PHYLOGEOGRAPHY OF THREE SOUTHERN AFRICAN ENDEMIC ELEPHANT-SHREWS AND A SUPERMATRIX APPROACH TO THE MACROSCELIDEA

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DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification. The experimental work has been conducted in the Department of Botany and Zoology, University of Stellenbosch. Specimens were collected under permits issued by Northern Cape Nature (Permit No. 0938/05), Cape Nature (Permit No. 373/2003), Department of Economic Affairs, Environment and Tourism (No. WRO 43/03WR and WRO 13/03WR) and South African National Parks.

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ABSTRACT

The order Macroscelidea has a strict African distribution and consists of two extant subfamilies, Rhynchocyoninae with a single genus that includes three species, and the Macroscelidinae represented by the remaining three genera, *Elephantulus* that includes 10 species, and the monotypic *Macroscelides* and *Petrodromus*. On the basis of molecular, cytogenetic and morphological evidence, *Elephantulus edwardii* (Cape rock elephant-shrew), the only strictly South African endemic species, was shown to comprise two closely related taxa. A new *Elephantulus* taxon, described here is reported for the first time. It has a restricted distribution in the central Nama Karoo of South Africa. Apart from important genetic distinctions, *Elephantulus sp. nov.* has several relatively subtle morphological characters that separate it from *E. edwardii*.

Molecular sequences from the mitochondrial cytochrome *b* gene and the control region of *E. edwardii sensu stricto* suggests the presence of a northern Namaqua and central Fynbos clade with four evolutionary lineages identified within the latter. The geographic delimitation of the northern and central clades corresponds closely with patterns reported for other rock dwelling vertebrate species indicating a shared biogeographic history for saxicolous taxa in South Africa. *Elephantulus rupestris* (western rock elephant-shrew) and *Macroscelides proboscideus* (round-eared elephant-shrew) are two taxa with largely overlapping distributions that span the semi-arid regions of South Africa and Namibia. Based on mitochondrial DNA sequence data *E. rupestris* has a structured genetic profile associated with a habitat of rocky outcrops compared to *M. proboscideus* that inhabits gravel plains, where the pattern is one of isolation-by–distance. Chromosomal changes, apart from heterochromatic differences, are limited to variation in diploid number among elephant-shrew species. These range from 2n=26 (*E. edwardii; E. rupestris*; *Elephantulus sp. nov.; E. intufi; E. brachyrhynchus* and *M. proboscideus*) to 2n=28 in both *Petrodromus tetradactylus* and *E. rozeti* to 2n=30 in *E. myurus*. Cross-species chromosome painting (Zoo-Fluorescence *in situ* hybridization or zoo-FISH) of *E. edwardii* flow-sorted probes that correspond to the five smaller sized autosomes (8-12) and the X chromosome showed no evidence of synteny disruption among *Elephantulus sp. nov.*, *E. intufi*, *E. myurus*, *P. tetradactylus* and *M. proboscideus*, and reinforced the G-banding observations underscoring the conservative karyotypes in these species.

A comprehensive phylogeny including all described elephant-shrew species is presented for the first time. A multigene supermatrix that included 3905 bp from three mitochondrial (12S rRNA, valine tRNA, 16S rRNA) and two nuclear segments (Von Willebrand factor [vWF] and exon 1 of the interphotoreceptor retinoid binding protein [IRBP]) was analysed. Cytogenetic characters, previously described morphological, anatomical and dental features as well as allozyme data and penis morphology were evaluated and mapped to the molecular topology. The molecular findings did not support a monophyletic origin for the genus *Elephantulus* and suggests that both the monotypic *Petrodromus* and *Macroscelides* should be included in *Elephantulus*. Molecular dating suggests that an arid-adapted Macroscelidinae lineage dispersed from east Africa at ~11.5 million years ago via the African arid corridor to south-western Africa. Subsequent speciation events within the Macroscelidinae are coincidental with three major periods of aridification of the African continent.

OPSOMMING

Die orde Macroscelidea het 'n verspreiding beperk tot Afrika en sluit twee bestaande subfamilies in, die Rhynchocyoninae wat drie spesies binne 'n enkele genus insluit en die Macroscelidinae verteenwoordig deur drie genera, *Elephantulus* (10 spesies) en die monotipiese *Macroscelides* en *Petrodromus*. Gebaseer op molekulêre, sitogenetiese en morfologiese bewyse, bestaan *E. edwardii*, tot op datum die enigste streng endemiese Suid-Afrikaanse klaasneusspesie, uit twee nabyverwante taksa. Die nuwe *Elephantulus* takson, hierin beskryf, het 'n beperkte verspreiding in die sentraal Nama Karoo van Suid-Afrika. Afgesien van belangrike genetiese bewyse wat die beskrywing van die nuwe spesie ondersteun, word *Elephantulus sp. nov.* gekenmerk deur 'n aantal subtiele morfologiese karakters wat dit onderskei van *E. edwardii*.

Binne *E. edwardii sensu stricto*, het mitochondriale molekulêre volgordes beduidende substruktuur aangedui regoor die spesies se verspreiding. Die data het die teenwoordigheid van 'n noordelike Namakwa en sentrale Fynbos klade aangetoon met vier evolusionêre lyne binne die laasgenoemde. Die geografiese skeiding van die noordelike en sentrale klades stem grootliks ooreen met patrone in ander rotsbewonende vertebraat spesies, wat op 'n gedeelde biogeografiese verlede in Suid-Afrika dui. *Elephantulus rupestris* (westelike klipklaasneus) en *Macroscelides proboscideus* (ronde-oor klaasneus) is twee taksa met verspreidings wat grootliks oorvleuel in die semi-woestyn streke van Suid-Afrika en Namibië. Mitochondriale DNS volgorde-bepaling dui op 'n gestruktueerde genetiese profiel binne *E. rupestris,* geassosieer met 'n habitat van rotskoppies, in vergelyking met 'n isolasie-deur-afstand patroon wat *M. proboscideus,* wat op gruisvlaktes aangetref word, karakteriseer. Chromosoom verandering, afgesien van heterochromatiese verskille, is beperk tot 'n strukturele verandering van 'n diploïede getal van 26 (*E. edwardii*; *E. rupestris*; *Elephantulus sp. nov.*; *E. intufi*; *E. brachyrhynchus* en *M. proboscideus*) tot 2n=28 in beide *Petrodromus tetradactylus* asook *E. rozeti* en 2n=30 in *E. myurus*. Kruis-spesies chromosoom fluoressent hibridisasie ("zoo-FISH") van die vloei-sorteerde merkers toegewys tot die vyf kleiner grootte outosome (8-12) asook die X chromosoom van *E. edwardii* tot metafase chromosome van *Elephantulus sp. nov.*, *E. intufi*, *E. myurus*, *P. tetradactylus* en *M. proboscideus* het geen bewyse getoon van sintenie-verbreking nie en versterk G-bandbepaling waarnemings wat die konserwatiewe kariotipes in hierdie spesies ondersteun.

'n Volledige evolusionêre filogenie, verteenwoordigend van alle erkende klaasneusspesies, word vir die eerste keer voorgestel. As sulks is 'n multigeen supermatriks wat gebaseer is op 3905 bp van drie mitochondriale (12S rRNA, valien tRNA, 16S rRNA) en twee nukluêre segmente (Von Willebrand faktor [vWF] en ekson 1 van die interfotoreseptor-retinoïed-bindende proteïen [IRBP]) ingesluit. As toevoeging, is nuwe sitogenetiese data, voorheen beskryfde morfologiese, anatomiese en dentale karakters sowel as data van allosieme-analises en penis morfologie ge-evalueer en nie-molekulêre ondersteuning aangedui op die molekulêre topologie. Die molekulêre bevindinge ondersteun nie 'n monofiletiese oorsprong vir *Elephantulus* nie en stel voor dat beide die monotipiese *Petrodromus* en *Macroscelides* ingesluit moet word in die genus *Elephantulus*. Molekulêre datering stel voor dat 'n dor-aangepasde Macroscelidinae lyn versprei het vanaf oos Afrika ~11.5 miljoen jaar gelede deur die "droeë Afrika korridor" tot in

suidwestelike Afrika. Verdere spesiasie gebeurtenisse binne die Macroscelidinae kan nouliks geassosieer word met drie groot periodes van verdorring in Afrika.

For Trevor and Joseline

with love

"I keep the subject of my inquiry constantly before me, and wait till the first dawning opens gradually, by little and little, into a full and clear light."

Isaac Newton

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AIS = Alleles in Space

AMOVA = Analysis of Molecular Variance

BAL = Basal length

BI = Bayesian Inference

bp = base pairs

BSA = Bovine Serum Albumin

CAS = California Academy of Sciences

Cyt *b* = Cytochrome *b*

CFM = Cape Fold Mountains

E = Ear length

EED = *Elephantulus edwardii*

EIN = *Elephantulus intufi*

EMY = *Elephantulus myurus*

EPI = *Elephantulus pilocaudata*

ERU = *Elephantulus rupestris*

ESN = *Elephantulus sp. nov.*

Fib7 = Seventh intron of the fibrinogen gene

HF c.u. = Hindfoot length

IRBP = Interphotoreceptor retinoid binding protein

IM = Iziko Museum

IUCN = World Conservation Union

M = Migration

min = minutes

MCMC = Markov Chain Monte Carlo

MMK = McGregor Museum

MP = Parsimony

MPR = *Macroscelides proboscideus*

ML = Maximum Likelihood

MYA = Million Years Ago

MY = Million Years

NOR(s) = Nucleolar organizer region(s)

PTE = *Petrodromus tetradactylus*

POB = Postorbital breadth

RL = Rostrum length

SAMOVA = Spatial Analysis of Molecular Variance

T = Divergence Time

T = Tail length

TL = Total length

TM = Transvaal Museum

TMRCA = Time to most recent common ancestor

ul = Microlitre

VWF = Von Willebrand Factor

ZB = Zygomatic breadth

CHAPTER 1

INTRODUCTION

1. The natural history of elephant-shrews

Elephant-shrews, or sengis, are an endemic group of African small mammals with all extant species (with the exception of *Elephantulus rozeti*) confined to sub-Saharan Africa (Corbet & Hanks 1968) (Fig.1.1). This enigmatic assemblage has intrigued researchers in the past and has been described as "secretive", "unusual" and of "evolutionary interest" (van der Horst 1946; Rathbun 1979).

The family Macroscelididae (order Macroscelidea) comprises six subfamilies, these include the extant Macroscelidinae and Rhynchocyoninae (Patterson 1965), the extinct Tertiary Metoldobotinae† and Herodotinae† (Simons *et al.* 1991) and the Mylomygalinae† and Myohyracinae† that differentiated prior to the Miocene (Butler & Hopwood 1957; Butler 1984). The Macroscelidea fossil record is remarkably complete with fossils described from all recognized subfamilies: the Metoldobotinae† (e.g. *Metoldobotes stromeri*†), Myohyracinae† (e.g. *Myohyrax oswaldi*† and *Protypotheroides beetzi*†), Mylomygalinae† (*Mylomygale spiersi*†) and the Herodotiinae† (including the genera *Chambius*†, *Herodotius*† and *Nementchatherium*†), as well as those from the extant subfamilies e.g. *Miorhynchocyon*† (Rhynchocyoninae) and *Pronasilio*† (Macroscelidinae) (Patterson 1965; Butler 1978; Butler 1984; Tabuce *et al.* 2001). Importantly, although extant species are confined to Africa, several fossils have been discovered on the Arabian Peninsula (Seiffert 2002).

Current taxonomy (following Corbet & Hanks 1968) calls for the recognition of two subfamilies, four genera and 15 species (Table 1.1). The surviving two natural groupings (subfamilies), the Macroscelidinae and Rhynchocyoninae are distinctly separated (Butler 1995), with Corbet & Hanks (1968) listing 30 phenetic characters that separate these two taxonomic entities. The Macroscelidinae is represented by three of the four extant genera: *Elephantulus* (10 species), *Macroscelides* (1 species) and *Petrodromus* (1 species). Both *Macroscelides* and *Elephantulus* are well represented in the fossil record with fossils dating back to the Late Pliocene (Butler & Greenwood 1976); no fossils are known for *Petrodromus* (Novacek 1984). The fossil species *Palaeothentoides africanus*† has characters that are common to both *Elephantulus* and *Rhynchocyon* although the overall description of this fossil led to its inclusion within Macroscelidinae (Patterson 1965). The second subfamily, Rhynchocyoninae, is monogeneric and includes three extant and two extinct species. *Rhynchocyon* fossils are recognized by features relating to the anterior part of the skull as well as five mandibular fragments (Butler 1969). Rhynchocyonines are characterized by longevity, morphological stability and bradytelic evolution (low rates of speciation) with only minor differences distinguishing fossil and recent *Rhynchocyon* taxa. It is important to note that the pattern of evolutionary stasis is not found in the macroscelidines (Novacek 1984). The bradytelic evolution in the rhynchocyonines

is perhaps best illustrated by a comparison between extinct and extant fossils recovered from Kenya where only minor changes occurred during the last 20 million years (MY) (Novacek 1984). It is therefore not surprising that certain authors (see e.g. Novacek 1984) refer to rhynchocyonines as “living fossils”.

With the exception of *Rhynchocyon* which has an east and central African distribution, all other extant genera (*Elephantulus*, *Macroscelides* and *Petrodromus*) are found within the southern African subregion (Fig. 1.1; Table 1.1). Seven elephant-shrew species occur in South Africa (*E. edwardii, E. myurus, E. rupestris, E. intufi, E. brachyrhynchus, P. tetradactylus* and *M. proboscideus*), with *E. edwardii* (Cape rock elephant-shrew) being the only strictly South African endemic. Six of the 10 *Elephantulus* species are found in southern Africa. Three of the six southern African species (*E. rupestris*, western rock elephant-shrew; *E. myurus*, eastern rock elephant-shrew and *E. edwardii*) use a habitat of rocky outcrops with the remaining three species (*E. brachyrhynchus*, short snouted elephant-shrew; *E. fuscus*, dusky elephant-shrew and *E. intufi*, bushveld elephant-shrew) having a more savanna grassland distribution. The remaining four species are the eastern African *E. fuscipes* (dusky-footed elephant-shrew) and *E. rufescens* (rufous elephant-shrew), the Somalian *E. revoili* (Somali elephant-shrew), and the north African *E. rozeti* (North-African elephant-shrew).

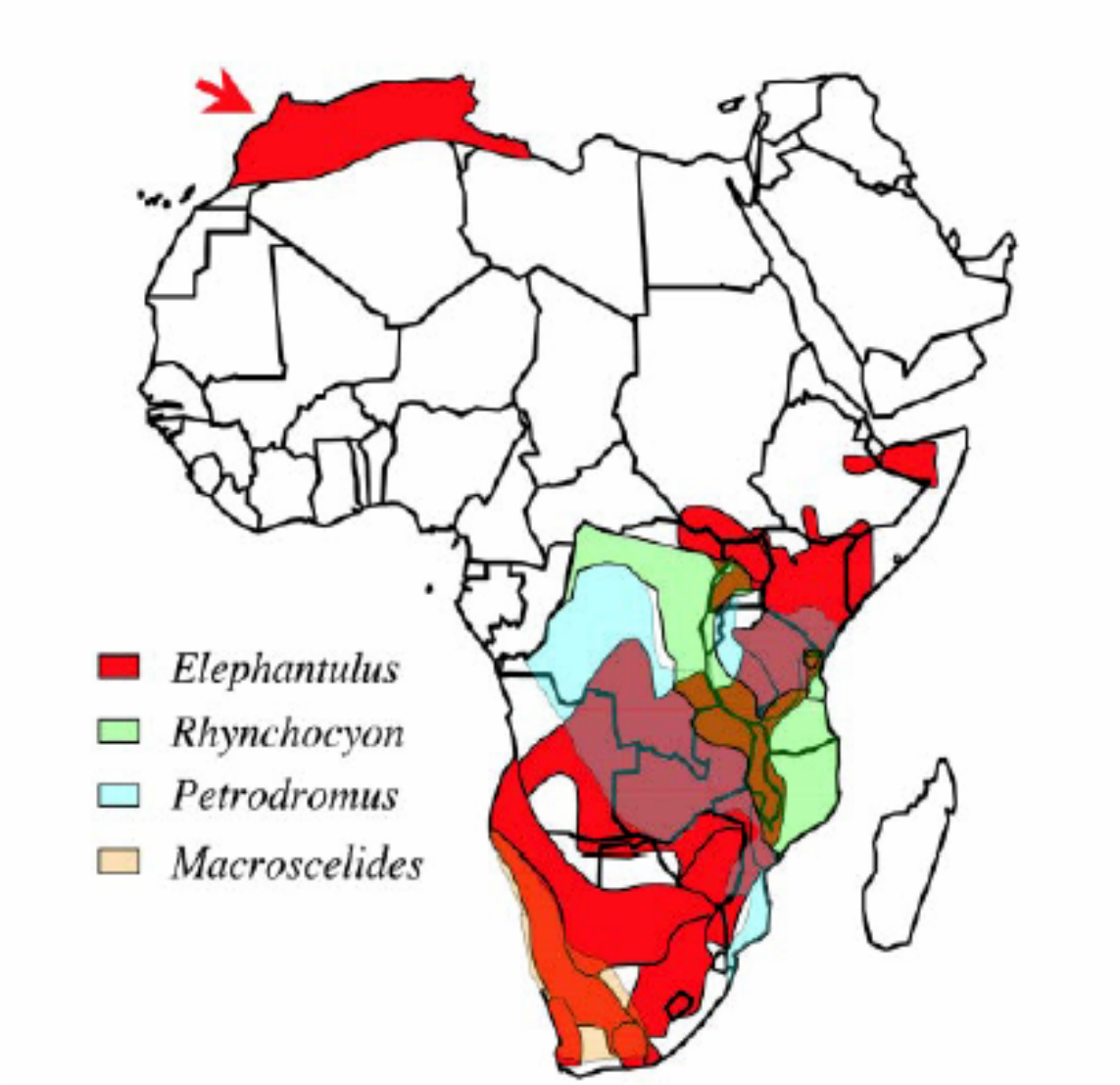

Figure 1.1 The distribution of the extant genera of the African endemic Macroscelidea. The red arrow indicates the distribution for *E. rozeti,* the only species found north of the Sahara dessert. The remaining species are distributed in the central, eastern and southern parts of Africa. Three (*Elephantulus, Macroscelides* and *Petrodromus*) of the four genera occur in South Africa. (Taken from Corbet & Hanks 1968).

Table 1.1 Taxonomic classification of the Macroscelidea as well as information on their habitat association and distribution (see Corbet & Hanks 1968; Global Mammal Assessment 2005, G. Rathbun unpubl. data; Skinner & Chimimba 2005).

Species	Original description	Habitat association	Distribution
Rhynchocyon chrysopygus Golden-rumped elephant-shrew	Günther 1881	Confined to lowland and montane forests, dense woodland and thickets	Coastal forests of Kenya
Rhynchocyon cirnei Checkered elephant-shrew	Peters 1847	Confined to lowland and montane forests, closed canopy woodlands and thick riverine bush	They occur from the north in the Democratic Republic of the Congo, Central African Republic, Tanzania, Uganda, to Mozambique and Malawi in the south
Rhynchocyon petersi Black-and-rufous elephant-shrew	Bocage 1880	Confined to evergreen and semi-deciduous forests, dense woodlands and coral rag scrub	Forests of the coastal region of Kenya and Tanzania
Macroscelides proboscideus Round-eared elephant-shrew	Shaw 1800	Open arid country with shrub bush and sparse grass cover on sandy or gravel plains	Confined to drier regions of the south-western part of Africa within the south-western arid zone. They occur throughout Namibia, the south-western parts of Botswana and the central and western parts of the Western Cape
Petrodromus tetradactylus Four-toed elephant-shrew	Peters 1846	Coastal, riparian and evergreen forests (dry, lowland and montane forests). Annual rainfall > 700 mm	Central, eastern and southern Africa. In southern Africa the species is confined to the north-eastern and eastern sectors. In South Africa they are confined to the north-east in the riparian forests of the Limpopo river and the northern and north-eastern parts of KwaZulu-Natal
Elephantulus rozeti North African elephant-shrew	Duvernoy 1833	Rocky areas in Mediterranean shrubland and subdesert zones	North-western Africa from south-western Morocco to Tunisia and Algeria
Elephantulus revoili Somali elephant-shrew	Hüet 1881	Dry shrubland and hot desert zones	Endemic to Somalia
Elephantulus rufescens Rufous elephant-shrew	Peters 1878	Dry savanna and tropical/subtropical shrubland	Eastern Africa from Tanzania through Kenya to Somalia and eastern Ethiopia and northwest as far as eastern Uganda and the extreme southern region of Sudan

Table 1.1 continued

Species	Original description	Habitat association	Distribution
Elephantulus brachyrhynchus Short-snouted elephant-shrew	Smith 1836	Dense grass with scrub bush and scattered trees. Dry tropical and subtropical savanna grasslands	Widely distributed throughout south, central and east Africa. In southern Africa they are confined to the north-east of Namibia, northern, north-eastern, eastern and southern parts of Botswana, western Mocambique, the North-West Province, northern and north-eastern Mpumalanga Province of South Africa extending to north-eastern Swaziland
Elephantulus intufi Bushveld elephant-shrew	Smith 1836	Prefers most arid terrain of all *Elephantulus* species. Open country (dry shrubland) with thin grass cover and low scrub bushes providing cover.	The northern parts of South Africa in the Bushveld but also utilizes the drier north-western parts of southern Africa occurring widely in Namibia (excluding the Namib desert), a narrow sector in south-western Angola, east, central and southern Botswana and the Northern Cape Province in South Africa
Elephantulus rupestris Western rock elephant-shrew	Smith 1831	Rocky outcrops and to a lesser extent dry shrublands	Endemic to southern Africa. Distributed though the Eastern Cape of South Africa, the Little Karoo and the western parts of the country extending into Namibia
Elephantulus fuscipes Dusky-footed elephant-shrew	Thomas 1894	Dry tropical and subtropical grasslands	South-western Sudan, north-eastern Democratic Republic of Congo and parts of Uganda
Elephantulus fuscus Dusky elephant-shrew	Peters 1852	Dry savanna	Recorded from north of the Zambezi River, from south-eastern Zambia, from southern Malawi and Mocambique. In the southern African subregion they are distributed in Mocambique only from the Tete, Vila Pery, Beira and lower Zambezi valley districts.

Table 1.1 continued

Species	Original description	Habitat association	Distribution
Elephantulus myurus Eastern rock elephant-shrew	Thomas & Schwann 1906	Rocky outcrops and to a lesser extent dry tropical/subtropical grasslands	Central south-eastern parts of southern Africa extending from South Africa to Zimbabwe, eastern Botswana and western Mocambique. Occurs outside the southern African subregion in Mocambique north of the Zambezi river. Distributed in South Africa in the North-West and the Northern Province, Mpumalanga, the Free State, the north of the Eastern Cape and in the mountainous regions of western KwaZulu-Natal and Lesotho
Elephantulus edwardii Cape rock elephant-shrew	Smith 1839	Rocky outcrops	Endemic to South Africa. Distributed through the south western and central parts of the Western Cape and Namaqualand to the Port Elizabeth region of the Eastern Cape

1.1 Evolutionary placement of macroscelids

Although not related to Rodentia (Murphy *et al.* 2001; Arnason *et al.* 2002), elephant-shrews are mouse-like in appearance and in their ecology. Macroscelids were subsequently placed among the insectivoran Menotyphla (Haeckel 1866) which groups tupaiids and macroscelids together. Subsequently Butler (1956) (supported by Patterson 1965; McKenna 1975; Novacek 1980) elevated the macroscelids to their own order. The taxonomic status of this order has received much attention with studies on living as well as fossil Macroscelidea included in the works of Butler & Hopwood (1957), Patterson (1965), Corbet & Hanks (1968), Butler & Greenwood (1976), Butler (1978) and Rathbun (1979).

The geographic origin of the Macroscelidea can be debated within three hypotheses. The first postulates an Asian origin for Macroscelidea. This hypothesis, which is based on cranial morphology and embryonic characters (Novacek *et al*. 1988) as well as the presence of a large, functional caecum (Woodall & Mackie 1987), places Macroscelidea as a sister group to Glires (lagomorphs and rodents) i.e., the Anagalida (McKenna 1975). The second hypothesis also points to a non-African origin based on the description of fossil postcrania that provides evidence for a close relationship between the North American Palaeocene–Eocene earliest known Macroscelidean relatives (apheliscines *Apheliscus*† and *Haplomylus*†) and the condylarths (early ungulate progenitors) (Zack *et al.* 2005). This hypothesis suggests that the restriction of the Macroscelidea to Africa from the middle Eocene to the present is either relictual, or indicative of a

Palaeocene or Eocene dispersal to Africa. The third hypothesis, based on dental characters of fossil Macroscelidea similarly points to a condylarthan origin, based on a close resemblance to paenungulates, however, an African origin for the group is proposed (Tabuce *et al.* 2001). This hypothesis followed the discovery of two macroscelid fossils from the subfamily Herodotinae†, *Chambius kasserinensis*† (Hartenberger 1986) from the Early-Middle Eocene in Tunisia, and *Herodotius pattersoni*† (Simons *et al.* 1991) from the Late Eocene of the Jebel El Quatrani Formation in Fayum, Egypt. Additional support for the latter hypothesis comes from an array of molecular studies (e.g. Springer *et al.* 1997; Stanhope *et al.* 1998a, b; Springer *et al.* 1999; Amrine-Madsen *et al.* 2003; Nikaido *et al.* 2003; Nishihara *et al.* 2005; Kriegs *et al.* 2006; Nishihara *et al.* 2006; Waters *et al.* 2007) which place Macroscelidea in the superordinal clade of African endemic eutherian mammals, the Afrotheria, which comprises six of the ~20 mammalian orders: the elephant (Order Proboscidea), hyrax (Hyracoidea), dugong and manatee (Sirenia), aardvark (Tubulidentata), elephant-shrew (Macroscelidea) and golden mole and tenrec (Tenrecoidea). Although cytogenetic markers (Froenicke *et al*. 2003; Yang *et al.* 2003; Robinson *et al.* 2004; Svartman *et al.* 2004) also provide unambiguous evidence in support of the afrotherian clade (but see Ruiz-Herrera & Robinson in press), morphological evidence is still generally lacking (Asher 1999; Werdelin & Nilsonne 1999; Carter 2001; Whidden 2002; Asher *et al.* 2003). Nonetheless, a skeletal synapomorphy for afrotherian mammals has more recently been identified (Sánchez-Villagra *et al.* 2005).

A large body of molecular data (Madsen *et al.* 2001; Murphy *et al.* 2001; Waddell *et al.* 2001; Murata *et al.* 2003; Nishihara *et al*. 2005) place the Macroscelidea within the “Afroinsectiphillia”, as do the cytogenetic data of Robinson *et al.* (2004), suggesting a phylogenetic grouping with Afrosoricida (tenrecs and golden moles) and Tubulidentata (aardvark). Collectively, the aardvark/elephant-shrew/tenrec and golden mole clade is termed “Afroinsectiphillia” (Waddell *et al.* 2001), whereas the elephant-shrew/tenrec and golden mole clade (excluding aardvark) is known as “Afroinsectivora”. However, if macroscelids are indeed more closely related to tubulidentates than to paenungulates, the similar postcranial, cranial and dental as well as soft-tissue features present in both macroscelids and paenungulates must be independently acquired (products of convergent evolution) from an “insectivore-like” ancestor. Alternatively, “herbivore/ungulate-like” features could be the primitive state in Afrotheria, and the shared morphological features of elephant-shrews and paenungulates could represent afrotherian synapomorphies. In such a scenario tenrecoids would have evolved “insectivore-like” adaptations from a “herbivore-like” progenitor involving evolutionary reversals (Seiffert 2002; Robinson & Seiffert 2004).

1.2 Evolutionary relationships within macroscelids

Although the ancestral position of the Macroscelidea has received much attention, information on the evolutionary relationships within the order is limited. Key studies of elephant-shrew phylogenetic relatedness include the dental, morphological and anatomical analyses of Corbet & Hanks (1968 revised by Corbet 1995), the allozyme analysis of both Tolliver *et al.* (1989) and Raman & Perrin (1997), sequence data from three mitochondrial DNA segments (12S rRNA, valine tRNA and 16S rRNA), as well as segments of two protein coding nuclear genes (exon 28 of the von Willebrand factor [vWF] and exon 1 of interphotoreceptor retinoid binding protein [IRBP]) (Douady *et al.* 2003). Although none of these studies had full species representation, they nonetheless shed light on interspecific sister-taxon relationships as well as the monopyletic/diphyletic origin of the subfamilies and genera.

Defining characters uniting the *Elephantulus* species (Novacek 1984) are generally lacking and consequently the genus is thought to be paraphyletic. At the generic level, *Elephantulus* is distinguished from *Macroscelides* and *Petrodromus* by the absence of specialized features that characterise these monotypic genera (Corbet & Hanks 1968; Corbet 1995). *Macroscelides proboscideus* (round-eared elephant-shrew) has a distinctly inflated auditory region with enlarged bullae visible from the top of the skull. From an external perspective the ears are rounded, broad, expanded and hairy inside (Kerley 1995). *Petrodromus tetradactylus* (four-toed elephant-shrew) is easily distinguished by its larger size, the absence of a big toe (hallux) on the hind feet, and two pairs of pectoral mammae present in females compared with the three pairs (two pairs pectoral and one pair abdominal) found in the other Macroscelidinae genera. The genus *Elephantulus* is monophyletic based on the allozyme studies of both Tolliver *et al.* (1989) and Raman & Perrin (1997); both studies were restricted to seven southern African elephant-shrews. When including the north African *E. rozeti*, the placement of this species argues for a diphyletic (Douady *et al.* 2003) origin for *Elephantulus* as proposed by Corbet (1995). *Elephantulus rozeti* clusters as sister taxon to the monotypic *Petrodromus* based on molecular data (Douady *et al.* 2003), whereas phenotypic characters (Corbet 1995) place this species within *Elephantulus*.

Molecular data group the rock elephant-shrews (*E. edwardii* and *E. myurus*) as sister taxa to *E. intufi* and *E. rufescens* (Douady *et al.* 2003). In contrast, the exact placement of *E. edwardii* remains inconclusive based on allozyme variation (Tolliver *et al.* 1989) where *E. edwardii* groups with either *E. myurus* or *E. brachyrhynchus*. The trichotomy of the three species groups (*E. edwardii* + *E. myurus*), (*E. rupestris* + *E. intufi*) and (*E. brachyrhynchus* + *E. fuscus* + *E. fuscipes*), as well as the grouping of the two pairs of southern species (*E. edwardii* + *E. myurus* and *E. rupestris* + *E. intufi*), could not be resolved by phenotypic data (Corbet 1995).

Known diploid numbers vary from 2n=26–30 in the macroscelids (Matthey 1954; Stimson & Goodman 1966; Wenhold & Robinson 1987; Tolliver *et al.* 1989; Robinson *et al.* 2004; Svartman *et al.* 2004) with information restricted to eight of the 15 elephant-shrew species. Reports are largely limited to unbanded preparations and in a few exceptions, to G-banding and C-banding patterns and the number of nucleolar organizer regions (NORs) present in each species (Wenhold & Robinson 1987; Robinson *et al.* 2004; Svartman *et al.* 2004). Most species have a diploid number of 2n=26 (*E. edwardii*; *E. rupestris*; *E. intufi*; *E. brachyrhynchus* and *M. proboscideus*) whereas *P. tetradactylus* (2n=28; Wenhold & Robinson 1987), *E. rozeti* (2n=28; Matthey 1954) and *E. myurus* (2n=30; Tolliver *et al.* 1989) differ by unique chromosomal changes. Chromosome painting has been limited to comparisons with human in two macroscelid species, *M. proboscideus* (Svartman *et al.* 2004) and *E. edwardii* (Robinson *et al.* 2004 – the specimen karyotyped by these authors was classified as *E. rupestris* but has subsequently been identified as *E. edwardii* by molecular sequencing; see Chapter 3).

1.3 Life history traits and ecology of macroscelids

Elephant-shrews are mainly insectivorous, notwithstanding their probable herbivorous ancestry suggested by the retention of a caecum facilitating hindgut fermentation in living Macroscelidea (Woodall & Mackie 1987) and the apparent adaptation to a herbivorous diet in the extinct Myohyracinae† (Butler 1984). They feed largely on ants, termites and other small invertebrates (Rathbun 1979; Kerley 1995). However, significant percentages of herbage and seeds/fruit are consumed by a number of extant species (Sauer & Sauer 1972; Rathbun 1979; Kerley 1995).

