 vs. 7	5.25	5.50	0.0442	0.0083
6 vs. 8	12.71	14.05	0.0773	0.0497
6 vs. 9	13.26	14.80	0.0884	0.0442
6 vs. 10	24.86	31.43	0.1713	0.0773
7 vs. 8	12.71	14.03	0.0746	0.0525
7 vs. 9	13.26	14.83	0.0912	0.0414
7 vs. 10	26.52	34.11	0.1796	0.0856
8 vs. 9	18.78	21.92	0.1105	0.0773
8 vs. 10	22.93	27.88	0.1354	0.0939
9 vs. 10	23.76	29.31	0.1492	0.0884

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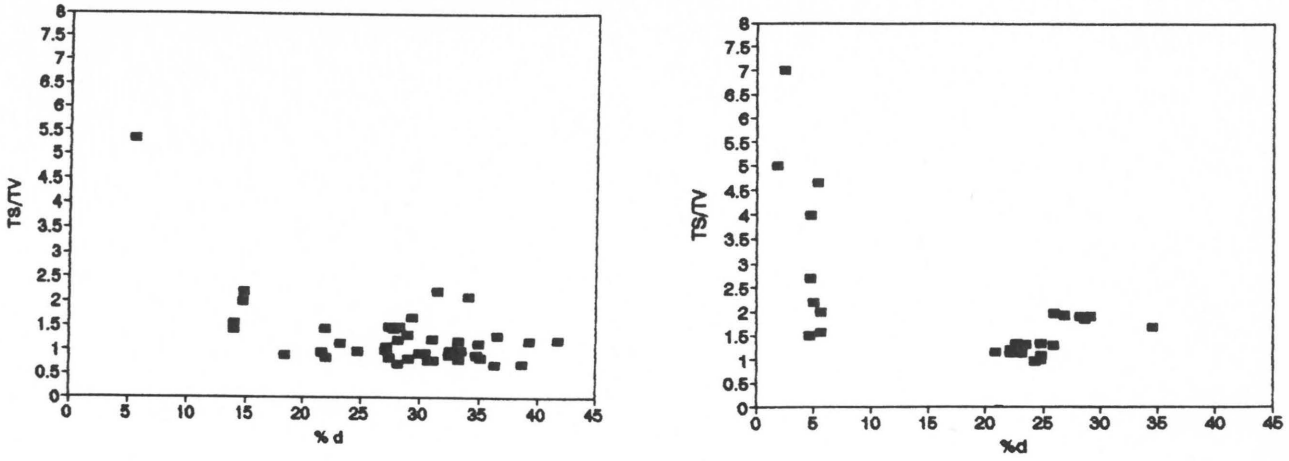
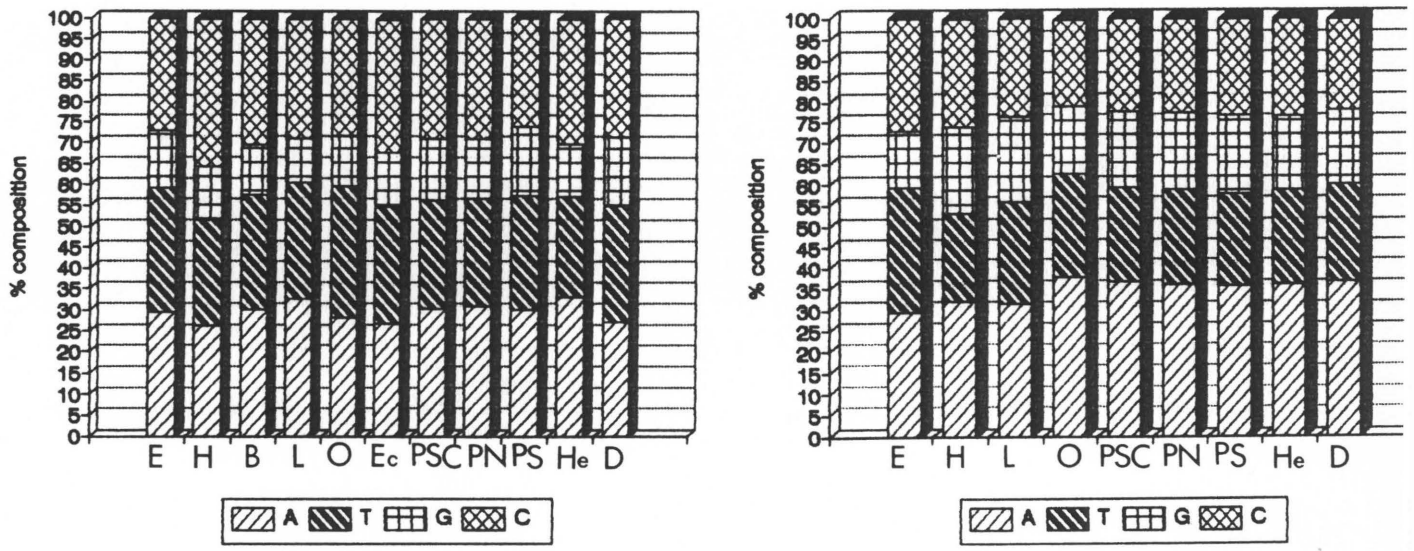
(b) 12S rRNA - Comparisons correspond to the order of sequences in Figure 16 b: 1 - *H. sapiens*, 2 - *L. africana*, 3 - *O. afer*, 4 - *P. c. capensis* (south/central), 5 - *P.c. capensis* (northern), 6 - *P. c. syriaca*, 7 - *H. brucei*, 8 - *D. arboreus*.

Comparison	% p	% d	TS rate	TV rate
1 vs. 2	26.99	34.53	0.1687	0.1012
1 vs. 3	18.07	20.84	0.0966	0.0841
1 vs. 4	20.99	24.82	0.1080	0.1019
1 vs. 5	20.99	24.79	0.1049	0.1049
1 vs. 6	20.68	24.34	0.1019	0.1049
1 vs. 7	20.99	24.82	0.1080	0.1019
1 vs. 8	19.75	23.13	0.1049	0.0926
2 vs. 3	21.65	25.89	0.1220	0.0945
2 vs. 4	22.96	28.23	0.1511	0.0785
2 vs. 5	23.26	28.65	0.1511	0.0816
2 vs. 6	23.56	29.15	0.1541	0.0816
2 vs. 7	21.45	25.96	0.1420	0.0725
2 vs. 8	22.05	26.84	0.1450	0.0755
3 vs. 4	19.02	22.15	0.1043	0.0859
3 vs. 5	19.02	22.12	0.1012	0.0890
3 vs. 6	20.86	24.79	0.1196	0.0890
3 vs. 7	19.94	23.47	0.1135	0.0859
3 vs. 8	19.33	22.62	0.1104	0.0828
4 vs. 5	1.81	1.84	0.0151	0.0030
4 vs. 6	2.42	2.47	0.0211	0.0030
4 vs. 7	4.53	4.70	0.0332	0.0121
4 vs. 8	4.83	5.02	0.0322	0.0151
5 vs. 6	3.02	3.12	0.0302	0.0000
5 vs. 7	5.14	5.37	0.0423	0.0091
5 vs. 8	4.53	4.69	0.0272	0.0181
6 vs. 7	4.53	4.71	0.0363	0.0091
6 vs. 8	5.44	5.67	0.0363	0.0181
7 vs. 8	5.44	5.66	0.0332	0.0211



(a) (b)

Figure 17 Transition/transversion (TS/TV) ratios contrasted against percentage sequence divergence (%d) for pairwise sequence comparisons of the (a) 362 nucleotide region of cytochrome b and the (b) 331 nucleotide region of 12S rRNA. Values are presented in Table 14.



(a) (b)

Figure 18 Base pair composition (%) of the (a) 362 bp region of cytochrome b and the (b) 331 bp region of 12S rRNA. Y-axis legends are as follows: E - empirical base frequencies (Felsenstein 1990), H - *H. sapiens* (Anderson *et al.* 1981), D - *D. bicornis* (cytochrome b data of Irwin *et al.* 1991), L - *L. africana* (cytochrome b data of Irwin *et al.* 1991), O - *O. afer*, Ec - *E. caballus*, PSC - *P. c. capensis* (south-central clade), PN - *P. c. capensis* (northern clade), PS - *P. c. syriaca*, He - *H. brucei*, D - *D. arboreus*.

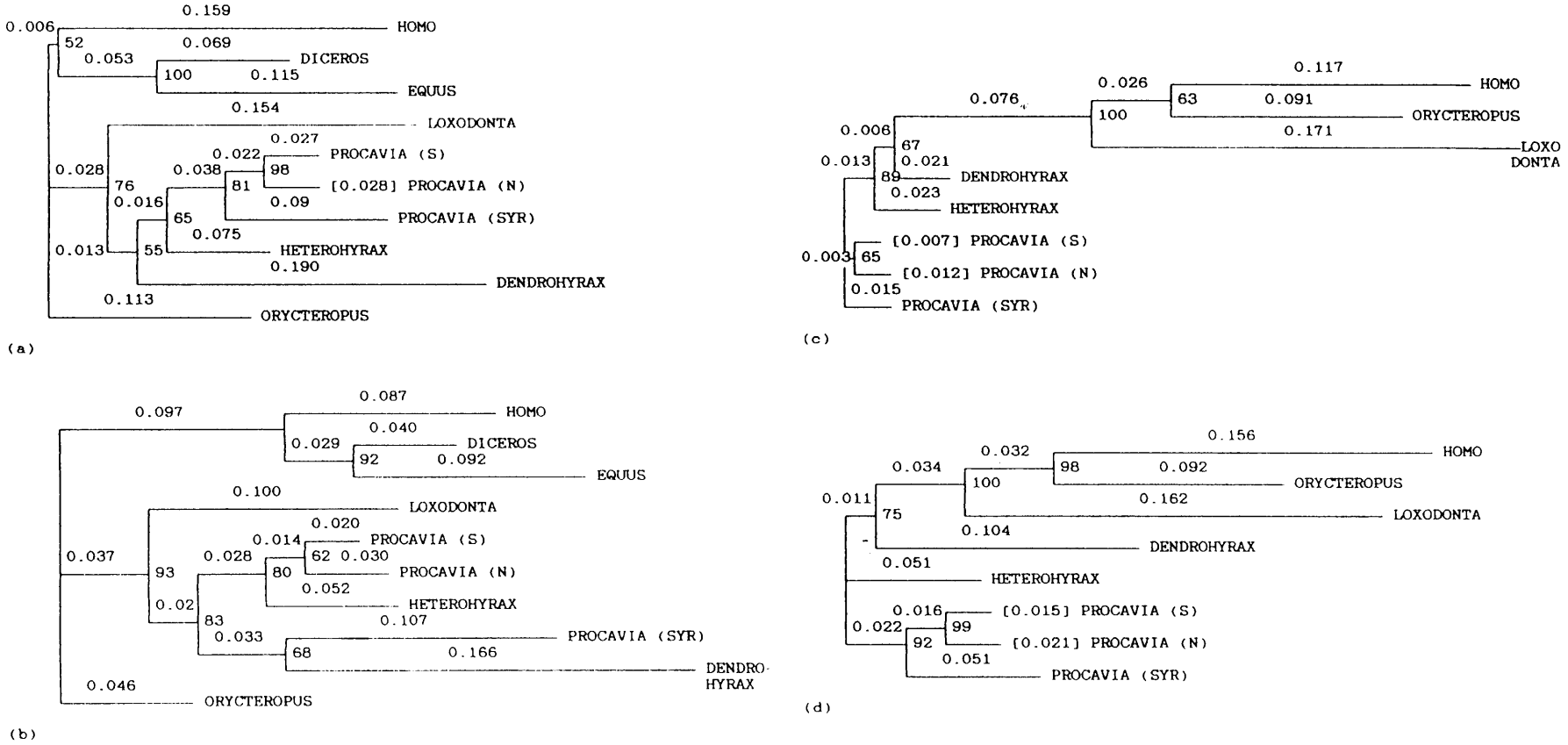


Figure 19 Neighbor-joining trees based on the percentage nucleotide sequence divergence between: (a) mtDNA cytochrome b sequences, (b) cytochrome b amino acid sequences, (c) 12S RNA sequences and (d) cytochrome b and 12 S rRNA combined. The values over each branch represent sequence divergence estimates corrected by the Kimura two parameter method. The bootstrap confidence limits (% occurrence in 1000 replicates) for internal branches are given at each node