The 15 elephant-shrew species are small-bodied and vary in size from ~100 mm to almost 300 mm and from just under 25 g to 500 g. They are highly cursorial (running), thus capable of rapid movement (Rathbun 1979). The hind limbs are much longer than the forelimbs and the ordinal name is literally derived from this feature: macro + scelidea = big thigh. Communication occurs both on an audible and visual level, with species-specific foot drumming behaviour and thin, shrill squeaks in the audible range being most common elements (Corbet & Hanks 1968). Most species possess well developed scent glands (Faurie & Perrin 1995) used in scent-marking. The eyes are large for the size of the head and all species are keen sighted picking up the slightest movement of possible prey items. The mobile trunk-like snout of elephant-shrews is well adapted to catching their insectivorous prey. The dental formula for all extant elephant-shrews is I 0-3/3 C 1/1 P 4/4 M 2/2-3 for both the upper and lower jaw (Corbet & Hanks 1968). Within the subfamily Macroscelidinae, *E. brachyrhynchus*, *E. fuscus* and *E. fuscipes* have a third molar (M^3) present on each side of the lower jaw (Corbet 1995). The most parsimonious conclusion is that the absence of the M^3 is a

primitive condition for the family, and has consequently been uniquely acquired by the ancestor of the sister group, *E. brachyrhynchus* + *E. fuscipes* + *E. fuscus* (Corbet 1995).

All species of elephant-shrews that have been studied are socially monogamous with intrapair overlapping home-ranges that are relatively stable over time and which in most cases most function as territories (Sauer 1973; Rathbun 1979; FitzGibbon 1995, 1997; Ribble & Perrin 2005; Rathbun & Rathbun 2006).This is exceptional as no other order of closely related mammals is thought to be entirely monogamous (Ribble & Perrin 2005). Same sex-aggression in elephant-shrews has been documented in captivity (Rathbun *et al*. 1981; pers. observation) as well as in the wild (Rathbun 1979). They follow an "absentee" strategy of maternal care (the young are born precocially) (Ralls *et al.* 1986) with no evidence of direct parental care (Rathbun & Rathbun 2006). Therefore the monogamous pair-association is not a reflection of males contributing care to offspring (obligate monogamy), but rather of female dispersal (facultative monogamy) (Ribble & Perrin 2005 and references therein). However, mammalian monogamy is scarce (less than 10% in mammals) (Kleiman 1977) and a currently unconfirmed adaptation must explain this behavioural strategy in elephant-shrews. The benefits of male mate guarding and/or defending of solitary females are regarded as the most likely explanations of this phenomenon (Lumpkin & Koontz 1986; Ribble & Perrin 2005; Rathbun & Rathbun 2006).The degree of social monogamy is density-dependent and home range size is variable (Rathhun & Rathbun 2006). One to two (rarely four) precocial (less precocial in *Rhynchocyon*) young are seasonally born when food is abundant (Sauer 1973; Rathbun 1979; Ribble & Perrin 2005). Reproduction is complex in this order with the process of female ovulation studied by van der Horst (1946) and Tripp (1971) among others. The male reproductive system supports the suggested relationship of elephant-shrews with paenungulates, and has been extensively studied by Woodall (1995a, b). Interestingly, males often remain sexually active throughout the year in spite the fact that they are seasonal breeders (Perrin 1997).

Elephant-shrews display peculiar metabolic rate i.e. a daily torpor in both summer and winter conditions (Lovegrove *et al.* 2001a, b; Mzilikazi *et al.* 2002; Mzilikazi & Lovegrove 2004); a physiological study on *E. myurus* and *E. rozeti* revealed that elephant-shrews' body temperature and oxygen consumption drop to a point characteristic of hibernation/deep torpor, but never exceeds the length of daily torpor (24 h) (Lovegrove *et al*. 2001a). Most species of elephant-shrews seem to be crepuscular (pers. observation) rather than strictly diurnal or nocturnal (see also Rathbun 1979). In contrast to the predominantly nocturnal activity documented for *E. edwardii* in Skinner & Chimimba (2005), Stuart *et al.* (2003) noted diurnal activity in their biological study on this species in the Western Cape. Macroscelids are also important in ecology, e.g. *E. edwardii* plays a role in the pollination of *Protea* species (Fleming & Nicolson 2003).

1.4 Conservation

In the face of an expanding human population, the major threat to elephant-shrew species is the degradation of their habitat caused by human interference (Perrin 1995). The negative effects of fragmentation and loss of habitat on the population dynamics of *Rhynchocyon* species (Rathbun 1995) suggest that many elephant-shrew (specifically *Rhynchocyon*) species are at risk given the possible decline in their geographical ranges. Consequently *R. chrysopygus* is listed on the IUCN redlist as endangered, *R. petersi* as vulnerable, *R. cirnei* as near threathened, and *E. fuscus*, *E. fuscipes* and *E. revoili* as data deficient (2006 IUCN redlist; http://www.iucnredlist.org). In addition, *P. tetradactylus* is listed as regionally endangered in South Africa (National Environmental Management: Biodiversity Act # 10 of 2004). It is noteworthy that the "vulnerable" conservation status of three southern African endemics (*E. edwardii*, *E. rupestris* and *M. proboscideus*) has recently been downgraded to "least concern" (IUCN redlist 2004; 2006). Consequently, both from a scientific and conservation perspective, it is important to clarify the evolutionary relationships and the processes driving speciation in the order Macroscelidea.

2. Phylogeography and landscape genetics

Phylogeography allows the investigation of fundamental links between population processes and regional patterns of diversity and biogeography (Bermingham & Moritz 1998; Avise 2000; Hedrick 2001). It has become standard practice to include geographical information with population genetic data to explain patterns over a spatial scale. Following from this, inferences can be made concerning past demography, effective population size, sex-specific gene flow, founder contributions, speciation, adaptive radiations and extinctions. To this end, genetic variation/isolation among groups can be objectively determined incorporating both geographical coordinates/distances, and molecular data using matrix comparisons such as a Mantel Test (Manly 1991), and software packages such as Spatial Analysis of Molecular Variance (SAMOVA; Dupanloup *et al*. 2002) and Alleles In Space (AIS; Miller 2005). It is further useful to separate the effects of recurrent gene flow/migration from the retention of ancestral haplotypes and to determine potential divergence times between identified lineages (e.g. as implemented in MDIV; Nielsen & Wakeley 2001).

Geographical distance, as considered in classical metapopulation studies (see Baquette 2004), does not explain the influence of environmental landscape features on genetic phylogeographic patterns. One possibility to overcome this shortcoming is to combine population genetic patterns with landscape ecology i.e., parameters which could act as possible barriers to the migration of individuals. Such an approach represents a current scientific trend termed landscape genetics (*sensu* Manel *et al.* 2003; see also Hewitt & Ibrahim 2001; Silverton & Antonovics 2001; Storfer *et al*. 2007). The ultimate aim of a landscape genetic

approach is to amalgamate traditional phylogeography (the combination of phylogeny and biogeography) with quantitative and qualitative landscape features to explain the evolutionary history of a taxon, as well as to provide information for future conservation efforts.

,An array of biotic factors such as life-history traits as well as abiotic factors including river flow patterns, mountain ranges, sea level fluctuations and the effects of climate change could form barriers to dispersal in species, and shape genetic diversity. Intuitively, however, one would expect species that occupy fragmented/discontinuous habitats to be characterized by a strong partitioning of genetic variation. Indeed, previous studies have reported distinct evolutionary lineages for rock-dwelling species such as the rock hyrax (*Procavia capensis;* Prinsloo & Robinson 1992), the red rock rabbit (*Pronolagus rupestris*; Matthee & Robinson 1996), the thick- toed gecko (*Pachydactylus rugosus;* Lamb & Bauer 2000) and the rock agama (*Agama atra*; Matthee & Flemming 2002). Concordant patterns in phylogenetically unrelated but ecologically similar species can expose historical features that shaped the biogeography of a region (Avise *et al*.1987; Avise & Hamrick 1996; Avise 2000; Avise 2002). For example, genetic population division in a number of invertebrates as well as vertebrates has shown the roles of ice ages in speciation in Europe (see Hewitt 1996; Hewitt 1999; Hewitt 2000; Hewitt 2004).

3. Museum material

The use of museum material to augment sampling in population genetic studies is becoming increasingly important in instances where a species has a high conservation status, or where it exhibits fluctuating and/or low population densities (e.g. Baker 1994). However, sequences obtained from these specimens should be carefully checked for contamination and the extraction and amplification of museum material often require specialised facilities and optimising of standard techniques (see e.g. Good & Sullivan 2001; Godoy *et al.* 2004; Rohland *et al.* 2004; Wisely *et al.* 2004; Stuart *et al.* 2006 for the use of museum tissue). The use of museum material in genetic studies, among many others, allows a strong case to be made for the upkeep and careful maintenance of museum collections, and the preservation of voucher specimens which in future can allow investigations on current *vs* historical patterns of gene flow.

Furthermore, genetics can provide unambiguous evidence of the distinctiveness of taxa that are morphologically cryptic. In other words, molecular genetics can contribute to the recognition of new species (e.g. Smith *et al.* 1990; Cushion *et al.* 2004; Kock *et al.* 2006; Davenport *et al.* 2006; Goodman *et al.* 2006; Gündüz *et al.* 2007; Olivieri *et al.* 2007; Smit *et al.* 2007) and the availability of museum voucher specimens can allow for the *a posteriori* identification of possible phenotypic differences among them.

4. Multigene supermatrix

Phylogenetic accuracy is supported through congruent patterns from multiple data sets (Cracraft & Helm-Bychowski 1991; Miyamoto & Cracraft 1991; Swofford 1991; Miyamoto & Fitch 1995). However, the most reliable phylogenetic tree for inferring evolutionary relationships within a group of taxa is likely to be representative of a combined data set that includes all available molecular, as well as morphological and/or palaeontological data (see Flores-Villela *et al*. 2000; Gatesy *et al*. 2002; Nylander *et al.* 2004 among others). A potential wealth of information from which evolutionary histories can be elucidated is provided with the rapidly accumulating volume of DNA data. However, the challenge is to find ways to optimally utilize existing sequence and other available data (Sanderson & Driskell 2003). It is not surprising, therefore, that many phylogenomic and comparative biological studies (see Bapteste *et al.* 2002; Rokas *et al.* 2003; Driskell *et al.* 2004) use sequence data obtained from public databases such as GenBank (http://www.ncbi.nlm.nih.gov) or Swiss-Prot (http://us.expasy.org/) to assemble large sequence alignments for analysis (Driskell *et al.* 2004; Yan *et al.* 2005).

Supertree/supermatrix phylogenetic analyses can readily be employed to convert multigene data sets into a single tree/matrix. In the total evidence approach to systematics, all characters and taxa are concatenated in a single matrix and the data analysed simultaneously (Miyamoto 1985; Kluge 1989; Nixon & Carpenter 1996). In a multigene supermatrix (e.g. Qiu *et al.* 2006; Domes *et al.* 2007) the sequences of all available individual genes are merged into a so-called supergene (Lartillot *et al.* 2007). It is important to note that the solution provided by either the supertree or supermatrix approach is stronger than that from either analysis separately, and supertree/supermatrix analyses can be profitably employed to help elucidate the phylogenetic relationships of the respective groups (Bininda-Emonds 2004).

In addition to molecular multigene supermatrices, other characters such as chromosomal, palaeontological and morphological data are useful in determining evolutionary relationships. Chromosomes are inherited in a Mendelian fashion making it possible to detect shared common ancestral states (synapomorphies) and therefore identify sister taxon groupings necessary to construct a phylogeny. However, the lack of theoretical discussion, consensus, and extensive information on the encoding and analysis of chromosomal characters has until fairly recently (but see Dobigny *et al*. 2004) limited the inclusion of cytogenetic data in cladistic studies. Two approaches (reviewed in Dobigny *et al.* 2004) can be used to construct a cytogenetic phylogeny. First, chromosomes (Modi & O'Brien 1987) or chromosomal segments (Ortells 1995) are treated as characters and the changes they underwent are scored as the character states. Secondly, since redundancy in the scoring of characters often occurs under such a scheme, the chromosomal changes

themselves are considered as the character/s and their presence/absence as the character state (Dobigny *et al*. 2004).

5. Comparative cytogenetics

5.1 Chromosomal changes and evolutionary speciation - background

Speciation is an ongoing process representing a continuum of variation ranging from clear phenotypic and genetic differences separating species to the slightest divergence between specimens within populations (Key 1981). When considering chromosomal rearrangements and their possible role in speciation, it is convention to adopt the Biological Species Concept (BSC). The BSC (Mayr 1942, 1963; also see Dobzhansky 1935, 1937a, b, 1970) defines a species as a group of "actually or potentially" interbreeding natural populations which are reproductively isolated from other such groups. According to the "hybrid-dysfunction" model of chromosomal speciation, hybrids with heterozygous chromosome rearrangements may produce unbalanced gametes (Ayala & Coluzzi 2005), and of all possible chromosomal rearrangements, it is only those few potentially negatively heterotic rearrangements that can influence the cladogenic process (i.e., are underdominant) that are important. These hybrids will have a low reproductive success. However, natural selection will "promote mutations that reduce the probability of intercrossing between populations carrying different rearrangements and thus promote their reproductive isolation" (Ayala and Coluzzi 2005), and rare mutations can be fixed in populations (Robinson & Roux 1985). The fixation of these mutants can affect the whole genome and become the driving force behind parapatric (contiguous), partly sympatric (geographically overlapping) and allopatric speciation between populations (White 1968, 1978; King 1993; Ayala & Coluzzi 2005). Examples of rare chromosomal changes having the capacity to malsegregate (disrupt) meiosis in hybrids are tandem fusions, reciprocal translocations, Robertsonian (centric) fusions or fissions, multiple centric fusions which share brachial homologies, X-autosome translocations and peri-/paracentric inversions (see White 1973). Further, it has been suggested that chromosomal polymorphisms can be strongly selected for their positive role in adaptation and traits such as those that impact on viability, development time, longevity, mating success, fecundity and body size (all of which have been linked to chromosomal inversion polymorphisms in *Drosophila* - see Hoffmann *et al.* 2004).

However, the "hybrid-dysfunction" model encounters a difficulty in explaining how the low fitness of the individual hybrid (which possesses the mutation and is subject to selection) will be prevented from successfully mating with specimens not carrying the mutation (Spirito 2000; Rieseberg 2001; Noor *et al*. 2001a, b; Machado *et al.* 2002; Navarro & Barton 2003a, b). Coluzzi (1982) proposed the "suppressed

recombination" model which points that mutations associated with chromosomal rearrangements are prevented from exchange between one population and another, whereas genetic flow will freely occur between colinear chromosomes. The mutations which are selected for their local adaptation will accumulate in the "protected chromosome regions" so that parapatric and/or partial sympatric speciation will be attained over time (Ayala & Coluzzi 2005). The "suppressed recombination" model has been suggested for a number of animal species e.g. *Anopheles* (Coluzzi 1982), *Drosophila* (Noor *et al*. 2001a, b; Machado *et al.* 2002) and primates (Navarro & Barton 2003a, b).

In conclusion, genome evolution tends to be a process which balances stability and variation that is essential for speciation. Genome stability can be influenced by the arrangement of chromosomes (i.e. chromosome territories) in the cell nucleus since it is evident that chromosomes that are closer to one another seem to undergo change more frequently than those further separated from each other (Meaburn & Mistelli 2007 and references therein). The genome consists of regions that tolerate rearrangements and hence hold potential for speciation, whereas other large blocks are conserved for their fundamental role in maintaining stability (Zhao *et al.* 2004). Alternatively, chromosomal or "non-genic" speciation could also be accomplished by numerous independent micromutations (single DNA base changes) (GC% hypothesis) in addition to the structural changes in chromosomes (macromutations) which would be more substantial than single base pair changes (Forsdyke 2004).

5.2 Chromosomal banding and chromosome painting

Banding patterns, that stain either euchromatin (G–banding; Seabright 1971) or heterochromatin (C-banding; Sumner 1990), reflect the intrinsic properties of the genome. These patterns present a sequence of bands along the length of the chromosome which differ both in width and staining intensity. Although banding patterns are usually identical for homologous chromosomes, G–banding can be applied to identify chromosomal differences among different species and the technique can, in certain instances, be used to assess primary homologies among taxa. Although banding has been extensively used for intra- and interspecific chromosome comparisons in the past (see e.g. Stanyon *et al.* 1987; Wenhold & Robinson 1987; Gilbert *et al*. 2006 for specific examples), cross-species chromosome painting (Fluorescence *in situ* hybridization – zoo-FISH) is more efficient for finer scale genomic investigations. FISH is a fluorescence-based detection system used to identify the binding sites of a DNA probe to a complementary DNA strand, usually represented by metaphase chromosomes dropped on a glass microscope slide. It involves the incubation of a single-stranded (denatured) DNA probe with a single-stranded (denatured) DNA sequence. The two complementary templates are allowed to re-anneal under experimental conditions and reform as a double-stranded molecule during hybridization. Chromosome painting has been successfully used to

examine synteny conservation across a large number of species (reviewed in Froenicke 2005; Ferguson-Smith & Trifonov 2007 among others).

6. Aims of the present investigation

1. To describe the phylogeographic population structure of (a) the Cape rock elephant-shrew (*E. edwardii*), (b) the western rock elephant-shrew (*E. rupestris*) and (c) the round-eared elephant-shrew (*M. proboscideus*) using two mitochondrial DNA markers (cytochrome *b* gene and the control region).

2. To (a) compare the spatial distribution of genetic variation observed in three southern African endemics, *E. edwardii*, *E. rupestris* and *M. proboscideus*, each with partly overlapping distributions and (b), search for correspondence in genetic patterns over spatial scales.

3. To provide the most comprehensive evolutionary history available for all extant elephant-shrews and test the monophyly of *Elephantulus* using a multigene supermatrix comprising the mitochondrial 12S rRNA, valine tRNA and 16S rRNA, as well as the nuclear protein coding segments of Von Willebrand factor (vWF) and the interphotoreceptor retinoid binding protein (IRBP). The molecular dataset is extended to include (a) novel cytogenetic characters (see fourth aim below), (b) published morphological, dental and anatomical characters (Corbet & Hanks 1968 revised by Corbet 1995), (c) data generated by electrophoretic analysis (Tolliver *et al.* 1989; Raman & Perrin 1997) and (d) data on penis morphology (Woodall 1995b).

4. To compare intraspecific and interspecific chromosomal changes in six species of Southern African Macroscelidea using standard G-banding, C-banding and the identification of nucleolar organizer regions (NORs). Fluorescence *in situ* hybridization (zoo-FISH) using *E. edwardii* flow-sorted chromosome-specific painting probes is employed to investigate fine-scale evolutionary relationships among these species.

CHAPTER 2

COALESCENCE METHODS REVEAL THE IMPACT OF VICARIANCE ON THE SPATIAL GENETIC STRUCTURE OF *ELEPHANTULUS EDWARDII* (AFROTHERIA, MACROSCELIDEA)

INTRODUCTION

The spatial distribution of genetic variation within species is, among other factors, determined by life history characteristics that include habitat choice, dispersal capabilities and behavioural attributes. Information pertaining to the phylogeographic structure of species occupying rocky habitats, particularly in southern Africa, is limited. Intuitively, however, one would expect these rock dwelling species to be characterized by strong genetic partitioning as a result of limited dispersal capabilities and patchiness of suitable habitat. Indeed, previous studies have reported distinct evolutionary lineages for rock-dwelling southern African species such as the red rock rabbit (*P. rupestris*; Matthee & Robinson 1996) and the rock agama (*A.atra*; Matthee & Flemming 2002). In an attempt to examine the processes underpinning the population structure in species occupying this particular habitat type, and to look for commonality of pattern across distinct evolutionary lineages which may be indicative of historical biogeographic barriers, and hence of importance in conservation planning, we determined the phylogeographic population structure of a South African endemic rock elephant-shrew, *E. edwardii*. Given that this species is similarly confined to rocky habitat with sparse vegetation, we reasoned that the Cape rock elephant-shrew may prove a useful index species in our search for congruent phylogeographic patterns in rock-dwelling taxa.

Smith (1839:14 in Roberts 1951) described *E. edwardii* (then known as *Macroscelides edwardii)* from the Olifants River, presumably near the present day settlement of Oudtshoorn (33.3S 22.2E) in the Western Cape of South Africa. Subsequent revisions by Corbet and Hanks (1968) and Meester *et al. (*1986) lumped *E. capensis* (Roberts 1924:62) and *E. karoensis* (Roberts 1938:234) within *E. edwardii,* thereby extending the geographic distribution to include two geographically isolated areas – one in the northern parts of the Western Cape, and the other in the Eastern Cape of South Africa (Fig. 2.1a & b). However, more extensive sampling revealed this supposedly disjunct pattern to be due to sampling artefact (see also Skinner & Chimimba 2005), with the species exhibiting a continuous distribution that includes the Succulent Karoo, Nama Karoo, and Fynbos vegetation biomes of South Africa (Fig. 2.1b & c; see Low & Rebelo 1996 for vegetation zone data).

The morphological identification of *E. edwardii* is problematic largely due to poorly defined distinguishing characteristics between the three rock dwelling *Elephantulus* species which is exacerbated by an overlap in their respective geographic distributions. There is a range overlap between *E. rupestris* (western rock elephant-shrew) and *E. edwardii* in the Northern Cape Province of South Africa, whereas *E. edwardii*, *E. rupestris* and *E. myurus* (eastern rock elephant-shrew) occur in sympatry in the Eastern Cape Province (Skinner & Chimimba 2005). Although *E. edwardii* is most commonly identified on dental characteristics,

Rautenbach and Nel (1980) noted that these vary considerably within the Western Cape Province populations.

Little is known about the basic biology of the Cape rock elephant-shrew which is fundamental to evaluating its recognition as vulnerable in the IUCN redlist (2004 IUCN redlist; http://www.iucnredlist.org). Clearly, given the difficulties associated with morphological identification, a genetic delimitation of *E. edwardii* would contribute significantly to develop accurate identification schemes for this species and, importantly from a conservation perspective, to more accurately define the geographic limits of this local endemic. Consequently our aims were twofold. First, to investigate the phylogeographic population structure of the Cape rock elephant-shrew across its geographic range as this would provide a fundamental link between population processes and regional patterns of diversity and biogeography. Among others, we applied a maximum likelihood and Bayesian coalescent approach to our data. Specifically, coalescent methods challenge the traditional practice of phylogeography in that they separate the effects of historical vicariance, isolation and ancestral polymorphism (incomplete lineage sorting) from migration (processes of gene flow) through the simultaneous estimation of population parameters such as migration rate, time of population divergence and time to most common recent ancestor (Nielsen & Wakely 2001). This study underlines the advantages of this method by showing the impacts of historical vicariance, ancestral polymorphism and restricted gene flow in shaping the current phylogeographic structure of *E. edwardii*. In addition, a novel interpolation-based graphical method (Alleles in Space; Miller 2005) is employed to provide a visual perspective of the spatial and temporal distribution of genetic structure over landscapes. Secondly, we attempt to place the genetic structure discovered in this species in the context of past landscape changes that involve geographical and climatological vicariance events in South Africa. We also search for congruence in the patterns documented for other rock-dwelling vertebrates with distributions that span the region of interest e.g. the red rock rabbit (*P. rupestris*; Matthee & Robinson 1996) and the rock agama (*A. atra*; Matthee & Flemming 2002).

MATERIALS AND METHODS

SAMPLE COLLECTION

To determine the phylogeographic population structure of *E. edwardii,* samples were collected from 28 localities across the species' range (see Fig. 2.1c). In addition to freshly collected tissue, museum specimens were used to augment our geographic coverage. In total, 106 specimens (Table 2.1) were sequenced which comprised 64 specimens housed in South African museums (the oldest of which dated to 1904; see appendix Table 1). To validate species status and to ensure the correct identification of *E. edwardii* specimens, barcoding was done by sequencing DNA from *E. rupestris* and *M. proboscideus*

specimens trapped well outside of the possible overlapping distribution areas (Table 2.2). Further, to investigate the status of *E. edwardii* as a single species, or complex of hitherto undescribed species, we sequenced fragments taken from the type specimens of *E. capensis* (TM 2312; Roberts 1924:62) and *E. karoensis* (TM 688; Roberts 1938:234), both taxa currently subsumed within *E. edwardii*. The type locality for *E. capensis* is given as Klawer, Van Rhynsdorp in the Western Cape (31.36S 18.42E), while that for *E. karoensis* is Deelfontein, north of Richmond in the central Karoo of South Africa (31.20S 23.85E).

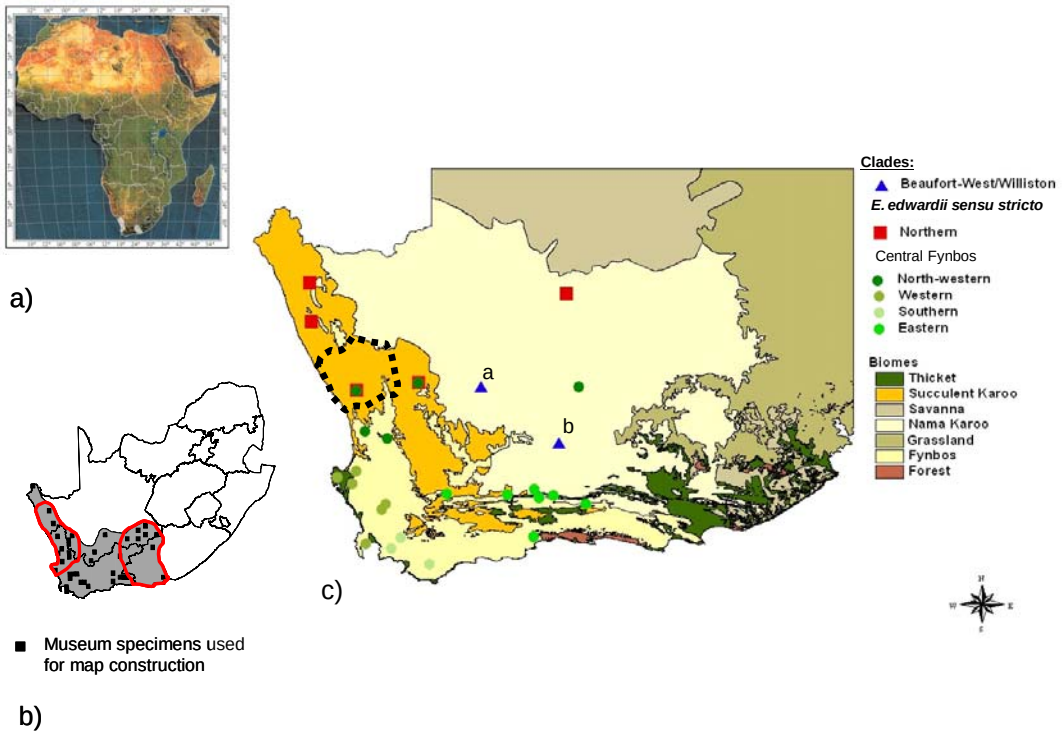

Figure 2.1 (a) Map of Africa (redrawn from Skinner & Chimimba 2005) showing the disjunct distribution previously proposed for the South African endemic *E. edwardii*. (b) Further sampling reported herein (see also Skinner & Chimimba 2005) shows a continuous distribution for this species stretching from Namaqualand in the Western Cape to Port Elizabeth in the Eastern Cape of South Africa (taken & redrawn from Friedmann & Daly 2004) (previously proposed disjunct distribution indicated in red); (c) Map positions of the 28 *E. edwardii* sampling localities (vegetation zones taken from Low & Rebelo 1996). The localities are coded as follows: Williston{a} and Beaufort-West{b} taxa (blue triangles), *E. edwardii sensu stricto* northern Namaqua (red squares) and the central Fynbos clades (north-western; western; southern and eastern lineages, green circles). The geographical position of the Knersvlakte region in the North-western Cape is roughly indicated by the dotted black circle.

Table 2.1 The number of *E. edwardii* specimens included for each of the sampling localities. We include their affinity to the three different genetic clades detected herein as well as finer scale structure within the central Fynbos clade. The source of the material is presented. (Transvaal Museum = TM; Iziko Museum = IM; Natal Museum = NM and Cape Nature = CN).

Clades	Lineage	Locality	cyt *b*	control region	combined dataset	Source material
Karoo		Beaufort-West	5	5	5	Museum specimen, TM
		Williston	2	2	2	Museum specimen, TM
Northern		Prieska	1	1	1	Museum specimen, TM
Namaqua		Melkboom, Namaqua National Park	1	1	1	Soft tissue
		Kamieskroon	1	1	1	Soft tissue
		Calvinia	1	1	1	Museum specimen, TM
		Nieuwoudtville	6	6	6	Soft tissue
		Klawer, Van Rhynsdorp	1	1	1	Museum specimen, TM
Central	North-western	Calvinia	1	1	1	Museum specimen, TM
Fynbos		Klawer, Van Rhynsdorp	3	5	3	Museum specimen, TM
		Travellers Rest, Clanwilliam	7	10	7	Soft tissue
		Cederberg Wilderness, Algeria	4	4	4	Soft tissue
		Cederberg Wilderness, Matjiesrivier	0	1	0	Museum specimen, IM
		Victoria-West	1	1	1	Soft tissue
	Western	Vredenburg	1	1	1	Museum specimen, TM
		Hopefield	3	4	3	Museum specimen, TM
		West Coast National Park	1	1	1	Soft tissue
		Elandsberg, Wellington	4	4	4	Soft tissue
		Paarl	1	1	1	Museum specimen, NM
		Strand	3	3	3	Museum specimen, TM
	Southern	Mizpah, Grabouw	2	4	2	Soft tissue
		Jonaskop, Villiersdorp	3	3	3	Soft tissue
		Fairfield, Napier	7	7	7	Soft tissue
	Eastern	Gamkaberg, Calitzdorp	4	5	4	Museum specimen, IM
		Besemfontein, Ladismith	4	6	3	Museum specimen, IM
		Cherrydouw, Oudtshoorn	6	8	6	Museum specimen, IM
		Doringrivier, Oudtshoorn	5	5	5	Museum specimen, IM

Table 2.1 continued

Clades / Lineage	Locality	cyt *b*	control region	combined dataset	Source material
	Ou Tol, Oudtshoorn	1	2	1	Museum specimen, CN
	Mossel Bay	3	3	3	Museum specimen, TM
	Uniondale	7	7	7	Museum specimen, TM
	Outeniqua, Natures Valley	0	2	0	Museum specimen, IM
Total no.		89	106	88	

* Soft = fresh DNA samples

Table 2.2 The number of elephant-shrew specimens analysed for each of the Southern African species. Sampling localities with geographical coordinates are also shown.

Species	Locality	Province	Country	Grid ref	No of specimens
M. proboscideus	Steytlerville	Eastern Cape	South Africa	33.30S 24.30E	2
	Goegap Nature Reserve, Springbok	Northern Cape	South Africa	29.22S 17.50E	2
	Paulshoek, Kamieskroon	Northern Cape	South Africa	30.38S 18.28E	1
	Tankwa Karoo National Park	Western Cape	South Africa	32.02S 20.05E	1
P. tetradactylus	Bonamanzi Game Park	KwaZulu-Natal	South Africa	28.00S 31.10E	1
E. brachyrhynchus	Spitskop Nature Reserve	Mpumalanga	South Africa	24.82S 30.12E	2
	Kgaswane Reserve	Northwest	South Africa	25.70S 27.18E	1
E. intufi	Molopo Nature Reserve	Northern Cape	South Africa	25.78S 22.90E	1
	Northern Namibia (Otjiwarongo)		Namibia	21.58S 16.93E	2
	Southern Namibia		Namibia	26.38S 17.98E	2
E. rupestris	Melkboom, Namaqua National Park	Northern Cape	South Africa	29.42S 17.55E	2
	Paulshoek, Kamieskroon	Northern Cape	South Africa	30.38S 18.28E	2
	Windhoek		Namibia	23.80S 17.10E	1
	Goegap Nature Reserve, Springbok	Northern Cape	South Africa	29.22S 17.50E	2
	Oudtshoorn	Western Cape	South Africa	33.30S 22.20E	1
E. myurus	Spitskop Nature Reserve	Mpumalanga	South Africa	24.82S 30.12E	2
	Hopetown	Northern Cape	South Africa	29.50S 24.13E	1
	Vryburg	Northwest	South Africa	27.00S 24.71E	2

DNA EXTRACTION AND SEQUENCING

Total genomic DNA was extracted from fresh tissue using a standard Proteinase K digestion followed by a phenol/chloroform extraction (Maniatis *et al.* 1982). To minimise destructive sampling to specimens housed in museum collections, dried tissue was preferentially taken from within the skull cavity using sterile forceps. DNA from museum specimens was extracted using a commercial kit (DNeasy Tissue Kit, Qiagen). The authenticity of this material was routinely confirmed in that extraction and PCR blanks were invariably negative, sequences were verified from at least two independent DNA extractions with a minimum of two independent PCR amplifications, and PCR amplicons from the same specimen always produced identical sequence (see Chapter 5 for a more detailed discussion of the protocols followed).