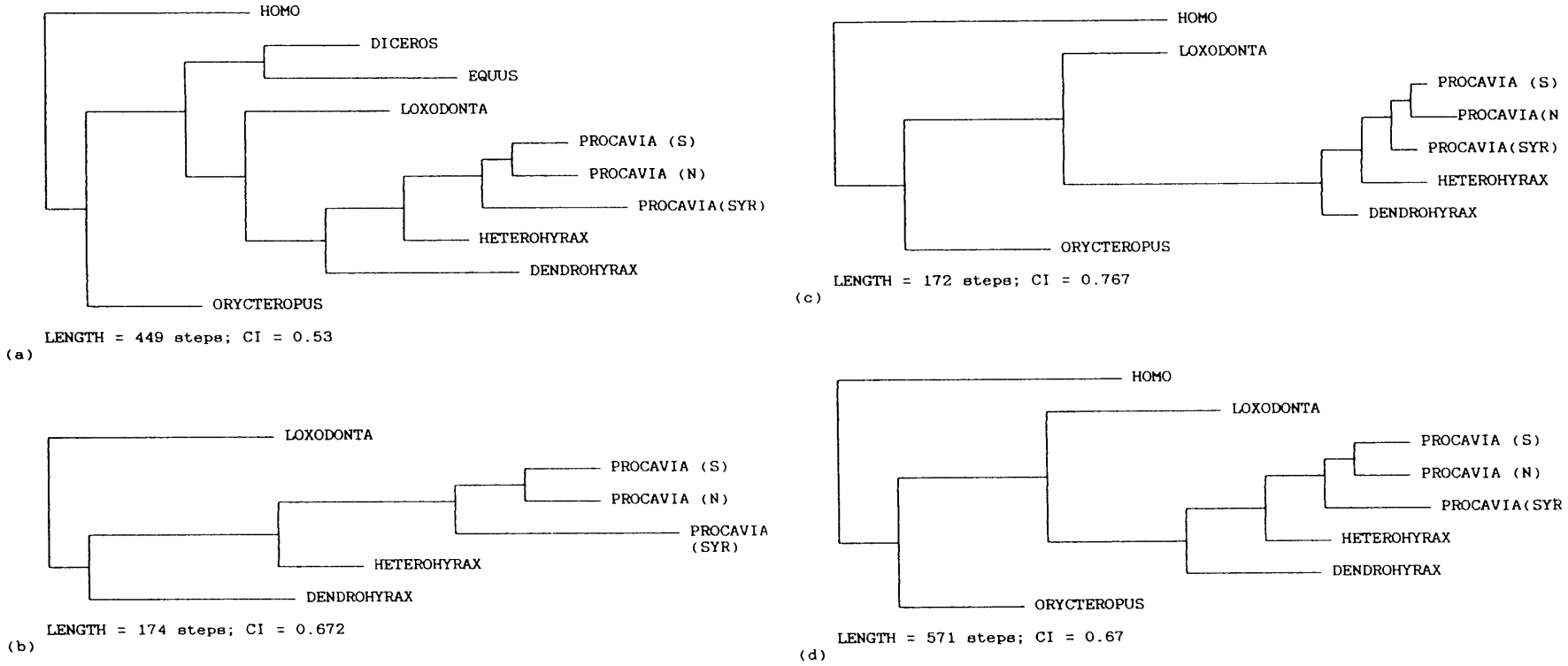
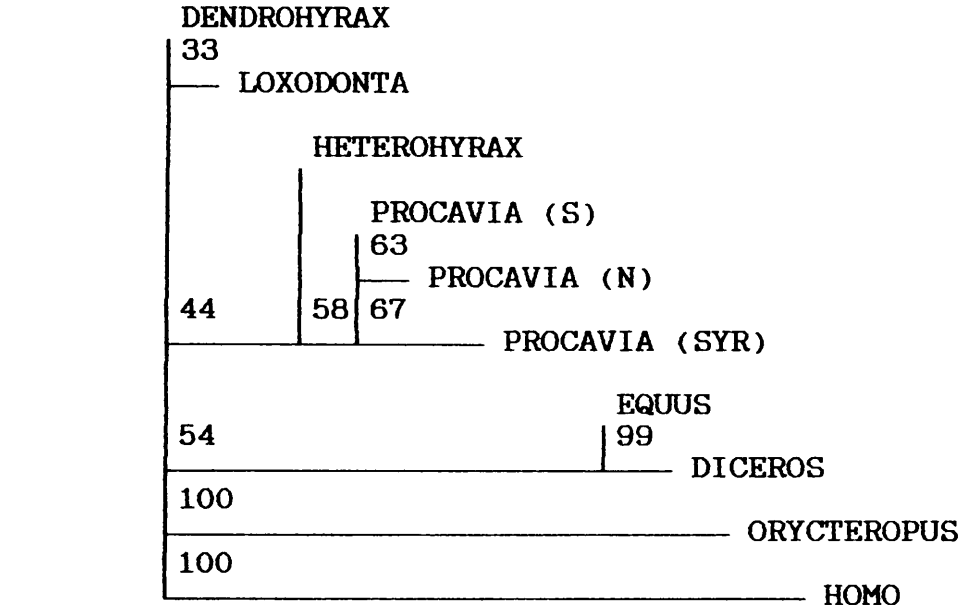
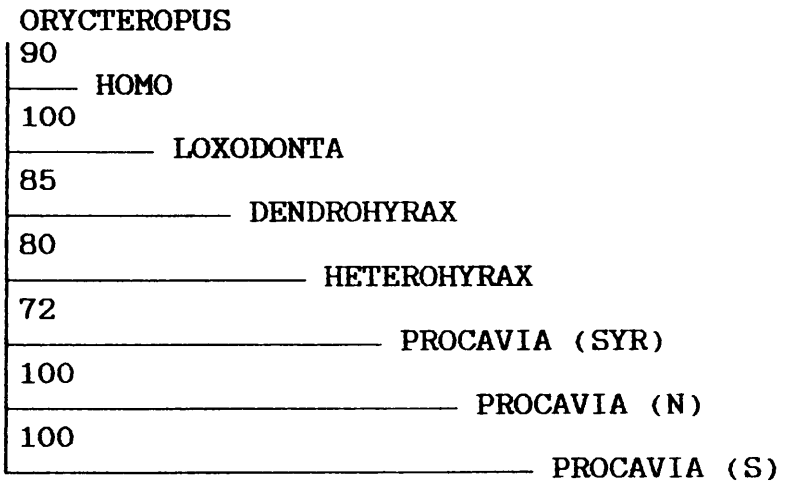


Figure 20 Single most parsimonious trees based on presence-absence character data for: (a) 140 informative cytochrome b sites (449 steps, CI 0.53); (b) 71 informative cytochrome b sites (174 steps, CI 0.67); (c) 67 informative 12S rRNA sites (172 steps, CI 0.77); (d) cytochrome b and 12S rRNA combined (571 steps, CI 0.67). In all instances the branch-and-bound option of PAUP (Swofford 1985) was used



(a)



(b)

Figure 21 Parsimony trees based on mtDNA cytochrome b (a) and 12S rRNA (b) data. The majority rule consensus trees were constructed using the bootstrap procedure (DNABOOT) in PHYLIP (Felsenstein 1990). The value at each node shows the number of times the group consisting of the species above and to the right of that node was found in 100 bootstrap replicates.

DISCUSSION AND CONCLUSIONS

Intraspecific mtDNA phylogeography

This investigation clearly revealed that the gain or loss of specific restriction sites was generally unique to each population studied. This created patchworks of mtDNA clones with haplotypes showing strong geographic patterning. The extent of intraspecific mtDNA structuring did, however, differ in the genera and probably reflects varying habitat constraints, dietary requirements and social structure, as well as historic geographic factors.

Intraspecific phylogeography of the Cape rock hyrax, Procavia capensis capensis

The mtDNA population structure of this widely distributed species was characterized by a high genotypic diversity suggestive of strong geographic partitioning. This was reflected by both the neighbor-joining trees (Figures 9 and 10) and the minimum-path network derived from the least number of mutational steps between clones (Figure 11). A pronounced genetic and geographic division was clearly evident in this species. This contrasted with the relatively small differences detected between populations comprising the two mitochondrially distinct clades.

Specimens from the Karoo National Park (locality 3, Figure 5) showed a close affinity to other populations in the south-central clade (Figure 11 a) while clone 2, the most frequently observed haplotype within the clade, was also geographically the most widespread occuring in six of the 13 sample populations. Strikingly, this clone was distinguished by only one or two site changes from most others within the clade, and thus forms the hub of the minimum path network. Given these relationships, and the low levels of sequence divergence between this and other closely related clones in geographically distant populations (populations 5-12; Figure 5), it is not unlikely that this reflects dispersion to these regions from some ancestral central Karoo stock in the recent evolutionary past, the patterns of which may have been similar to those witnessed during the 1930s (Kolbe 1967).

At that time Kolbe (1967) reported great increases in hyrax numbers (probably due to the combined effects of reduced predation and to vegetational, hydrological and climatological factors), which resulted in hyrax movement away from their rocky refuges into new and marginal habitats. The dispersion was from the eastern and south-eastern Karoo (the nearest sample population No. 3) north-eastwards along the Great Escarpment (Figure 22), a pattern that is broadly congruent with that of the minimum path network (Figure 11 a and Figure 22 inset).

Dispersal to the south was not clearly demarcated (Kolbe 1967). It is striking, however, that the southern-most locality analysed in the present investigation (population 2; Figure 5) was characterized by six mtDNA clones in a sample comprising seven individuals (Figure 11 a and Figure 22 inset), making it also the most genetically divergent population studied (see also Table 9). This could reflect

Figure 22 Radiation of *P. c. capensis* subsequent to large population increases during the 1930s. The shaded area represents hyrax movement as reported by Kolbe (1967). The radiation originated in the central Karoo (the nearest sample population in this study indicated by a circle) and spread north-eastwards along the Great Escarpment. The inset shows the geographic mtDNA relationships within the south-central clade and indicates the relatively close relationships between geographically distant clones distributed along major mountain ranges.

an extrinsic impediment to southward hyrax movement and should the 1930 hyrax dispersion have mimiced the situation in the evolutionary past, the increased time since common ancestry may have allowed for a greater accumulation of mutations in this geographic region. Given the relatively close relationship between clones 1 and 9 (Figure 11 a) however, some gene flow must have occurred between the regions represented by the De Hoop and Karoo populations (Figure 5).

In a few instances clones detected within the same sample populations are separated by several mutational steps (clones 1 and 2 in population 1; clones 3 and 5 in population 2; clones 2 and 11 in population 4 and clones 19 and 20 in population 12; Figure 11 a and Figure 22 inset). These divergent lineages (including those within the De Hoop population) may reflect recent colonizations superimposed on older populations, and historic population subdivision in which the subdivided units did not become extinct.

Likewise, the presence of the closely related clones 23 and 24 within almost all populations of the northern *P. c. capensis* clade (Figure 11 b) may indicate an ancestral state. Furthermore, the low sequence diversity and numbers of mutational steps between clones within this clade (Figure 11 b) indicate that colonization of the area is probably more recent than that of the south-central clade.

In contrast to the low levels of variation found within the two mtDNA clades, a marked genetic discontinuity (3.32%) is evident between them. Sequence divergence values of this magnitude are generally considered to be suggestive of long-term extrinsic barriers to gene flow (category I in Avise *et al.* 1987a) and have been detected, among others, in the horseshoe crab *Limulus polyphemus* (Saunders *et al.* 1986), the pocket gopher *Geomys pinetis* (Avise, Giblin-Davidson, Laerm, Patton & Lansman 1979) and the deer mouse *Peromyscus maniculatus* (Lansman *et al.* 1983a).

The geographic distribution of the two *P. c. capensis* mtDNA clades, as defined in this study, is presented in Figure 23 (a). Given the species' habitat specificity (rocky mountainous situations) it is informative to compare their delineation to the distribution of major mountain ranges in South Africa (Figure 23 b). From these data it is apparent that the discontinuity reflects historic hyrax dispersal along two separate routes, with the large south-central clade following the Great Escarpment and the northern clade being restricted to the northern mountain ranges, primarily the Soutpansberg-Magaliesberg axis, but also extending north-westwards to the Matopos in Zimbabwe.

A Geographic Information Systems analysis (GIS) was conducted in two discrete areas of contact between the two clades. Investigatory parameters included elevation and mean annual rainfall (Figures 24-26) for both areas, as well as ambient temperature (mean annual minimum and maximum) and vegetation type for the eastern Transvaal area (results not shown). Results obtained for the first area (Figures 24-25) supported the routing of hyrax along independent mountain ranges since the eastern Transvaal populations that are located on the Great Escarpment (Lydenburg, Pilgrims' Rest and Blyde River) form part of the south-central clade, while those to the north (Mica) and east (Middelburg) of the Drakensberg are representative of the northern clade (Figure 24). The Great Escarpment is characterized by higher rainfall (Figure 25), lower temperatures and unique vegetation types and is

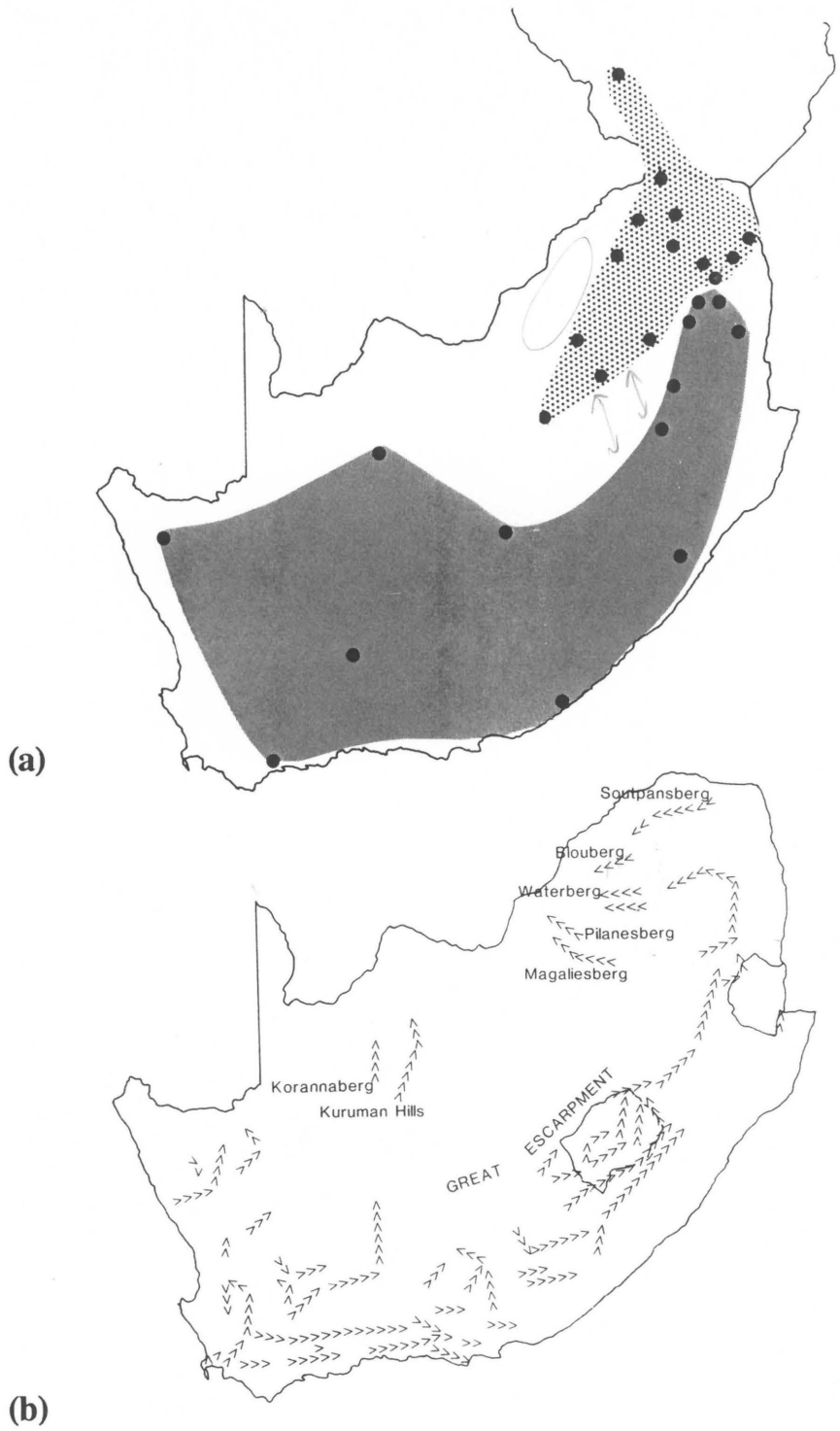


Figure 23 (a) Geographic distribution of two major rock hyrax mtDNA clades, the (■) south-central and (⁘) northern clade. (b) Mountain ranges of South Africa showing the extensive Great Escarpment and the Soutpansberg-Magaliesberg axis (redrawn from Bristow 1985).