Two mitochondrial DNA segments were chosen. First, we amplified and sequenced a large portion of the mitochondrial DNA protein coding cytochrome *b* gene (1052 bp). The second fragment comprised 337 bp on the 5' side of the hypervariable control region. In addition to the use of universal primers (Pääbo & Wilson 1988; Kocher *et al*. 1989; Irwin *et al*. 1991; Rosel *et al*. 1994) and, critical to the present investigation, elephant-shrew specific primers were designed for both segments to allow for the amplification of degraded museum material (Chapter 5). PCR amplification followed standard procedures. Given that PCR inhibitors are often present in museum extractions, successful amplification of older tissue required the addition of Bovine Serum Albumin (BSA; 4 ul of 0.001 g/ml to a 30 ul reaction). Amplifications were carried out in a GeneAmp PCR 2700 system (Applied Biosystems) with a thermal profile involving an initial denaturation step of 3 min at 95 C followed by 35 cycles at 95 C for 30 s, 50 C for 30 s and 72 C for 60 s. When amplifying DNA from museum specimens, the annealing steps varied from 46-52 °C for the cytochrome *b* gene, and 48-50 °C for the control region fragment.

Sequencing reactions were performed using BigDye chemistry (version 3; Applied Biosystems). Sequencing cocktails were cleaned using Centrisep spin columns (Princeton Separations) and the products were analysed on a 3100 AB (Applied Biosystems) automated sequencer. Electropherograms of the raw data were manually checked and edited with Sequence Editor™ software v1.0.3a (Applied Biosystems). Sequences were submitted to GenBank under accession numbers DQ901016-DQ901256.

DATA ANALYSES

Species verification

To confirm the species status of *E. edwardii*, genealogical relationships were constructed by maximum likelihood (ML) and parsimony (MP) methods using PAUP* (Swofford 2001) (1000 nonparametric bootstrap replicates estimated clade support), together with a Bayesian inference approach (BI) (20 x 10^6 generations)

as implemented in MrBayes 3.0 (Huelsenbeck & Ronquist 2001). The model of evolution that best fitted our combined data (employing Modeltest; Posada & Crandall 2001) was GTR + I (0.308) + G (1.076) with the base composition of A = 31.62%, C = 29.47%, G = 9.78% and T = 29.13%. Phylogenetic analyses included representative sequences of the seven South African distributed elephant-shrew species (*P. tetradactylus*, *M. proboscideus*, *E. brachyrhynchus*, *E. intufi*, *E. rupestris, E. edwardii* and *E. myurus*) (see Table 2.2) and were rooted with other members of the Afroinsectiphillia: *Orycteropus afer* (Y18475), *Echinops telfairi* (AB099484) and *Chrysochloris asiatica* (NC004920).

Spatial structure within E. edwardii

We explored the spatial distribution of mitochondrial variation within the Cape rock elephant-shrew using a Spatial Analysis of Molecular Variance (SAMOVA version 1.0; Dupanloup *et al.* 2002), as well as a hierarchical analysis of molecular variance (AMOVA implemented in Arlequin version 3; Excoffier *et al.* 2005). SAMOVA provides an objective means to maximise the proportion of total genetic variance due to differences between groups of populations. Conventional Φ statistics were calculated in Arlequin. Permutational procedures (1000 randomisations) were used to provide significance tests for these statistics under the null hypothesis of panmixia. We further used Monmonier's algorithm (implemented in Alleles in Space (AIS); Miller 2005) to search for barriers to gene flow where the greatest genetic distance between any two locations forms the initial barrier segment. An interpolation-based graphical procedure was used to detect genetic structure over landscapes. We used the default settings of '*midpoint of edges derived from Delaunay triangulation*' and '*residual genetic distances*' with a weight value of 1.0 as the visual spatial approach.

Haplotype networks/trees are more sensitive (Excoffier *et al*. 1992) than tree-based criteria to trace finer-scale population structure through space and time (Bermingham & Moritz 1998; Goldstein *et al*. 2000; Posada & Crandall 2001). To investigate the level of connectedness between haplotypes, we constructed an unrooted neighbour joining network implementing uncorrected "p" distances using SplitsTree 4 (Huson & Bryant 2006).

To test the validity of the evolutionary patterns imposed, we followed a maximum likelihood and Bayesian coalescent based approach (see Nielsen & Wakeley 2001; Griswold & Baker 2002; Bulgin *et al*. 2003; Bowie *et al*. 2006). This method, as implemented in MDIV (Nielsen 2002), estimates population divergence time and has the advantage of separating secondary contact/gene flow from the confounding effects of ancestral polymorphism (see Nielsen & Wakely 2001). To estimate the values of θ (theta), M (migration), T (divergence time) and TMRCA (time to the most recent ancestor) the pairwise comparison ran for 20×10^6

generations set on the finite-sites model with upper bounds of 10 migrants per generation and a population divergence time of 10 units. θ, *M* and *T* were plotted after multiple runs to confirm the best estimate of posterior distribution. Credibility intervals (95%) were calculated for each parameter.

We employed a standard molecular clock to date the divergence times among lineages in an attempt to place the evolutionary events within *E. edwardii* into a biogeography context. Several problems may, however, confound the use of such a clock leading to incorrect estimates of divergence times. First, we calculated both intra- as well as interpopulation divergences (uncorrected p-distances) using PAUP* (Swofford 2001). These were corrected for ancestral polymorphisms (see Arbogast *et al.* 2002) following Nei and Li (1979) where $d_A=d_{XY}-[(d_X+d_Y)/2]$. Secondly, we verified that our data evolved in a clock-like manner. This was done by comparing unconstraint ML trees (including branch lengths) with trees (including branch lengths) where a molecular clock was enforced. All ML searches were done under the optimal model of evolution as determined by Modeltest (TrN + I (0.8649) + G (0.6032)). Trees were compared using the Shimodaira-Hasegawa (SH) test in PAUP*.

The presence of clinal genetic structure, known as isolation-by distance, was evaluated using the regression of $(F_{ST}/(1- F_{ST})$ on the logarithm of geographical distance (Rousset 1997) implemented in the Mantel test (Manly 1991). Geographical distances were determined "as the crow flies", i.e. we estimated the shortest and most direct route between populations rather than along mountain ranges. We also included an "Isolation by Resistance" approach and determined the values of conductance (analogous to migration) and resistance (reciprocal of conductance) in Circuitscape (Mcrae 2006).

RESULTS

For ease of representation and discussion we present here the results obtained from the combined dataset (unless otherwise indicated) since the results from each of the two fast evolving mitochondrial fragments were largely congruent irrespective of whether the DNA fragments were considered singly or in combination. In total, our data set comprised 1389 characters (1052 bp of cytochrome *b* gene and 337 bp of 5' side of the control region).

Verification of species status

The monophyly of three species within the genera *Elephantulus* and *Macroscelides* with overlapping distribution ranges was confirmed using a subset of specimens selected to encompass the genetic variation within *E. edwardii* (six specimens), *E. rupestris* (eight specimens) and *M. proboscideus* (seven specimens) (see Table 2.2). Our analyses revealed no shared haplotypes between them. The average uncorrected

sequence divergence for the combined mtDNA data separating *M. proboscideus* and *E. rupestris* was 21.3% and 19.3% between *E. rupestris* and *E. edwardii. Elephantulus edwardii* and *M. proboscideus* were separated by 24.3%.

Divergences among species were an order of magnitude higher than within species. The average sequence divergence separating specimens within *M. proboscideus* is 1.5% (0.53 – 2.5%), 0.9% between *E. rupestris* specimens (0.22 – 1.5%), and 1.5% between *E. edwardii* specimens (0.23 – 3.18%).

Sequencing of the 5' end of the control region of the *E. karoensis* and *E. capensis* type specimens (currently synonimized with *E. edwardii*) identified *E. capensis* as falling within *E. edwardii* which is consistent with the synonomy proposed by Meester *et al*. (1986). However, in contrast to the current taxonomy, the *E. karoensis* type specimen grouped within *E. rupestris* (Fig. 2.2). The apparent misidentification of the *E. karoensis* type was subsequently confirmed by an examination of morphological and dental characters which suggest that Robert's (1938:234) classification was misled by the specimen's subadult pelage. Although the two lingual cusps on the premolar P^2 are poorly developed in this subadult, and therefore more closely resemble the dentition of *E. edwardii*, the specimen showed a lingual cusp on the first upper premolar (P^1) which is diagnostic of *E. rupestris*. In addition, the pointed, rather than rounded shape of the ears and the tuft length at the tail-tip of this specimen, similarly reflects its affinity to *E. rupestris*.

The Karoo clade

The sequencing of seven of the 64 *E. edwardii* museum specimens drawn from the central Karoo localities of Williston (30.25S 20.80E) and Beaufort-West (32.40S 22.60E; Fig. 2.1c) questioned the species affiliations of these specimens. The mean sequence divergence for specimens from these two localities differ from the major *E. edwardii* clade by 12.1% (11.9 – 12.2%) in the case of the former, and by 11.5% (11.3 – 11.8%) in the latter. These values are an order of magnitude, or higher, than the intraspecific divergences detected within the species *E. edwardii* (1.5%) and the taxa from Beaufort-West (0.4%) and Williston (0.5%). The five Beaufort-West specimens differ by 7.4% from the two Williston specimens. We interpret these data as reflecting the presence of one, or possibly two distinct and hitherto undescribed taxa (species?) from the Beaufort-West and Williston areas of the South African central Karoo. Both lineages are monophyletic and sister to *E. edwardii sensu stricto* clearly indicating a close evolutionary relationship to this strictly South African endemic (Fig. 2.2). Moreover, the Williston and Beaufort-West taxa are phylogenetically distant from the co-occurring and phenotypically similar *E. rupestris* or *M. proboscideus*.

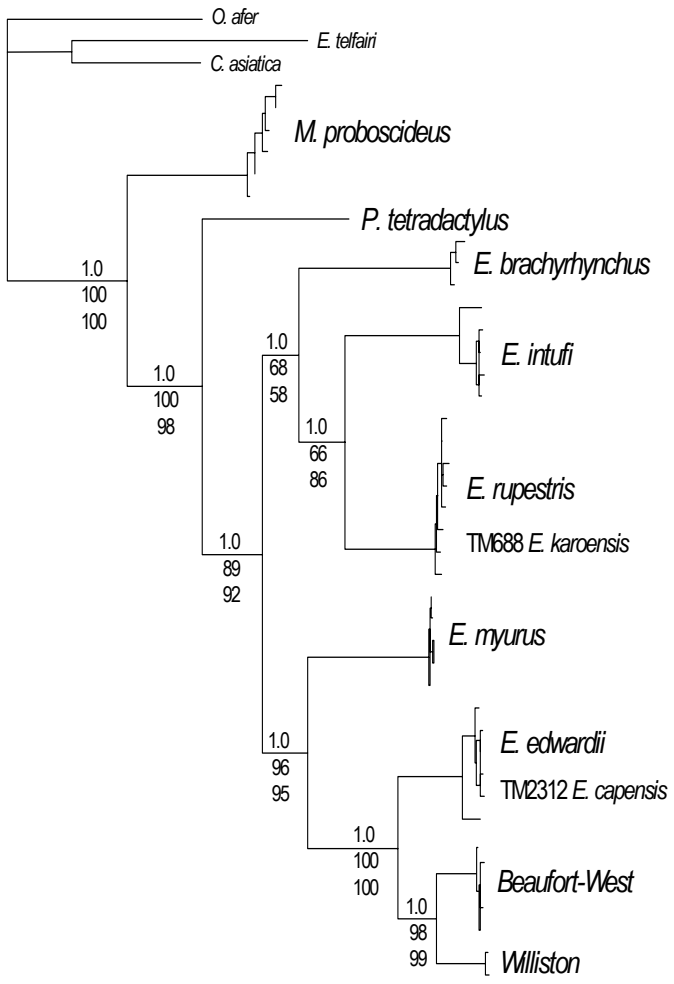

Figure 2.2 Bayesian tree (with branch lengths) based on the combined cytochrome *b* and control region showing the phylogenetic position of the different elephant-shrew species including the distinct Karoo clade represented by the localities of Beaufort-West and Williston. The tree is rooted on members of the Afroinsectiphillia. Values above the nodes infer BI posterior probabilities from a 20 million generation run and values below the nodes represent nonparametric bootstrap support for MP (top) and ML (bottom) for 1000 replicates. The phylogenetic positions of the type specimens for the previously recognized (but currently subsumed) *E. karoensis* (TM688) and *E. capensis* (TM2312) are shown.

Phylogeography: E. edwardii sensu stricto

To investigate both the deeper as well as more recent patterns of genetic divergence within *E. edwardii sensu stricto*, we reanalysed our data excluding the seven divergent Karoo specimens from Beaufort-West and Williston discussed above (see Fig. 2.1c). Our combined data set included 81 specimens from 26 localities across the range of *E. edwardii* for which we had comparable cytochrome *b* and control region data (see Table 2.1). Our analysis identified 60 haplotypes of which 11 were shared; the remaining 49 haplotypes were private / unique.

When considering sampling localities as populations, AMOVA resulted in a highly significant Φ_{ST} value (0.834; $p < 0.001$) indicating substructure in our data. The spatial distribution of variation was maximised when two evolutionary lineages were selected ($\Phi_{CT} = 0.704$; $p < 0.001$; see Fig. 2.1c for lineages). These lineages represent a northern Namaqua clade (Prieska; Namaqua National Park; Kamieskroon; Calvinia; Van Rhynsdorp; Nieuwoudtville) and a central Fynbos clade. The unrooted neighbour joining network (Fig. 2.3a) similarly shows these two clades where a remarkable 26 nucleotide differences (and 100% parsimony and maximum likelihood bootstrap support) separate them. Additionally, no shared haplotypes were detected between these clades. However, northern Namaqua and central Fynbos haplotypes were found in sympatry at two localities (Calvinia and Van Rhynsdorp; see Fig. 2.1c) situated on the border between these two groups. The average sequence divergence that separates the northern clade from the remaining central clade is 1.97% (corrected for ancestral polymorphism; see Table 2.3).

Table 2.3 The average uncorrected p distances (given as percentages) for 1389 bp of sequence corrected for ancestral polymorphism between the five evolutionary lineages (see Fig. 2.1) detected in *E. edwardii sensu stricto*.

	Northern	Central			
		North-western	Western	Southern	Eastern
Northern					
North-western	1.89				
Western	2.01	0.35			
Southern	1.88	0.52	0.75		
Eastern	2.08	0.52	0.28	0.88	

Within the central Fynbos clade, a further four lineages is evident (Fig. 2.3a). Although not as pronounced as the northern Namaqua and central Fynbos clades, an average of 0.55% sequence divergence (corrected for ancestral polymorphism; see Table 2.3) separated them. SAMOVA, excluding the northern Namaqua

clade, confirmed these distinct lineages with Φ_{CT} being maximised for four groups (Φ_{CT} = 0.580; p < 0.001): a north-western (Calvinia; Van Rhynsdorp; Clanwilliam; Cederberg; Victoria-West), western (Vredenburg; Hopefield; West Coast National Park; Wellington; Paarl; Strand), southern (Grabouw; Villiersdorp; Napier) and eastern (Calitzdorp; Ladismith; Oudtshoorn; Mossel Bay; Uniondale) group.

The graphical representation of our results, as found with AIS (Miller 2005), is presented in Fig. 2.3b. The presence of the five lineages is illustrated over the landscape inhabited by *E. edwardii* with the northern Namaqua clade corresponding to the left (most divergent) peak. A north/south division was the first barrier segment to be identified with Monmonier's algorithm which represents the fragmentation between the northern Namaqua and central Fynbos clades (Miller pers. com.).

The Mantel test indicated no isolation-by-distance between the northern Namaqua and central Fynbos clades (p < 0.001). This would imply that the separation between these clades might be due to fragmentation and vicariance, a finding supported by nested clade analyses (Templeton 1998; Posada *et al*. 2000; appendix Fig. 1 & appendix Table 2). However, isolation-by-distance contributed to the separation of the four lineages within the central Fynbos clade (p > 0.05). The evaluation and use of resistance/conductance values from the "Isolation by Resistance" analysis among the five evolutionary lineages corroborated the above findings and confirmed the northern Namaqua clade as genetically most divergent. On average, the values between this northern clade and the central Fynbos clade (resistance = 0.417; conductance = 2.401) suggested higher resistance and lower conductance compared to those calculated between the four lineages within the latter (resistance = 0.203; conductance = 5.226).

Molecular clock and coalescent approach

We corrected our pairwise differences for ancestral polymorphism (see Table 2.3) for the application of a molecular clock. On average, correcting for ancestral polymorphism reduced sequence divergences by 0.35%. We also verified that our data evolved in a clock-like manner. The null hypothesis of a constant evolutionary rate across lineages could not be rejected (p = 0.182; likelihood score of 2902.524 for the unconstrained (best) tree and 2915.105 for the clock-enforced tree). In further support of our application of a molecular clock, congruent time estimates were obtained by two independent methods; a relaxed Bayesian molecular clock and the MDIV Bayesian coalescent approach (see below).

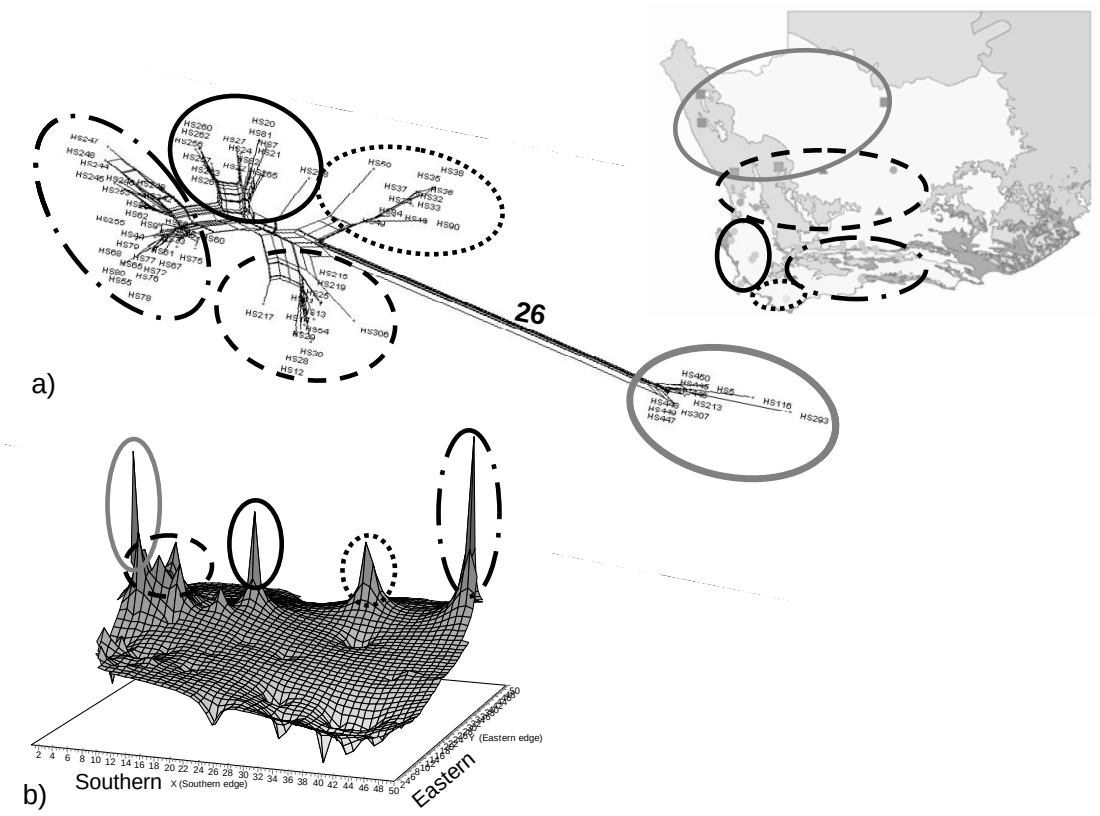

Figure 2.3 (a) The five evolutionary lineages are circle coded and are representative of the northern Namaqua (solid thick grey), north-western (dashed black), western (solid black), southern (square dotted black) and eastern (dash dotted black) Fynbos lineages on the geographical map. The mitochondrial neighbour joining network shows the northern Namaqua clade connected to the central Fynbos clade by 26 mutational steps. Separation was on average two mutational steps between the four lineages within the central Fynbos clade. (b) A graphical interpolation-based representation of the genetic structure over the distribution of *E. edwardii*.

Douady *et al.* (2003) estimated the divergence between *M. proboscideus* and *E. edwardii* at 21.3 ± 3.3 million years ago (MYA) using both mitochondrial and nuclear data. Their divergence time was estimated with a relaxed Bayesian molecular clock employing the Paenungulate divergence date determined by Springer *et al*. (2003). Applying their divergence time to the average sequence divergence estimate in our study separating *M. proboscideus* and *E. edwardii* (24.3% corrected for ancestral polymorphism), we estimate a rate of evolution of 1.14 ± 0.18 % per MY (24.3%/21.3 MYA) in elephant-shrews. These values placed the separation of the northern Namaqua and central Fynbos groups at 1.73 MYA (range 1.4 – 2.0

MYA) (the mutation rate was estimated at 1.14 ± 0.18 % per MY and the average uncorrected sequence divergence (corrected for ancestral polymorphism) separating lineages was 1.97%).

Coalescent analyses were restricted to a population comparison of the northern Namaqua clade (n=11) and its geographically neighbouring lineage within the central Fynbos clade, the north-western group (n=16). Estimated values and 95% credibility intervals are θ = 12.38 (6.29 – 28.05), M = 0.04 (0.00 – 1.52) and T = 2.16. Low migration rates in combination with the retention of ancestral polymorphism would indicate a past fragmentation event (also found with nested clade analyses; data not shown). The T-value was converted into a population divergence time of 1.69 MYA using a mutation rate of 1.14% per MY (or a substitution rate of 1.58 $x10^{-5}$ per site per year) and a generation time of one year. This estimate is consistent with that of our molecular clock. The estimated value of genealogical divergence TMRCA = 3.08 from the most recent common ancestor resulted in a divergence time of 2.41 MYA, ~0.7 MYA before the population divergence.

DISCUSSION

The 15 recognized species of elephant-shrews (Order Macroscelidea) are grouped within four genera (*Rhynchocyon*, *Elephantulus*, *Macroscelides* and *Petrodromus*) all of which are endemic to Sub-Saharan Africa (Perrin 1997; Douady *et al.* 2003; Skinner & Chimimba 2005). The only exception to this strict Sub-Saharan endemicity involves the intrusion of a single *Elephantulus* species, *E. rozeti*, into northern Africa. Of the genera, *Elephantulus* is the most species-rich comprising ten taxa of which six occur within southern Africa (*E. brachyrhynchus*, *E. fuscus*, *E. intufi*, *E. rupestris, E. myurus* and *E. edwardii)*.

Verification of the species status

The three elephant-shrew species that have overlapping distributions in the southern and western regions of South Africa (*E. edwardii, E. rupestris* and *M. proboscideus*) are morphologically very similar. Although *M. proboscideus* (this species prefers gravel plains to rocky outcrops) is ecologically separated from *E. edwardii* and *E. rupestris* (which prefer rocky outcrops) on habitat, the two rock elephant-shrew species are virtually impossible to distinguish in the field. Our molecular sequence data unequivocally confirmed the status of these three species. Sequence divergences (cytochrome *b*) separating species range from 19.7% between *E. rupestris* and *E. edwardii,* to 24.7% between *M. proboscideus* and *E. edwardii*. Although sequence data used in isolation are not good indicators of species status, it should be noted that the values separating these elephant-shrew species fall in the upper range when viewed comparatively in mammals (see Johns and Avise 1998 for a summary of divergences separating species; Bradley & Baker 2001; Colangelo *et al*. 2005 for specific examples). Perhaps more importantly, no haplotypes are shared among these three species, and they form distinct monophyletic groups (Fig. 2.2).

Phylogeography

Life history characters determine the spatial distribution of genetic variation in species. Pertinent factors with respect to the Cape rock elephant-shrew include that it is monogamous, territorial and strictly confined to rocky habitat (Skinner & Chimimba 2005). Given these traits, one would intuitively expect significant population substructure – a characteristic confirmed by our spatial analysis of genetic variation. In summary, our analyses provided evidence of three distinct *E. edwardii* clades discussed more fully below.

(i) The Karoo clade – evidence in support of an undescribed species

Seven museum specimens from two localities in the central Karoo of South Africa (Beaufort-West and Williston) consistently grouped separately from the remainder of the *E. edwardii* specimens (see Fig. 2.2). Animals from these two localities are, on average, separated by 12.2% uncorrected cytochrome *b* sequence divergence from *E. edwardii sensu stricto*. Based on other mammalian species (see Johns & Avise 1998; Bradley & Baker 2001; Colangelo *et al*. 2005) these values suggest genetic distinctiveness for these specimens. Their cytochrome *b* and control region haplotypes are unique from those of other elephant-shrews with overlapping ranges (*E. edwardii*, *E. rupestris* and *M. proboscideus*), and show closest evolutionary affinity to *E. edwardii*. Our cursory analysis of phenotypic and dental characteristics suggest no notable differences in terms of the latter, but several phenotypic characters (e.g. tuft length on tail; ventral and dorsal pelage and flank colour; colour and shape of eye ring) hold promise for distinguishing these specimens from elephant-shrew species with overlapping distributions. However, given that these unique lineages have currently been detected at only two localities (Beaufort-West and Williston), more comprehensive sampling is required to accurately determine the geographic extent and phenotypic plasticity of this apparently unique elephant-shrew assemblage.

(ii) The northern Namaqua clade

The most distinct lineages within the Cape rock elephant-shrew (*E. edwardii sensu stricto*) were derived from specimens collected at the Namaqua National Park, Kamieskroon, Prieska, Calvinia, Nieuwoudtville and Van Rhynsdorp (see Fig. 2.1c), all of which occur along the Atlantic seaboard in close proximity to the South African / Namibian border. Although this group is represented by only 11 *E. edwardii* specimens, the number of mutational steps (26) separating this lineage from the other clades to the south far exceeds those between other lineages. The southern limit of this group coincides with the so-called “Knersvlakte” region (see Fig. 2.1c) in the north-western Cape of South Africa which is thought to be coincidental with the limits of upliftment of the great western escarpment which occurred ~18 MYA (Moon & Dardis 1988).

Importantly, isolated genetic assemblages were similarly described for other rock-dwelling species that have distributions that span this region. These include the red rock rabbit *P. rupestris* (Matthee & Robinson 1996), the rock agama *A. atra* (Matthee & Flemming 2002) as well as the *Goggia* and *Pachydactylus* gecko genera (Branch *et al.* 1995, 1996; Bauer *et al.*1997; Bauer 1999; Lamb & Bauer 2000). We argue that past fragmentation occurring ~1.7 MYA is largely responsible for separating the Namaqua (northern) lineage from the remainder of the central Fynbos Cape rock elephant-shrew populations. The sympatric presence of northern and north-western haplotypes at two sampling localities in the vicinity of the Knersvlakte (Calvinia and Van Rhynsdorp) can best be ascribed to one of two possible scenarios. First, incomplete lineage sorting (ancestral polymorphism) is responsible for the observed pattern or, secondly, that the populations were formerly geographically isolated/fragmented (with a possibility of intermediate haplotypes becoming extinct) and after subsequent range expansions a secondary contact zone formed. In both described cases the absence of gene flow and/or shared haplotypes between these suggests reproductive isolation (speciation) which should be verified through additional studies including mating experiments and hybrid fertility testing.

Various topographical and climatic events might have either singly, or in concert, caused the isolation and / or local extinction of taxa confined to rocky habitat in this region. We further speculate that the isolation would have been repeatedly enforced through multiple events. First, at ~2 MYA (Linder 2003 and references therein) a series of Pleistocene marine transgressions inundated the western coastal plains with rises in sea-levels of between 45-50m, 75-90m and 27m respectively (Hendey 1970a,b). Secondly, changes in river-flow patterns could also have served as isolating mechanisms. For example, in this region the proto-Berg River or Langebaanweg River, which were the precursors of the present Great Berg River, changed course during the 45-50m marine transgression (Hendey 1970a, b). Thirdly, Deacon and Lancaster (1988) argued that climate changes during the past 3 MY were particularly harsh on the western side of the continent resulting in temperature fluctuations and associated wet-dry cycles (Brain & Meester 1964; Brain 1985; Van Zinderen-Bakker & Mercer 1986; Lindesay 1998a, b). Lastly, recent Kalahari sandflows occurred from the north to the south which would have acted as a physical barrier separating populations (Deacon & Lancaster 1988; Haacke 1989; Lancaster 1989). Noteworthy evolutionary changes in other vertebrates and African hominids similarly occurred at ~1.7 MYA, co-incident with one of three estimated major peaks of aridification in Africa in the Plio-Pleistocene epoch (deMenocal 1995). In addition, Matthee and Flemming (2002) placed the phylogeographic discontinuity between the *A. atra* clades across the Knersvlakte at roughly this time (2.2 – 4.4 MYA) which is consistent with the divergence of the northern and central haplotyopes from their common ancestor (2.41 MYA).

(iii) The central Fynbos clade

The habitat across the distribution of *E. edwardii* is certainly not homogeneous. The areas occupied by the northern Namaqua group can best be described as terrestrial islands (marginal habitat *sensu* Prinsloo & Robinson 1992) surrounded by hostile plains (Moon & Dardis 1988). In comparison, the central clade is found within the relative safety of the Cape Fold Mountains (CFM) with cover provided by fynbos vegetation. The CFM is the major topological component of the renowned Cape Floristic Kingdom found at the southern tip of Africa with the high degree of diversity and endemism in this region being credited to environmental fluctuations from the Pliocene and Pleistocene epochs (Midgley *et al*. 2001; Linder 2003). *Elephantulus edwardii* is important in the ecology of the Cape Floristic Kingdom where the species plays a major role in the pollination of *Protea s*pecies (Fleming & Nicolson 2003).

It is noteworthy that the phylogeographic trends described for the rock agama *A. atra* in the Cape Fold Belt (Swart 2006), sharing the habitat requirements and overlapping in distribution with *E. edwardii*, are largely consistent with the divergence patterns found for the *E. edwardii* central Fynbos clade. Within the central clade, shallow genetic structure was evident with four evolutionary lineages identified: the north-western, western, southern and eastern lineages. We argue that the geographic occurrence of these four lineages broadly resembles the climatic differences and vegetation types related to the position of mountain ranges shaping the Cape Fold Belt. Our analyses indicate that the shallow phylogeographic patterns within these lineages are the result of restricted gene flow with isolation by distance. Environmental fluctuations within the Pleistocene epoch (2.0 - 0.1 MYA) are mostly credited for these events with glacial and interglacial conditions alternating at ~100 000 year intervals (Lindesay 1998a,b). When applying a molecular clock (based on corrected sequence divergences; see Table 2.3), the separation between these lineages varies from 0.7 MYA years for the split between the southern and eastern lineages, to 0.24 MYA for the separation of the western and eastern lineages. It is striking that no shared haplotypes exist among the four central *E. edwardii* lineages. Within groups, however, haplotypes were commonly shared between populations resulting in the absence of deep structure.

In summary therefore, our data support the recognition of *E. edwardii* as a distinct species in which we show three major clades (Karoo, northern Namaqua and central Fynbos). We argue for distinctiveness of the Karoo assemblage based on genetic divergence and phenotypic characters. The mitochondrial analyses proved useful in identifying genetic variation, past fragmentation, and the impact of ancestral polymorphism in shaping the genetic profiles of the northern Namaqua and central Fynbos clades. Finally, this investigation underscores the importance of museum material in phylogeographic studies and specifically in

identifying cryptic species (Good & Sullivan 2001; Godoy *et al*. 2004; Rohland *et al*. 2004), especially in instances where access to material is constrained by low population densities and the conservation status of the species.

CHAPTER 3

A NEW SPECIES* OF ELEPHANT-SHREW (AFROTHERIA: MACROSCELIDEA: *ELEPHANTULUS*) FROM SOUTH AFRICA

*Disclaimer: International Commission on Zoological Nomenclature Article 8.2. "A work that contains a statement to the effect that it is not issued for public and permanent scientific record, or for purposes of zoological nomenclature, is not published within the meaning of the Code".

INTRODUCTION

The order Macroscelidea (elephant-shrews) is nested within Afrotheria, an endemic African clade of mammals that comprises six orders whose recognition is based almost exclusively on DNA sequences and other genomic data (Proboscidea: elephants, Sirenia: dugong and manatee, Hyracoidea: hyraxes, Afrosoricida: tenrecs and golden moles, Tubulidentata: aardvark and Macroscelidea: elephant-shrews) (e.g. Springer et al. 1997; Stanhope et al. 1998a, b; Springer et al. 1999; Amrine-Madsen et al. 2003; Nikaido et al. 2003; Robinson et al. 2004; Svartman et al. 2004; Nishihara et al. 2005; Kriegs et al. 2006; Nishihara et al. 2006; Waters et al. 2007). Fifteen species are recognized within the Macroscelidea all of which, with the exception of a single species, *E. rozeti*, have a strict sub-Saharan distribution (Corbet and Hanks 1968). Elephant-shrews (acronym sengis) are small bodied, capable of rapid movement (jumping and running), insectivorous and display social monogamy (Rathbun 1979). Two extant subfamilies, the Macroscelidinae and Rhynchocyoninae, are recognised within the order. The Macroscelidinae includes three of the four currently recognized genera: the monotypic *Macroscelides* which is a south-western African gravel plain specialist, the monotypic *Petrodromus* (with a south, east and central African forest distribution) and *Elephantulus* that includes 10 species found throughout a diverse array of habitats (Corbet and Hanks 1968). The second subfamily, Rhynchocyoninae, is represented by three extant east and central African forest species within *Rhynchocyon*, a genus that is considered to include a new species from the Udzungwa Mountains in Tanzania (Rovero and Rathbun 2006; Rovero et al. in press).