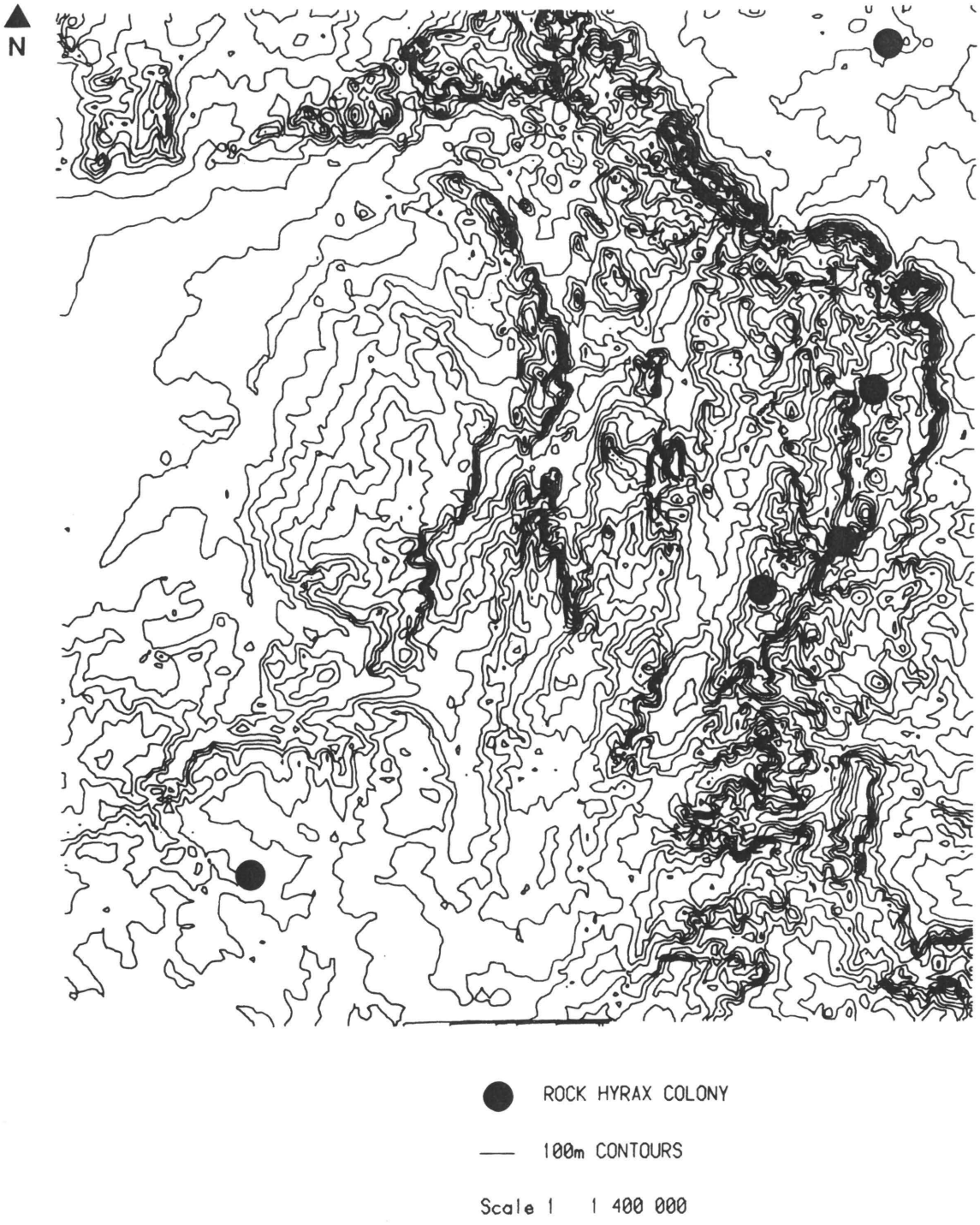


Figure 24 Relief map of the eastern Transvaal region bounded by the Springbok flats in the north-west, Mica in the north-east, Barberton in the south-east and Middelburg in the south-west. The hyrax populations included in the study area are (from north to south): Mica (northern clade), Blyde River, Pilgrims' Rest and Lydenburg (south-central clade), and Middelburg (northern clade).

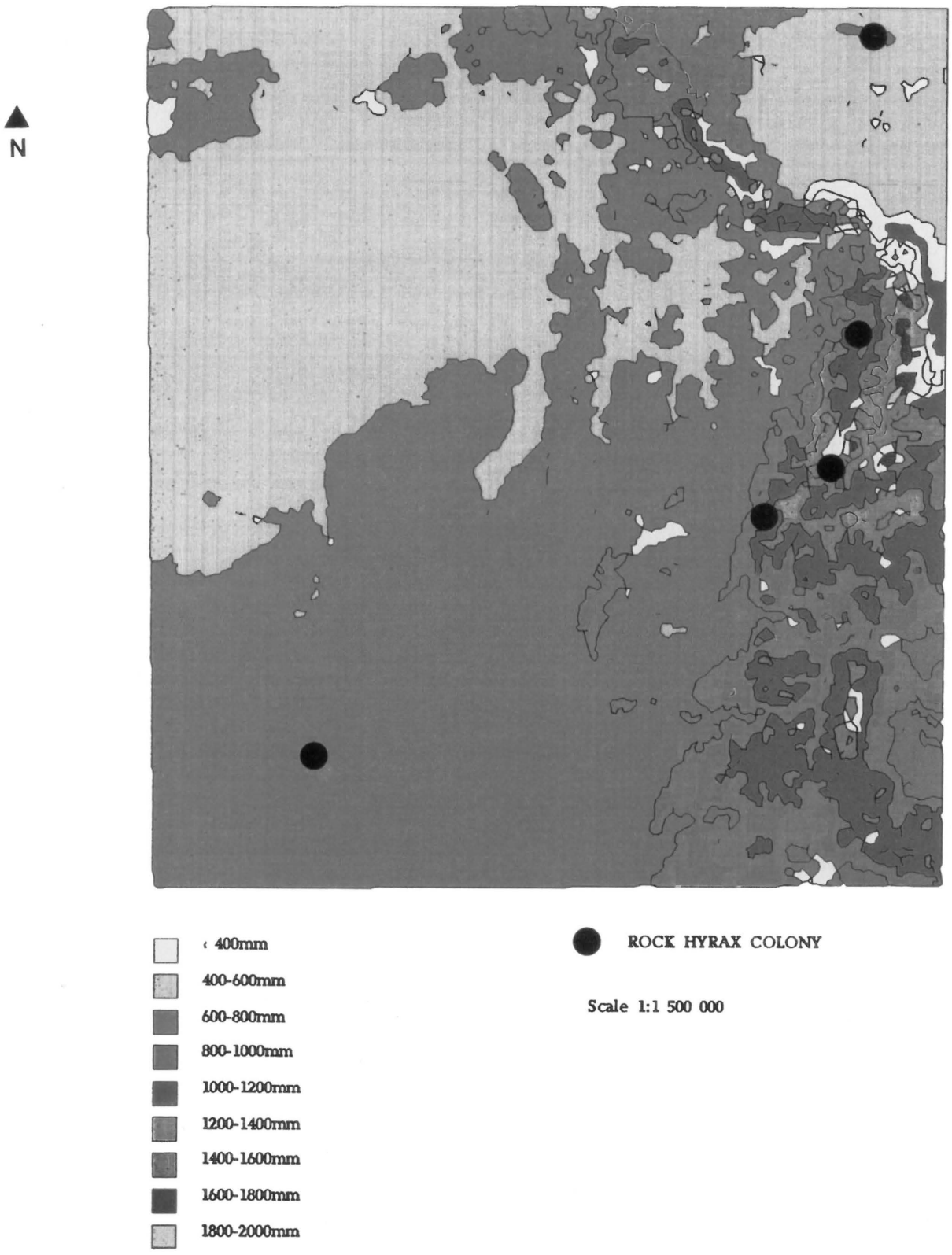


Figure 25 Annual average rainfall recorded for the eastern Transvaal area. Hyrax populations included in the survey area are (from north to south): Mica (northern clade), Blyde River, Pilgrims' Rest and Lydenburg (south-central clade), and Middelburg (northern clade).

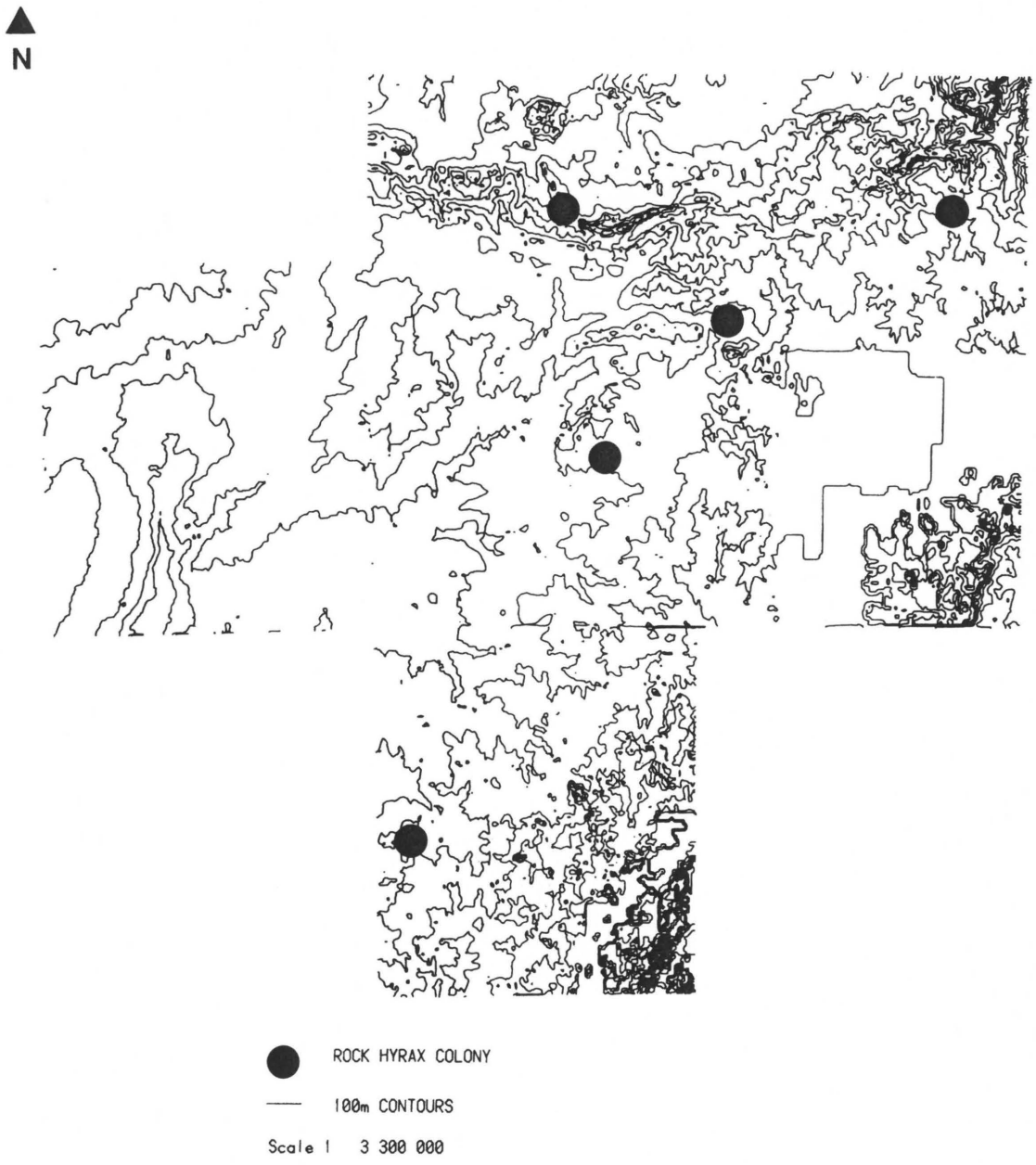


Figure 26 Relief map of the zone of contact between the two rock hyrax clades bounded by the Magaliesberg in the north-west, Middelburg in the north-east and Bloemfontein in the south. Hyrax populations included in the survey area are (from north-to-south): Middelburg, Rustenburg, Suikerbosrand and Vredefort (northern clade), and Bloemfontein (south-central clade).

thus clearly distinct from the Transvaal Lowveld and eastern Highveld, both of which experience lower rainfall (Figure 25) and higher temperatures. The second area of analysis encompassed an extensive region of contact in the western and southern Transvaal, and in the northern Orange Free State. In this case however, the separation of the two clades appears to be related to the absence of suitable habitat, with the relief map (Figure 26) clearly revealing a dearth of kopje and mountainous terrain. These findings also indicate that the range maps (Skinner & Smithers 1991) reflect a true situation and not a sampling hiatus.

Therefore, while contemporary geographic influences may explain the maintenance of the genetic break, it is hypothesized that the reasons for the initial separation of the two lineages can be traced to global temperature changes and the effects of vicariance which occurred approximately 1.7 million years ago. Two evolutionary scenarios can be proposed. First, the geographic distribution of clones 23 and 24 of the northern clade (Figure 11 b) suggest that colonization of this area may have taken place from the north. This is supported by the close relationship between lineage 23 and clones found within the Matopos population in Zimbabwe, and it seems likely that the northern *P. c. capensis* mtDNA clade may represent a southern extension of the sister taxon, *P. c. johnstoni*. As currently understood however, *P. c. johnstoni* extends only as far south as Malawi, although the transition between the two has never been rigorously assessed (Bothma 1971; Honacki *et al.* 1982; Smith 1985; Figure 1 a). Clearly therefore, an evaluation of the mtDNA affiliations of *P. c. johnstoni* specimens from Malawi, or Kenya, to lineages comprising the northern clade will be informative. A close association between specimens would support the hypothesis that the northern clade simply reflects a southern extension of *P. c. johnstoni*, and that the existing range maps of these taxa are grossly inadequate.

Alternatively, should there be no close association, the presence of two clades in what has conventionally been regarded as a single species, *P. c. capensis*, may reflect local genetic divergence. It has been argued that the past two million years, in particular, has witnessed exceptional climatic instability, and that the direct and indirect effects of glacial advances and retreats significantly altered species' distributions (Avise 1989). A major plunge in global temperatures occurred approximately 2.6 million years ago which was followed, during the Pleistocene, by oscillations between glacial and interglacial conditions (Figure 27, Brain 1985). These global temperature fluctuations profoundly influenced the southern African climate which, in turn, altered the region's vegetation (Van Zinderen Bakker 1978).

Upper Pleistocene and Holocene deposits from the Boomplaas Cave in the southern Cape Province indicate that rock hyrax numbers oscillated in synchrony with climatic changes (Klein 1983). Given the hyrax's poor thermoregulatory capabilities (Taylor & Sale 1969; Bartholomew & Rainey 1971; Louw, Louw & Retief 1972; McNairn & Fairall 1979) and the sensitivity of various biological processes to fluctuations in temperature and food availability, it is conceivable that during the glacial maxima hyrax distributions receded into small refugial populations that, in turn, expanded during

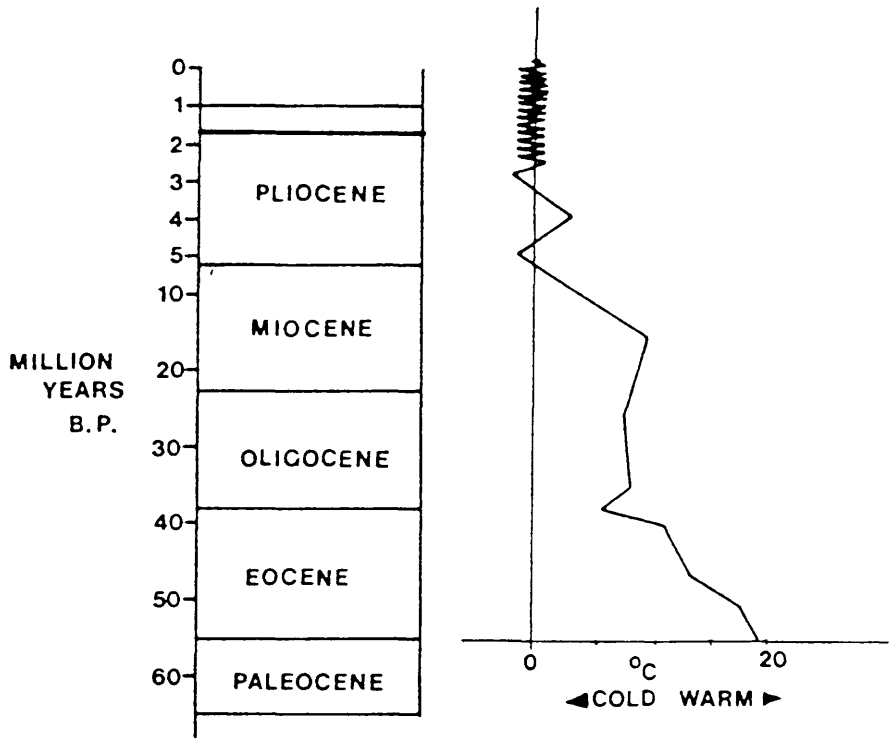


Figure 27 The global temperature trend during the Cainozoic Era. The curve was deduced from deep-sea core analysis. Note the progressive decline in temperature until the end of the Miocene and the oscillation between glacial and interglacial episodes during the latter part of the Pliocene (Brain 1985).

subsequent interglacial periods. This type of fragmentation could promote genetic differentiation between local demes and, in extreme cases, lead to speciation.

Quite clearly, however, additional research is required to resolve these competing hypotheses. In addition to assessing the mtDNA affinities between *P. c. johnstoni* and the *P. c. capensis* northern clade, the mtDNA data set should be bolstered by an assessment of nuclear DNA variation (for example protein electrophoresis), as well as by the rigorous morphometric analysis of skull and toothrow characteristics. If these investigations strengthen the recognition of the northern *P. c. capensis* mtDNA clade as a separate species, it is conceivable that, in addition to the geographic factors implicated in this study as maintaining the genetic break between the clades, competitive exclusion may contribute to the maintenance of their genetic integrity in areas of sympatry.

Intraspecific phylogeography of the yellow-spotted hyrax, Heterohyrax brucei

Although *H. brucei* and *P. c. capensis* occur sympatrically (Hoeck 1975), an observation that was confirmed in this study when populations in the northern and eastern Transvaal were sampled, the observed mtDNA structuring within the two species was different. Genotypic diversity within *H. brucei* was slightly lower than in *P. c. capensis* but, nonetheless, was indicative of a relatively high degree of mtDNA specificity among individuals. The discontinuous genetic divergence pattern in this taxon contrasts with the continuity within the northern *P. c. capensis* clade, and it seems likely that this may reflect differing habitat requirements. *Heterohyrax* is considered a specialist browser (Turner & Watson 1965) and, by definition, would be less tolerant of change than the habitat generalist, *Procavia* (Sale 1965). Consequently, past climatic fluctuations and concomitant vegetational changes may have had an even greater impact on range expansion in *Heterohyrax* than *Procavia*.

The two *H. brucei* mtDNA clades (Figure 9), which are calculated to have diverged approximately 1.5 million years ago, are largely supported by current subspecies delineations. The southern African subspecies, *H. b. ruddi* and *H. b. granti* have, in the past, been delineated using differences in the length of the upper toothrow (Bothma 1964). They can, to some extent, also be distinguished on the basis of dorsal colour patterns (Bothma 1966). Interestingly however, a third colour morph within *H. b. ruddi* was also noted (Bothma 1966), which confirmed an earlier observation that specimens from the Soutpansberg and Blyde River regions probably represent intergrades between *H. b. ruddi* and *H. b. granti* (Bothma 1964).

On the basis of geographic position the Soutpansberg (clone 46), Blyde River (clone 43), Ohrigstad (clone 44) and Buffelshoek (clone 45) populations would, conventionally, have been regarded as *H. b. ruddi* but their mtDNA affinities clearly placed them within *H. b. granti* (clone 42 of the Munnik population; Figure 12). This association is further strengthened by the GIS analysis of the elevation and rainfall data for the seven South African *H. brucei* collection localities sampled

in the present study. Bothma (1966) had previously found *H. b. granti* to show preference for areas with high annual rainfall, an observation supported herein. Vhembe and Pafuri (*H. b. ruddi*; populations 2 and 3, Figure 6) are characterized by low elevation (on average 418m above sea level) and low mean annual rainfall (330mm), while the five remaining localities (corresponding to *H. b. granti* and Bothma's "intergrades"; No's 4-8, Figure 6) are all high lying (1200m) and experience higher rainfall (670mm); their association with *H. b. granti* therefore comes as no surprise. It is noteworthy however, that the populations thought to represent "intergrades" between *H. b. ruddi* and *H. b. granti* (Soutpansberg and Blyde River), as well as a population previously considered as *H. b. ruddi* (Buffelshoek), group within *H. b. granti* and this may indicate that a more rigorous phenotypic delineation of the subspecies is required.

Intraspecific variation in the southern tree hyrax, Dendrohyrax arboreus

Dendrohyrax arboreus is classified as rare in the South African Red Data Book (Smithers 1986) which consequently precluded extensive sampling of the species. As a result, only two populations were included in this investigation. Although the small sample size does not allow for meaningful discussion of the mtDNA phylogeography, the data are nonetheless informative.

The two sample populations, Pirie Forest and Alexandria State Forest (Figure 6), which are separated by less than 150 km, were characterized by two distinct mtDNA lineages that were calculated to have diverged approximately 1.8 million years ago. Although natural forest in South Africa is currently highly fragmented, it would appear to have been more extensive in the past and has been characterized by repeated cycles of expansion and contraction (Axelrod & Raven 1978). While detailed information regarding the extent of these forests at the time of divergence of the two mtDNA lineages is lacking, the extent of the afromontane forest immediately before, during and after the last glacial maximum are known. This led Lawes (1990) to propose a scenario for the radiation of the forest dwelling samango monkey which, given the similarities in habitat requirements, may also be directly applicable to the evolutionary history of *D. arboreus* in South Africa.

In essence, Lawes' (1990) hypothesis invoked changes to the extent and distribution of afromontane and coastal lowland forests in southern Africa over the past 100 000 years (Figure 28). Extensive forests are thought to have occurred in South Africa due to increased rainfall during interglacial periods and these probably ranged from the eastern Cape into the eastern Transvaal (Van Zinderen Bakker 1978; Figure 28 a). During the last glacial maximum (18 000 BP), cooler and drier conditions prevailed and the forests receded (Figure 28 b). The period following deglaciation is thought to have been more arid, making the re-establishment of forests problematic (Figure 28 c).

Lawes (1990) proposed that *Dendrohyrax*, like the samango monkey, may have radiated from East Africa via the extensive afromontane forest belt (Figure 29 a). As with the samango, *D. arboreus*

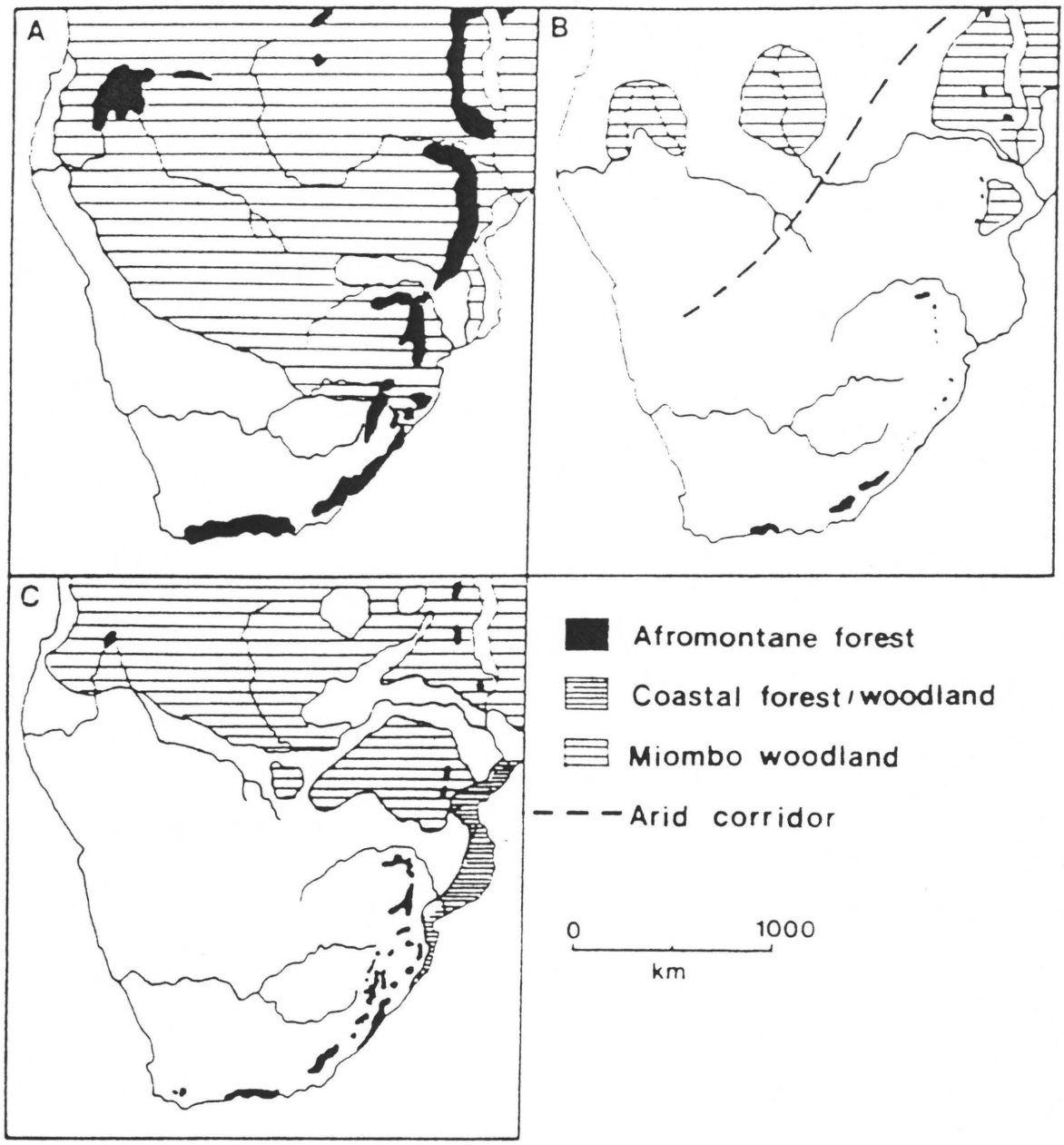


Figure 28 The extent of afromontane forest distribution in southern Africa (a) before the last glacial maximum, (b) during the last glacial maximum and (c) after deglaciation (Lawes 1990).