Information on the number of existing species per biome, region, and/or continent is important in making informed conservation decisions (Medellín and Soberón 1999). This information is, however, incomplete even for supposedly well-known groups of animals such as mammals (Morell 1996). For example, new mammalian species continue to be recognized such as the giant elephant-shrew from East Africa (Rovero et al. in press), the Laotian rock rat (*Laonastes aenigmamus*), a rodent species from the Khammouan region of Laos (Jenkins et al. 2004) and the African forest elephant (*Loxodonta cyclotis*), previously thought to be a subspecies of the African elephant (*L. africana*) (Roca et al. 2001).

The description of a novel species should preferably be based on a number of character types including molecular, morphological and anatomical data. However, these species are often cryptic, or have only a few subtle characters that distinguish them from sibling species. In these instances genetics has become a powerful tool in providing the first clues in the recognition of new species (e.g. *Laniarius spp.* (shrike), Smith et al. 1990; *Pneumocystis wakefieldiae* (rat), Cushion et al. 2004; *Microcebus spp.* (mouse lemur), Olivieri et al. 2007; *Microgale jobihely* (shrew tenrec), Goodman et al. 2006; *Spermophilus taurensis* (Taurus ground squirrel), Gündüz et al. 2007). This is exemplified by elephant-shrews where the genetic distinctiveness of a

lineage from the central South African Nama-Karoo (Karoo clade) was suggested by the analysis of mitochondrial sequences from elephant-shrew specimens (Smit et al. 2007). This novel lineage clustered as the sister to *E. edwardii* within a larger clade that also included *E. myurus* (Smit et al. 2007).

The Karoo clade (herein proposed to represent a previously unrecognised species of elephant-shrew, see Chapter 2) overlaps in distribution with the Cape rock elephant-shrew (*E. edwardii;* Fig. 3.1b), the western rock elephant-shrew (*E. rupestris;* Fig. 3.1c) and the round eared elephant-shrew (*M. proboscideus*) in the South African Karoo. All of the southern African rock elephant-shrew species (including *E. myurus* which does not occur in this region) are morphologically very similar, but are each phenotypically distinct from *M. proboscideus* (Corbet and Hanks 1968).

This study extends the investigation of Smit et al. (2007) through the addition of 10 specimens and provides evidence for the formal recognition of a new elephant-shrew species from South Africa. A multidisciplinary approach is followed that includes genetic sequencing of mitochondrial and nuclear segments and comparative cytogenetics. This study further assesses several phenotypic characters (principally those of Corbet and Hanks 1968) for their usefulness in species identification, and in so doing expands the existing macroscelid key (Corbet 1974) to include the morphological identification of the new species described herein, and its delimitation from the phenotypically similar and largely sympatric *E. rupestris* and *E. edwardii*.

MATERIALS AND METHODS

Sample collection

Seventeen specimens of the new species were sequenced which included three specimens live-trapped in the field, seven specimens from the Transvaal Museum (TM; South Africa), five specimens from the McGregor Museum (MMK; South Africa) and two specimens from the California Academy of Sciences (CAS; USA) (Table 3.1). The 10 additional specimens of the new form of *Elephantulus* were compared to the two rock elephant-shrew taxa with which it co-occurs, *E. edwardii* and *E. rupestris*, using sequence data from the complete mitochondrial protein coding cytochrome *b* gene and the 5' side of the hypervariable control region of representative specimens. In addition, 360 bp of the 7th intron of the nuclear fibrinogen gene was sequenced for all specimens of the new species and representatives of *E. edwardii* (n=7) and *E. rupestris* (n=8).

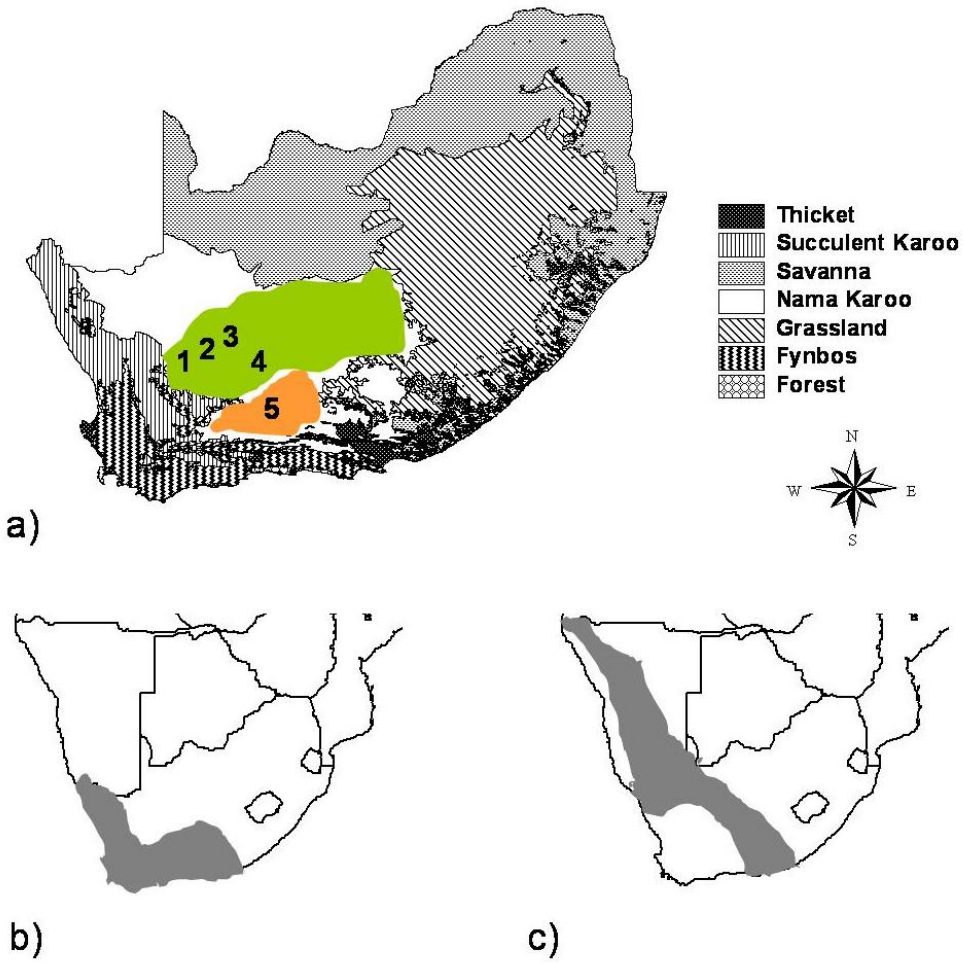

Figure 3.1 (a) Map of South Africa indicating the various vegetation biomes based on Low and Rebelo (1996). The various collection localities representing the Karoo lineage are indicated: {1} Calvinia, {2} Williston, {3} Carnarvon, {4} Loxton and {5} Beaufort-West. The approximate positions of the Upper Karoo (green) and the Lower Karoo Bioregion (orange) vegetational units in the Nama Karoo are indicated. Distributions of the two rock elephant-shrews that overlap in range with the new form of *Elephantulus* namely (b) *E. edwardii* and (c) *E. rupestris* are given on a southern African scale (ranges taken and redrawn from the Global Mammal Assessment sengi maps; G. Rathbun unpubl. data).

DNA extraction and sequencing

DNA from museum specimens was extracted using a commercial kit (DNeasy Tissue Kit, Qiagen). Total genomic DNA was extracted from fresh tissue collected in the field using a standard Proteinase K digestion followed by a phenol/chloroform extraction (Maniatis et al. 1982). Specific primers for elephant-shrews were designed and used in conjunction with the universal primers of Pääbo and Wilson (1988), Kocher et al. (1989), Irwin et al. (1991), Rosel et al. (1994) and Seddon et al. (2001).

Table 3.1 Specimens of the new *Elephantulus* species, *E. edwardii* and *E. rupestris* included in the present study. Specimens for which mitochondrial (m), nuclear (n) and cytogenetic (c) data were available are indicated. (California Academy of Sciences = CAS; McGregor Museum = MMK; Transvaal Museum = TM and Stellenbosch University = HS). See appendix Table 3 for more information.

Species	Locality		Country	Latitude, longitude	no of specimens			Source material
					m	n	c	
Elephantulus new *sp.*	Beaufort-West	Western Cape	South Africa	32º12'S, 22º19'E	5	3		Museum specimen TM29496 - TM29498; TM29528-TM29529
	Williston	Northern Cape	South Africa	31º06'S, 21º 21'E	2			Museum specimen TM27303 - TM27304
	Carnarvon	Northern Cape	South Africa	30º30'S, 22º06'E	5	4		Museum specimen MMK/M/2167 - MMK/M/2171
	Calvinia	Northern Cape	South Africa	31º48'S, 19º49'E	3	3	3	Soft tissue MMK/M/7305 - MMK/M7307
	Loxton	Northern Cape	South Africa	31°28'S, 22°22'E	2			Museum specimen CAS27648 - CAS27649
E. edwardii	Melkboom, Namaqua National Park	Northern Cape	South Africa	29°25'S, 17°33'E		1		Soft tissue HS5
	Kamieskroon	Northern Cape	South Africa	30°07'S, 17°34'E	1			Soft tissue HS116
	Clanwilliam	Western Cape	South Africa	32°06'S, 18°29E	1			Soft tissue HS22
	Cederberg	Western Cape	South Africa	32°14'S, 19°03'E	1		2	Soft tissue HS12, 14
	Wellington	Western Cape	South Africa	32°48'S, 18°30'E	1	3		Soft tissue HS20, 81, 82
	Grabouw	Western Cape	South Africa	33°29'S, 18°24'E	1		1	Soft tissue HS84
	Napier	Western Cape	South Africa	33°29'S, 18°17'E	1	3	3	Soft tissue HS33, 36, 38
E. rupestris	Melkboom, Namaqua National Park	Northern Cape	South Africa	29°15'S, 17°33'E	1	2		Soft tissue HS1, 2
	Paulshoek, Kamieskroon	Northern Cape	South Africa	30°23'S, 18°17'E	1	3		Soft tissue HS114, 117, 120
	Windhoek		Namibia	23°48'S, 17°06'E	1			Soft tissue HS63
	Goegap Nature Reserve, Springbok	Northern Cape	South Africa	29°13'S, 17°30'E	2	2		Soft tissue HS137, 156
	Oudtshoorn	Western Cape	South Africa	33°18'S, 22°12'E	1	1		Soft tissue HS161

To minimise destructive sampling of museum specimens, dried tissue was preferentially taken from within the skull cavity using sterile forceps. PCR blanks were invariably clean and re-extraction and amplification always produced the same sequence. GenBank blast searches confirmed their status as elephant-shrews.

PCR amplification followed standard procedures. Amplification was carried out in a GeneAmp PCR 2700 system (Applied Biosystems) with a thermal profile involving an initial denaturation step of 3 min at 95 ˚C followed by 35 cycles at 95 ˚C for 30 s, 50 ˚C for 30 s and 72 ˚C for 60 s. Successful amplification of museum tissue required the addition of BSA (4 ul of 0.001 g/ml to a 30 ul reaction). Amplicons were electrophoresed in 1% agarose gels. Sequencing reactions were performed using BigDye chemistry (version 3; Applied Biosystems). Sequencing cocktails were cleaned using Centrisep spin columns (Princeton Separations) and the products analyzed on a 3100 AB automated sequencer. Electropherograms of the raw data were manually checked and edited with Sequence Editor™ software v1.0.3a (Applied Biosystems). Sequences have been submitted to GenBank under accession numbers DQ901212 - DQ901218; DQ901250 - DQ901256; EU076240 - EU076283.

Data analysis

Analyses of the sequence data followed Smit et al. (2007). In short, maximum likelihood (ML) and parsimony (MP) analyses were performed in PAUP* (Swofford 2001) (1000 nonparametric bootstrap replicates estimated clade support) together with a Bayesian Inference approach (BI) (20 x 10^6 generations) as implemented in MrBayes v3.1 (Huelsenbeck and Ronquist 2001). The optimal evolutionary models for the various data partitions were determined in Modeltest (Posada and Crandall 2001) (GTR + I + G model for the combined mitochondrial DNA and Trn + I model for the nuclear dataset).

Chromosome and standard karyotype preparation

Metaphase chromosome spreads were obtained from fibroblast cultures (*E. edwardii* n=6; New species n=3) established from tail biopsies and cultivated in tissue culture medium supplemented with 15% foetal calf serum and maintained at 37 ºC and 5% CO_2. Metaphase chromosomes were harvested following conventional procedures and subjected to G-banding (Seabright 1971), C-banding (Sumner 1990) and silver staining (Goodpasture and Bloom 1975). The chromosomes (2n=26) were numbered in decreasing size and arranged following Robinson et al. (2004). The specimens analyzed cytogenetically are listed in Table 3.1. Trapping and tissue collection protocols were approved by the ethics committee at Stellenbosch University (clearance number 2006B01008).

Phenotypic comparison

The morphological distinction between the new species and *E. edwardii* and *E. rupestris*, the elephant-shrew species with which it co-occurs and which are morphologically very similar to it, was based on an analysis of 15 specimens of the new form of *Elephantulus* and 25 adult specimens for each of *E. edwardii* and *E. rupestris*; all *E. edwardii* and *E. rupestris* specimens examined in the study are housed in the mammal collections of the TM. The characters examined follow Corbet and Hanks (1968) and include the colour of the pelage, dental morphology, a number of standard external body measurements as well as selected cranial measurements (see Table 3.2). Cranial measurements of the new species as well as of *E. edwardii* and *E. rupestris* were taken manually. External measurements for the new species, *E. edwardii* and *E. rupestris* were recorded directly from the TM and MMK museum labels (with the exception of three field collected specimens of the new species taken manually). Since only DNA was available from the CAS specimens they were not included in the morphological comparisons. Adults were defined by the presence of a fully erupted permanent dentition (Skinner and Chimimba 2005). Five of the 15 specimens of the new species were classified as subadult/juvenile and therefore excluded from the metric analyses and qualitative dental comparisons. Measurements were taken with digital calipers. External measurements included total length (TL), tail length (T), ear length measured from the notch of the ear (E) and hind foot length from the heel to the end of the longest claw (HF c.u.). Means and ranges are reported separately for sexes. Four cranial measurements were recorded (see Fig. 3.2)– these include greatest length of skull (GLS: from the anterior most point of the premaxilla (rostrum) to the posterior most point of the skull i.e. posterior point of the occipital bone, along the longitudinal axis of skull), rostrum length (RL: from the anterior most point of the premaxilla to the anterior most point of the suture at the border between the nasal and frontal bones), zygomatic breadth (ZB: greatest distance between the outer margins of the zygomatic arches) and least interorbital breadth (LIB: least distance dorsally between the orbits). There was no significant sexual dimorphism within either the new species, *E. edwardii* or *E. rupestris* as determined by a Mann Whitney U-test and the sexes were therefore combined for analyses of the external and cranial measurement data (Kruskal Wallis ANOVA and posthoc multiple comparisons of mean ranks for all groups). All statistical analyses were done in Statistica v8.0. The qualitative dental characters were evaluated for their usefulness in distinguishing *E. edwardii* from *E. rupestris*. These include the presence of lingual and labial cusps on P^1 and P^2 as well as the shape of P^2.

Table 3.2 External and cranial measurements of the new species, *E. edwardii* and *E. rupestris*. External measurements for *E. edwardii* and *E. rupestris* are from specimen labels whereas all cranial measurements were taken by HAS.

	New species						*E. edwardii*						*E. rupestris*								
	Males			Females			Males			Females			Males			Females			Kruskal Wallis ANOVA		
	Mean ± SD	n	Range	Mean ± SD	n	Range	Mean ± SD	n	Range	Mean ± SD	n	Range	Mean ± SD	n	Range	Mean ± SD	n	Range	H	n	p
TL (mm)	246 ± 14.2	6	226 - 266	242 ± 8.5	4	232 - 252	241 ± 7.8	2	235 - 246	252 ± 16.0	11	220 - 271	268 ± 15.8	9	248 - 297	271 ± 15.4	7	247 - 288	14.030	39	<0.001
T (mm)	129 ± 14.6	6	112 - 151	130 ± 5.7	4	122 - 135	127 ± 2.8	2	125 - 129	133 ± 10.5	11	112 - 146	143 ± 9.7	9	131 - 160	137 ± 17.6	7	107 - 160	6.114	39	0.047
E (mm)	29 ± 2.1	6	25 - 31	30 ± 1.3	4	29 - 32	27 ± 2.1	2	25 - 28	28 ± 1.9	11	25 - 30	25 ± 2.4	9	21 - 29	26 ± 2.4	7	24 - 31	13.955	39	<0.001
HF c.u.(mm)	34 ± 1.4	6	32 - 36	34 ± 0.6	4	34 - 35	35 ± 0.7	2	34 - 35	36 ± 1.3	11	34 - 38	37 ± 2.4	9	33 - 40	37 ± 1.8	7	35 - 39	10.494	39	0.005
Mass (g)	45 ± 4.3	6	40 - 52	49 ± 8.6	4	38 - 59	41 ± 7.1	2	36 - 46	39 ± 7.8	5	31 - 51	64 ± 6.5	6	58 - 76	64 ± 9.0	4	54 - 76	*		
GLS (mm)	34.0 ± 0.7	6	33.2 - 35.1	34.7± 0.4	4	34.2 - 35.1	34.0 ± 1.5	4	31.8 - 34.7	35.0 ± 1.8	17	30.3 - 37.3	37.4 ± 1.1	13	36.0 - 39.5	37.2 ± 1.4	8	35.4 - 39.7	28.482	52	<0.001
RL (mm)	15.3 ± 0.4	6	14.8 - 15.7	15.6 ± 1.1	4	14.8 - 17.3	16.0 ± 0.6	4	15.1 - 16.5	16.5 ± 1.7	17	13.5 - 19.0	17.9 ± 1.0	13	16.0 - 19.9	18.0 ± 1.0	8	17.0 - 20.1	21.822	52	<0.001
ZB (mm)	19.0 ± 0.7	6	18.3 - 20.0	19.1± 0.1	4	19.0 - 19.3	19.5 ± 0.5	4	19.0 - 20.0	19.0 ± 0.6	17	18.2 - 20.2	20.1 ± 0.6	13	19.2 - 21.4	20.2 ± 0.5	8	19.2 - 20.8	16.353	52	<0.001
LIB (mm)	7.10 ± 0.4	6	6.5 - 7.5	7.50 ± 0.4	4	7.0 - 7.8	7.50 ± 0.5	4	7.1 - 8.2	7.30 ± 0.4	17	6.9 - 8.0	7.70 ± 0.5	13	7.1 - 8.4	7.60 ± 0.5	8	7.0 - 8.5	6.906	52	0.032

*Mass was excluded from statistical analyses due to limited sample size.

TL (total length); T (tail length); E (ear length); HF c.u. (hind foot length); GLS (greatest length of skull); RL (rostrum length); ZB (zygomatic breadth); LIB (least interorbital breadth)

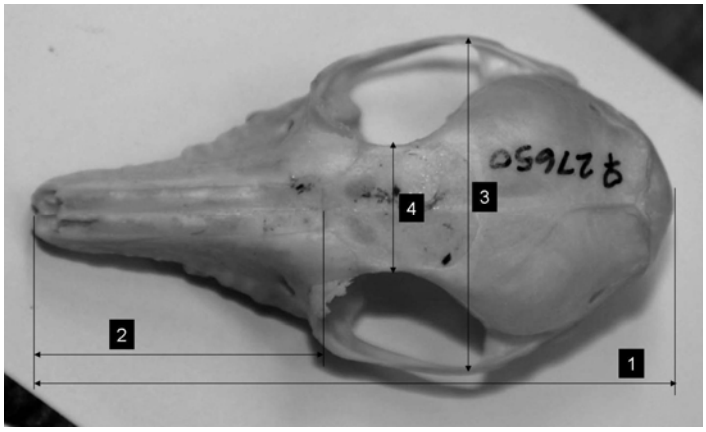

Figure 3.2 Cranial measurements superimposed on the dorsal view of a representative *Elephantulus* skull (*E. edwardii*; CAS 27650). 1) Greatest length of skull (GLS), 2) rostrum length (RL), 3) zygomatic breadth (ZB) and 4) least interorbital breadth (LIB).

RESULTS

Elephantulus pilocaudata, new species (H. A. Smit)

Holotype

Adult female captured at Vondelingsfontein Farm on 19 September 2006 by HAS.

Material

Voucher specimen placed in the McGregor Museum, Kimberley (MMK), South Africa (MMK/M/7305). Fresh DNA sample (heart, liver) stored at Stellenbosch University (HS451).

Type locality

Vondelingsfontein Farm, Calvinia, Northern Cape Province, South Africa (31º48'S, 19º49'E, 1449m above sea level).

Paratypes

TM 27303 (adult male), TM 27304 (subadult female) collected at Goraas Farm (31º06'S, 21º 21'E), Williston, Northern Cape Province, South Africa on 10 February 1977 by I. Rautenbach et al.

TM 29496 (adult female), TM 29497 (subadult male), TM 29498 (adult male), TM 29528 (adult male), TM 29529 (subadult male) collected at the Karoo National Park (32º12'S, 22º19'E), Beaufort-West, Northern Cape Province, South Africa on 21/22 January 1979 by I. Rautenbach et al.

MMK/M/2167 (male), MMK/M/2168 (male), MMK/M/2169 (male), MMK/M/2170 (female), MMK/M/2171 (female) collected at Carnarvon Commonage (30º30'S, 22º06'E), Carnarvon, Northern Cape Province, South Africa in 1983 by H. Erasmus.

MMK/M/7306 (juvenile male), MMK/M/7307 (juvenile male), collected at Vondelingsfontein Farm, Calvinia (31º48'S, 19º49'E), Northern Cape Province, South Africa on 19 September 2006 by HAS.

Distribution

Elephantulus pilocaudata is confined to rocky habitat with an elevation of ≥1300m above sea level. This species is restricted (endemic) to the Upper and Lower Karoo Bioregions of the Nama Karoo, South Africa (Fig. 3.1a).

Etymology

The specific epithet refers to a morphological character (one of a suite of traits collectively diagnostic) – the tail-tip is considerably more tufted in this species than in its sister species *E. edwardii*, but less so compared to *E. rupestris*. "Pilo" = hair and "cauda" = tail; gender feminine (see Fig. 3.3). It is recommended that the vernacular name should be "Karoo rock elephant-shrew", representative of its geographic occurrence in the Nama Karoo of South Africa.

Description

The upper parts of the body and forehead are grey-brown tinged yellow and grizzled with blackish brown. This extends to the flanks and contrasts sharply with the gradual change in colour evident between the dorsal and flanking regions of *E. edwardii* and *E. rupestris*, the two southern African rock elephant-shrew species with which it shares overlapping ranges (see Fig. 3.3a & b). There is a dorsal defuse black-brown pencil line along the midline of the proboscis that becomes lighter towards the forehead. The vibrissae are black. Ears are proportionately large, broad at the base with rounded tips. Post-auricular region is tawny-rufous tinged with pale yellow-brown rather than orange and extends behind the neck; it is less conspicuous than in *E. rupestris* but slightly more so than in *E. edwardii*. The under parts are mottled/blotchy grey. The eye ring is yellow-cream and more prominent at the bottom, almost broken above to the right with the inner hair of the ear margins being similar in colour. The tail is entirely black distally but proximally black above and paler below. The dark-coloured hair that covers the tail is more dense towards the tip (<4 mm) where it ends in a definite tuft which is more pronounced than in *E. edwardii* (<4mm), but less so than in *E. rupestris* (>6mm) (see Fig. 3.3d). Total, tail, hind feet and ear length as well as body mass of adults are reported in Table 3.2. Tail length exceeds head-and-body length and is in this respect similar to *E. edwardii* and *E. rupestris*. The dental formula is I 3/3 C 1/1 P 4/4 M 2/2 = 40.

Comparisons

A number of distinct characters distinguish *E. pilocaudata* from other elephant-shrew species. These include mitochondrial and nuclear sequence differences, fixed cytogenetic characters as well as several subtle morphological features.

Mitochondrial and nuclear evidence

The monophyly of *E. pilocaudata* is supported by all methods of analysis (Fig. 3.4) and is consistent with the phylogeny based on mitochondrial and nuclear markers reported by Smit et al. (2007). The sequence divergences separating *E. pilocaudata* from *E. edwardii* and *E. rupestris* are given in Table 3.3 and are comparable to those distinguishing other well recognized species within this clade (see Smit et al. 2007). An uncorrected p-distance of 13.8% calculated from the combined mitochondrial protein coding cytochrome *b* gene and the control region sequences separates *E. pilocaudata* from its sister species *E. edwardii*. In the case of the seventh intron of the fibrinogen gene (Fib 7), an uncorrected p-distance of 4.2% separates *E. pilocaudata* from *E. edwardii*. Of further interest are two monophyletic groupings within *E. pilocaudata* which correspond to the geographical localities of Beaufort-West and Carnarvon/Calvinia/Williston/Loxton (see Fig. 3.1a). These two groups are well supported by bootstrap values and posterior probabilities. In addition, a 75 bp insertion is present in the control regions (data not included) of all Carnarvon/Calvinia/Williston/Loxton specimens and this distinguishes the clade from the Beaufort-West lineage.

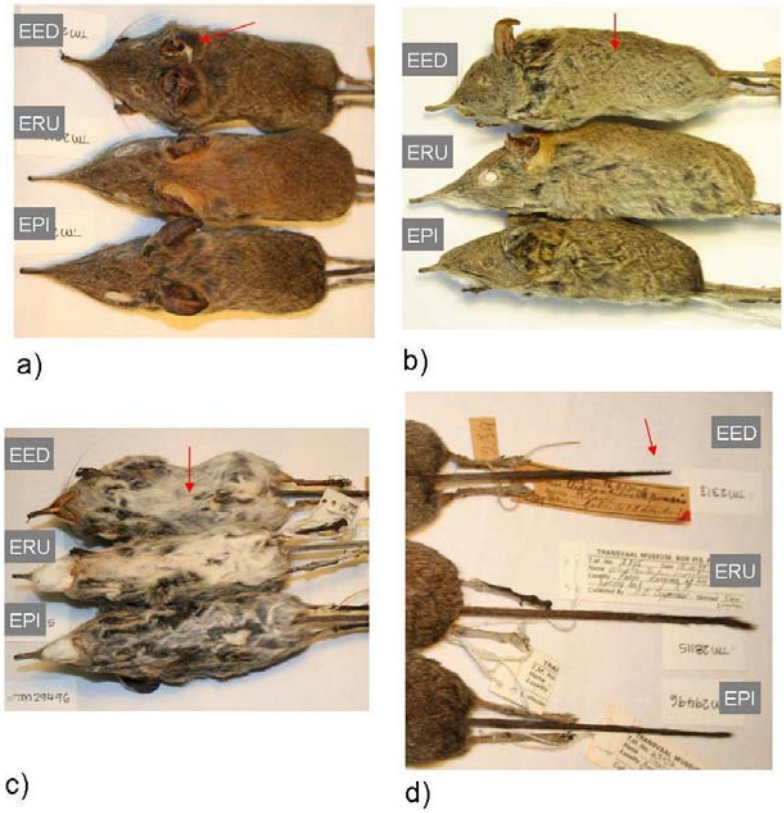

Figure 3.3 Differences in (a) dorsal, (b) flank and (c) ventral pelage between *E. edwardii* (EED), *E. rupestris* (ERU) and *E. pilocaudata* (EPI). (d) The *E. pilocaudata* tail is considerably more tufted towards the tip than in *E. edwardii* but less so than in *E. rupestris*.

Table 3.3 Uncorrected sequence divergences separating *E. pilocaudata* from *E. edwardii* and *E. rupestris*, the rock elephant-shrew species with which it co-occurs in parts of its range. Values are based on (a) 1381 bp of mitochondrial and (b) 360 bp of nuclear sequence data. Values in bold represent intraspecific genetic variation.

	E. rupestris	*E. edwardii*	*E. pilocaudata*	
			Beaufort -West	*Calvinia, Carnarvon, Williston, Loxton*
a) mitochondrial				
E. rupestris	1.10	22.88	22.17	
E. edwardii		1.57	13.80	
E. pilocaudata – Beaufort-West			0.45	9.84
E. pilocaudata - Calvinia, Carnarvon, Williston, Loxton				2.58
b) nuclear				
E. rupestris	0.2	17.14	15.45	
E. edwardii		0.15	4.19	
E. pilocaudata			0.01	

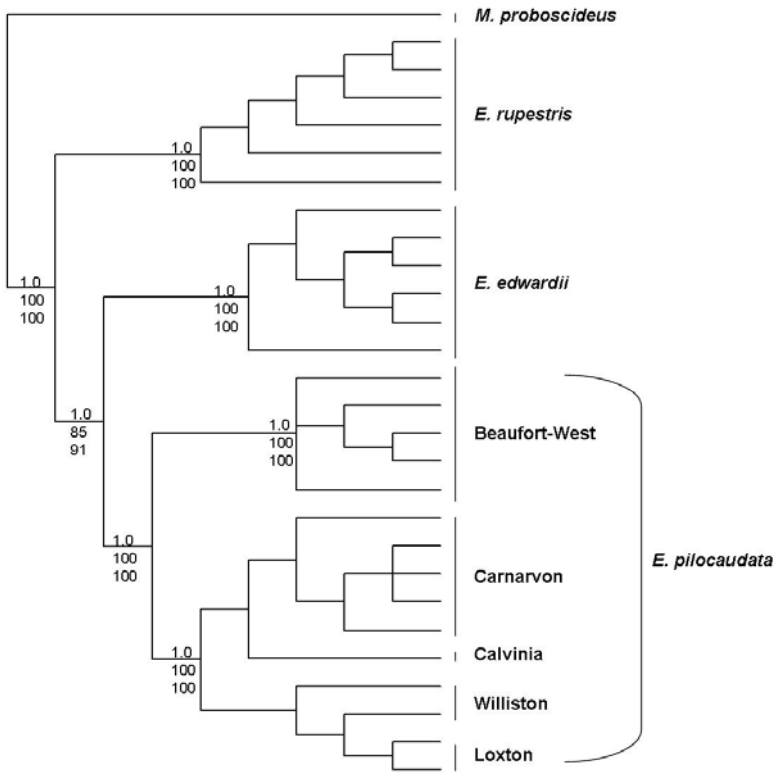

Figure 3.4 Bayesian tree based on the combined sequences from the cytochrome *b* gene and control region showing the phylogenetic relatedness of *E. pilocaudata* to *E. edwardii* and to *E. rupestris,* the two rock elephant-shrew species with which it co-occurs (see Table 3.1 for geographic localities). The tree is rooted on *M. proboscideus*. Values above the nodes indicate posterior probabilities from a 20 million generation run and values below the nodes represent nonparametric bootstrap support for MP (top) and ML (bottom) for 1000 replicates. The monophyly of each species was supported by a posterior probability of 1.0 and 100% bootstrap support.

Cytogenetic evidence

Elephantulus pilocaudata has a diploid number of 26 which is consistent with that of *E. edwardii* and most other Macroscelidinae (see Table 3.4). These include *E. rupestris, E. brachyrhynchus, E. intufi* and *M. proboscideus* (Wenhold and Robinson 1987; Tolliver et al. 1989; Robinson et al. 2004; Svartman et al. 2004). However, several fixed cytogenetic differences separate *E. pilocaudata* and *E. edwardii*.

Table 3.4 Diploid chromosome numbers (2n) reported for Macroscelidinae species.