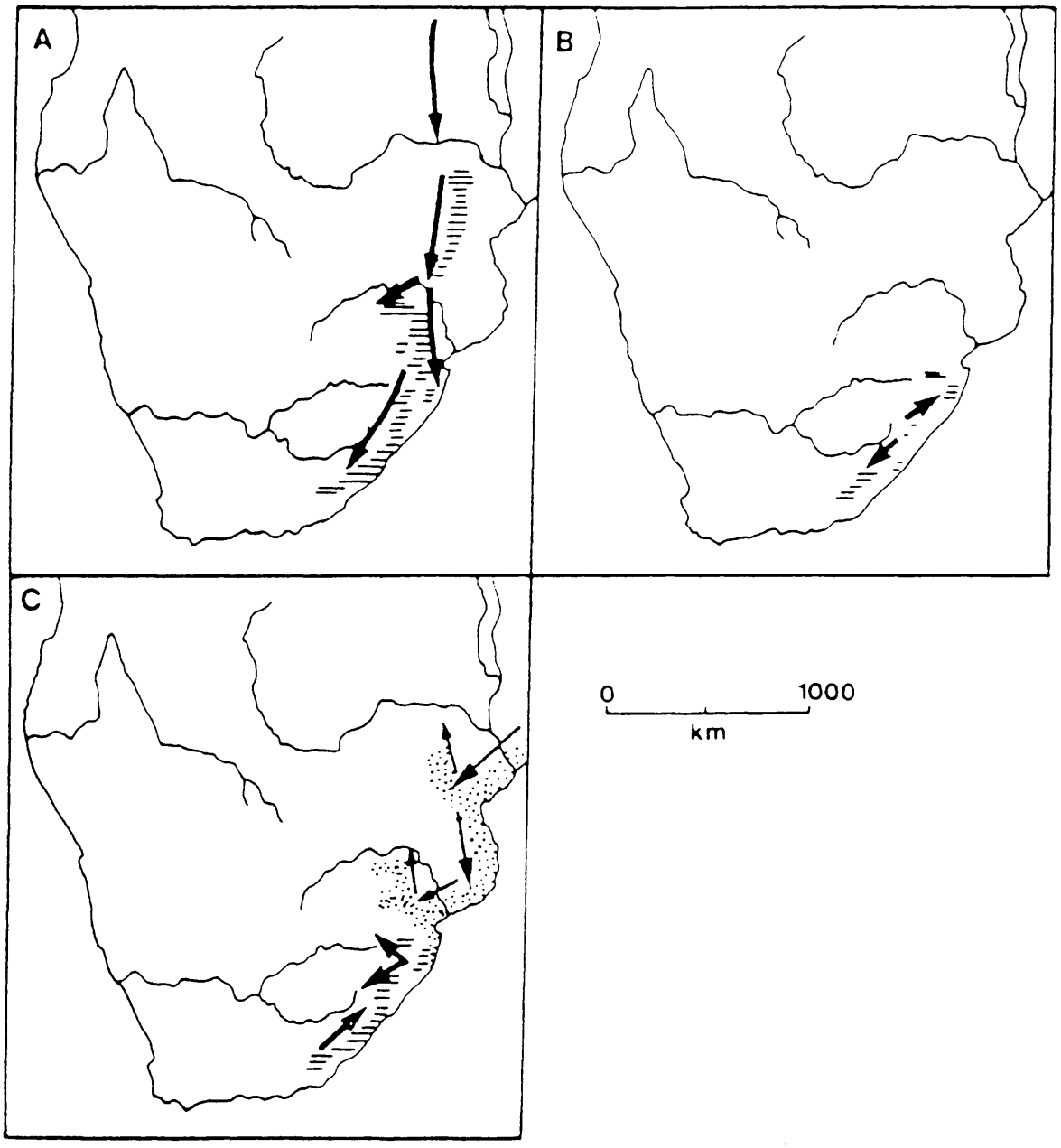


Figure 29 The proposed routes of samango monkey radiation into southern Africa. (a) 100 000 to 20 000 BP, (b) isolation of eastern Cape populations during the last glacial maximum (25 000 - 12 000 BP) and (c) proposed re-colonization following the last glacial maximum (Lawes 1990).

may have been isolated in the eastern Cape forests (Figure 29 b) and recolonization from this refugial stock could have occurred during the subsequent interglacial period 12 000 years B.P. (Figure 29 c). Therefore, *D. arboreus* populations that are currently isolated may have been in contact with each other in their relatively recent past. If this was true, the more ancient lineage divergence indicated by the mtDNA data (1.8 million years B.P.) probably represents a sampling artefact and additional sampling would provide a more definitive picture of the mtDNA phylogeography of this species.

Interspecific relationships of the Hyracoidea

MtDNA RFLP analysis

The data sets obtained by RFLP and sequencing analysis are largely congruent. It is noteworthy however, that *H. brucei* appears, on RFLP analysis, to be more closely related to *P. c. capensis* than this species is to *P. c. syriaca* (Figure 13). This association is thought to be spurious and is probably caused by small sample size, since only one *P. c. syriaca* population was sampled (n = 2). This is underscored by the fact that interspecific sequence divergence estimates (uncorrected for intraspecific variation) indicate the two *P. capensis* groups to be closely allied, and average within-group variation is known to decrease estimates of variation between groups (Nei & Miller 1990). Clearly however, sampling of *P. capensis* throughout its African and Arabian range would substantially clarify relationships between the different recognized forms, as well as their relationship to the closely related *H. brucei*.

MtDNA sequencing

Phylogenetic assessments based on nucleotide sequence from two mtDNA genes yielded trees with similar branching orders (Figures 19-21). While the neighbor-joining tree derived from amino acid sequences of the cytochrome b fragment (Figure 19 b) was identical to that based on the RFLP data (Figure 13), the position of *P. c. syriaca* in all other trees clearly reflected a closer phylogenetic relationship to the *P. c. capensis* clades. *Dendrohyrax* was invariably placed as the most basal taxon within the Hyracoidea, while *Procavia* appears to be the most recently derived form. This, to a large extent, agrees with the fossil evolutionary history (Bothma 1964). Interestingly, *Heterohyrax* is considered to be morphologically intermediate between *Procavia* and *Dendrohyrax* (Bothma 1964), an association that is supported by the present study, although a much closer affiliation of the rock dwelling forms is suggested.

The rate of change in the cytochrome b gene has been reported to be higher than the overall rate for the mitochondrial genome (Meyer *et al.* 1990; Irwin *et al.* 1991; Geffen, Mercure, Girman, Macdonald & Wayne 1992). In the present study the disparity is more pronounced at deeper divergence times even though calculations are based on the suggested 2.5% sequence divergence per million years (Meyer *et al.* 1990). For example, the divergence between two *P. c. capensis* clones (representative of the mtDNA clades within this taxon) is calculated at 2.1 million years on sequence data *cf.* 2.17 million years B.P. from the RFLP analysis. The divergence of these lineages from *P. c. syriaca* was dated at five million years B.P. (*cf.* 3.5 million years on RFLPs) while the *Procavia* and *Heterohyrax* lineages separated approximately six million years ago (*cf.* 4.4 million years on RFLPs). The highest degree of disparity exists for the rock hyrax: tree hyrax divergence which is estimated to have occurred nearly 12 million years ago on sequence data compared to the 6.25 million years based on the mitochondrial RFLP study. These discrepancies are thought to indicate that the mutation rate across the cytochrome b gene is not constant, an observation supported by Irwin *et al.* (1991) who found that the gene comprises highly variable transmembrane domains which are interspersed with conserved regions. Consequently, the rate of evolution in the 362 nucleotide region, sequenced in this study, could be higher than the suggested rate for the entire cytochrome b gene, and thus have inflated the sequence divergence estimates in this study. If this were so, the divergences between evolutionary lineages may be more recent, and could approach those calculated from the RFLP data.

The molecular based estimates of divergence and the order of lineage separation are at variance with those of the fossil record. Palaeontological data suggest an early separation of the *Procavia* lineage, dated at approximately seven million years B.P. (Churcher 1954). This is contrary to its position as the most recent lineage based on the molecular data. Likewise, the mid-Pliocene ($\pm$ 3 million years B.P.) divergence of *Heterohyrax* from *Dendrohyrax* is refuted by the molecular data. This anomalous situation may reflect inaccuracies in the fossil record compounded by the fact that first fossil occurrences often represent minimum estimates of divergence times between taxa (Novacek & Norell 1982); alternatively, molecular and morphological evolutionary change may simply lack synchrony.

Sequence evolution in the Hyracoidea has progressed mainly through silent third codon transitions and transversions, while replacement changes were found to increase with an increase in divergence time (Appendix VI). Of the 362 cytochrome b nucleotides analysed 191 were variable, 55% of which occurred at third codon positions. Only six replacement changes were detected between the *P. c. capensis* clades, whereas 33-36 replacements separated *Dendrohyrax* from the other hyrax species. As was to be anticipated, the overall level of change was higher for the cytochrome b gene than 12S rRNA (Attardi 1985) while no insertion/deletion changes were detected in the protein coding gene, confirming a trend noted by (Brown 1983). Insertion/deletion changes were identified in the 12S rRNA gene (Figure 16).

Although only a limited assessment (Appendix VII) of intraspecific sequence variation was undertaken, the comparison of 180 nucleotides of the cytochrome b gene revealed little variation within the two *P. c. capensis* clades, a result mirrored by the RFLP analysis. Somewhat surprisingly, given the RFLP sequence divergence between the two *D. arboreus* clones (d = 3.63%), the analysis of the 180 bp cytochrome b fragment indicated the two lineages to be invariant. This unexpected result cannot readily be explained but clearly merits further investigation. In contrast to the other taxa, the two *H. brucei* specimens (clones 39 and 46; Figure 12) showed considerable sequence variation (Appendix VII) more in keeping with their divergence estimate of d = 6.33%. Nonetheless, the use of a single haplotype (clone 39) was considered justified for interspecific comparisons since it was the most frequently observed clone among the *H. brucei* specimens analysed in this study and could, furthermore, unequivocally be assigned to the subspecies *H. b. ruddi.*

Although the main aim of the present investigation was not an assessment of higher order relationships, the inclusion of several outgroup species (used to root phylogenies based on the mtDNA sequences) yielded data that was informative with respect to the debate on the closest extant relatives of the Hyracoidea. Since its description, its status and evolutionary relationships have been questioned. The Hyracoidea's external resemblance to the Rodentia was quickly proved a misconception (Romer 1968), as was the suggested close relationship between it and the extinct South American †Notoungulata (Ameghino 1897 in Shoshani 1991). Associations supported by recent investigations involve a Hyracoidea-Perissodactyla relationship (McKenna 1975; Fischer 1989) and a possible affinity between the Hyracoidea and Tethytheria (Proboscidea and Sirenia; Simpson 1945; De Jong *et al.* 1981; Kleinschmidt *et al.* 1986; Shoshani 1986).

The proposed Hyracoidea:Perissodactyla relationship rests on morphological evidence: molar teeth of the Hyracoidea are thought to resemble those of the rhinoceros and horse (Osborn 1907), while Fischer (1989) considers the Hyracoidea as secondary plantigrades derived from cursorial ancestors and suggests that the nails of the hyrax are actual hoofs. Simpson (1945), however, classified the Hyracoidea within the superorder Paenungulata; this grouping is supported by similarities in the upper incisors and foot structure (Simpson 1945), placental features (Novacek & Wyss 1986) and several skull and muscle characters (Shoshani 1986). Additionally, immunodiffusion studies (Shoshani 1986) and the analysis of amino acid sequences (De Jong *et al.* 1981; Kleinschmidt *et al.* 1986) indicate a common origin for the Hyracoidea and Proboscidea orders. Most recently, a cladistic investigation which included fossil forms (†*Hyracotherium, Moeritherium* and the Desmostylia) similarly favoured a Hyracoidea-Tethytheria relationship, although this grouping was highly vulnerable to collapse when minor homoplasy was introduced (Novacek 1992a).

The analysis of nucleotide sequences from the two mtDNA genes used in this study support a Hyracoidea:Proboscidea relationship (Simpson 1945; Romer 1966; De Jong *et al.* 1981; Kleinschmidt *et al.* 1986; Shoshani 1986). The suggested affinity between the aardvark and the Paenungulata on α-crystallin sequences (De Jong *et al.* 1981) was not sustained by this analysis of cytochrome b

sequences. Additionally, the data do not suggest the Paenungulata to be a separate offshoot from the combined ungulate stem group (proposed by De Jong *et al.* 1981), but rather that the Hyracoidea, Proboscidea and Perissodactyla may have evolved from the same primitive stock. This latter observation is supported by several other investigations (McKenna 1975; Novacek 1982; Goodman, Czelusniak & Beeber 1985; Kleinschmidt *et al.* 1986; Novacek 1992a).

In conclusion, although the nucleotide sequence data of the two mtDNA genes provide support for a close hyracoidean-proboscidean evolutionary relationship, this must be tempered by the fact that morphological and molecular characters may change at different evolutionary rates (Goodman, Weiss & Czelusniak 1982; Ammerman & Hillis 1992; Novacek 1992b). Given this consideration, and the rapid ("bushlike") radiation of eutherian mammals at the end of the Mesozoic (Novacek 1982), the short periods of common ancestry that probably characterized these divergent lineages may have confounded the recovery of informative molecular synapomorphies (Ammerman & Hillis 1992) making a definitive assessment of the Hyracoidea's higher order relationships problematic, and possibly futile. Nonetheless, I believe that the present investigation has advanced our understanding of the phylogenetic relationships of the Hyracoidea. More importantly however, the analysis of phylogeographic structure has contributed significantly to the detection of biogenetic diversity within this enigmatic mammalian order, and these results may assist with the future conservation of regional faunas in South Africa.

SUMMARY

Identical diploid chromosome numbers (2n = 54) were found in representatives of the South African *Procavia, Heterohyrax* and *Dendrohyrax* species and this probably reflects the ancestral condition for the order. Selected *Procavia* specimens, representative of two distinct mitochondrial DNA (mtDNA) clades, were determined to be karyotypically invariant. While convincing G-band homology could not be demonstrated for the entire chromosomal complement of the three genera, probably due to poor banding resolution, homology was nonetheless evident in many instances. The changes responsible for the difference in diploid number between the elephant (2n = 56) and the Hyracoidea (2n = 54) could not be identified. Constitutive heterochromatin was found to be strictly centromeric in *Procavia* and *Heterohyrax*, while the greater amounts and unique distribution of heterochromatin in *Dendrohyrax* is likely to be a recently derived character.

Restriction fragment length polymorphisms (RFLPs) in mtDNA of 141 specimens representative of the three hyrax species were assayed using a suite of 10 restriction endonucleases. The Hyracoidea, with their limited dispersal capabilities, showed strong geographic patterning of mtDNA haplotypes and large phylogenetic gaps distinguished several mtDNA clusters. In *H. brucei* and *D. arboreus* (representing the southern-most extremes of the African distribution of these species), marked genetic differentiation was evident; this extends even to geographically close populations. In *H. brucei,* the mtDNA phylogeography is largely congruent with recognized subspecific boundaries and was probably promoted by the constraint of habitat specificity and its effects on gene flow. Likewise, mtDNA gene flow in *D. arboreus* is thought to have been disrupted by the fragmentation of its forest habitat.

A marked genetic break delineated a south-central and northern clade within *P. c. capensis*. The relationship between clones within the two discrete clades reflects a continuous genetic divergence pattern which was thought to have developed as a result of recent colonizations from refugial stocks. It seems likely that these events were promoted by population flushes brought about by the high reproductive capacity of this species under favourable environmental conditions.

The two mtDNA clades within *P. c. capensis* were estimated to have diverged approximately two million years B.P. The south-central clade is distributed throughout the Cape Province, Natal, southern Orange Free State and eastern Transvaal, while the northern clade is limited to the northern Orange Free State, the western, central and north-eastern Transvaal and western Zimbabwe.

The lack of suitable habitat in the western Transvaal, north-eastern Cape and north-western Orange Free State was identified as contributing to the maintenance of the two clades in these areas, but similar extrinsic barriers were not evident in other regions of the zone of contact. This was supported by GIS analysis of elevation in two separate regions of contact between the clades. Furthermore, in areas where suitable intervening habitat spans the break between them, contemporary climatic differences are implicated in the maintenance of mtDNA integrity.

The phylogeographic relationships within, and between the two clades clearly illustrate historic rock hyrax dispersal along separate routes. The distribution of the south-central clade follows the Great Escarpment, while the northern clade is restricted to the northern mountain ranges, from the Magaliesberg in the south-western Transvaal to the Matopos in western Zimbabwe and the Lebombo mountains in the north-eastern Transvaal. Two evolutionary scenarios were proposed which could possibly account for these data. The northern clade may represent a southern extension of a currently recognized sister taxon *P. c. johnstoni* or, alternatively, it may have resulted from a relatively recent local event possibly mediated by fluctuations in global climatic patterns. Neither of the two hypotheses could be excluded and it was suggested that assessments of mtDNA affinities of the northern clade to *P. c. johnstoni* specimens from Malawi or Kenya would, in conjunction with the analysis of nuclear DNA variation and skull morphometrics, contribute significantly to resolving the taxonomic status of this clade.

The analysis of 692 nucleotides of the cytochrome b and 12S rRNA mtDNA genes indicated *D. arboreus* to be the most primitive representative of the extant Hyracoidea, while the northern *P. c. capensis* clade appeared, from the surveyed taxa, to be the most recently derived form. Both RFLP and sequencing data indicated a close phylogenetic relationship between *Heterohyrax* and *Procavia*, species with similar habitat preference and social structure. A high degree of congruence was evident between the different data sets (RFLP and sequencing), and phylogenetic trees generated using different philosophical approaches (phenetic and cladistic) yielded similar topologies. Additionally, the present study supported a close phylogenetic relationship between the Hyracoidea and the Proboscidea, and a common evolutionary origin for ungulate-related taxa.

OPSOMMING

Die diploïede chromosoomgetal (2n = 54) is identies in al die verteenwoordigers van die Suid-Afrikaanse *Procavia*-, *Heterohyrax*- en *Dendrohyrax*-spesies en dit dui waarskynlik op die voorouerlike toestand vir die orde. Uitgekose *Procavia* eksemplare, wat verteenwoordigend van twee onderskeibare mitochondriale DNA (mtDNA) groepe is, was kariotipies identies. Oortuigende G-band homologie kon waarskynlik weens swak bandresolusie nie vir die totale chromosoomkomplement van die drie genera vasgestel word nie, maar in etlike gevalle was duidelike homologie wel sigbaar. Die veranderinge wat vir die verskil in diploïede chromosoomgetal tussen die olifant (2n = 56) en die Hyracoidea (2n = 54) verantwoordelik was, kon nie geïdentifiseer word nie. In *Procavia* en *Heterohyrax* was die verspreiding van heterochromatien uitsluitlik sentromeries van aard. Daarteenoor is die groter hoeveelheid en unieke verspreiding van heterochromatien in *Dendrohyrax* waarskynlik 'n nuut-verworwe eienskap.

Restriksie fragment lengte polimorfismes (RFLP's) in mtDNA van 141 diere, wat verteenwoordigend van die drie dassiespesies is, is met 'n reeks van 10 restriksie-ensieme ontleed. Die Hyracoidea wat deur beperkte verspreidingsvermoë gekenmerk word, het sterk geografiese groepering van mtDNA haplotipes vertoon en groot filogenetiese afstande het verskeie mtDNA groeperings onderskei. In *H. brucei* en *D. arboreus* (verteenwordigend van die mees suidelike verspreiding van dié spesies in Afrika) was opvallende genetiese onderskeiding sigbaar; dit geld selfs tussen geografies nabygeleë bevolkings. In *H. brucei* stem die mtDNA filogeografie grotendeels met aanvaarde subspesie grense ooreen en dit is waarskynlik deur die beperkinge van habitatspesifisiteit en die invloed wat dit op geenvloei het bevorder. Dit is moontlik dat mtDNA-geenvloei in *D. arboreus* op 'n soortgelyke wyse deur die fragmentering van woudhabitat onderbreek word.

'n Opvallende genetiese breuk het 'n suid/sentrale- en noordelike-groep binne *P. c. capensis* onderskei. Die verhouding tussen haplotipes binne die twee afsonderlike groepe het 'n kontinue genetiese divergensiepatroon, wat as gevolg van onlangse kolonisering vanaf toevlugsbevolkings ontstaan het, vertoon. Dit is moontlik dat dié gebeure deur bevolkingsontploffings, wat aan die hoë voortplantingspotensiaal van die spesie onder gunstige omgewingstoestande toegeskryf kan word, teweeggebring is.

Die twee mtDNA-groepe binne *P. c. capensis* het na raming ongeveer twee miljoen jaar gelede vanuit 'n gesamentlike voorouer ontwikkel. Die suid/sentrale-groep is regdeur die Kaapprovinsie, Natal, die Oranje-Vrystaat en die Oos-Transvaal versprei, terwyl die noordelike-groep tot die noordelike OVS, wes-, sentraal- en noordoos-Transvaal en die westelike deel van Zimbabwe beperk is. Daar word voorgestel dat die gebrek aan geskikte habitat in Wes-Transvaal, noordoos-Kaapland en die noordwes OVS bydra tot die handhawing van die twee groepe in dié gebiede, maar soortgelyke ekstrinsieke skeidings is nie in ander dele van die kontaksone waargeneem nie. Hierdie waarneming is deur GIS analise van reliëf in twee afsonderlike gebiede van die kontaksone tussen die twee groepe

bevestig. Daarbenewens word geïmpliseer dat huidige klimaatsverskille tot die handhawing van die mtDNA integriteit van die twee groepe bydra in gebiede waar geskikte habitat die kontaksone oorbrug.

Die filogeografiese verwantskappe binne- en tussen die twee groepe dui op geskiedkundige dassieverspreiding langs afsonderlike roetes. Die verspreiding van die suid/sentrale-groep volg die Eskarpement, terwyl die noordelike groep tot die noordelike bergreekse beperk is en vanaf die Magaliesberge in die suidweste tot by die Matopos in die weste van Zimbabwe en die Lebomboberge in noordoos-Transvaal strek. Twee moontlike evolusionêre verklarings word aangevoer. Dit is moontlik dat die noordelike-groep 'n suidelike uitbreiding van die sustertakson *P. c. johnstoni* verteenwoordig, of alternatiewelik kan dit die resultaat van relatief onlangse plaaslike genetiese skeiding, wat deur die wisseling in wêreldwye klimaatspatrone teweeggebring is, wees. Nie een van die twee moontlikhede kon uitgesluit word nie en daar word voorgestel dat bepaling van die mtDNA verwantskap tussen die noordelike-*P. c. capensis*-groep en *P. c. johnstoni* afkomstig van Malawi of Kenia, tesame met 'n analise van nukluêre DNA variasie en skedelmorfometrie, 'n beduidende bydra sal lewer tot die opklaring van die taksonomiese status van die noordelike-*P. c. capensis*-groep.

Die ontleding van 692 nukleotiede van die sitochroom b en 12S rRNA mtDNA gene dui daarop dat *D. arboreus* die mees primitiewe- en die noordelike *P. c. capensis* die mees onlangs verworwe vorme binne die Hyracoidea is. Beide RFLP- en nukleotiedopeenvolgingsdata dui aan dat *Heterohyrax* en *Procavia*, spesies met soortgelyke habitatsvoorkeur en sosiale struktuur, filogeneties naverwant is. 'n Hoë vlak van ooreenstemming kom duidelik tussen die verskillende datastelle (RFLP en nukleotiedopeenvolging), asook tussen filogenetise stambome wat o.g.v. verskillende filosofiese benaderings (feneties en kladisties) geskep is, na vore. Daarbenewens ondersteun die huidige studie 'n naverwante filogenetiese verhouding tussen die Hyracoidea en Proboscidea asook 'n gesamentlike evolusionêre oorsprong van ungulaat-verwante groepe.

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APPENDIX I

GAZETEER

LOCALITY	COORDINATES
Alexandria State Forest	33°44'S 26°22'E
Bloemfontein (Naval Hill)	29°06'S 26°14'E
Blouberg	23°07'S 28°56'E
Blyde River - Belvedere - Burkes' Luck - Lowveld Lookout	24°42'S 30°54'E 24°40'S 30°49'E 24°36'S 30°48'E
De Hoop Nature Reserve	34°28'S 20°27'E
East London (Beacon Bay)	32°58'S 27°58'E
Ermelo (Farm Spitzkop)	26°27'S 29°55'E
Gravelotte (Farm Blackhills)	23°50'S 30°52'E
Haifa (Israel)	32°30'N 35°00'E
Howick (Farm Goodhope)	29°26'S 30°05'E
Karoo National Park	32°21'S 22°35'E
Kuruman - Farm Woodstock - Farm Witberg	27°35'S 23°26'E 27°12'S 22°30'E
Letaba (KNP - Masorini, Sheswani, Shikumbu, Shilawuri)	23°51'S 31°20'E
Lydenburg (Farm Smutskuilen)	25°08'S 30°35'E
Maqili (KNP)	25°26'S 31°19'E
Marken (Farm Hottentots-Holland)	23°40'S 28°20'E
Matopos (Zimbabwe)	20°34'S 28°30'E
Mica (Farm Trucking)	24°10'S 30°48'E
Middelburg (Cycad Hiking Trail)	25°45'S 29°30'E
Munnik	23°34'S 29°45'E
Ohrigstad	24°45'S 30°32'E
Pafuri (KNP)	22°26'S 31°17'E
Pilgrims' Rest (TGME)	24°52'S 30°47'E
Pirie Forest (Ciskei)	32°45'S 27°17'E
Potgietersrust (Farm Bellevue)	23°55'S 28°44'E

Continued/

LOCALITY	COORDINATES
Rustenburg Nature Reserve	25°43'S 27°11'E
Shilowa (KNP)	23°30'S 31°35'E
Soutpansberg (Buzzard Mountain)	23°02'S 29°44'E
Springbok	29°40'S 17°53'E
Suikerbosrand Nature Reserve	26°30'S 28°15'E
Vhembe Nature Reserve	22°12'S 29°20'E
Volksrust (Farm Waterval)	27°24'S 29°45'E
Vredefort (Farm Mooihoek)	27°07'S 27°15'E
Zebediela (Farm Buffelshoek)	24°12'S 29°10'S

Abbreviations : KNP - Kruger National Park

TGME - Transvaal Gold Mining Estates

APPENDIX II

Procavia capensis capensis

LOCALITY	MUSEUM ACCESSION NUMBERS
Blackhills	TM 42 327 - 42 329
Bloemfontein	TM 41 700 - 41 705
Blouberg	TM 42 326
Blyde River	TM 42 656 - 42 657
Buzzard Mt	TM 42 315
East London	KM 32 024
Ermelo	TM 43 200
Howick	TM 41 699
Karoo N.P.	TM 41 667
Kuruman	TM 41 706 - 41 714
Lydenburg	TM 42 478
Marken	TM 42 312
Matopos	Not yet available
Mica	TM 42 479 - 42 484
Middelburg	TM 42 474 - 42 477
Munnik	TM 42 306 - 42 309
Pilgrims' Rest	TM 43 772 - 43 775
Potgietersrus	TM 42 313 - 42 314
Rustenburg	TM 41 285 - 41 287
Suikerbosrand	TM 42 991 - 42 995
Vhembe	TM 41 288 - 41 292, 42 319, 42 324, 42 325
Volksrust	TM 42 436 - 42 438
Vredefort	TM 42 880

Continued/

Procavia capensis syriaca

LOCALITY	MUSEUM ACCESSION NUMBERS
Haifa	TM 41 995 - 41 996

Heterohyrax brucei

LOCALITY	MUSEUM ACCESSION NUMBERS
Blyde River	TM 43 776
Buffelshoek	TM 42 317 - 42 318
Buzzard Mt	TM 42 316
Matopos	Not yet available
Munnik	TM 42 310
Pafuri (KNP)	TM 42 357 - 42 359
Vhembe	TM 41 293 - 41 297, 42 319 - 42 323

Dendrohyrax arboreus

LOCALITY	MUSEUM ACCESSION NUMBERS
Pirie Forest	KM 32 022 - 32 033

ABBREVIATIONS: KM - Kaffrarian Museum, King Williams Town

TM - Transvaal Museum, Pretoria

APPENDIX III

CATALOGUE OF TISSUES, SKINS AND SKULLS FROM SAMPLE SPECIMENS

Specimen number *	Locality	Sex	H	L	O	U	I	E**
Pc 3.6	Vhembe	F	X	X	X	X	-	-
Pc 3.7	Vhembe	F	X	X	-	-	-	X
Pc 3.8	Vhembe	M	X	X	-	-	-	X
Pc 3.9	Vhembe	F	X	X	-	X	-	-
Pc 3.10	Vhembe	M	X	X	-	X	X	-
Pc 4.3	Pilgrims' Rest	F	X	X	-	X	-	X
Pc 4.4	Pilgrims' Rest	F	X	X	-	X	-	X
Pc 5.1	Karoo N.P.	F	X	X	-	-	-	X
Pc 5.3	Karoo N.P.	M	-	X	-	X	-	X
Pc 5.4	Karoo N.P.	F	-	X	-	-	-	X
Pc 5.7	Karoo N.P.	F	X	X	-	-	-	X
Pc 5.9	Karoo N.P.	F	-	X	-	-	-	-
Pc 5.11	Karoo N.P.	F	X	X	-	-	-	-
Pc 5.13	Karoo N.P.	F	-	X	-	-	-	-
Pc 5.14	Karoo N.P.	M	-	X	-	-	-	-
Pc 5.15	Karoo N.P.	M	X	X	-	-	-	X
Pc 5.16	Karoo N.P.	M	X	X	-	-	-	X
Pc 7.1	Howick	F	X	X	-	-	-	X
Pc 7.2	Howick	F	X	X	-	-	-	X
Pc 7.3	Howick	M	X	X	X	-	-	X
Pc 7.4	Howick	M	X	X	X	-	-	X
Pc 7.5	Howick	M	X	X	-	-	-	X
Pc 7.6	Howick	F	X	X	X	X	-	X
Pc 7.7	Howick	M	X	X	X	-	-	X
Pc 7.8	Howick	M	X	X	X	-	-	X
Pc 8.1	East London	M	X	X	-	X	X	-