Species	2n	Reference
Elephantulus edwardii Cape rock elephant-shrew	26	Tolliver et al. 1989
Elephantulus rupestris Western rock elephant-shrew	26	Wenhold and Robinson 1987; Tolliver et al. 1989;
Elephantulus myurus Eastern rock elephant-shrew	30	Ford and Hamerton 1956; Tolliver et al. 1989
Elephantulus brachyrhynchus Short-snouted elephant-shrew	26	Stimson and Goodman 1966; Tolliver et al. 1989
Elephantulus intufi Bushveld elephant-shrew	26	Tolliver et al. 1989
Elephantulus rozeti North African elephant-shrew	28	Matthey 1954
Macroscelides proboscideus Round-eared elephant-shrew	26	Wenhold and Robinson 1987; Tolliver et al. 1989; Svartman et al. 2004
Petrodromus tetradactylus Four-toed elephant-shrew	28	Wenhold and Robinson 1987; Tolliver et al. 1989

The *E. edwardii* (EED) and *E. pilocaudata* (EPI) G-banded karyotypes are shown in Fig. 3.5a & b. The *E. edwardii* karyotype presented herein is identical to that of Robinson et al. (2004) (reported as *E. rupestris* by these authors but subsequently identified in the present study as *E. edwardii* based on sequence data). A comparison of the G- and C-banded chromosomes of *E. edwardii* and *E. pilocaudata* are shown in Fig. 3.6a & b. The *E. edwardii* and *E. pilocaudata* karyotypes are largely identical at the level of G-band resolution obtained in these analyses. Differences in the amount of heterochromatin and a centromere shift in chromosomes 3 and 4 of the two species account for the positional changes in the respective karyotypes (discussed below; see Fig. 3.7). Silver staining of nucleolar organizer regions (NORs) in *E. edwardii* and *E. pilocaudata* and published data on *E. rupestris* (Wenhold and Robinson 1987) show the presence of two pairs of NOR bearing chromosomes in both *E. edwardii* (Fig. 3.6c) and *E. rupestris* which contrast with the 10 NORs (corresponding to five autosomal pairs) detected in *E. pilocaudata* (Fig. 3.6d). Taken collectively these data argue for an absence of gene flow between *E. pilocaudata* and *E. edwardii*. This further underpins the uniqueness of *E. pilocaudata* based on the sequence data and strengthens the case for its recognition as a distinct species.

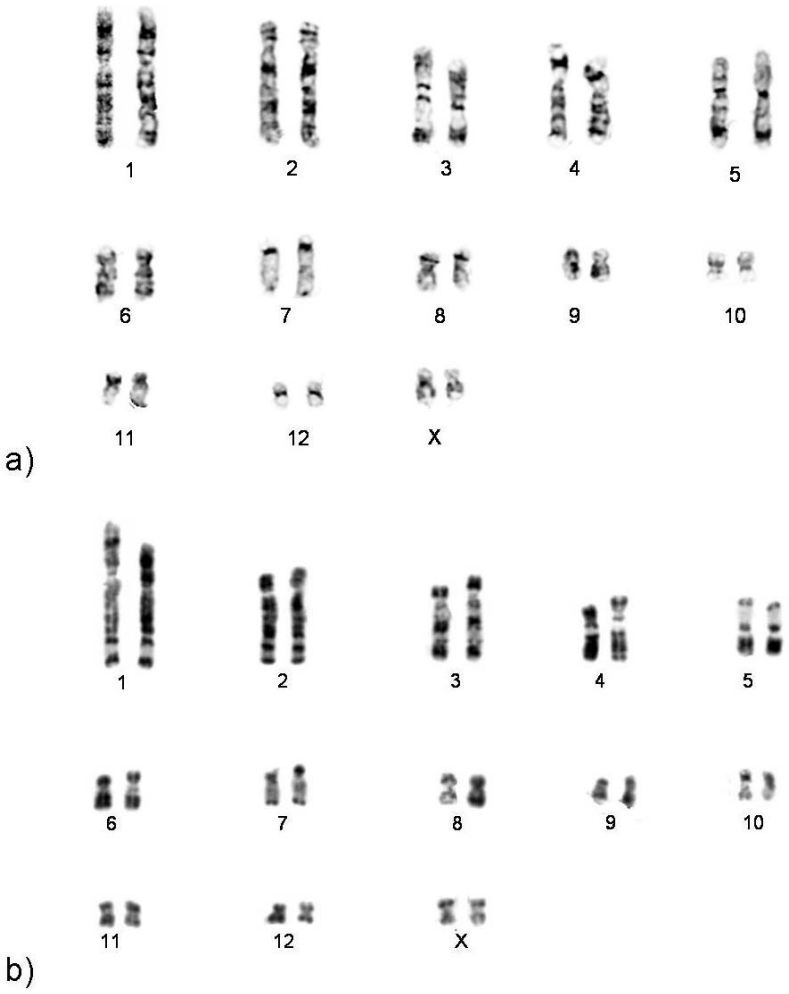

Figure 3.5 G-banded karyotypes of (a) *E. edwardii* and (b) *E. pilocaudata*. Chromosomes are ordered according to size and centromere position.

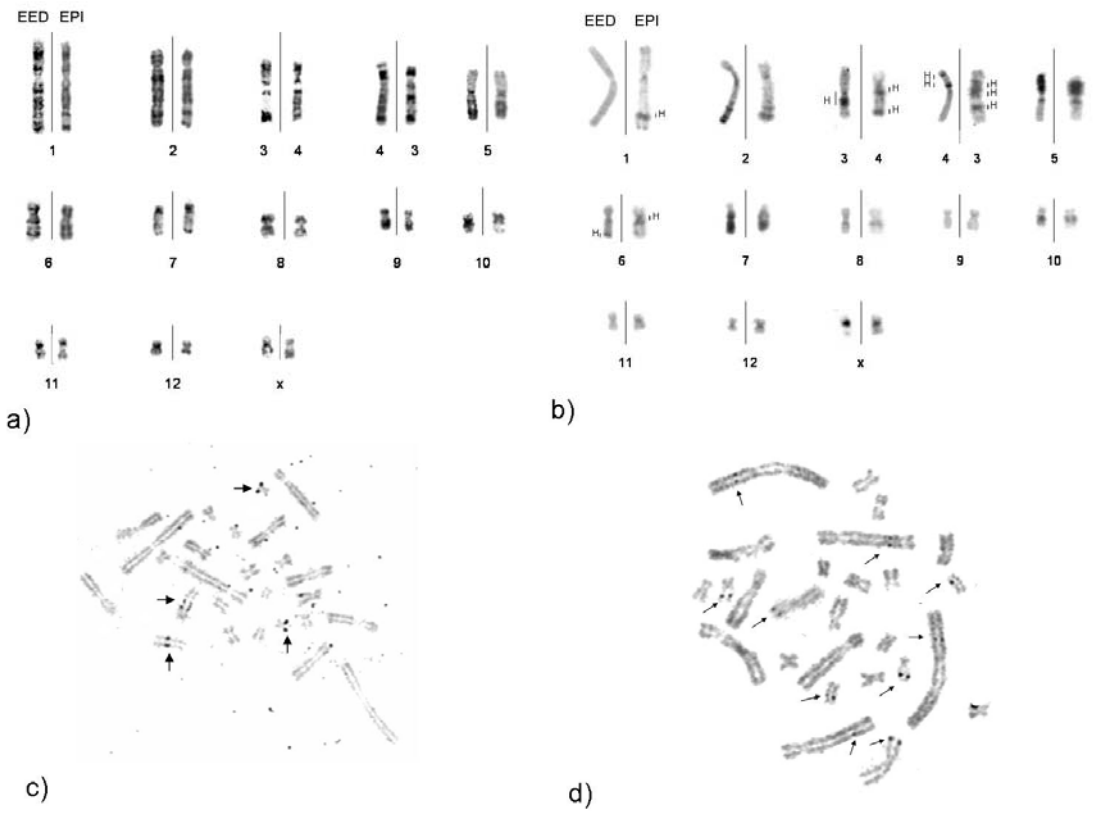

Figure 3.6 (a) Half-karyotype G-band comparisons of *E. edwardii* EED (left) and *E. pilocaudata* EPI (right). (b) Half-karyotype C-band comparisons of *E. edwardii* EED (left) and *E. pilocaudata* EPI (right); chromosome identification was done by sequential banding. Both centromeric and interstitial C-bands are evident. Nucleolar organizer regions (NORs) in representative cells of (c) *E. edwardii* (n = 4) and (d) *E. pilocaudata* (n = 10).

A comparison of the *E. pilocaudata* chromosome EPI 4 and its homologue in *E. edwardii* EED 3 is presented in Fig. 3.7a. A reconstruction shows that EED 3 and EPI 4 differ through a centromeric shift and heterochromatic amplification in the long arm of EED 3, as well as by the presence of a heterochromatic band near the distal end of EPI 4 (Fig. 3.7b). It is noteworthy that while EPI 4 appears to be similar in morphology and G-banding pattern to ERU 3 (Wenhold and Robinson 1987), the latter does not show the same C-bands as either *E. edwardii* or *E. pilocaudata*.

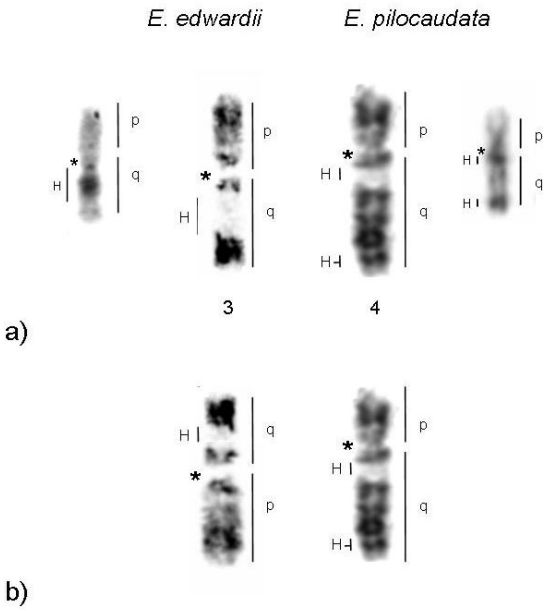

Figure 3.7 (a) Side-by-side comparison of the G- and C-banded *E. pilocaudata* chromosome EPI 4 and its homologue in *E. edwardii* EED 3. (C-banded chromosomes are presented in a contracted state to the left and right of the G-banded chromosomes of each species). (b) A reconstruction shows that the chromosomes differ through a centromeric shift and heterochromatic amplification in the long arm of EED 3, as well as by the presence of a heterochromatic band near the distal end of EPI 4. In this reconstruction EED 3 is inverted and the heterochromatic block in the q arm trimmed to match the size of the corresponding region in EPI 4.

Phenotypic characteristics

Although *E. pilocaudata* is phenotypically very similar to *E. edwardii* and *E. rupestris,* a suite of subtle features (no single diagnostic trait) support its recognition as a distinct species (see Fig. 3.3 and Table 3.5). The most reliable of these are presented below. The descriptions of *E. edwardii* and *E. rupestris* follow Corbet and Hanks (1968).

(i) The dorsal pelage of *E. pilocaudata* and *E. edwardii* is similar being darker greyish-brown tinged yellow and grizzled with blackish brown (rather than reddish brown) but is paler greyish-brown in *E. rupestris* (Fig. 3.3a). The inconspicuous tawny-rufous (tinged with yellow-brown) patches behind the ears in both *E. pilocaudata* and *E. edwardii* contrast sharply with the prominent orange buff patches of *E. rupestris* (Fig. 3.3a). The dorsal colouring extends to the flanks in *E. pilocaudata* as opposed to the presence of a gradual change from dorsal pelage (grey-brown) to the flanks (entirely grey) in both *E. edwardii* and *E. rupestris* (Fig. 3.3b). The

ventral pelage is distinctly different in all three species appearing mottled/blotched yellow darker grey in *E. pilocaudata*, grey in *E. edwardii* and white (less grey) in *E. rupestris* (Fig. 3.3c).

(ii) The tail-tuft, a characteristic that separates *E. pilocaudata* from *E. edwardii* as well as *E. rupestris*, is noticeably more dense (<4mm) in *E. pilocaudata* than in *E. edwardii* (<4mm), but less so than in *E. rupestris* (>6mm) (Fig. 3.3d); there is no consistent difference in tail colour between *E. pilocaudata*, *E. edwardii* and *E. rupestris*; the tail is black above and tends to be paler on the ventral surface towards the base but is completely black distally in all three species. Tail length exceeds head and body length in *E. pilocaudata*, *E. edwardii* and *E. rupestris* but more so in *E. rupestris*.

(iii) The light buffy colour above the mouth at the base of the nose and posterior to the angle of the mouth and dorsal on the cheek in *E. pilocaudata* appears absent in both *E. edwardii* and *E. rupestris*.

(iv) The eye ring (external marking around the eye) is broken to the right above in *E. pilocaudata* and yellow-cream compared to the solid whitish-grey eye ring of *E. edwardii* and white eye ring of *E. rupestris;* however, it should be noted that the shape of the eye-ring was not always consistent between specimens within the three species.

(v) The ears of all three species are proportionately large; the ears have rounded tips in *E. pilocaudata* and *E. edwardii* that are sharpened in *E. rupestris;* although the supratragus and tragus is slightly developed in both *E. pilocaudata* and *E. edwardii*, this character is absent in *E. rupestris* (see Corbet and Hanks 1968 for an illustration of the tragus and supratragus in *E. edwardii*).

No phenotypic distinctions could be made between specimens of the two monophyletic lineages detected within *E. pilocaudata* (the Beaufort-West and the Calvinia/Carnarvon/Williston groups, see above). External body and cranial measurements are reported in Table 3.2. There are no statistically supported differences in external measurements between *E. pilocaudata* and *E. edwardii*. However, *E. rupestris* is larger than both *E. pilocaudata* and *E. edwardii* in overall size as measured by total length (TL; $p<0.001$; $p=0.029$), greatest length of skull (GLS; $p<0.001$; $p<0.001$), rostrum length (RL; $p<0.001$; $p=0.007$) and zygomatic breadth (ZB; $p<0.001$; $p=0.011$) (see Table 3.4). *Elephantulus rupestris* is similarly significantly different from *E. pilocaudata* in tail length (T; $p=0.050$), ear length (E; $p<0.001$) and hind foot length (HF c.u.; $p=0.004$). The Kruskal Wallis ANOVA p-value of the cranial measurement least interorbital breadth (LIB) reported *E. rupestris* as significantly different to the other two species ($p=0.031$; see Table 3.4), however, the posthoc p-

values were nearing significance (*E. rupestris* vs *E. pilocaudata*; p=0.078 and *E. rupestris* vs *E. edwardii*; p=0.095). Mass was excluded from the statistical analyses due to limited sample size.

The adult dental formula I 3/3 C 1/1 P 4/4 M 2/2 = 40 is identical in *E. pilocaudata*, *E. edwardii* and *E. rupestris* (and most other Macroscelidinae). A set of qualitative dental characteristics clearly separates *E. edwardii* and *E. pilocaudata* from *E. rupestris*. These characters include (i) the absence of lingual cusps on P^1 in *E. edwardii* and *E. pilocaudata* and their presence in *E. rupestris*, (ii) anterior labial cusps well developed but posterior cusps poorly so in P^1 and P^2 of *E. edwardii* and *E. pilocaudata*; both anterior and posterior labial cusps are well developed in *E. rupestris* and (iii) P^2 is sectorial in *E. edwardii* and *E. pilocaudata* but with variable lingual cusps in contrast to a molariform upper P^2 with two lingual cusps present in *E. rupestris* (see Table 3.5) (for *E. edwardii* and *E. rupestris* - see Corbet and Hanks 1968 also for illustrations of dental features; Skinner and Chimimba 2005). Root characteristics of the lower P^1 were not examined in the present study as verification of this character would have damaged the skulls preserved in museum collections.

Key to the Elephantulus species

(Adapted from Corbet 1974 and expanded to include data presented above).

1. Pectoral gland present, a naked or short-haired patch in centre of thorax 2
   Pectoral gland absent 4

2. Prominent brown mark behind eye; two lower molars, i.e. ten lower teeth 3
   No brown mark behind eye; three lower molars *E. fuscipes*

3. Hair of tail becoming long towards the tip, forming a brush; tail about 120% of head and body; I^2 equal in size to I^1 and I^3 *E. revoili*
   Hair of tail not forming a brush; tail about equal to head and body; I^2 smaller than I^1 *E. rufescens*

4. Tail shorter than head and body; three lower molars, i.e. eleven lower teeth *E. brachyrhynchus**
   Tail not shorter than head and body; two lower molars 5

5. P^1 with a lingual cusp; P^2 molariform, with two well-developed lingual cups; ventral pelage superficially white 6
   P^1 lacking a lingual cusp; P^2 sectorial with or without small lingual cusps; ventral pelage showing grey, except in the North African *E. rozeti* 7

* should be further split into *E. brachyrhynchus* and *E. fuscus*

6. Size larger; upper toothrow over 18.7 mm; tail about 115% of head and body, distinctly tufted towards the tip, predominantly black above; white eye-ring narrow, broken above and below the eye; P^2 and P^3 with three cusps, arranged in a triangle, behind the principal cusp *E. rupestris*

Size smaller; upper toothrow under 18.7 mm; tail about 106% of head and body, not distinctly tufted, speckled above; white eye-ring conspicuous and unbroken; P^2 and P^3 with only two cusps, arranged transversely, behind the principal cusp *E. intufi*

7. Ectotympanic parts of bullae inflated to same level as entotympanic parts; I^2 equal to I^1 and I^3 (Southern Africa) 8

Ectotympanic parts of bullae much less inflated that entotympanic parts; I^2 larger than I^1 and I^3 (North Africa) *E. rozeti*

8. P^2 with one, occasionally two, lingual cusps; supratragus small and fairly thick; premaxillary suture slightly sinuous; tail bicoloured throughout its length, yellow-brown above, entirely short-haired *E. myurus*

P2 without a lingual cusp; supratragus large and thin; premaxillary suture straight; tail black above, distal half black all round and slightly tufted 9

9. Tail less tufted towards tip (hairs <4mm); dorsal pelage (grey-brown tinged with yellow) separated from grey flanks; ventral pelage appears grey *E. edwardii*

Tail considerably more tufted towards tip (hairs <4mm); dorsal pelage (grey-brown tinged with yellow) extends to flanks; ventral pelage appears mottled/blotched yellow grey *E. pilocaudata*

Notes on Conservation Status

Information on the conservation status of the species is lacking. Importantly, that only 17 specimens of the new species (three live trapped by HAS; two trapped by Dr Galen Rathbun and 12 museum specimens) have been collected despite extensive field work in the Nama Karoo indicates that *E. pilocaudata* is regionally limited and rarely encountered, and efforts should be made to assess its relative abundance and to determine potential threats to its habitat.

Table 3.5 Morphological differences distinguishing *E. pilocaudata* (present study) from *E. edwardii* and *E. rupestris* (taken from Corbet and Hanks 1968; also for illustrations of cranial and dental features).

	E. pilocaudata	*E. edwardii*	*E. rupestris*
Tail	Black above; pale below at base but distal-half black all around; considerably more tufted towards tip than *E. edwardii* but less than *E. rupestris* (<4mm)	Black above; pale below at base but distal-half black all around; tufted towards tip (<4mm)	Black above; slightly lighter on the under surface towards the base; elongated brush at tip (>6 mm)
Dorsal pelage	Grey-brown, tinged yellowish and grizzled with blackish brown; extending to flanks	Grey-brown, tinged yellowish and grizzled with blackish brown; becoming grey on flanks	Grey-brown, although paler (greyer) than in *E. pilocaudata* and *E. edwardii*, becoming grey on flanks
Flank colour	Similar to dorsal pelage	Grey	Grey
Ventral pelage	Appears mottled/blotched yellow grey	Appears grey	Appears white (less grey)
Buffy patches behind ears	Tawny-rufous/yellow-brown hair patch; less conspicuous than in *E. rupestris*	Tawny-rufous/yellow-brown hair patch; less conspicuous than in *E. rupestris*	Rufous/yellow-orange hair patch extending to neck - prominent
Cheek colour	Light buff	Absent (appears grey)	Absent (appears grey)
Ears	Proportionally large; broad at base with rounded tips; supratragus and tragus slightly developed	Proportionally large; broad at base with rounded tips; supratragus and tragus slightly developed	Proportionally large; sharper tips than *E. edwardii* and *E. pilocaudata*; supratragus and tragus not developed
Eye ring (external marking around eye)	Broken to the right above (not consistent); prominent at bottom; yellow-cream	Solid; white-grey	Distinct; broken (above and below); white
Suture between premaxilla and maxilla	Straight	Straight	Sinuous
Skull	Swollen ectotympanic; less inflated entotympanic bullae	Swollen ectotympanic; less inflated entotympanic bullae	Ectotympanic not inflated; inflated entotympanic bullae
Upper P^1	Lacking lingual cusp; reduction of all but one principal cusp	Lacking lingual cusp; reduction of all but one principal cusp	With lingual cusp
Upper P^1 and P^2	Well developed anterior but poorly developed posterior labial cusps	Well developed anterior but poorly developed posterior labial cusps	Anterior and posterior well developed
Upper P^2	Sectorial	Sectorial	Molariform
Upper P^2 lingual cusp	Single lingual cusp present or absent	Single lingual cusp present or absent	Two lingual cusps present

DISCUSSION

The assignment of the monophyletic Karoo clade to either of the available species names within *E. edwardii* (*E. capensis* or *E. karoensis* - both names had previously been synonomised within *E. edwardii,* Corbet and Hanks 1968; Meester et al. 1986) was conclusively ruled out by Smit et al. (2007). DNA sequencing of the *E. capensis* type specimen (TM 2312, GenBank DQ901249; Roberts 1924:62) placed the specimen firmly within *E. edwardii* while sequence from the *E. karoensis* type specimen (TM 688, GenBank DQ901238; Roberts 1938:234) was found to cluster within *E. rupestris* (Smit et al. 2007).

In this dissertation compelling evidence is provided for the recognition of a new species of *Elephantulus*, *E. pilocaudata*. The description is based on the analysis of mitochondrial and nuclear DNA sequences and comparative cytogenetic data. An identification scheme is provided that distinguishes *E. pilocaudata* from other rock elephant-shrew species with which it co-occurs. The recognition of *E. pilocaudata* increases the number of elephant-shrew species within *Elephantulus* (subfamily Macroscelidinae) to 11. The southern African rock elephant-shrews are subsequently considered to include *E. pilocaudata, E. edwardii, E. rupestris* and *E. myurus*. Of these, *E. pilocaudata* and *E. edwardii* are endemic to South Africa further underscoring the region's rich elephant-shrew biodiversity. Seven of the 15 extant species (and three of the four genera) occur within its borders. The new species is possibly regionally limited to the Nama Karoo which borders on two Biodiversity Hotspots, the Succulent Karoo to the west and the Cape Floristic Kingdom to the south (Low and Rebelo 1996). The vegetation biome is subdivided into the Bushmanland and Upper and Lower Karoo Bioregion vegetational units (Mucina and Rutherford 2006; see Fig. 3.1a). It is noteworthy that specimens that group within the Calvinia/Carnarvon/Williston/Loxton clade are referable to the Upper Karoo Bioregion, whereas specimens that have the Beaufort-West genetic profile all occur in the Lower Karoo Bioregion.

CHAPTER 4

RELATIVE IMPORTANCE OF HABITAT ASSOCIATION IN SHAPING GENETIC PROFILES: IMPACT ON TWO SOUTHERN AFRICAN ELEPHANT-SHREW TAXA

Introduction

A central theme in the biodiversity sciences is to understand current patterns of species abundance and distribution, and to predict the likely impact of environmental change on these. Typically, molecular tools are used to describe the genetic structure of species across their ranges and this information is matched with what is known about the history of a region (including climatological and geomorphological data; see e.g. Holzhauser et al., 2006; Smit et al., 2007; Spear et al., 2005). This history is largely responsible for alterations in those processes that shape species patterns such as colonisation, fragmentation and range dynamics and matched with species genetic structure, provides important insights into current biodiversity patterns (Hewitt and Ibrahim, 2001). However, current processes such as gene flow, species' biology and landscape variables similarly influence the spatial distribution of genetic variation. In this respect the integration of the previously independent fields of genetics, ecology and spatial statistics (termed landscape genetics *sensu* Manel et al., 2003) add valuable insights to understanding species' spatial genetic patterns (see e.g. Hewitt and Ibrahim, 2001; Storfer et al., 2007).

Important considerations in landscape genetics are matrix quality (habitat homogeneity) and connectivity (*sensu* Baquette, 2004). For species with continuous distributions, this structural (landscape) connectivity often leads to functional (genetic) connectivity because of migration and gene flow. However, in instances where species have a naturally disjunct distribution, genetic patterns often reflect this fragmented distribution (Storfer et al., 2007 and references therein). Following from this, habitat occupation should be an important driving force in shaping genetic variation. Relatively few studies have correlated genetic pattern to habitat occupation (but see brown bears *Ursus,* Talbot and Shields, 1996; seahorses *Hippocampus,* Lourie et al., 2005; surgeon fish *Acanthurus,* Rocha et al., 2002 for exceptions), and even in these instances, it is often difficult to disentangle the specific effects of habitat association from species' biology and life history characteristics such as territoriality, mating system and dispersal.

The aim of this study is to determine the genetic population profiles of two closely related taxa (see e.g. Collin, 2001; Dawson et al., 2002; Paulay and Meyer, 2006) and to investigate the relative importance of habitat association and other landscape features as driving forces in shaping the spatial pattern of genetic variation. This is done in an attempt to distinguish these effects from those caused by species biology (see e.g. Bird et al., 2007). The focus was on two southern African endemic (Corbet and Hanks, 1968) elephant-shrew species, the round-eared elephant-shrew (*M. proboscideus*) and the western rock elephant-shrew (*E. rupestris*). For the purpose of this dissertation they are considered to have largely similar life history traits (this certainly is true when considering differences among species that are unrelated). Life-history traits of *E. rupestris* are assumed to be identical to that of *E. myurus* (social structure has been studied in *E. myurus*

which similarly uses a habitat of rocky outcrops [Ribble and Perrin, 2005]). These two species have largely overlapping distributions in South Africa and Namibia (see Fig. 4.1a and 4.2a; Perrin, 1997; Skinner and Chimimba, 2005) that include the semi-arid Succulent Karoo, Nama Karoo and Fynbos vegetation biomes (Low and Rebelo, 1996; Mucina and Rutheford, 2006). Both these species are relatively small sized (*E. rupestris* 54.0 – 77.0g; *M. proboscideus* 31.0 – 47.0g), insectivorous (or omnivorous as recorded for *M. proboscideus*; Kerley, 1995), territorial, live solitarily (Rathbun 1979) or in facultative (not obligatory) pairs, and show social uniparental monogamy (Rathbun and Rathbun, 2006). However, these two species are markedly different in habitat occupation. *Macroscelides proboscideus* inhabits desert and semi-arid zones comprising open country with shrub bush and sparse grass cover on gravel plains. Shelter may include burrows or small holes under rocks and scree. Conversely, *E. rupestris* is closely associated with rocky outcrops where holes and piles of boulders provide shelter (Skinner and Chimimba, 2005: 22-34).

Materials and Methods

Taxonomic sampling

Two subspecies are currently recognized in *M. proboscideus* which respectively show a South African/Namibian distribution (*M. p. proboscideus* Shaw, 1800:536) and a northern Namib Desert distribution (*M. p. flavicaudatus* Lundholm, 1955:285). For comparative purpose only *M. p. proboscideus* is included. In the case of *E. rupestris* (Smith, 1831:11) there are no currently recognized subspecies. A number of species were previously described based on phenotypic differences but these are all synonymised within *M. p. proboscideus* and *E. rupestris* (Corbet and Hanks, 1968; Ellerman et al., 1953; Meester et al., 1986; Roberts, 1951). To verify the monophyly and current taxonomy of these two elephant-shrew species, partial sequence from the type specimens (housed in the Transvaal Museum (TM), South Africa) of all the previously recognized taxa (see Table 4.1) was included.

Table 4.1 Type specimens housed in the Transvaal Museum (TM; South Africa) currently synonomised within *M. p. proboscideus* or *E. rupestris*.

Species	Type	Description	Date collected	Specimen no.	Locality	Country
M. p. proboscideus	*M. proboscideus hewitti*	Roberts 1929	1918	TM 2358	Cradock	South Africa
	M. proboscideus langi	Roberts 1933	1927	TM 5128	Van Rhynsdorp	South Africa
	M. proboscideus chiversi	Roberts 1933	1932	TM 6924	Upington	South Africa
	M. typicus ausensis	Roberts 1938	1937	TM 8255	Aus	Namibia
	M. typicus harei	Roberts 1938	1937	TM 8256	Brandvlei/Van Wyksvlei	South Africa
	M. typicus brandvleiensis	Roberts 1938	1937	TM 8258	Brandvlei	South Africa
	M. typicus calviniensis	Roberts 1938	1937	TM 8262	Calvinia	South Africa
E. rupestris	*E. vandami vandami*	Roberts 1924	1918	TM 2361	Cradock	South Africa
	E. barlowi	Roberts 1938	1927	TM 8265	Aus	Namibia
	E. kobosensis	Roberts 1938	1937	TM 8268	Rehoboth	Namibia
	E. rupestris tarri	Roberts 1938	1937	TM 8275	Helmeringhausen	Namibia
	E. barlowi gordoniensis	Roberts 1946	1941	TM 9431	Upington	South Africa
	E. barlowi okombahensis	Roberts 1946	1941	TM 9433	Damaraland	Namibia
	E. vandami montanus	Roberts 1955	1951	TM 10502	Kaokoland	Namibia

The phylogeographic structure of *M. p. proboscideus* was determined using a sample of 56 specimens from 23 localities throughout the taxon's range (see Fig. 4.1a and Table 4.2a). Similarly, 70 *E. rupestris* specimens were included from 32 localities (see Fig. 4.2a and Table 4.2b). The use of museum tissue is becoming increasingly important in assessing genetic profiles (Godoy et al., 2004; Good and Sullivan, 2001; Rohland et al., 2004; Smit et al., 2007), specifically in instances where access to material is constrained by varying population densities, and the costs and time involved in fieldwork. This is exemplified by the two elephant-shrew taxa included in the present investigation. Consequently >75% of the material analysed here is derived from museum specimens, the oldest of which dates back to 1918.

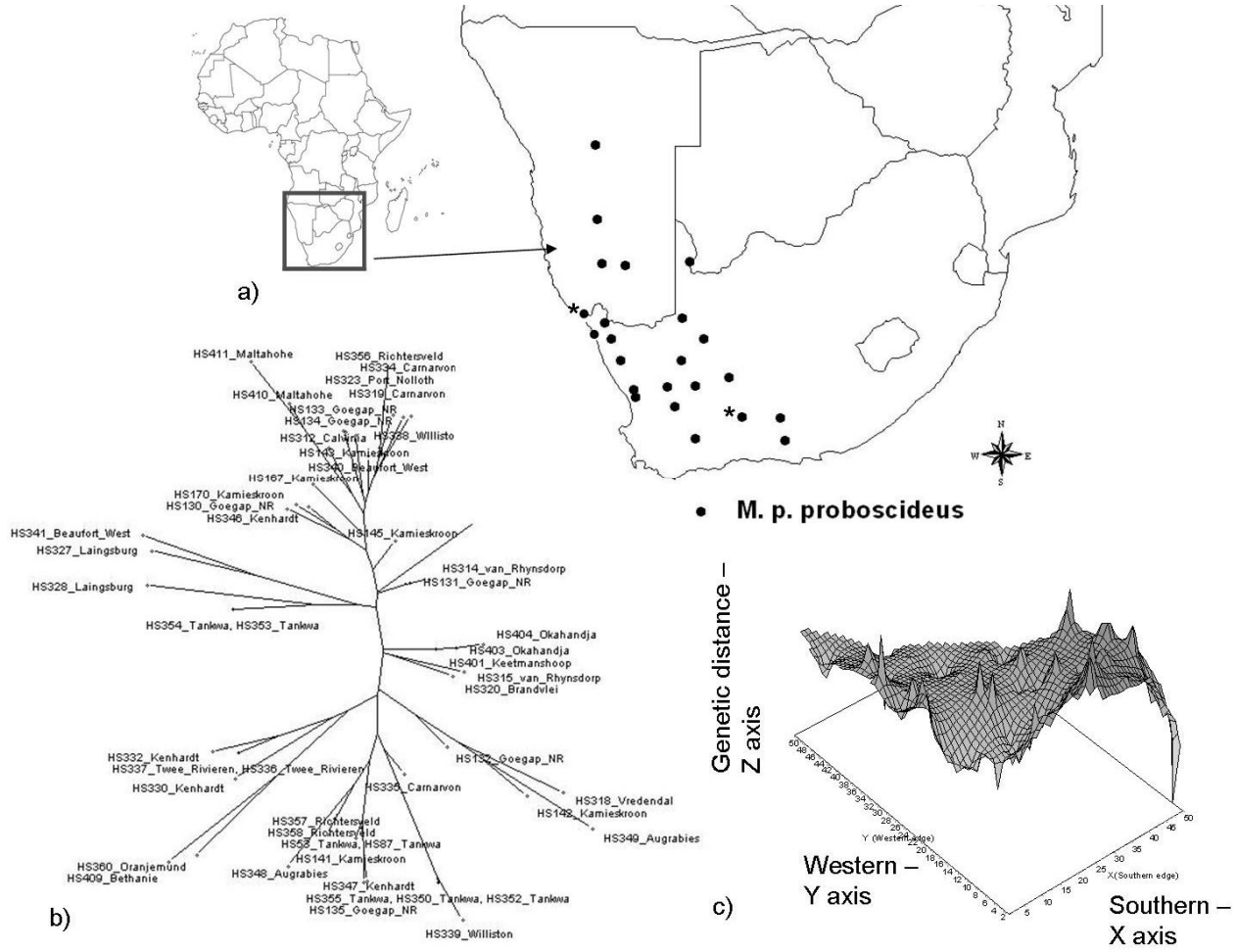

Figure 4.1 (a) Sampling localities (n=23) included over the entire range of *M. p. proboscideus* throughout Namibia and South Africa. Asterisks (*) refer to those localities with the largest genetic distances. (b) Unrooted neighbour joining haplotype tree based on 1398 bp of sequences from the combined cytochrome *b* gene and control region and (c) results of a genetic landscape interpolation (AIS) analysis with largest genetic distance corresponding to height (Z-axis) and a spatial scale with geographic locations (X and Y axes).