Continued/

Specimen number *	Locality	Sex	H	L	O	U	I	E**
Pc 9.1	Bloemfontein	M	X	X	-	X	-	X
Pc 9.2	Bloemfontein	F	X	X	-	-	-	X
Pc 9.3	Bloemfontein	F	X	X	-	X	X	-
Pc 9.4	Bloemfontein	F	X	X	-	X	-	X
Pc 9.5	Bloemfontein	M	X	X	-	X	-	-
Pc 9.6	Bloemfontein	F	X	X	-	X	-	-
Pc 9.7	Bloemfontein	M	X	X	-	X	-	X
Pc 9.8	Bloemfontein	M	X	X	-	X	-	-
Pc 10.1	Kuruman (a)	F	X	X	-	X	-	X
Pc 10.2	Kuruman (a)	M	X	X	-	X	-	X
Pc 10.3	Kuruman (a)	F	X	X	X	-	-	-
Pc 10.4	Kuruman (a)	M	X	X	X	-	-	X
Pc 10.5	Kuruman (b)	F	X	X	-	X	-	X
Pc 10.6	Kuruman (b)	F	X	X	X	X	-	-
Pc 10.7	Kuruman (b)	F	X	X	-	X	-	-
Pc 10.8	Kuruman (b)	F	X	X	-	-	-	-
Pc 10.9	Kuruman (b)	F	X	X	X	X	-	-
Pc 10.10	Kuruman (b)	M	X	X	-	X	-	-
Pc 10.11	Kuruman (b)	M	X	X	-	-	-	-
Pc 11.1	Munnik	M	X	X	X	X	-	X
Pc 11.2	Munnik	F	X	X	-	X	-	x
Pc 11.3	Munnik	F	X	X	-	X	X	x
Pc 11.4	Munnik	F	X	X	-	X	-	X
Pc 11.5	Munnik	F	-	X	-	-	-	x
Pc 11.7	Munnik	F	X	X	-	X	-	-
Pc 12.1	KNP Sheswani	F	X	X	-	X	-	X
Pc 12.2	KNP Masorini	M	X	X	X	-	-	-
Pc 12.3	KNP Shikumbu	F	X	X	X	X	-	X

Continued/

Specimen number *	Locality	Sex	H	L	O	U	I	E**
Pc 12.4	KNP Shilawuri	M	X	X	X	X	X	-
Pc 12.5	KNP Maqili	F	X	X	X	X	X	-
Pc 12.6	KNP Maqili	F	X	X	-	-	-	X
Pc 13.1	Marken	F	X	X	-	X	-	-
Pc 14.1	Potgietersrus	M	X	X	-	X	-	-
Pc 14.2	Potgietersrus	F	X	X	-	X	-	-
Pc 15.1	Soutpansberg	M	X	X	X	X	-	-
Pc 16.1	Blouberg	F	X	X	-	X	X	-
Pc 17.1	Gravelotte	M	X	X	-	X	-	-
Pc 17.2	Gravelotte	F	X	X	-	X	X	-
Pc 17.3	Gravelotte	F	X	X	-	X	-	-
Pc 17.4	Gravelotte	M	X	X	-	-	-	-
Pc 18.1	Volksrust	M	X	X	X	X	-	-
Pc 18.2	Volksrust	F	X	X	X	X	-	x
Pc 18.3	Volksrust	F	X	X	-	X	-	-
Pc 18.4	Volksrust	F	X	X	-	-	-	-
Pc 19.1	Middelburg	M	X	X	-	X	-	-
Pc 19.2	Middelburg	M	X	X	-	X	-	-
Pc 19.3	Middelburg	M	X	X	-	X	-	-
Pc 19.4	Middelburg	F	X	X	-	X	-	-
Pc 19.5	Middelburg	M	X	X	-	-	-	-
Pc 20.1	Lydenburg	F	X	X	-	X	X	-
Pc 22.1	Mica	F	X	X	-	X	X	x
Pc 22.2	Mica	M	X	X	-	X	-	-
Pc 22.3	Mica	F	X	X	-	X	-	x
Pc 22.4	Mica	F	X	X	-	X	-	-
Pc 22.5	Mica	F	X	X	-	X	-	-
Pc 23.1	KNP Shilowa	F	X	X	-	X	-	x

Continued/

Specimen number *	Locality	Sex	H	L	O	U	I	E**
Pc 23.2	KNP Shilowa	F	X	X	-	-	-	-
Pc 24.1	Blyde River	M	X	X	X	X	X	-
Pc 24.2	Blyde River	F	X	X	-	X	-	-
Pc 25.1	Vredefort	M	X	X	-	-	-	-
Pc 25.2	Vredefort	F	X	X	-	-	-	-
Pc 25.3	Vredefort	F	X	X	-	X	X	-
Pc 25.4	Vredefort	F	X	X	X	-	-	-
Pc 26.1	Suikerbosrand	M	X	X	-	X	X	-
Pc 26.2	Suikerbosrand	F	X	X	-	X	X	-
Pc 26.3	Suikerbosrand	M	X	X	-	-	-	-
Pc 26.4	Suikerbosrand	F	X	X	-	X	X	-
Pc 26.5	Suikerbosrand	F	X	X	-	X	X	-
Pc 27.1	Ermelo	M	X	X	-	-	-	-
Pc 27.2	Ermelo	M	X	X	-	X	X	-
Pc 27.3	Ermelo	F	X	X	-	-	-	-
Pc 27.4	Ermelo	M	X	X	-	-	-	-
Ps 1	Haifa	F	X	X	-	X	X	X
Ps 2	Haifa	M	X	X	-	X	X	X
Hb 1.7	Vhembe	M	X	X	X	X	-	-
Hb 1.10	Vhembe	M	X	X	-	X	X	-
Hb 2.2	Blyde River	F	X	X	-	X	-	x
Hb 2.3	Blyde River	M	X	X	-	-	-	X
Hb 3.1	Munnik	F	X	X	-	X	-	-
Hb 4.1	Zebediela	F	X	X	-	X	-	-
Hb 4.2	Zebediela	M	X	X	-	X	X	-
Hb 5.1	Soutpansberg	F	X	X	X	X	-	-
Hb 7.1	Ohrigstad	M	X	X	-	-	-	-

Continued/

Specimen number *	Locality	Sex	H	L	O	U	I	E**
Da 1.1	Pirie Forest	F	X	X	X	X	X	X
Da 1.2	Pirie Forest	M	X	X	X	X	X	X

* - Pc - *Procavia capensis capensis*, Ps - *P. c. syriaca*, Hb - *Heterohyrax brucei*, Da - *Dendrohyrax arboreus*. These numbers are collection numbers only and do not correspond to locality designations used in the thesis. Specimen records for the following localities are not available or incomplete: Pc 1 - De Hoop Nature Reserve, Pc 2 - Rustenburg Nature Reserve, Pc 3 - Vhembe, Pc 4 - Pilgrims' Rest, Pc 6 - Springbok, Pc 28 - Matopos, Hb 1 - Vhembe, Hb 6 - KNP Pafuri, Hb 8 - Matopos, Da 2 - Alexandria State Forest.

** - H - heart, L - liver, O - other soft tissue (spleen, kidney, muscle), U- skull, I - skin, E - ear biopsy, X - collected, x -collected from fetus, "-" - not collected.

- other abbreviations: Kuruman (a) - Farm Woodstock, Kuruman (b) - Farm Witberg, KNP - Kruger National Park, F - Female, M - Male.

APPENDIX IV

RESTRICTION FRAGMENT SIZES

Restriction enzyme	Type	Fragment sizes (kb)
*Ava*I	a	9.1 5.05 2.35
	b	9.1 3.7 2.35 1.35
	c	7.5 5.05 2.35 2.05
	d	9.1 5.05 1.57 0.82
	e	6.8 5.05 2.85 2.35
	f	5.4 5.05 3.7 2.35
	g	8.6 4.95 1.57 0.82 0.56
*Bam*HI	a	9.0 3.4 1.8 1.16 0.83
	b	9.0 6.7 0.83
	c	9.0 4.2 3.4
	d	9.0 3.4 3.27 0.83
	e	14.5 1.8
	f	9.0 7.4
	g	9.8 3.4 3.27
	h	9.0 3.2 1.8 1.16 0.83 0.2
	i	13.5 1.8 1.0
	j	9.0 3.9 3.7
*Dra*I	a	14.8 1.8
	b	8.3 6.5 1.8
	c	10.65 4.15 1.8
	d	13.8 1.8 1.0
	e	7.7 4.1 1.88 1.8 1.13
	f	6.5 5.1 3.2 1.8
	g	7.5 3.9 1.88 1.8 1.13 0.4
	h	12.3 2.5 1.8

Continued/

Restriction enzyme	Type	Fragment sizes (kb)
*Hinc*II	a	9.6 3.65 3.4
	b	5.3 4.85 3.27 2.65
	c	7.0 3.65 3.4 3.05
	d	12.5 2.35 1.08 0.7
	e	11.0 3.65 1.85
	f	7.0 3.65 3.4 2.52 0.5
	g	9.6 3.65 2.35 1.08
	h	10.6 3.65 2.35
	i	5.3 3.05 2.65 2.35 2.2 0.9
	j	9.6 3.65 2.35 0.7 0.58
	k	6.4 3.65 3.0 2.35 1.17
	l	5.7 3.65 3.4 3.05 1.3
	m	10.15 3.27 2.65
	n	4.9 2.9 2.65 2.35 2.2 0.9 0.5
	o	3.9 3.65 3.05 1.5 1.3 1.17 1.08 0.9
*Hind*III	a	8.6 2.7 2.4 2.0 0.5
	b	8.6 2.93 2.7 2.4
	c	9.1 2.7 2.4 2.0
	d	9.1 7.5
	e	10.98 2.7 2.4
	f	9.1 6.3 1.1
	g	10.5 6.0
	h	14.2 2.4
	i	16.5
	j	8.27 2.7 2.4 2.0 0.8
	k	11.9 2.7 2.0
	l	8.6 5.3 2.7
	m	13.9 2.7
	n	8.27 2.7 2.0 1.65 0.93 0.83

Continued/

Restriction enzyme	Type	Fragment sizes (kb)
*Hind*III	o	10.25 5.75 0.5
*Pvu*II	a	16.5
	b	10.5 6.0
	c	7.0 3.25 2.8 2.6 0.8
	d	7.0 6.0 3.25
	e	13.5 3.0
*Sac*I	a	13.5 2.9
	b	9.4 6.9
	c	11.0 2.9 2.48
	d	9.4 3.9 3.0
	e	16.5
	f	10.0 6.5
*Sca*I	a	5.6 3.2 3.1 2.2 1.2 0.75 0.65
	b	6.5 4.0 3.0 1.15 0.88 0.51
	c	6.2 2.5 2.42 1.78 1.35 1.15 0.88 0.51
	d	6.2 5.3 2.2 1.78 1.2
	e	4.6 2.9 1.78 1.52 1.2 1.1 0.97 0.88 0.8 0.75
	f	3.3 3.0 2.5 2.42 1.78 1.35 1.15 0.88 0.51
	g	6.2 5.3 2.2 1.2 1.1 0.75
	i	6.2 5.3 3.4 1.2 0.65
	j	6.2 5.3 2.2 1.2 1.0 0.75 0.1
	k	6.5 5.0 3.0 2.1
	l	6.0 4.0 3.0 1.25 0.88 0.51 0.42
	m	6.0 3.2 2.9 2.2 1.1 0.65 0.42 0.13 0.1
	n	6.0 3.2 2.9 2.2 1.2 0.65 0.42 0.13
	o	4.48 2.9 1.78 1.52 1.2 1.1 0.97 0.88 0.8 0.75 0.13
	p	6.5 3.3 3.2 1.5 1.35 0.88

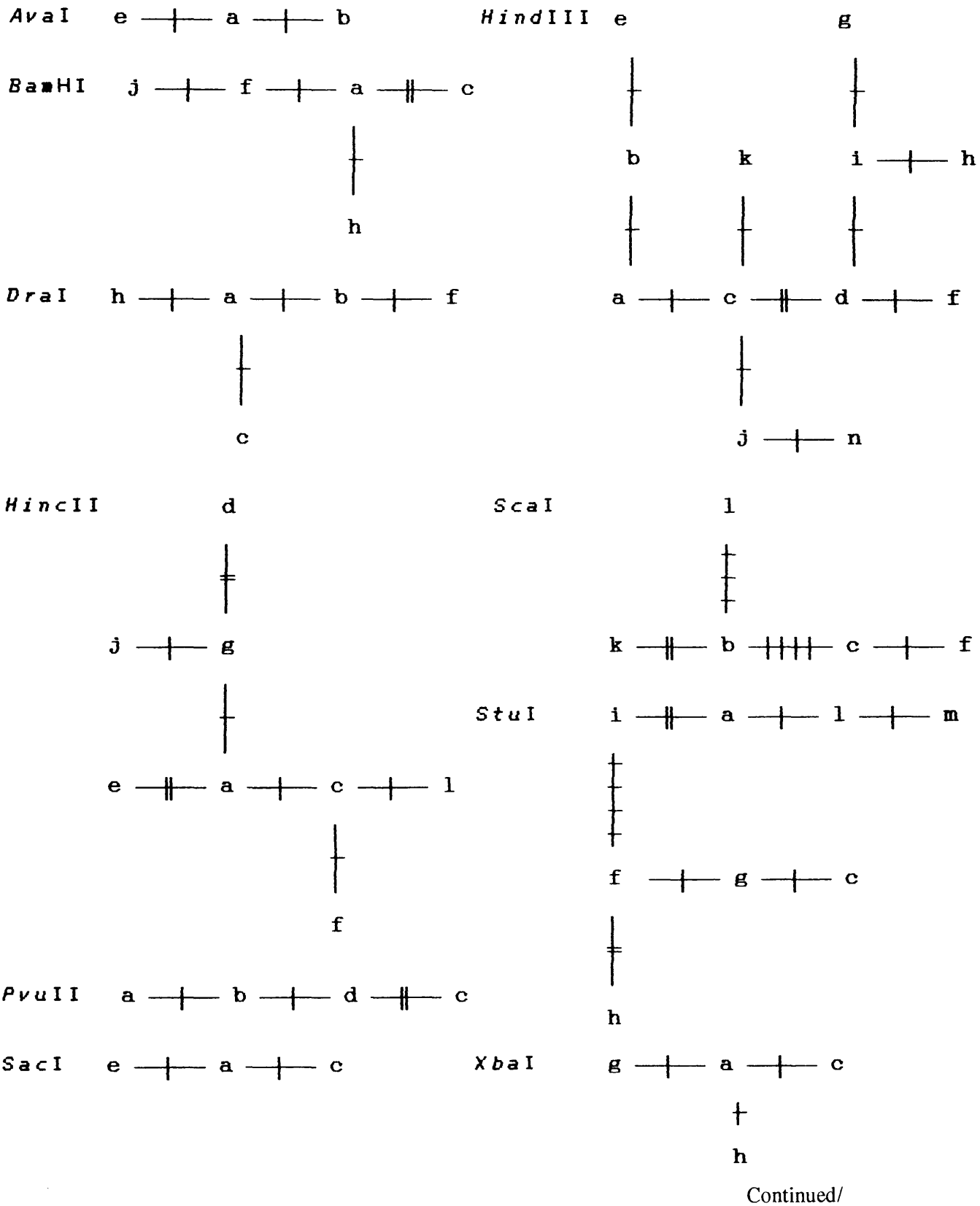
Continued/

Restriction enzyme	Type	Fragment sizes (kb)
*Stu*I	a	3.78 3.35 3.25 1.87 1.56 1.5 0.56 0.33
	b	5.1 4.7 4.1 1.56 1.56
	c	3.85 3.35 3.25 1.87 1.56 1.42 0.91 0.07
	d	7.2 3.5 2.5 1.56 1.56
	e	7.4 5.4 3.35
	f	7.2 3.25 1.87 1.56 1.42 0.91
	g	3.85 3.42 3.25 1.87 1.56 1.42 0.91
	h	4.5 3.68 3.25 1.87 1.56 1.42
	i	5.1 3.78 3.25 1.87 1.56 0.56
	j	8.0 5.1 3.25 0.56
	k	9.8 4.1 1.56 1.56
	l	3.78 3.42 3.25 1.87 1.56 1.5 0.56 0.33 0.16 1.0
	m	3.78 3.42 3.35 1.87 1.56 1.5 0.56 0.33 0.16
	n	8.4 3.25 2.4 1.42 0.7
	o	3.85 3.78 2.3 2.2 2.0 1.56 0.88
	p	9.1 3.25 2.4 1.42
*Xba*I	a	9.29 4.9 1.22 1.17
	b	9.29 6.0 1.22
	c	9.29 2.55 2.32 1.22 1.17
	d	9.58 5.8 1.17
	e	16.5
	f	15.2 1.4
	g	10.51 4.9 1.17

APPENDIX V

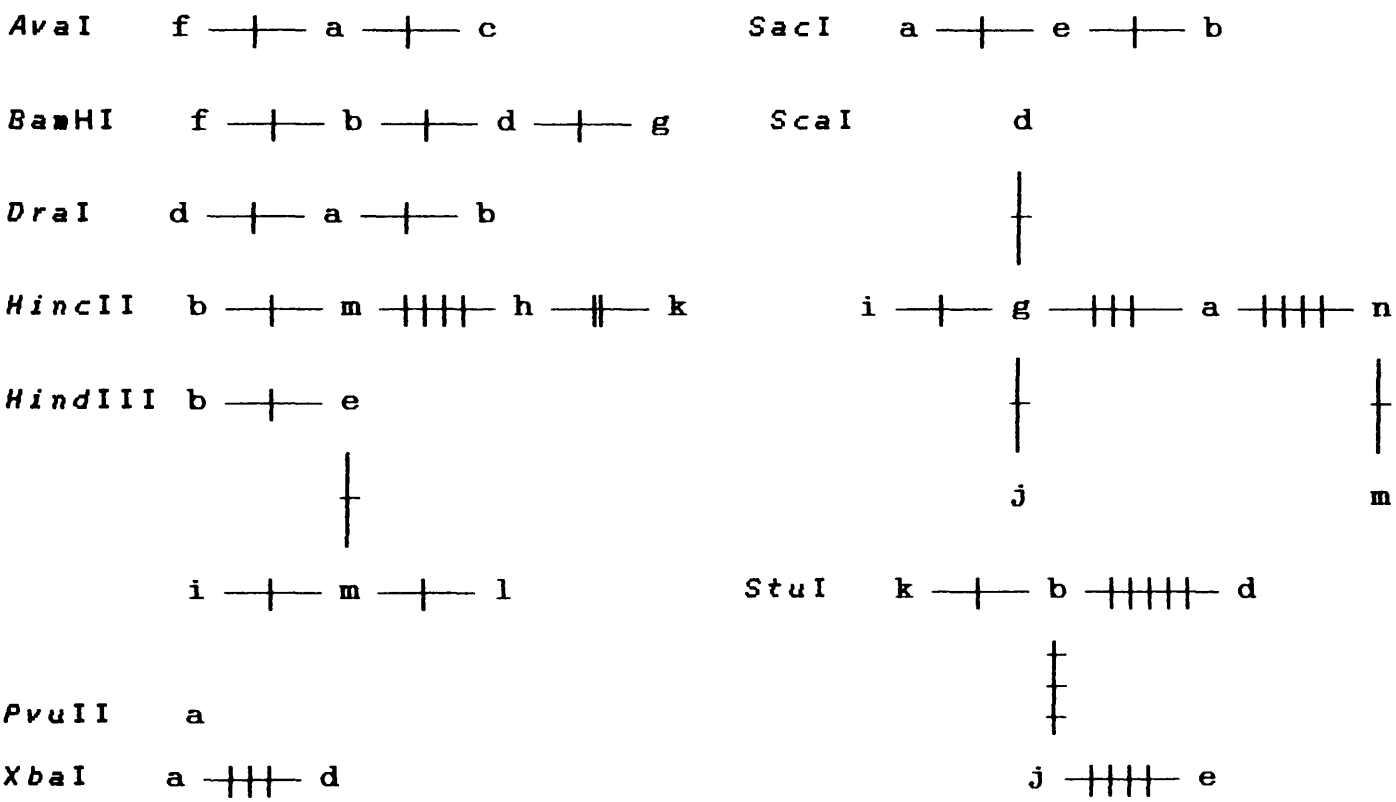
SCHEMATIC REPRESENTATION OF THE MINIMUM NUMBER OF MUTATIONAL STEPS BETWEEN RESTRICTION FRAGMENT PATTERNS PRODUCED BY DIGESTION WITH 10 RESTRICTION ENDONUCLEASES FOR THREE SOUTH AFRICAN HYRAX SPECIES.

Procavia capensis capensis

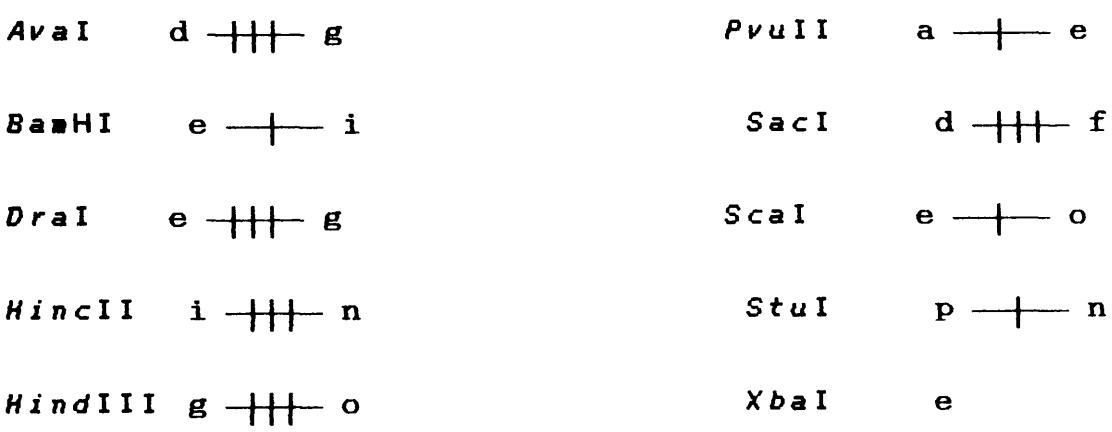


Continued/

Heterohyrax brucei



Dendrohyrax arboreus



APPENDIX VI

AMINO ACID SEQUENCES OF A 362 BP REGION OF THE MTDNA CYTOCHROME B GENE FOR FIVE REPRESENTATIVES OF THE HYRACOIDEA AND FIVE OUTGROUP SPECIES.

```
HOMO          CPLMKLINHSFIDLPTPSNISAWWNFGSLLGACLILGITTGLFLAMHYSP
DICEROS       HPLIKIINHSFIDLPTPSNISAWWNFGSLLGICLILGILTGLFLAMHYTP
LOXODONTA     HPLLKIINKSFIDLPTPSNMSTWWNFGSLLGACLITGILTGLFLAMHYTP
ORYCTEROP     HPLIKIINHSFIDLPTPSNISAWWNFGSLLGICLIIGIVTGLFLAMHYTS
EQUUS         HPLIKIINHSFTDLPTPSNISSWWNFGSLLGICLILGILTGLFLAILPSR
PROCSOUTH     HPLLKTINDAFIDLPTPSNISTWWNFGSLLGACLIIGILTGLFLAMHYTS
PROCNORTH     YPLLRTINDAFIDLPTPSNISAWWNFGSLLGACLIIGILTGLFLAMHYTS
PROCSYR       HPLFRTINEVFIDLPTMSKIQAWWMLGSLLGACLMIGILTGLFLALHYTP
HETEROHYR     HPLMKTINDANIDLPTPPNISTWWNFGSLLGACLIIGILTGLFLAMHYTS
DENDROHYR     LPLFKVMNNVFIDLPTPSNISAWWNLGSLLGACLIIGILTGLFLALHYTP
               ** . .*    **** ... .** .***** **. ** ******.  .

HOMO          DASTAFSSIAHITRDVNYGWIIRYLHANGASMFFICLFLHIGRGLYYGSF
DICEROS       DTTTAFSSVAHICREVNYGWIIRYLHANGASMFFICLFIHMGRGLYYGSY
LOXODONTA     DTMTAFSSMSHICRDVNYGWIIRGLHSNGASIFFLCLYTHIGRNIYYGSY
ORYCTEROP     DTSTAFSSVTHICRDVNYGWLIRYLHANGASMFFICLFIHIGRGIYYGSY
EQUUS         AATTAFSSVTHICRDVNYGWIIGYLHANGASMFFICLFIHVGRGLYYGSY
PROCSOUTH     DTMTAFASVTHICRDVNHGWVIRYLHANGASLFFVCLYAHIGRGIYYGSY
PROCNORTH     DIMTAFASVTHICRDVNHGWIIRYLHANGASLFFMCLYAHIGRGIYYGSY
PROCSYR       DTMTAFASVTHVCREVNHGWIIWYLHANGASLFFVCLCAHIGRGIYYGSY
HETEROHYR     DTTTAFASVTHICRDVNYGWIIRYHHAIGASMFFMCLYAHIGRGIYYGSY
DENDROHYR     VTTAAFSSVTRVCRAVNYGWAVCCLHANGASVFFICLYARNGRRIHYDSY
                 .**.*.... * ** ** .   *. ***.**.**  . ** . *.*.

HOMO          LYSETWNIGIILLLATMATAF
DICEROS       TFLKTWNIGVILLLTVMATAF
LOXODONTA     LYSETWNIGIMLLLITMATAF
ORYCTEROP     LYSETWNIGIILLFTTMATAF
EQUUS         TFLETWNIGIILLFTVMATAF
PROCSOUTH     LYSETWNIGVLLLLTTMATAF
PROCNORTH     LYSETWNIGVLLLLTTMATAF
PROCSYR       LYPETWNSGVLLLLTTMATAF
HETEROHYR     LYSETWNIGVLLLLTTMATAF
DENDROHYR     LYLETWNSGILLVLATMATAF
               .  *** *..*..  *****
```

* - 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 VII

INTRASPECIFIC VARIATION OF 180 BP REGION OF THE MTDNA CYTOCHROME B GENE

H. brucei

```
ACTCCGATGTTTCAGGTTTCTGAGTATAGGTAAGATCCGTAGTAGATACC
ACTCCAATGTTTCAGGTTTCTAGGTATAGGTATGAGCCGTAGTAAATGCC
***** ***************  ********* ** ******** ** **

TCGTCCAATATGAGCGTAGAGGCATATGAAGAATATGGATGCTCCGATGG
TCGTCCAATGTGGGCGTACAGGCATATGAAGAATACTGATGCTCCGTTGG
********* ** ***** ****************  ********* ***

CGTGATGATATCGGATTATTCAGCCGTAGTTTACGTCTCGGCAGATGTGG
CATGGAGATATCGGATTGTTCAGCCATAGTTTACATCTCGGCAAACGTGA
* **  *********** ******* ******** ******** * ***

GTTACTGACGCGAATGCGGTTGTTGTGTCTGAT
GTTACTGACGCGAATGCGGTTGTTGTATCTGAT
************************** ******
```

P. c. capensis (northern clade)

```
ACTCCGATGTTTCAAGTTTCTGAGTACAGGTAGGATCCGTAGTAAATACC
ACTCCGATGTTTCAAGTTTCTGAGTACAGGTAGGATCCGTAGTAAATACC
**************************************************

TCGTCCGATGTGGGCATATAAACATATGAAGAATAGGGATGCTCCGTTGG
TCGTCCGATGTGGGCATATAAACATATGAAGAATAGGGATGCTCCGTTGG
**************************************************

CATGCAGGATATCGGATTATCCATCCATGGTTTACGTCTCGACAAATGTG
CATGCAGGATATCGGATTATCCACCCATGGTTTACCTCTCGACAAATGTG
********************** ********** **************

GGTTACTGATGCGAATGCAGCTTATC
GGTTACTGATGCGAATGCAGCTTATC
*************************
```

P. c. capensis (south/central clade)

```
CTATTGGTTGTTAGTAAGAGTAAAACTCCGATGTTTCAAGTTTCTGAATA
CTATTGGTTGTTAGTAAGAGTAAAACTCCAATGTTTCAAGTTTCTGAGTA
CTATTGGTTGTTAATAAGAGTAAAACTCCAATGTTTCAAGTTTCTGAATA
************* *************** ***************** **

CAGATAGGATCCGTAGTAAATACCTCGTCCGATGTGGGCATATAAGCAAA
TAGGTAGGATCCGTAGTAAATACCTCGTCCGATGTGGGCATATAAGCAAA
CAGGTAGGATCCGTAGTAAATACCTCGTCCGATGTGGGCATATAAGCAAA
 ** **********************************************

CGAAGAATAGGGATGCTCCATTGGCATGTAGGATATCGGATTACCCACCC
CGAAGAATAGGGATGCTCCGTTGGCATGTAGGATATCGGATTACCCATCC
CGAAGAATAGGGATGCTCCATTGGCCTGTAGGTTATCGGATTACCCACCC
******************* ***** ****** ************** **

ATGGTTCACGTCTCGGCAAATGTGGGTTACTGATGCGA
ATGGTTTACGTCTCGGCAAACGTGGGTTACTGACGCGA
ATGGTTCACGTCTCGGCAAATGTGGGTTACTGACGCGA
****** ************* ************ ****
```