Table 4.2 Sample localities, evolutionary lineages, number of specimens sampled and source material for (a) *M. p. proboscideus* and (b) *E. rupestris* (Transvaal Museum = TM, South Africa and Iziko Museum = IM, South Africa). See appendix Table 3 for voucher numbers and geographical coordinates of sampling localities.

a)

Country	Clade*	Locality	No. of specimens	Source material
Namibia		Okahandja	2	Museum tissue, TM
		Maltahohe	2	Museum tissue, TM
		Bethanie	1	Museum tissue, TM
		Keetmanshoop	1	Museum tissue, TM
		Oranjemund	1	Museum tissue, TM
South Africa		Port Nolloth	1	Museum tissue, TM
		Richtersveld National Park, Richtersveld	3	Museum tissue, TM
		Twee Rivieren, Kalagadi Transfrontier Park	2	Museum tissue, TM
		Augrabies National Park, Augrabies	2	Museum tissue, TM
		Goegap Nature Reserve, Springbok	6	Soft tissue
		Kamieskroon	6	Soft tissue
		Kenhardt	4	Museum tissue, TM
		Brandvlei	1	Museum tissue, TM
		Carnarvon	3	Museum tissue, TM
		Williston	2	Museum tissue, TM
		Calvinia	1	Museum tissue, TM
		Vredendal	1	Museum tissue, TM
		Van Rhynsdorp	2	Museum tissue, TM
		Tankwa National Park, Ceres	7	Soft tissue; Museum tissue, TM
		Laingsburg	2	Museum tissue, TM
		Karoo National Park, Beaufort West	3	Museum tissue, TM
		Aberdeen	1	Museum tissue, TM
		Steytlerville	2	Soft tissue
Total no.			56	

* No structure detected within *M. p. proboscideus*

Table 4.2 continued

b)

Country	Lineage	Locality	No. of specimens	Source material
Namibia	Kaokoland	Kaokoland	3	Museum tissue, TM
	Namibia	Damaraland	1	Museum tissue, TM
	Namibia	Kamanjab	1	Museum tissue, TM
	Namibia	Walvis Bay	1	Museum tissue, TM
	Namibia	Karibib	3	Museum tissue, TM
	Namibia	Windhoek	1	Museum tissue, TM
	Namibia	Rehoboth	3	Museum tissue, TM
	Namibia	Helmeringhausen	2	Museum tissue, TM
	Namibia	Berseba	1	Museum tissue, TM
	Namibia	Brukkaros	1	Museum tissue, TM
	Namibia	Aus	3	Museum tissue, TM
South Africa	Northern Cape B	Lutzputs	1	Museum tissue, TM
	Northern Cape B/Namibia	Upington	3	Museum tissue, TM
	Northern Cape B	Louisvale	2	Museum tissue, TM
	Northern Cape A	Prieska	1	Museum tissue, TM
	Northern Cape B	Augrabies	1	Museum tissue, TM
	Northern Cape B	Komaggas	1	Museum tissue, TM
	Northern Cape B	Nababeep	1	Museum tissue, TM
	Northern Cape A/B	Springbok	12	Soft tissue; Museum tissue, TM
	Northern Cape A/B	Kamieskroon	7	Soft tissue
	Northern Cape B	Kenhardt	1	Museum tissue, TM
	Northern Cape A	Carnarvon	1	Museum tissue, TM
	Northern Cape A/B	Calvinia	2	Museum tissue, TM
	Eastern Cape	Touwsriver	1	Museum tissue, TM
	Eastern Cape	Karoo National Park, Beaufort West	3	Museum tissue, TM
	Eastern Cape	Cherrydouw, Oudtshoorn	2	Museum tissue, IM
	Eastern Cape	Oudtshoorn	1	Soft tissue
	Eastern Cape	Gamkaberg, Calitzdorp	2	Museum tissue, IM
	Eastern Cape	Elandskloof, Ladismith	2	Museum tissue, IM
	Eastern Cape	Port Elizabeth	1	Museum tissue, TM
	Eastern Cape	Albany, Grahamstown	3	Museum tissue, TM
	Eastern Cape	Cradock	2	Museum tissue, TM
Total no.			70	

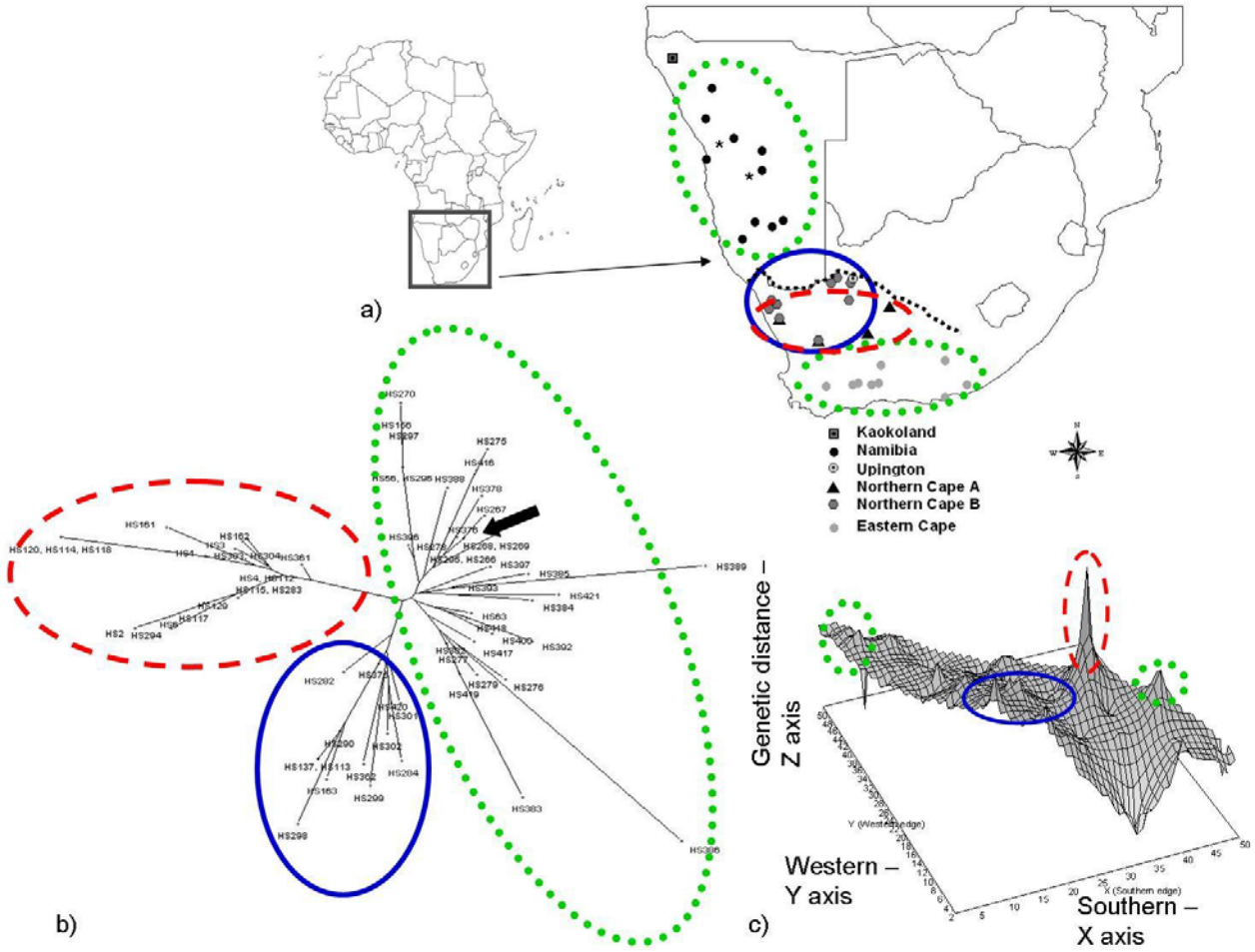

Figure 4.2 (a) Sampling localities (n=32) covering the entire range of *E. rupestris* throughout Namibia and South Africa. The different lineages for *E. rupestris* are shape and colour coded (Namibia/Eastern Cape – broken green line "·····", Northern Cape A – dashed red line "- - -" and Northern Cape B – solid blue line "—"). The broken grey line "·····" indicates the position of the Orange River. Asterisks (*) refer to those localities with largest genetic distances. (b) Unrooted neighbour joining haplotype tree based on 1398 bp of the combined cytochrome *b* gene and control region. The most divergent lineage, Kaokoland, is excluded from the tree to provide better resolution. Lineages are coded following (a). The Upington specimen (HS376) that groups within Namibia/Eastern Cape is indicated with a small arrow (see text). (c) The genetic landscape interpolation (AIS) with genetic distance (height = Z-axis) corresponding to the different clades identified within *E. rupestris* over spatial scale (X and Y axes).

DNA extraction, PCR and sequencing

A commercial DNA extraction kit (DNeasy Tissue Kit, Qiagen) was used for all DNA extractions. Tissue from museum specimens was preferentially taken from within the skull cavity using sterile forceps thereby avoiding external damage to specimens. To verify the authenticity of data from museum specimens, standard ancient DNA protocols (Rohland et al., 2004) were routinely followed. At least two independent PCR reactions were done to verify sequences. Extraction and PCR blanks were always negative and sequences were verified with BLAST searches in GenBank. In addition, the DNA amplicons from different specimens routinely returned sequences that differed between specimens, making it highly unlikely that these resulted from cross-contamination.

All laboratory protocols used are described in Smit et al. (2007). In brief, PCR reactions were carried out in a GeneAmp PCR 2700 system (Applied Biosystems) involving an initial denaturation step of 3 min at 95 ˚C followed by 35 cycles at 95 ˚C for 30 s, primer and tissue specific annealing temperature for 30 s and 72 ˚C for 60 s. Amplifications were completed with a final 5 min extension step at 72 ˚C. To counteract PCR inhibitors present in the historical tissue, amplifications of museum derived material routinely included BSA (4 µl of 0.001 g/ml to a 30 µl reaction). Sequencing reactions were performed using BigDye chemistry (version 3; Applied Biosystems). Centrisep spin columns (Princeton Separations) were used to clean sequencing cocktails. The products were analyzed on a 3100 AB automated sequencer. Electropherograms of the raw sequences were checked by eye and edited with Sequence Editor™ software version 1.0.3a (Applied Biosystems) (see detailed protocol in Chapter 5).

Two mitochondrial DNA markers were employed to describe spatial genetic variation; the protein coding cytochrome *b* gene as well as the 5' portion of the control region. Amplification of sequences from fresh material used universal primers (Irwin et al., 1991; Kocher et al., 1989; Pääbo and Wilson, 1988; Rosel et al., 1994). However, to improve successful PCR amplification of museum material, species-specific primers spanning no more than 300 bp were designed. Sequences were submitted to GenBank under accession numbers EF141697 - EF141822 (*M. proboscideus*) and EF141557 - EF141696 (*E. rupestris*).

Data analyses

To verify the monophyly of these two taxa, standard tree building algorithms as implemented in PAUP* (Swofford, 2001) were used. The most parsimonious tree was explored using the heuristic search option with 100 random additions of taxa and TBR branch swapping. Maximum likelihood analyses were performed specifying the optimal model selected under the LRT criteria implemented in Modeltest version 3.06

(Posada and Crandall, 2001). Nodal support was assessed using 1000 parsimony and maximum likelihood bootstrap replications.

For analysis below the species level, haplotypes were connected with Splitstree 4 (Huson and Bryant, 2006) in an unrooted neighbour joining tree. Uncorrected p-distances were calculated between all pairs of individuals in PAUP*. The spatial distribution of mitochondrial variation within these two elephant-shrew species was explored using a Spatial Analysis of Molecular Variance (SAMOVA version 1.0; Dupanloup et al., 2002), as well as a hierarchical analysis of molecular variance (AMOVA implemented in Arlequin version 3.1; Excoffier et al., 2005). SAMOVA provides an objective means of maximising the proportion of total genetic variance due to differences between groups of populations (populations were used as a synonym for sampling localities). Conventional Φ statistics were calculated in Arlequin. Permutational procedures (1000 randomisations) were used to provide significance tests for these statistics under the null hypothesis of panmixia. Monmonier's algorithm was used (implemented in Alleles in Space (AIS); Miller, 2005) to search for barriers to gene flow where the greatest genetic distance between any two locations form the initial barrier segment. An interpolation-based graphical procedure was used to detect genetic structure over landscapes using the default settings of *midpoint of edges derived from Delaunay triangulation* and *residual genetic distances* with a weight value of 1.0 as the visual spatial approach.

The shortest and most direct route between localities (as-the-crow-flies) was used to explore the presence of clinal genetic structure (isolation-by-distance). Again, sampling localities were treated as populations. A Mantel test (Manly, 1991) indicated whether a positive correlation exists between geographic distance and uncorrected sequence distance using the regression of $(F_{ST}/(1- F_{ST})$ on the logarithm of geographical distance (Rousset, 1997).

A maximum likelihood and Bayesian coalescent approach was followed (see Nielsen and Wakeley, 2001; implemented in MDIV; Nielsen, 2002) to separate recurrent gene flow from the effects of ancestral polymorphisms, and estimate population divergence times. Estimates of θ (theta), M (migration), T (divergence time) and TMRCA (time to the most recent common ancestor) were based on pairwise comparison (20 X 10^6 generations, with the exception of one run for 100 X 10^6 generations) set on the finite-sites model with upper bounds of 10 migrants per generation, and a population divergence time of 10 units. The statistics θ, M and T were plotted after multiple runs to confirm the best estimate of posterior distribution. Credibility intervals (95%) were calculated for each parameter.

Results

Monophyly of species

The monophyly of *M. p. proboscideus* and *E. rupestris* was verified by comparing 371 bp of the control region from type specimens of 14 of the previously recognized but currently subsumed species (see Table 4.1) to the sequences from specimens included in the phylogeography study. In addition, representative sequences of the other five South African elephant-shrew species (*P. tetradactylus*, *E. brachyrhynchus*, *E. intufi*, *E. edwardii* and *E. myurus*) (DQ901184 - DQ901211; DQ901219 - DQ901237; DQ901239 - DQ901248) were included. The phylogenetic trees were rooted on members of the Afroinsectiphillia: *O. afer* (Y18475), *E. telfairi* (AB099484) and *C. asiatica* (NC004920). Irrespective method of analyses (parsimony or maximum likelihood), the monophyly of both *M. p. proboscideus* and *E. rupestris* was supported by 100% bootstrap support (data not shown; see also Chapter 1 and Chapter 7), confirming the current taxonomy.

Population structure

For ease of representation, and given that the results were largely congruent for both species when cytochrome *b* and the control region were analyzed singly and/or in combination, only the results for the combined analyses are presented below.

M. p. proboscideus

The combined *M. p. proboscideus* data set comprised 1398 bp (1027 bp of cytochrome *b* and 371 bp on the 5' side of the control region) representing 56 specimens from 23 localities. A total of 51 haplotypes were identified of which 47 were private/unique, and four were shared between specimens (h=0.99). The highest sequence divergence separating specimens was 1.57% between those from Beaufort-West (central South Africa) and Oranjemund (Namibia) (see Fig. 4.1a).

At a first glance the spatial pattern observed for *M. p. proboscideus* resembled a random as opposed to a structured pattern (see unrooted neighbour joining tree; Fig. 4.1b). However, when implementing SAMOVA, Φ_{CT} increased to the level where the number of groups equalled the number of populations (sampling localities) indicative of an isolation-by-distance process. This result was confirmed by the Mantel test which rejected the null hypothesis of no association between genetic and geographic distances (r=0.306; p=0.002).

The genetic landscape analysis (Fig. 4.1c) indicated non-homogeneous genetic distances (height = Z-axis) over a spatial (X and Y axes) scale. The effects of ancestral polymorphism against recurrent processes of migration were explored with a Bayesian coalescent approach among *M. p. proboscideus* neighbouring

localities. Gene flow (~5 migrants per generation) was preferred to the retention of ancestral haplotypes in all runs (data not shown) reflecting moderate levels of current migration resulting in the isolation-by-distance pattern shown above.

E. rupestris

The data set comprised 1287 bp (960 bp of the cytochrome *b* gene and 327 bp control region) from 70 specimens representative of 32 localities. Eight of the 62 inferred haplotypes were shared among specimens with the remainder (54 haplotypes) being unique/private (*h*=0.99).

The highest number of mutational steps between haplotypes (10; corresponding to 1.63% uncorrected sequence divergence) was found between the northern most locality of Kaokoland and the remainder of samples; this association was supported by a Bayesian posterior probability of 1.0 (data not shown). SAMOVA similarly maximised the portion of genetic variance at two groups which corresponded to Kaokoland and the remainder of the localities (Φ_{CT}=0.469; p=0.034). This result was confirmed by the spatial landscape interpolation (AIS) with Monmonier's algorithm placing the genetic barrier between Kaokoland and the remainder of localities (data not shown).

The Kaokoland samples were subsequently excluded to investigate whether genetic subdivision characterizes other areas across the distribution of *E. rupestris*. This was done to prevent swamping by this locality thereby obscuring a potential more shallow structure across the remainder of the distribution (following Smit et al., 2007). The highest sequence divergence separating specimens was 1.48% between those collected from Rehoboth (Namibia) and Karibib (Namibia) (Fig. 4.2a). The unrooted neighbour joining tree revealed the presence of three distinct haplogroups which loosely correspond to geographic sampling regions. These are Northern Cape A (Springbok, Kamieskroon, Prieska, Calvinia, Carnarvon), Northern Cape B (Upington, Komaggas, Kenhardt, Nababeep, Lutzputs, Springbok, Kamieskroon, Augrabies, Calvinia, Louisvale) and Namibia/Eastern Cape (comprising localities from Namibia and the Eastern Cape) (Fig. 4.2b). The genetic structure over geographical scale (X and Y axes) largely corresponded to the observed clades within *E. rupestris* (Fig. 4.2c). The largest genetic distances (height = Z-axis) were found in the central Karoo (within the Northern Cape A clade).

The grouping of Namibian localities with those from the Eastern Cape was unexpected. Bayesian analysis did not statistically support this grouping and no haplotypes are shared among the Eastern Cape and Namibian sampling localities. An AMOVA which specified the Eastern Cape localities as a distinct group separate from Namibian ones returned a significant Φ_{ST} value underscoring the distinctiveness of these two

regions (Φ_{ST}=0.545; p<0.001). Finally, the retention of ancestral haplotypes was preferred to gene flow (0.54 migrants per generation) when only these two groups were considered in a coalescent analysis (see Table 4.3). Given this evidence, Namibia and Eastern Cape are deemed as two distinct groups.

Coalescent Bayesian modelling was performed for four clades (Northern Cape A, Northern Cape B, Namibia and Eastern Cape). (Kaokoland was not included since it did not share haplotypes with any other lineage). Theta values neared zero but did not converge to zero in all comparisons. Incomplete lineage sorting (ancestral polymorphism) was identified between clades with levels of gene flow (migration) in all analyses being negligible (less than 1 migrant per generation). Estimates of θ, M and T as well as detailed accounts of processes involved are provided in Table 4.3. In addition, the time to most recent common ancestor (TMRCA) was estimated for each comparison (Table 4.3).

Discussion

Many biotic and abiotic factors shape the spatial distribution of genetic variation across a landscape. Consequently it is imperative to consider all of these variables and to tease their relative contributions apart when attempting to interpret a specific genetic pattern. Arguably one of the most important considerations should be the connectedness of the landscape where one might expect very different genetic patterns for species with a continuous as opposed to a disjunct distribution (e.g. Taylor, 1993). To minimise the effect of life history traits, two species with very similar biology (within a broader small mammal context) were purposefully selected, the rationale being that differences in their phylogeographic patterns should be largely due to landscape variables directly or indirectly affecting dispersal rather than biology. Two distinct genetic patterns characterized the species included here explained through landscape variables including the use of different kinds of habitat and the influence of biogeographic processes on these.

Table 4.3 Results of MDIV for *E. rupestris*. In all comparisons gene flow was negligible and the effects of ancestral polymorphism evident.

Comparison	***θ***	Confidence interval	***M***	Confidence interval	***T***	Divergence time (MYA)	TMRCA (MYA)	Process
Northern Cape A vs Northern Cape B	10.87	5.75 - 22.81	0.08	0.00 - 1.44	0.80	0.59	0.87	Ancestral polymorphism
Namibia vs Eastern Cape	17.86	9.93 - 29.90	0.54	0.02 - 8.68	0.38	0.46	0.86	Ancestral polymorphism
Northern Cape A vs Namibia	17.77	9.64 - 31.73	0.12	0.00 - 1.66	0.58	0.70	1.05	Ancestral polymorphism
Northern Cape A vs Eastern Cape	10.05	6.73 - 20.48	0.10	0.00 - 1.24	0.86	0.59	0.84	Ancestral polymorphism
Northern Cape B vs Namibia	21.66	11.96 - 34.24	0.14	0.02 - 2.32	0.44	0.65	0.89	Ancestral polymorphism
Northern Cape B vs Eastern Cape	12.44	6.49 - 23.94	0.10	0.00 - 1.54	0.72	0.61	0.8	Ancestral polymorphism

M. p. proboscideus

Macroscelides p. proboscideus is characterized by a phylogeographic pattern typical of isolation-by-distance (IBD) signifying that geographical distance accounts for the observed genetic pattern (Wright, 1943). The round-eared elephant-shrew inhabits plains with a coarse gravel substrate. Although gravel plains in themselves are fragmented rather than continuous, they are nonetheless typically more continuous when compared to a habitat of rocky outcrops (L. Mucina pers. com). In other words, dispersal would be less impaired than in a species which use patchier habitat such as rocky outcrops. Why *M. p. proboscideus* exhibits IBD rather than a random (panmictic) population structure may be explained by its life-history traits including a small body size, social monogamy and territoriality (see e.g. Sauer and Sauer, 1972). Vertebrate species characterized by a random pattern are typically highly mobile and include bats, (Natterer's bat *Myotis nattereri*; Rivers et al., 2005), birds (Red grouse *Lagopus lagopus scoticus*; Piertney et al., 2000) and marine vertebrates (Heaviside dolphin *Cephalorhynchus heavisidii*; Jansen van Vuuren et al., 2002). In sharp contrast, small mammals (including elephant-shrews) are limited in the distance that they can disperse. IBD is not an uncommon phenomenon in other small mammal species with continuous distributions such as the hispid cotton rat (*Sigmodon hispidus*; Pfau et al., 2001) and the house mouse (*Mus musculus*; Dallas et al., 1995). Genetic patterns might differ between sexes (see e.g. Johnson et al., 2003; Seielstad et al., 1998), however this pattern is unlikely for elephant-shrews given their social structure where individuals form monogamous pairs and remain within territories (Sauer and Sauer, 1972; Rathbun and Rathbun, 2006).

E. rupestris

In sharp contrast, the spatial distribution of genetic variation in *E. rupestris* was characterized by significant substructure across the species' range. This result was anticipated given that the species is strictly confined to rocky outcrops in a sea of inhospitable plains. The spatial habitat occupied is therefore of lower connectivity (this is typical of the classical metapopulation model; Baquette, 2004). Several clades or groups were identified within the range of this species, the most distinct being confined to northern Kaokoland on the border between Namibia and Angola (see Coetzee, 1969 for vegetation and relief maps of the region). There are no haplotypes shared between this lineage and the remainder of the specimens. Although our genetic analysis for this northern group is based on a limited sample (n=3), and should therefore be considered preliminary, it is nonetheless noteworthy that this break coincides with the genetic pattern found in the rock-dwelling gecko, *Pachydactylus scherzi* (Bauer, 1999), possibly reflecting a similar effect of fragmentation of the rocky areas in northern Damaraland and Kaokoland.

An additional four haplogroups can be recognized across the remainder of the species' distribution. These correspond broadly to the Namibian, Northern Cape (A + B) and Eastern Cape regions. These lineages share few haplotypes, and a coalescent approach suggests a pattern of ancestral polymorphism with limited gene flow between them. Haplotypes belonging to the Northern Cape A and Northern Cape B clades are found in the Succulent and Nama Karoo (*sensu* Mucina and Rutherford, 2006). These areas are noted for isolated rocky outcrops with extensive plains separating them. Although relatively few haplotypes are shared between the Northern Cape A and Northern Cape B groups, there is a large overlap in the geographic distribution of these clades. Bayesian coalescent modelling indicated the retention of ancestral polymorphism between these lineages at the localities of Springbok, Kamieskroon and Calvinia. Historically, populations may have been confined to isolated refugia for extended periods allowing the accumulation of genetic differences among them, effectively causing two genetically divergent lineages with largely overlapping ranges (populations would have expanded during times when suitable habitat became available; see e.g. Saarma et al., 2007).

In species where populations are naturally more remote, physical barriers are more likely to impede gene flow than in a species with a continuous distribution (see e.g. Taylor, 1993). Examples include *E. rupestris* and *A. atra* (southern rock agama), an organism with similar habitat preferences. Where the distributions of these two species overlap*, E. rupestris* haplotype distribution closely match the clades described for *A. atra* (Matthee and Flemming, 2002): a northern (corresponding to the Namibian group in *E. rupestris*), north-central (corresponding to Northern Cape A and Northern Cape B lineages in *E. rupestris*) and south-eastern clade (overlapping with *E. rupestris*' Eastern Cape clade).

Biogeographic change

Both *M. p. proboscideus* and *E. rupestris* occur across a wide range of vegetation biomes with a rainfall gradient that overlays the distribution of these two species. Higher levels of precipitation are found in the east (Fynbos and Albany thicket biomes) compared to the west (Succulent and Nama Karoo) (Mucina and Rutherford, 2006). This may, to some extent, indicate a large tolerance to changes in vegetation brought about by climatic oscillations. Divergence times between the *E. rupestris* clades (Namibian, Northern Cape (A + B) and Eastern Cape) date between 0.5 – 0.7 MYA (see Table 4.3) in the Quaternary. At this time in the Interpleniglacial (0.80 – 0.25 MYA), which served as a transition period between the Last Interglacial (1.3 – 0.8 MYA) and the Last Glacial Maximum (0.25 - 0.15 MYA), the temperatures cooled and the region became drier than present (Lindesay 1988b), which could account for subsequent genetic divergences. Nonetheless, historical factors such as physical barriers to gene flow e.g. upliftment and river flow patterns,

either singly or in combination, undoubtedly contribute to shaping the current distributions and genetic profiles of the species.

For *E. rupestris* the estimates of genetic distance were largest in the vicinity of the central Karoo indicating lower levels of gene flow and higher levels of isolation/fragmentation (see Fig. 4.2c). This region coincides with the localities from where the divergent Karoo clade (represented by *Elephantulus sp. nov.*; Chapter 3) has been described (Smit et al., 2007). The region is situated within the Nama Karoo vegetation biome where the Upper (north) and Lower (south) Karoo bioregion vegetation units subdivide (Mucina and Rutherford, 2006). In addition, a sharp elevation difference accompanies the subdivision of the Upper and Lower Karoo bioregions which is possibly a remnant of two periods of interior upliftment (~20 MYA and ~5 MYA) in southern Africa which caused a rise of ~200m in the eastern escarpment (McCarthy and Rubidge, 2005). Subsequent changes in vegetation and climate following upliftment could have influenced the evolutionary history of species inhabiting the Lower and Upper Karoo bioregions.

In general, rivers are widely regarded as effective barriers to gene flow (Riverine Barrier Hypothesis, Alexio, 2004; Colwell, 2000; Patton et al., 2000), however, this view is not without its detractors (Ayres and Clutton-Brock, 1992; Gascon et al., 2000). The Orange River, which currently forms the border between South Africa and Namibia, has formed an absolute barrier to dispersal in some rock-dwelling reptiles (e.g. *Narudasia festiva*, *Typhlosaurus* and *Pachydactylus* groups; Bauer, 1999; Lamb and Bauer, 2000; *A. atra*; Matthee and Flemming, 2002) although not in others (Bauer, 1999; Lamb and Bauer, 2000). In *E. rupestris* a partition exists between clades south (Northern Cape clades) and north of the Orange River (Namibian clade) (see Fig. 4.2c) that is not reflected in the genetic profile of *M. p. proboscideus*. This is supported by the absence of genetic subdivision in the widespread “plain” burrow-dwelling yellow mongoose (*Cynictis penicillata*) (Jansen van Vuuren and Robinson, 1997).

In conclusion, this study shows that an increase in structural connectivity of the landscape is positively correlated with an increase in genetic connectivity reflected in contemporary gene flow. Furthermore, physical barriers tend to have a more pronounced effect on species with a naturally clustered distribution (*E. rupestris*) compared to one where the distribution is more continuous (*M. p. proboscideus*).

CHAPTER 5

REFINEMENT OF DNA EXTRACTION AND PCR LABORATORY PROTOCOLS INCREASES SUCCESS WITH MUSEUM MATERIAL

The use of museum material in DNA-based studies is becoming increasingly important (Good & Sullivan 2001; Godoy *et al.* 2004; Rohland *et al.* 2004), especially where access to fresh material is constrained by low population densities and/or the conservation status of species. This is reflected in elephant-shrews (Macroscelidea; Afrotheria) where three of the 15 described species are listed as worthy of special conservation concern, with the status of a further three compromised by insufficient data (IUCN redlist 2006; http://www.iucnredlist/org). Although non-invasive sampling addresses conservation concerns, this does not address instances where population numbers are so low as to preclude sampling, as is the case for many of the southern African elephant-shrew species. This study therefore relied on 155 museum specimens representing three species and two genera with the oldest specimen dating back to 1904 (*E. edwardii,* 57 specimens; *E. rupestris*, 55; *M. proboscideus,* 43 specimens).

Successful amplification of museum tissue can be particularly problematic and depends on the age and correct preservation of the material. Furthermore, DNA retrieved from museum material is most often degraded precluding amplification of shorter fragments that is frequently impossible with so-called universal primers designed for non-degraded DNA. This study reports a number of modifications to extraction and PCR procedures and re-emphasizes the need for species-specific primers when working with degraded DNA (this study included 27 novel species-specific primers which amplified overlapping regions of ~350 bp in length; see Table 5.1 & Fig. 5.1). Genotyping errors when dealing with ancient material can be problematic in population genetic studies (see Bonin *et al.* 2004 & references therein). However, the methods that are described here resulted in an almost negligible error rate (0.5% based on comparisons of 3944 bp) obtained from good quality genomic DNA.

Tissue from museum specimens should, where possible, be taken from inside the skull cavity. This method minimises damage to museum specimens (tissue sampling from museum specimens can be destructive, which together with the often low amplification success rate, might cultivate an indifferent approach towards the use of this material in DNA studies). Moreover, this approach has the potential to allow researchers greater access to type material. An added advantage is that tissue inside the skull cavity often escapes treatment with chemicals that degrade and destroy DNA (such as borax) and therefore offers higher chance of successful amplification when compared to skin snips.

Table 5.1 List of primers developed for degraded DNA and PCR cycling parameters used in the present study. The sequences are reported 5'→3'.

Species	DNA	Primer identification	Reference/sequence	Annealing temp. (˚C)	GenBank accession nr*
E. edwardii	cytochrome *b*	ESL1	ATCCAACATTTCATGATG	50	DQ901016 - DQ901091; DQ901212 - DQ901218
	cytochrome *b*	ESL2	GACARATATCRTTCTGAGG	46	
	cytochrome *b*	ESL3	CYTATTTGSYTAYGCTAT	46	
	cytochrome *b*	ESH1	ATAAYTAATGGGCTAGTA	46	
	cytochrome *b*	ESH2	TGTTATACTATTAGGAC	46	
	cytochrome *b*	ESH3	CTACGGAGAAGCCRCC	50	
	control region	ESD1	GTTGCTAGTGGTTTCTCGAAGC	49	DQ901092 - DQ901183; DQ901250 - DQ901256
	Fibrinogen 7	Fib7ES1	GATGGCTGGTACGTACT	57	EU07267 - EU76283
	Fibrinogen 7	Fib7ES2	AGGCGTATGTGGCAATTCAT	57	
	Fibrinogen 7	Fib7ES3	TTTAAATTTTACTCTCTGA	52	
	Fibrinogen 7	Fib7ES4	GTGTTTGAAGTTATTAGAA	52	
E. rupestris	cytochrome *b*	ERL1	ACCACAGGACTATTCCTA	49	EF141627 - EF141696
	cytochrome *b*	ERL2	CCTTTCACTTCATCCTTCC	51	
	cytochrome *b*	ERL3	CAGAATGRTACTTCCTATT	46	
	cytochrome *b*	ERH1	CAATAATGAATGGAAGGATGA	49	
	cytochrome *b*	ERH2	ACGCCTCCTAGTTTSTTA	51	
	cytochrome *b*	ERH3	CTATAATTTAATGATGTGRT	46	
	control region	ERN1	AACACCCAAAGCTGATATTC	51	EF141557 - EF141626
	control region	ERD1	TAGAAACCCCCACGATG	51	
M. proboscideus	cytochrome *b*	MPL1	AATCACACCCATTACTCAAAA	49	EF141764 - EF141822
	cytochrome *b*	MPL2	TATCTACTACGGCTCCTA	49	
	cytochrome *b*	MPL3	AGACCCAGACAATTATA	46	
	cytochrome *b*	MPH1	GGCTACTCCGATGTTT	49	
	cytochrome *b*	MPH2	GTATAATTGTCTGGGTCT	49	
	cytochrome *b*	MPH3	CTAGGATTAATAKGAARTA	46	
	control region	MPN1	CCACCATCAGCACCCAA	55	EF141697 - EF141763
	control region	MPD1	GTATAGTTCCGGTATAGAAACCCC	55	

* Genbank accession numbers are reported for specimens used in primer construction.

Museum extractions involved a hot lysis protocol and the use of a commercial DNA extraction kit (DNeasy Tissue Kit, Qiagen). Approximately 25 mg tissue was digested following the manufacturer's recommendations. In cases where samples had not fully lysed during the recommended 24 h incubation, an additional 10 µl proteinase K was added every 24 h and incubated at 56 ˚C to a maximum of four days. To enhance PCR amplification and to increase the amount of template available, a PCR without primers (primerless PCR) (Stemmer 1994; Weber *et al.* 2000) was used prior to real-time PCR amplification. This method, which is not commonly used, differs through the exclusion of primers. A primerless PCR has the advantage of multiplying shorter fragments of degraded template DNA. For a 100 µl reaction, 8 µl template DNA, 16 µl 10x buffer, 8 µl 10x $MgCl_2$, 8 µl 2mM dNTPs, 2 units of *Taq* DNA polymerase (Southern Cross

Biotechnologies) and 2 µl of 0.001 g/ml BSA were added. Amplifications were carried out in a GeneAmp PCR 2700 system (Applied Biosystems) with a thermal profile involving an initial denaturation step of 5 min at 94 ˚C followed by 5 cycles at 94 ˚C for 30 s, 45 ˚C for 30 s and 72 ˚C for 30 s. This was followed by 40 cycles at 94 ˚C for 30 s, 50 ˚C for 60 s and 72 ˚C for 50 s and the amplicons then cooled to a maintained temperature of 4 ˚C.

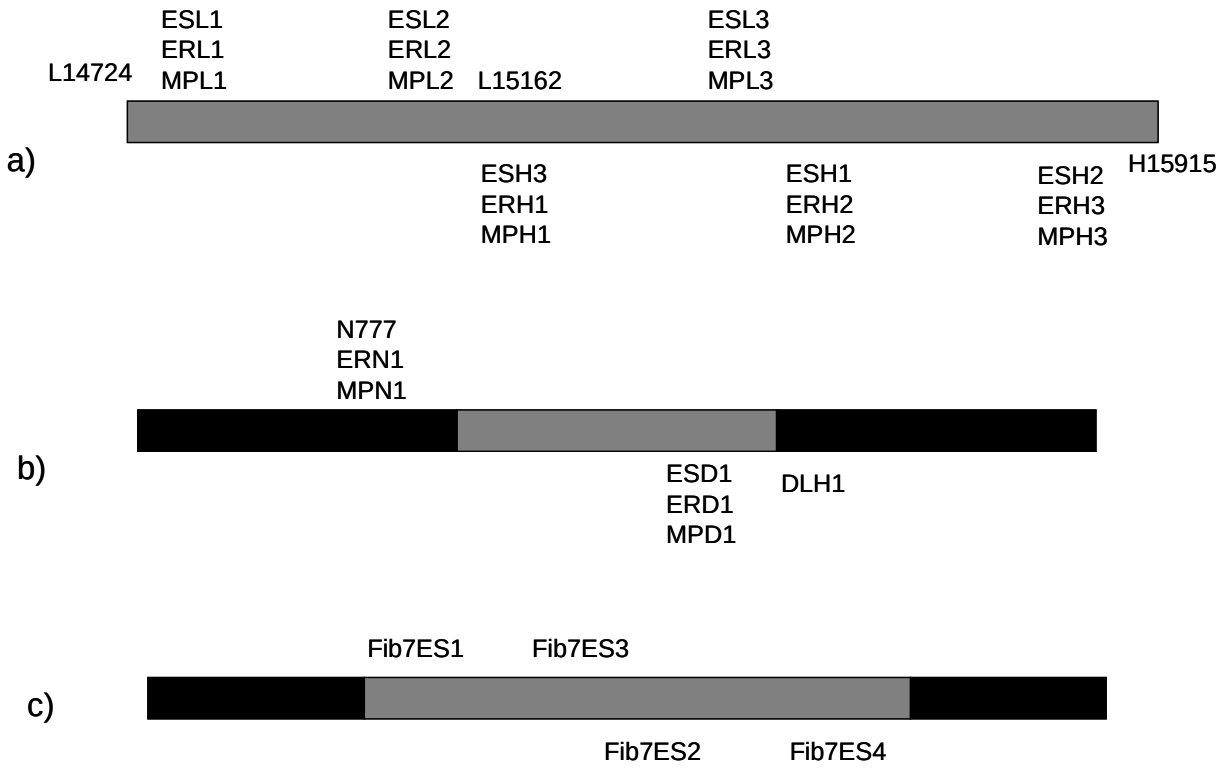

Figure 5.1 Spatial design of elephant-shrew specific primers for the (a) mitochondrial cytochrome *b* gene, (b) the control region and (c) the seventh intron of the nuclear fibrinogen gene. Forward primers are indicated above and reverse primers below. Relevant regions amplified are indicated in grey.

Standard PCR reactions were performed using 10 µl primerless PCR product as template DNA in the presence of 3 µl 10x/ $MgCl_2$ buffer, 3 µl 2mM dNTPs, 2 µl (10 pmol.µl $^{-1}$) of each primer, 0.2 µl of *Taq* DNA polymerase and adjusted to a final volume of 30 µl with sterile distilled water. Given that PCR inhibitors are often present in museum extractions, BSA was added (4 µl of 0.001 g/ml) to the PCR since it serves as a blocking agent that stabilizes inhibiting agents (Kreader 1996; Nagai *et al.* 1998). Amplifications started with a denaturation step of 5 min at 94 ˚C followed by 35 cycles at 94 ˚C for 45 s, and followed by primer-dependent annealing temperature for 45 s and 72 ˚C for 45 s. Importantly, PCR reactions were completed by 2 cycles of primer-dependent annealing for 5 min and an extension at 72 ˚C for 5 min before cooling

down and maintained at 4 ˚C. The primer-dependent annealing temperature was determined by the lowest primer-specific annealing temperature of the primer pair combination in use. The focus of this study was on the mitochondrial DNA cytochrome *b* gene and the control region. However, the inclusion of nuclear markers has become a virtual requirement in population level studies (Zhang & Hewitt 2003) and species-specific primers for the seventh intron of the fibrinogen gene (Fib7) were consequently included. Table 5.1 provides a summary of the primer sequences, annealing temperatures, species targeted and GenBank accession numbers.

In conclusion, the use of museum specimens to gain information on the genetic status of populations (spatially and temporarily) is becoming important (Smit *et al.* 2007) but the inclusion of this material is, unfortunately, often hampered by low amplification success rates. In this report a sampling technique where tissue is preferentially taken from within the skull cavity, minimising damage to museum specimens, is described. Certain modifications to conventional DNA extraction and PCR methodologies, including a PCR without primers (which markedly improves both the quality as well as quantity of template DNA), are proposed. Finally, this investigation emphasizes the importance of species-specific primers that span short (< 350 bp) stretches of target DNA.

CHAPTER 6

COMPARATIVE CYTOGENETICS

INTRODUCTION

Mammalian karyotyping, as well as the identification and understanding of the processes underlying chromosomal evolution has progressed considerably in recent years (see e.g. Carbone *et al.* 2006). Modern advances in sequencing and comparative genomics have shown that the genome is a composite of regions that are prone to reorganization and genomic tracts that do not show the same levels of evolutionary plasticity (reviewed in Ruiz-Herrera *et al*. 2006), and that rates of karyotypic evolution differ dramatically between eutherian orders (O'Brien & Stanyon 1999; Weinberg 2004; Froenicke 2005; Gilbert *et al.* 2006). For example, a maximum eutherian rate of 5.3 changes per 10 MY has been documented for the Indian muntjak while the minimum rate, represented by the dolphin and ferret, is 0.63 changes per 10 MY (Froenicke 2005). The rate of chromosomal evolution (0.7 per 10 MY) observed in golden moles, a representative of the Afroinsectiphillia (aardvark; elephant-shrews; golden moles and tenrecs), falls within the lower range for eutherian orders (Gilbert *et al*. 2006). Similarly, in contrast to a rapid rate of chromosomal change shown for the dog, mouse and rat genomes (Gregory *et al.* 2002; Zhao *et al*. 2004; Lindblad-Toh *et al.* 2005) among others, little interchromosomal repatterning has occurred in other members of the Afroinsectiphillia (Robinson *et al.* 2004) since their divergence from common ancestry 77 - 66 MYA (Springer *et al.* 2003). Further, based on the retention of a large number of primitive syntenies it can be concluded that Afroinsectiphillia has retained many characteristics of the primitive karyotype thought to be present in the common ancestor of eutherian mammals (Robinson *et al.* 2004). Within the Afroinsectiphillia, tenrecs have the highest diploid variation ranging from 30 to 56 (Gilbert *et al*. in press) whereas the aardvark (Tubulidentata) has a low diploid number of 2n=20 (Yang *et al.* 2003). Known chromosome numbers in golden moles range from 2n=28 in *Calcochloris obtusirostris* to 2n=36 in *Amblysomus robustus* (Bronner 2000; see also Gilbert *et al.* 2006; Gilbert *et al.* in press). Chromosomal variation in the elephant-shrews (Order Macroscelidea) is equally constrained where diploid chromosome numbers vary from 2n=26, for example in *E. rupestris* (Wenhold & Robinson 1987), to 2n=30 in *E. myurus* (Tolliver *et al.* 1989).

The use of cytogenetic markers to resolve the evolutionary relationships within the Macroscelidea has been limited to a small number of studies reporting mostly diploid numbers and unbanded karyotypes. Furthermore, although chromosome painting has been applied to test the phylogenetic position of the Macroscelidea within the Afrotheria (Robinson *et al.* 2004; Svartman *et al.* 2004), the use of FISH to resolve the evolutionary relationships within macroscelids has been neglected. The value of chromosome painting in identifying syntenies between species and at higher evolutionary levels is reflected by the large number of studies employing this technique. In fact, painting data are available for more than 60 species of 12 of the 18 eutherian orders (Froenicke 2005; Svartman *et al.* 2006), albeit in most instances these are limited to unidirectional painting schemes.

The overarching aim herein was to report banded karyotypes and to identify regions of homology of six species of southern African elephant-shrews (*E. edwardii, Elephantulus sp. nov.* (see Chapter 3)*, E. intufi, E. myurus, P. tetradactylus* and *M. proboscideus*) based on conventional banding (G- and C-banding), as well as the identification of nucleolar organizer regions (NORs). In addition, cross-species chromosome painting (Zoo-Fluorescence *in situ* hybridization or zoo-FISH) using a subset of flow-sorted *E. edwardii* painting probes was employed to resolve problematic chromosomal comparisons among species. Technical limitations (discussed below) precluded the use of a full suite of painting probes but, fortunately, a combination of good G-band resolution for the larger autosomes and FISH results on the smaller autosomes (less well resolved with G-banding) provided a robust approach to studying chromosomal evolution in these species. All *E. edwardii* flow-sorted probes were characterized but subsequently only a subset of these were hybridised to metaphase chromosome spreads of *E. intufi*, *Elephantulus sp. nov.* (see Chapter 3), *E. myurus*, *P. tetradactylus* and *M. proboscideus*.

METHODS

Specimen collection and metaphase preparation

Material was obtained from six South African elephant-shrew species collected under permit. The six species are representative of three genera, *Elephantulus*, *Macroscelides and Petrodromus*: *E. edwardii* (2n=26), *Elephantulus sp. nov.* (2n=26), *E. intufi* (2n=26), *E. myurus* (2n=30), *M. probosicdeus* (2n=26) and *P. tetradactylus* (2n=28) (see Table 6.1 for numbers of specimens collected, and their collection localities). Metaphase spreads were obtained from fibroblast cultures established from tail and/or ear clips. Fibroblasts were cultured in DMEM or Amniomax (Gibco) medium supplemented with 15% foetal calf serum; incubation was at 37 °C and with 5% CO_2. G-banding by trypsin (see Seabright 1971) and C-banding by barium hydroxide (see Sumner 1990) followed conventional procedures. Nucleolar organizer regions (NORs) were detected using silver nitrate (Goodpasture & Bloom 1975).

Table 6.1 Number of specimens karyotyped and their sampling localities. See appendix Table 3 for voucher numbers.

Species	Locality		Country	Grid ref	No. of speci-mens
E. edwardii	Cederberg	Western Cape	South Africa	32.23S 19.05E	3
	Napier	Western Cape	South Africa	33.49S 18.29E	5
	Grabouw	Western Cape	South Africa	33.49S 18.40E	1
	Tankwa Karoo National Park	Western Cape	South Africa	32.02S 20.05E	1
Elephantulus sp. nov.	Calvinia	Northern Cape	South Africa	31.80S 19.82E	3
E. intufi	Molopo Nature Reserve	Northern Cape	South Africa	25.78S 22.90E	1
P. tetradactylus	Bonamanzi Game Park	KwaZulu-Natal	South Africa	28.00S 31.10E	1
M. proboscideus	Steytlerville	Eastern Cape	South Africa	33.30S 24.30E	2
E. myurus	Ellisras	Limpopo	South Africa	23.52S 27.61E	2
	Spitskop Nature Reserve	Mpumalanga	South Africa	24.82S 30.12E	2

Flow-sorting and probe labelling

Chromosome-specific painting probes were prepared from flow-sorted Hoechst 33258 and chromomycin A-3 stained chromosomes of *E. edwardii* (EED) (2n=26) provided by the Cambridge Resource Centre for Comparative Genomics, UK. These were separated by size and AT:GC ratio. The flow-karyotype and characterised peaks are shown in Fig. 6.1. The two homologues of EED 5 were sorted separately (see Fig. 6.1) reflecting different amounts of heterochromatin present in the short arms of these chromosomes (see Chapter 3; Fig. 3.4a). The flow-sorts were amplified using 6MW primers and oligonucleotide-primed PCR (DOP-PCR) (Telenius *et al.* 1992) and labelled with either biotin and/or digoxigenin-dUTP (Roche). The assignment of each flow-sorted peak to specific *E. edwardii* chromosomes was done by the hybridization (painting) of each fluorescently labelled flow-sort onto 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories) banded metaphase spreads. Double-colour hybridizations were used to resolve ambiguities where more than one chromosome was represented in a specific flow-sorted peak.

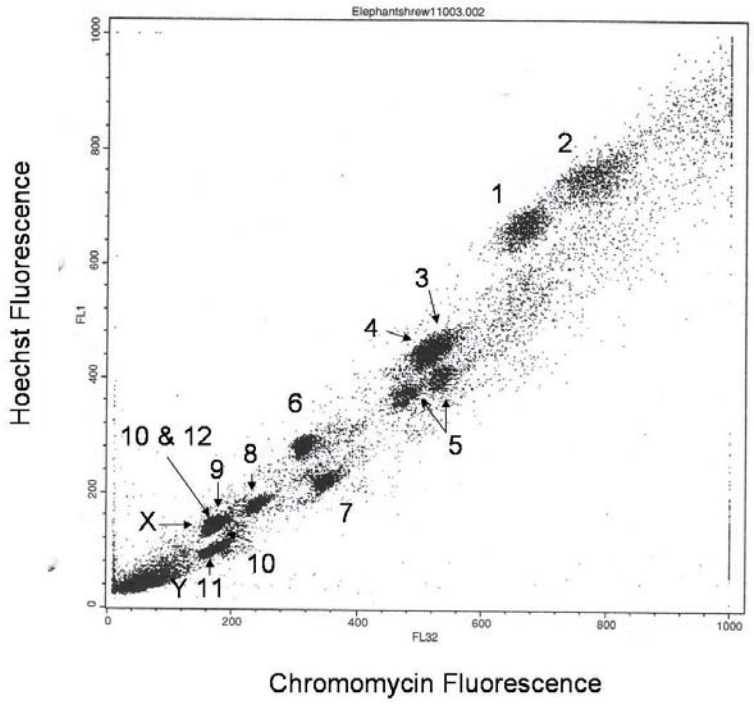

Figure 6.1 Flow-sorted karyotype of *E. edwardii* showing the assignment of peaks to specific chromosomes as ordered in the *E. edwardii* karyotype (Chapter 3; Fig. 3.4a).

Chromosome painting and probe detection

Cross-species chromosome painting followed Yang *et al*. (1997a, b, 2003) with modification. Biotin- and/or digoxigenin-dUTP labelled chromosome specific paints (100-150 ng) were precipitated at -80 C for 3 hours and included 6 µl labelled-probe, 6 µl unlabelled non-specific (largely heterochromatic) probe, 6 µl salmon sperm (Invitrogen), 6 µl Mouse Cot-1 (Invitrogen), 6 µl Sodium Acetate and 14 µl 100% ethanol. Precipitation proved highly useful in minimising non-specific hybridization. Furthermore, the addition of non-specific probe DNA improved the resolution of the painting results (discussed below). Precipitated probes were rinsed once with 70% ethanol, dried and made up to 14 µl with hybridization buffer (50% deionized formamide, 10% dextran sulphate, 2X SSC, 0.5 mol/L phosphate buffer, pH 7.3 and 1X Denhardt's solution). The probes were denatured at 65 C for 10 min and then preannealed by incubation at 37 C for 60 min.

Metaphase chromosomes were cleared of extraneous cytoplasm by a pre-treatment with pepsin when necessary (see e.g. Raap *et al.* 1989); this increases probe accessibility. Slides were heat-aged for 1 hour and denatured by incubation in 70% formamide/30% 2X SSC solution at 65 C for 20 sec, dehydrated in ice-cold 70% ethanol, and dehydrated through a 70, 80, 90 and 100% ethanol series. Hereafter, the

preannealed probes were applied to the metaphase preparations, covered and sealed with rubber cement and then incubated for 48h at 37 C. Increasing incubation time up to 96h improved the hybridization of more problematic probes. After incubation, post hybridization washes followed two 5 min incubations in 50% formamide/50% 2X SSC and then by two 5 min rinses in 2X SSC at 43 C. Fluorochromes were used to visualize labelled paints - Cy3-avidin (Amersham Biosciences) for biotin-dUTP and FITC (Roche) for digoxigenin-dUTP. Slides were mounted in Vectashield mounting medium and counterstained with DAPI. The images were captured with a CCD camera coupled to an Olympus BX60 fluorescence microscope and analysed using Genus 3.7 software (Applied Imaging).

RESULTS AND DISCUSSION

Karyotypes

Karyotypes for each species were prepared with the chromosomes arranged according to size and centromere position. Diploid numbers and number of fundamental arms are listed in Chapter 3; Table 3.4. The G-banded karyotype of *E. intufi* (EIN) (2n=26) and *E. myurus* (EMY) (2n=30) are shown in Fig. 6.2a & b respectively. The *M. proboscideus* (MPR) (2n=26) karyotype (Fig. 6.2c) is identical to that reported by Svartman *et al.* (2004) and by Wenhold & Robinson (1987); the *P. tetradactylus* (PTE) (2n=28) karyotype (Fig. 6.2d) is similar to that presented by Wenhold & Robinson (1987) although there is some variation in chromosome numbering among these studies. The banded karyotypes of *E. edwardii* (EED) (2n=26) and *Elephantulus sp. nov.* (ESN) (2n=26) have been presented separately (see Chapter 3; Fig. 3.4). Other karyotypic information on the macroscelids is limited to banded karyotypes reported for *E. rupestris* (ERU) (2n=26; Wenhold & Robinson 1987) and reports of diploid numbers of *E. brachyrhynchus* (2n=26; Stimson & Goodman 1966; Tolliver *et al.* 1989) and *E. rozeti* (2n=28; Matthey 1954).

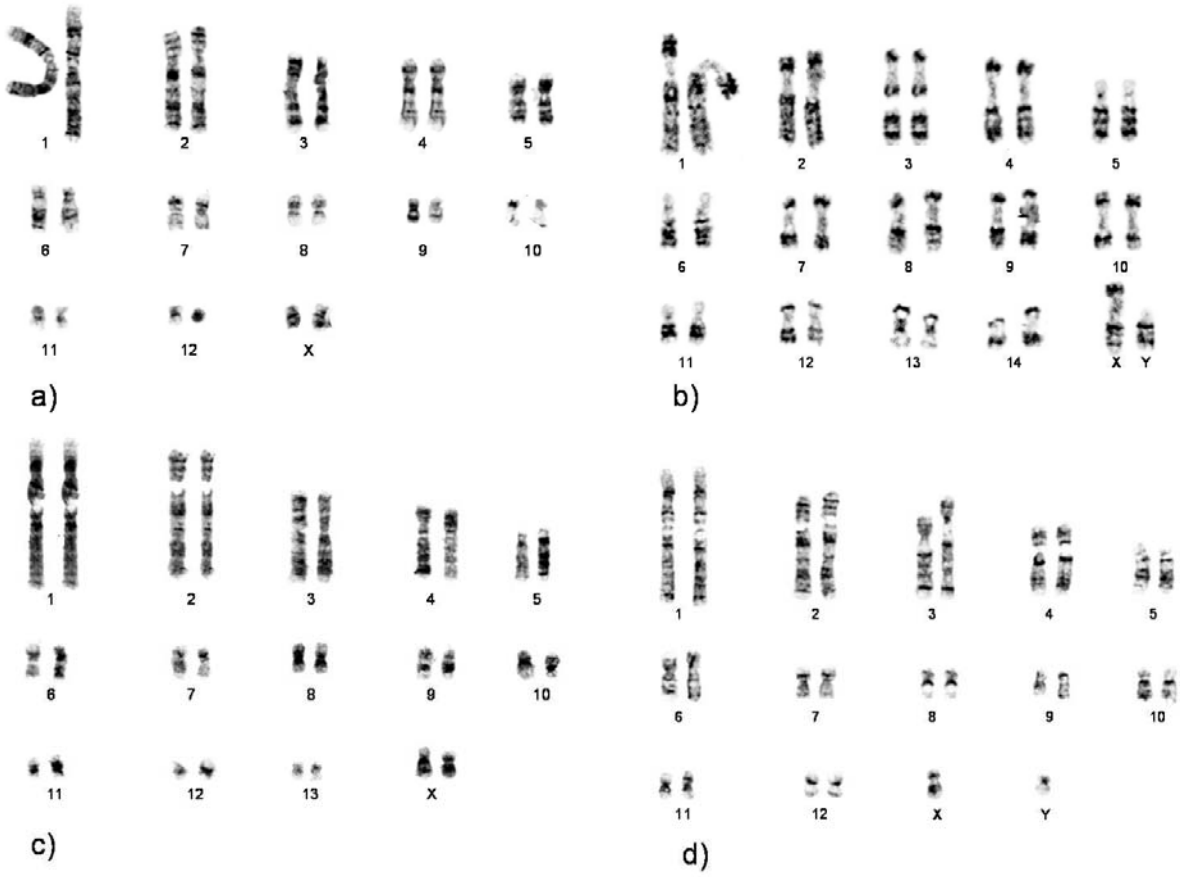

Figure 6.2 G-banded karyotypes of (a) *E. intufi,* (b) *E. myurus,* (c) *P. tetradactylus* and (d) *M. proboscideus.*

Half-karyotype comparisons showing the G-banded chromosomes of five of the six species analysed in this study are shown in Fig. 6.3. Differences in the amount of heterochromatin account for the positional changes in the respective karyotypes of *E. edwardii, Elephantulus sp. nov., E. intufi, P. tetradactylus* and *M. proboscideus*. With minor exceptions, related to heterochromatic differences (Fig. 6.4), the G-banded chromosomes of *Elephantulus sp. nov., E. intufi, M. proboscideus* and *E. edwardii* are identical at this level of resolution. However, *P. tetradactylus* (2n=28) and *E. myurus* (2n=30) show structural changes that are unique to each species (autapomorphies). The chromosomes of *E. myurus* are excluded from the banding comparisons due to the addition of large blocks of heterochromatin in the centromeric regions of this species which made direct comparisons difficult (Fig. 6.5).

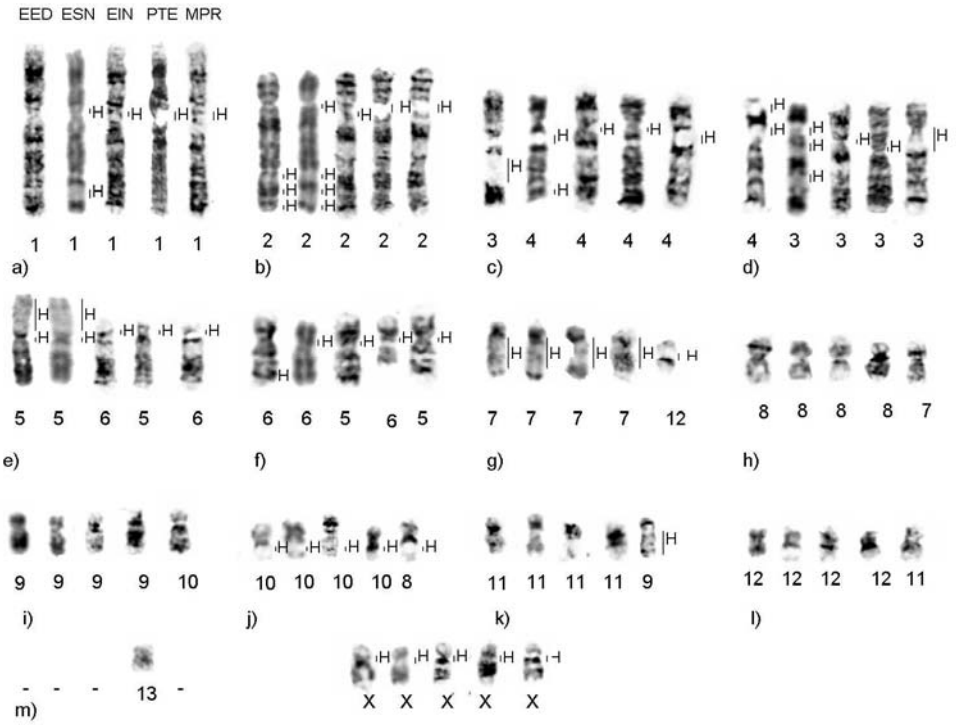

Figure 6.3 Half-karyotype G-band comparisons between *E. edwardii* EED, *Elephantulus sp. nov.* ESN, *E. intufi* EIN, *P. tetradactylus* PTE and *M. proboscideus* MPR. Heterochromatic regions (H) are indicated.

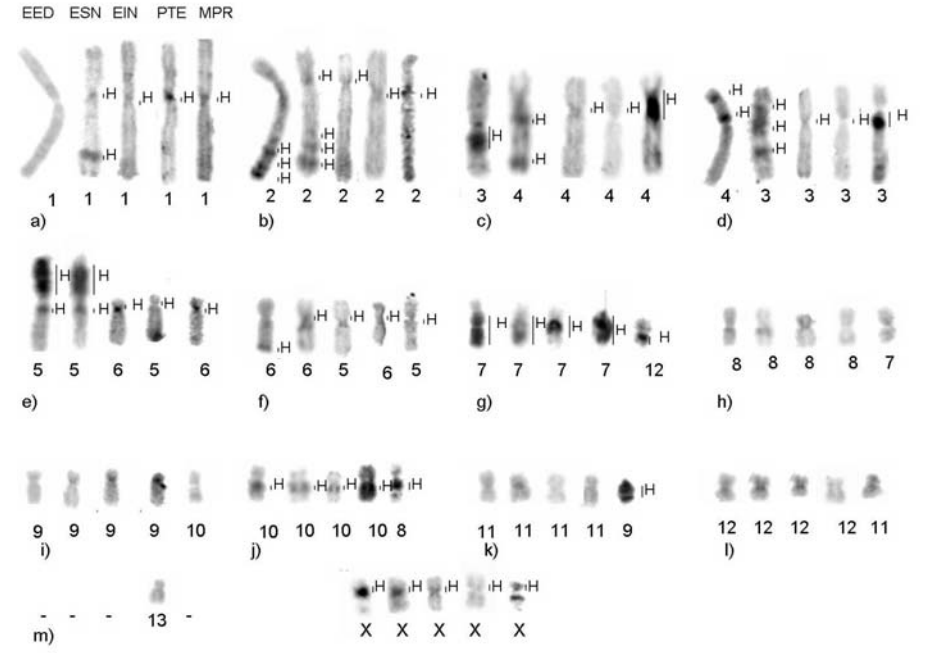

Figure 6.4 Half-karyotype C-band comparisons between *E. edwardii* EED, *Elephantulus sp. nov.* ESN, *E. intufi* EIN, *P. tetradactylus* PTE and *M. proboscideus* MPR. Chromosome identity was determined using sequential banding. Heterochromatic regions (H) are indicated.

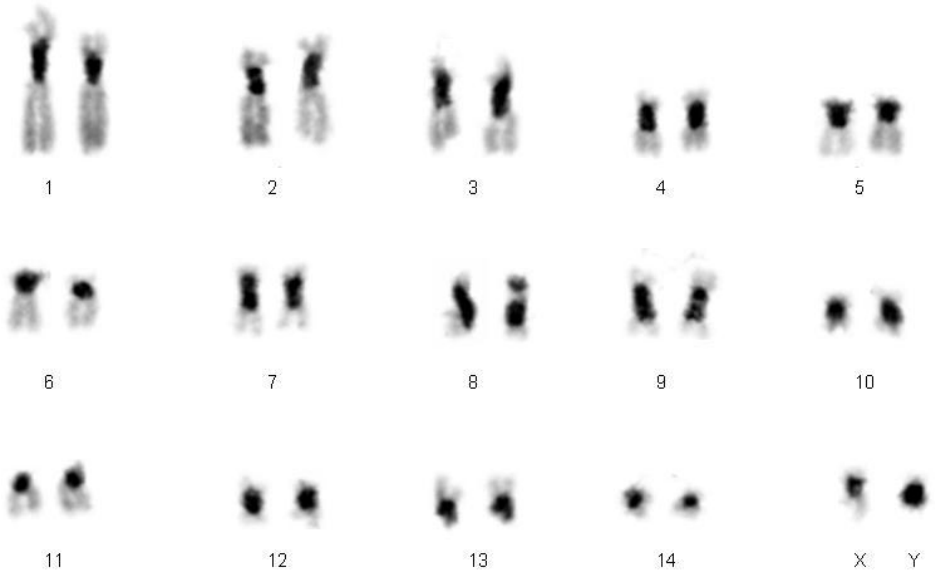

Figure 6.5 C-banded karyotype of *E. myurus.* (This species was not included in the half-karyotype C-band comparison shown in Fig. 6.4 due to difficulties making direct comparisons).

The sister taxon grouping of *E. edwardii* + *Elephantulus sp. nov.* presented in Chapter 3 is supported by three heterochromatic bands near the distal end of the long arms of EED 2 and ESN 2 (Fig. 6.3b) as well as the amplification of heterochromatin on the distal end of the short arms of both EED 5 and ESN 5 (Fig. 6.3e) that associate these taxa to the exclusion of all other elephant-shrew species studied. Furthermore, *E. edwardii* is defined through a centromeric shift and heterochromatic amplification in the long arm of EED 3 (Fig. 6.3c –see Chapter 3; Fig. 3.6) as well as the presence of a distal band of heterochromatin in the long arm of EED 6 (Fig. 6.3f), all of which are lacking in other species (i.e., they represent autapomorphies for the recognition of *Elephantulus sp. nov.* as a distinct species – see Chapter 3). Additionally, the presence of a heterochromatic band near the distal end of both ESN 1 (Fig. 6.3a) and ESN 4 (Fig. 6.3c) similarly support the distinctness of this elephant-shrew species. Heterochromatic differences in EED 4, ESN 3, EIN 3, PTE 3 and MPR 3 account for the change in chromosomal morphology from subtelocentric to submetacentric in PTE 3 and EIN 3 (Fig. 6.3d). Lastly, *M. proboscideus* differs from *Elephantulus* and *Petrodromus* in the size of the heterochomatic block present in MPR 12 (Fig. 6.3g).

Differences in 2n and structural changes

Petrodromus tetradactylus (2n=28) differs from the 2n=26 chromosomal group (*E. edwardii, Elephantulus sp. nov., E. intufi* and *M. proboscideus*) through the presence of two chromosomes (PTE 6 and PTE 13) that

are fused in the 2n=26 group of species (represented by EED 6, ESN 6, EIN 5 and MPR 5). Given the phylogenetic placement of *P. tetradactylus* within the phylogenetic tree based on nuclear and mitochondrial sequencing (Douady *et al.* 2003) it is possible to hypothesize that this rearrangement represents a fission (most parsimonious explanation) which differs from the tandem fusion proposed by Wenhold & Robinson (1987). However, a tandem fusion would, in turn, support the tree topologies of the cladistic analyses of both Tolliver *et al.* (1989) and Raman & Perrin (1997) as well as dental, anatomical and morphological analyses of Corbet (1995), which show the placement of *P. tetradactylus* in respect to a monophyletic *Elephantulus*. Furthermore, given the sister-taxon relationship of *P. tetradactylus* + *E. rozeti* in the sequence-based phylogeny of Douady *et al*. (2003), this rearrangement (irrespective whether it was a fusion/fission) could possibly represent a shared derived character (synapomophy) for their placement as sister taxa given the shared diploid number of 28 of *P. tetradactylus* and *E. rozeti*. However, it should be noted that karyotypic information for *E. rozeti* is limited to its diploid number (Matthey 1954) and no banding data exist to support the hypothesis. Moreover, the standard karyotype of *E. myurus* (2n=30) differs from the 2n=26 group through the presence of a medium-sized subtelocentric and small telocentric pair of autosomes (Tolliver *et al*. 1989). It seems likely that this rearrangement represents an autapomorphy (not parsimony-informative) distinguishing it from its sister taxon, *E. edwardii* (2n=26) (see phylogenies of Tolliver *et al.* 1989; Corbet 1995; Raman & Perrin 1997; Douady *et al.* 2003; Smit *et al.* 2007). However, the direction of chromosomal change as well as the identification of the rearrangements remains to be resolved.

Nucleolar Organizer Regions (NORs)

Silver staining showed one pair of NOR bearing chromosomes in *E. intufi* (Fig. 6.6a) and *E. myurus* (Fig. 6.6b). Similarly, both *P. tetradactylus* (Wenhold & Robinson 1987) and *M. proboscideus* (Svartman *et al.* 2004) are reported to possess only one NOR-bearing autosomal pair (Fig. 6.6c & d respectively). Two pairs of NORs were identified in the genomes of *E. edwardii* (Chapter 3; Fig. 3.5c) and *E. rupestris* (Wenhold & Robinson 1987) and 10 NORs (5 pairs) were documented in *Elephantulus sp. nov.* (see Chapter 3; Fig. 3.5d). One of the NOR bearing chromosome pairs in *E. edwardii, E. rupestris* and *Elephantulus sp. nov.* has tentatively been identified in this study as being homologous to those in *E. intufi, E. myurus, P. tetradactylus* and *M. proboscideus*, based on the size and morphology of the smaller autosome on which it is present.

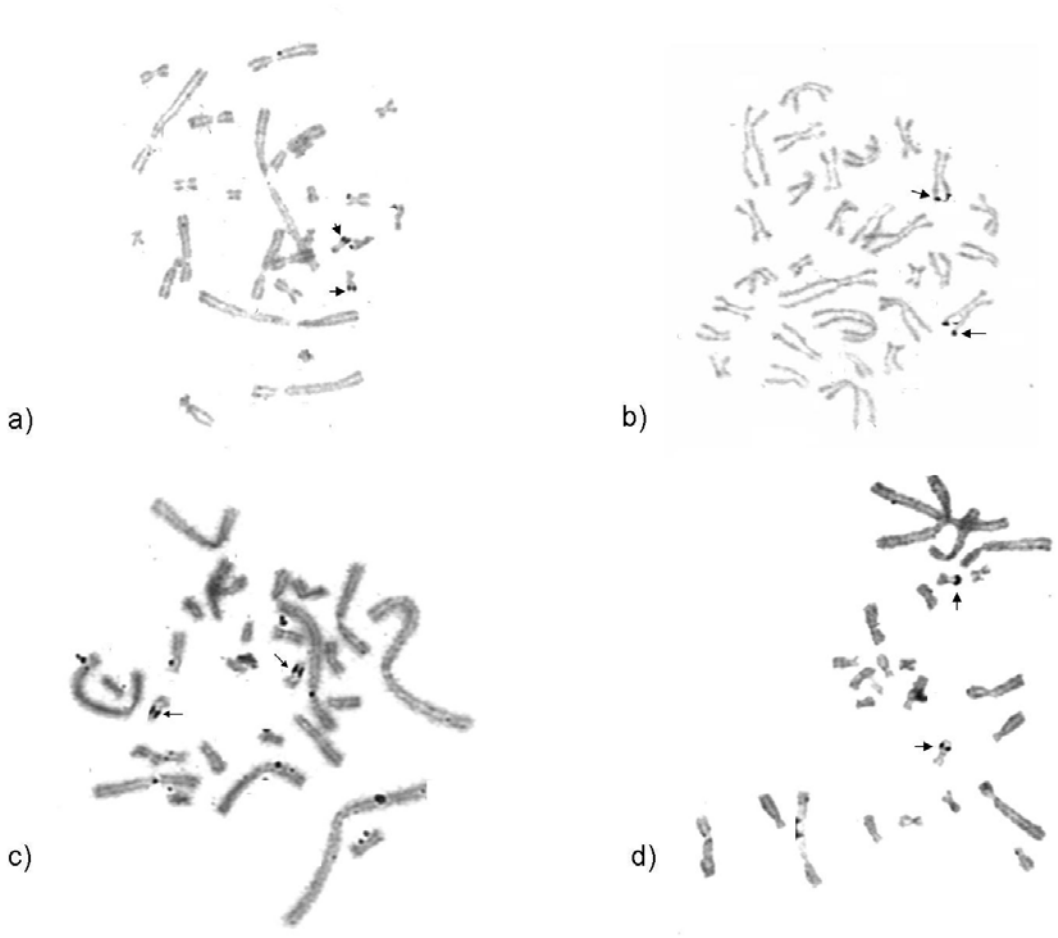

Figure 6.6 Representative metaphase cells showing the number of NORs (arrowed) in (a) *E. intufi* (n=2), (b) *E. myurus* (n=2), (c) *P. tetradactylus* (n=2) and (d) *M. proboscideus* (n=2) (see Fig. 3.5c & d for the location of NORs in *E. edwardii* and *Elephantulus sp. nov.* respectively).

Chromosome painting

The characterisation of flow-sorted *E. edwardii* paints is presented in Fig. 6.1. Although all flow-sorts were characterised, only a subset of these were used in cross-species chromosome painting due to technical difficulties encountered with the hybridization of the larger autosomes. This is thought to be due to the presence of large amounts of heterochromatin in these autosomes (pairs 1-7) in all elephant-shrew species studied herein. Consequently chromosome painting (zoo-FISH) was successful in only the smallest autosomes (pairs 8–12) importantly, however, these are the chromosomes with less well resolved G-banding patterns making zoo-FISH particularly useful in these instances. The hybridization patterns of painting probes EED 8-12 and the X to chromosomes of *Elephantulus sp. nov.* (Fig. 6.7), *E. intufi* (Fig. 6.8), *P. tetradactylus* (Fig. 6.9), *M. proboscideus* (Fig. 6.10) and *E. myurus* (Fig. 6.11) are shown. In all instances

only a single chromosomal pair was hybridized showing that no interchromosomal rearrangement has occurred since common ancestry confirming their homology across these species. That said, however, homologies detected using whole chromosome painting probes cannot rule out intrachromosomal shuffling (e.g. inversions and transpositions), and consequently these data do not preclude their presence in the genomes of these species.

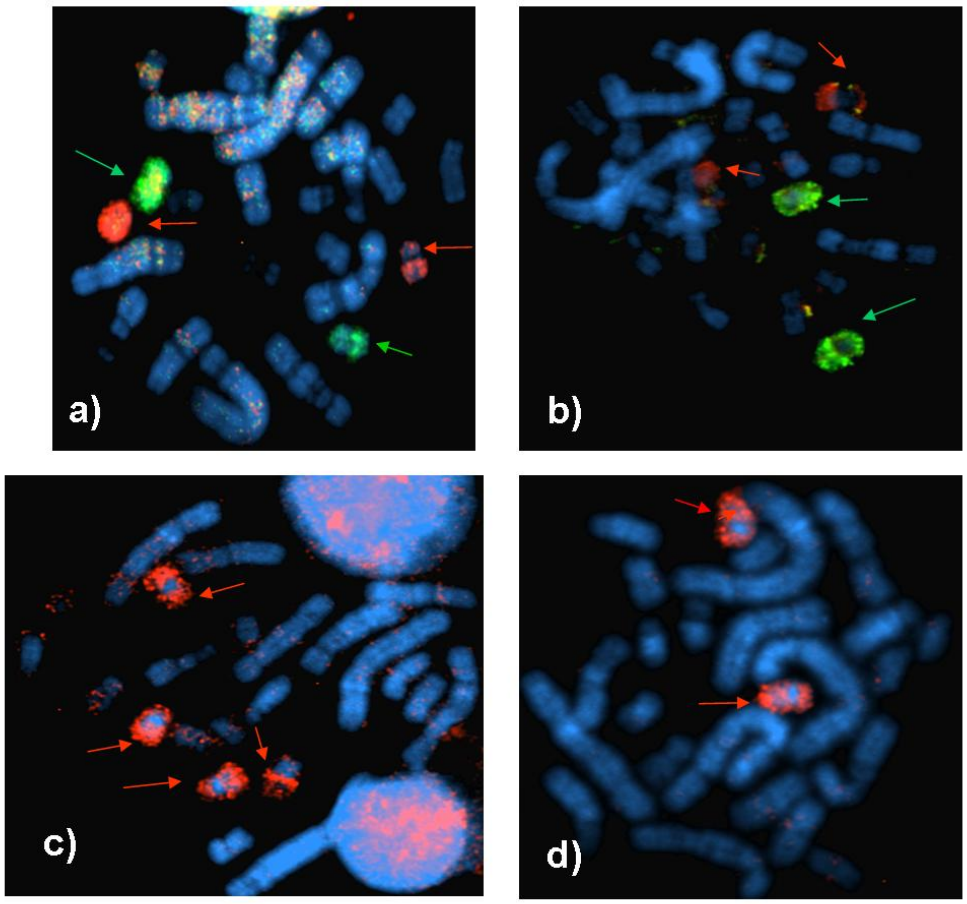

Figure 6.7 Examples of cross-species chromosome painting using (a) EED 8 (green) and EED 9 (red), (b) EED 10 (green) and EED 11 (red), (c) EED 10 and EED 12, (d) EED X on metaphase chromosome spreads of a female *Elephantulus sp. nov.*.

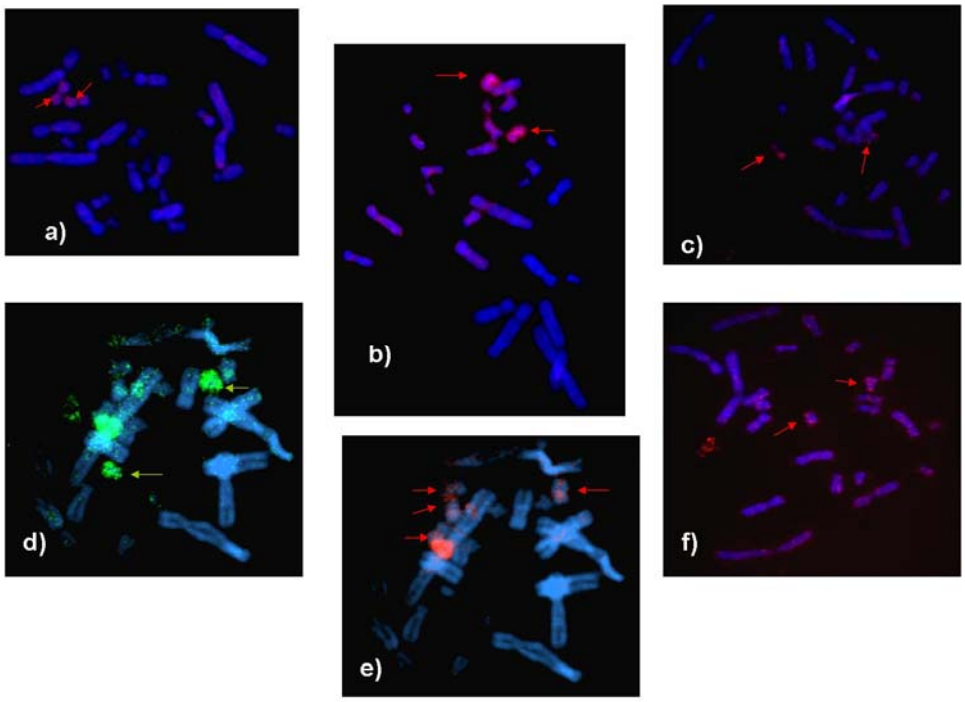

Figure 6.8 Examples of cross-species chromosome painting using (a) EED 8, (b) EED 9, (c) EED 10, (d) EED 11, (e) EED 10 and EED 12, (f) EED X on metaphase chromosome spreads of a female *E. intufi*.

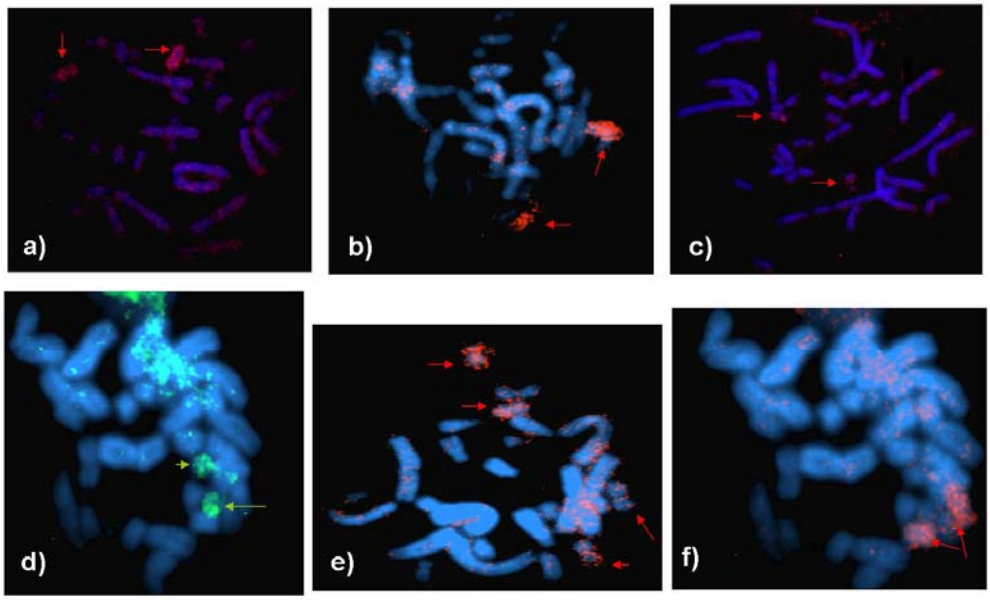

Figure 6.9 Examples of cross-species chromosome painting using (a) EED 8, (b) EED 9, (c) EED 10, (d) EED 11, (e) EED 10 and EED 12, (f) EED X on metaphase chromosome spreads of a female *P. tetradactylus*.

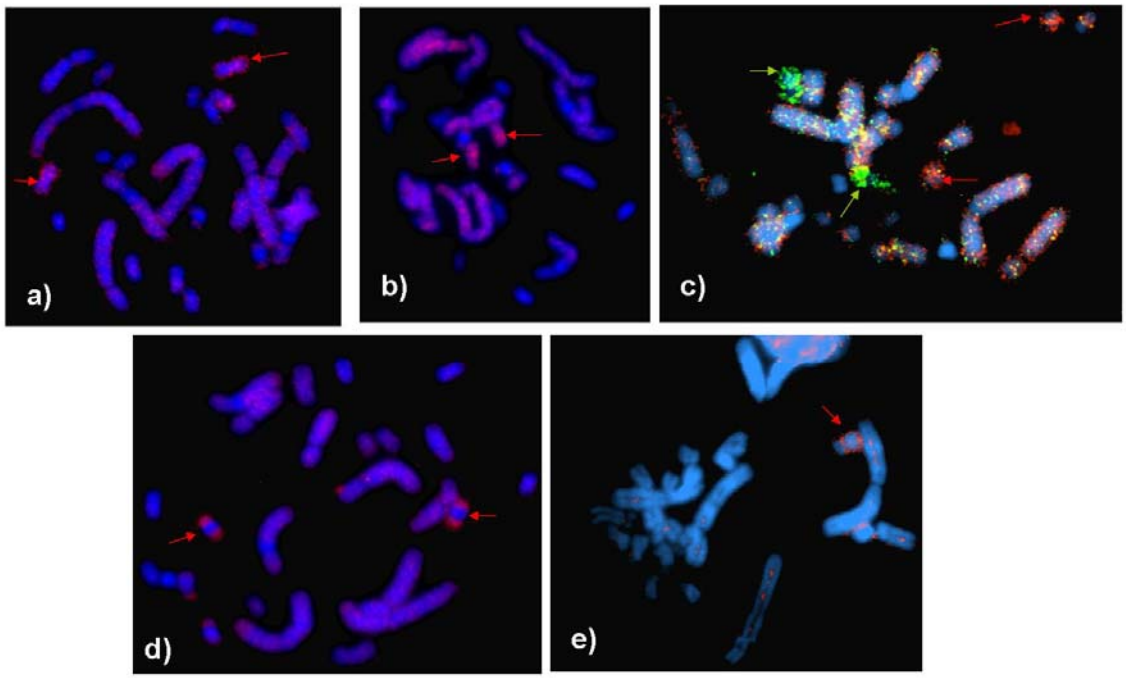

Figure 6.10 Examples of cross-species chromosome painting using (a) EED 8, (b) EED 9, (c) EED 10 (green) and EED 12 (red), (d) EED 11, (e) EED X on metaphase chromosome spreads of a male *M. proboscideus*.

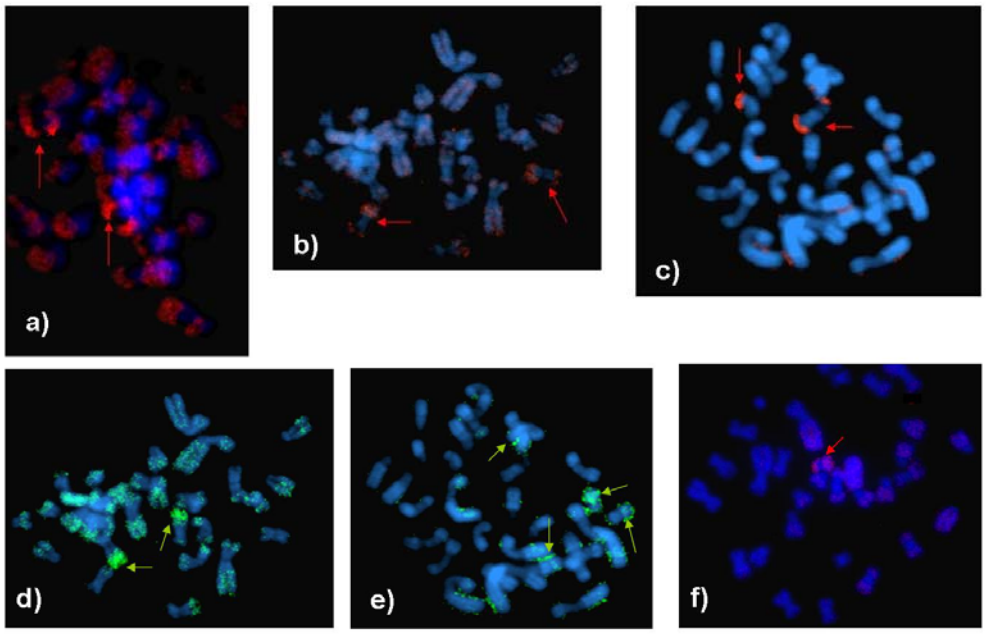

Figure 6.11 Examples of cross-species chromosome painting using (a) EED 8, (b) EED 9, (c) EED 10, (d) EED 11, (e) EED 10 and EED 12, (f) EED X on metaphase chromosome spreads of a male *E. myurus*.

The *E. myurus* karyotype possesses the largest amounts of centromeric heterochromatin of all elephant-shrew species analysed (Fig. 6.5). Painting experiments using EED 10 and 11 show hybridization to only half of each corresponding autosome in *E. myurus,* a result best explained by the addition of heterochromatic blocks in these regions (Fig. 6.11d). The addition of mouse COT-1 (rich in highly repetitive sequences, see e.g. Dugan *et al.* 2005), salmon sperm and an unlabelled non-specific *E. edwardii* probe to the probe mixture prior to precipitation, suppressed cross-hybridisation with similar repetitive sequences from other non-targeted chromosomes and this greatly facilitated the interpretation of the painting results.

In conclusion, this study sheds light on interspecific chromosomal relationships within the macroscelids and extends the investigation of the low diploid numbered Afroinsectiphillia and hence the search for the ancestral eutherian karyotype. The improved resolution provided by chromosome painting of the smaller sized autosomes confirmed G-banding results i.e. the retention of these as single homologous entities across all species examined. Moreover, zoo-FISH showed that the five smaller autosomes (i.e., those homologous to EED 8–12) of *P. tetradactylus* (2n=28) and *E. myurus* (2n=30) are not involved in rearrangements which account for the differences in diploid number between these two species when compared to the 2n=26 chromosomal group. Based on the conserved karyotypic nature of the extant species, the ancestor common to the Macoscelidinae (*Elephantulus, Macroscelides* and *Petrodromus*) was most likely characterised by 2n=26. Karyotypic information on the Rhynchocyoninae (genus *Rhynchocyon*) is unknown and these data are critical for further advances in determining the ancestral macroscelid karyotype.

CHAPTER 7

MORE THE MERRIER: EXPLOITING MULTIPLE CHARACTERS IN A HOLISTIC APPROACH TO ELEPHANT-SHREW RELATIONSHIPS

INTRODUCTION

Elephant-shrews represent a monophyletic order (Macroscelidea) comprising 15 extant species classified into two subfamilies and four genera. These placental mammals are small bodied, cursorial and saltatorial with a diet comprising mainly of invertebrates (Rathbun 1979). The evolutionary placement of elephant-shrews has been contentious. Haeckel (1866) included them with the insectivoran Menotyphla based on their morphology and insectivorous diet. Following this initial classification, they have been placed as a sister group to the Glires (lagomorphs and rodents) i.e., the Anagalida (McKenna 1975) on cranial morphology, embryonic characters (Novacek *et al*. 1988) and the presence of a large, functional caecum (Woodall & Mackie 1987). Most recently the outcomes of a plethora of genetic studies (e.g. Springer *et al.* 1997; Stanhope *et al.* 1998a, b; Springer *et al.* 1999; Amrine-Madsen *et al.* 2003; Nikaido *et al.* 2003; Robinson *et al.* 2004; Svartman *et al.* 2004; Nishihara *et al.* 2005; Kriegs *et al.* 2006; Nishihara *et al.* 2006; Waters *et al.* 2007) have resulted in the placement of elephant-shrews within the Afrotheria. Their placement within this group of African endemics is further supported by the close resemblance of the dental characters of fossil elephant-shrews to condylarths (primitive fossil ungulate (paenungulate) progenitors) (Tabuce *et al.* 2001; Zack *et al.* 2005).

In contrast to their evolutionary placement, relationships within the order have received much less attention. The two extant subfamilies, the Macroscelidinae and Rhynchocyoninae, are separated by no less than 30 phenetic characters (Butler 1995; Corbet & Hanks 1968). The Macroscelidinae include three of the four currently recognized genera; these are the monotypic *Macroscelides* which is a south-western African gravel plain specialist, the monotypic *Petrodromus* (with a south, east and central African forest distribution) and *Elephantulus* which includes 10 species found throughout a diverse array of habitats (refer to Chapter 1). The second subfamily, Rhynchocyoninae, is represented by three extant east and central African forest species all within a single genus, *Rhynchocyon*. Studies on the phylogenetic relationships between currently recognised elephant-shrews include the dental, morphological and anatomical analyses of Corbet & Hanks (1968 revised by Corbet 1995), and the allozyme analyses of both Tolliver *et al*. (1989) and Raman & Perrin (1997). To date, a single molecular phylogeny based on three mitochondrial and two nuclear fragments has been constructed for this group (Douady *et al.* 2003), however, this study suffered from incomplete species representation.

The evolutionary placement of *Elephantulus* is problematic. Corbet (1995) described synapomorphic dental, morphological and anatomical characters of all Macroscelidinae that supported *Elephantulus* as a monophyletic group. In contrast, Douady *et al*. (2003) suggested a diphyletic origin for *Elephantulus*, with *E. rozeti* grouping as a sister taxon to the monotypic *Petrodromus*. In addition, several *Elephantulus* species

have never been considered and their phylogenetic placement consequently remains moot. In an attempt to resolve the evolutionary status of *Elephantulus*, a supermatrix approach is followed here. This type of phylogenetic analysis allows the exploitation of multiple datasets as well as multigene matrices (Miyamoto 1985; Kluge 1989; Nixon & Carpenter 1996).

This study is the first of its kind to provide a comprehensive evolutionary phylogeny for the African Macroscelidea that includes complete taxon representation, including the recently proposed species from the South African Nama Karoo (*Elephantulus sp. nov.*; Chapter 3). A supermatrix approach is adopted where all available gene data are combined (see e.g. Flores-Villela *et al*. 2000; Gatesy *et al*. 2002; Nylander *et al.* 2004) into a supergene *sensu* Lartillot *et al.* (2007) (also see e.g. Willows-Munro *et al.* 2005; Qiu *et al.* 2006; Domes *et al.* 2007). Finally cytogenetic characters (G-banding, C-banding and nucleolar organiser regions (NORs); see Chapter 6) as well as the data of Tolliver *et al.* (1989), Corbet & Hanks (1968 revised by Corbet 1995), Woodall (1995) and Raman & Perrin (1997) are mapped to the molecular tree topology to test the robustness of the retrieved evolutionary relationships.

MATERIALS & METHODS

Specimen collection, DNA extraction and sequencing

All currently recognised elephant-shrews were included to elucidate relationships within the group (see Table 7.1 for species information and GenBank accession numbers). The study of Douady *et al.* (2003) was extended through the inclusion of museum representatives of six species (specimens are housed in the Natural History Museum, London, UK – museum numbers are given in parenthesis): *E. fuscipes* (1935.3.22.1), *E. revoili* (1897.8.9.6), *E. fuscus* (1907.1.11.11), *Rhynchocyon petersi petersi* (1975.857), *R. cirnei reichardi* (1897.10.1.26) and *R. chrysopygus* (1928.12.7.2). The inclusion of *E. rupestris* and *E. brachyrhynchus* together with *Elephantulus sp. nov.* (Chapter 3) completed the full taxon representation. Members of the Afrotheria - aardvark, tenrec, rock hyrax and dugong - were used as outgroups of successive relatedness to the ingroup.

Table 7.1 African distribution, source material and GenBank accession numbers for elephant-shrews included in this aspect of the investigation. GenBank accession numbers are also provided for the outgroup taxa. See appendix Table 3 for voucher numbers and geographical coordinates of sampling localities.

		GenBank Accession Nr			
		Mitochondrial	Nuclear		
Species	Elephant-shrew distribution in Africa	12S rRNA, valine tRNA, 16S rRNA	IBRP exon 1	VWF exon 28	Source of material/sequences
Dugong dugon Dugong		U60185; AF17921	U48583	U31608	Lavergne *et al.* 1996; Porter *et al.* 1996; Springer *et al*. 1997; Stanhope *et al.* 1998a
Procavia capensis Cape rock hyrax		U60184; U97335	U48586	U31619	Lavergne *et al.* 1996; Porter *et al.* 1996; Springer *et al.* 1997; Stanhope *et al.* 1998a
Orycteropus afer Aardvark		U97338	U48712	U31617	Porter *et al.* 1996; Springer *et al.* 1997; Stanhope *et al.* 1998a
Echinops telfairi Lesser hedgehog tenrec		AF069540	*	*	Stanhope *et al.* 1998a
Rhynchocyon chrysopygus Golden-rumped elephant -shrew	Kenya	EU136152	*	*	Museum specimen, Natural History Museum 1928.12.7.2
R. cirnei Checkered elephant-shrew	Central African Republic; Democratic Republic of the Congo; Uganda; Tanzania; Mozambique; Malawi; Zambia	EU136154	*	*	Museum specimen, Natural History Museum 1897.10.1.26
R. petersi Black and rufous elephant -shrew	Kenya; Tanzania	EU136153	*	*	Museum specimen, Natural History Museum 1975.857
Macroscelides proboscideus Round-eared elephant-shrew	Botswana; South Africa; Namibia	AY310883	AY310900	AY310890	Douady *et al.* 2003
		EU136155	*	EU136137	Soft tissue

Table 7.1 continued

Species	Elephant-shrew distribution in Africa	GenBank Accession Nr			Source of material/sequences
		Mitochondrial	Nuclear		
		12S rRNA, valine tRNA, 16S rRNA	IBRP exon 1	VWF exon 28	
Petrodromus tetradactylus Four-toed elephant-shrew	Kenya; Democratic Republic of the Congo;Tanzania; Mozambique; Malawi; Zambia; Zimbabwe; South Africa; Angola	AY310883	AY310897	AY310890	Douady *et al.* 2003
		EU136156	EU136145	EU136138	Soft tissue
Elephantulus brachyrhynchus Short-snouted elephant -shrew	Democratic Republic of the Congo; Sudan; Uganda; Kenya; Tanzania; Malawi; Mozambique; Zambia; Zimbabwe; South Africa; Namibia; Angola	AY310879	*	*	Douady *et al.* 2003
		EU136160	EU136146	EU136139	Soft tissue
		EU136161	EU136147	*	Soft tissue
E. edwardii Cape rock elephant-shrew	South Africa	EU136166	*	EU136144	Soft tissue
E. fuscipes Dusky footed elephant-shrew	Democratic Republic of the Congo; Sudan; Uganda	EU136157	*	*	Museum specimen, Natural History Museum 1935.3.22.1
E. fuscus Dusky elephant-shrew	Mozambique; Malawi; Zambia	EU136158	*	*	Museum specimen, Natural History Museum 1907.1.11.11
E. intufi Bushveld elephant-shrew	Botswana; South Africa; Namibia; Angola	AY310884	AY310898	AY310891	Douady *et al.* 2003
		EU136162	EU136148	EU136140	Soft tissue
E. myurus Eastern rock elephant-shrew	Zimbabwe; Botswana; South Africa; Mozambique	AY310882	AY310896	AY310889	Douady *et al.* 2003
		EU136165	EU136151	EU136143	Soft tissue
E. revoili Somali elephant-shrew	Somalia	EU136159	*	*	Museum specimen, Natural History Museum 1897.8.9.6
E. rozeti North African elephant-shrew	Morocco; Algeria; Country: Libyan Arab Jamahiriya; Tunisia	AY310881	AY310895	AY310888	Douady *et al.* 2003

Table 7.1 continued

Species	Elephant-shrew distribution in Africa	GenBank Accession Nr			Source of material/sequences
		Mitochondrial	Nuclear		
		12S rRNA, valine tRNA, 16S rRNA	IBRP exon 1	VWF exon 28	
E. rufescens Rufous elephant-shrew	Somalia; Ethiopia; Sudan; Uganda; Kenya; Tanzania	U97339	U48584	U31612	Porter *et al.* 1996; Springer *et al.* 1997; Stanhope *et al.* 1998a
E. rupestris Western rock elephant-shrew	Zimbabwe; Botswana; South Africa; Namibia	EU136163	EU136149	EU136141	Soft tissue
		EU136164	EU136150	EU136142	Soft tissue
Elephantulus sp. nov.	South Africa	EU136167	*	*	Soft tissue
Listed as *E. edwardi*i in Douady *et al*. 2003		AY310885	AY310899	AY310892	Douady *et al.* 2003

* not sequenced

Total genomic DNA was extracted from fresh tissue using a standard proteinase K digestion (10 mg/ml) followed by a phenol/chloroform extraction (Maniatis *et al.* 1982). In the case of museum material, tissue was preferentially taken from within the skull cavity and the DNA extracted using a commercial kit (DNeasy Tissue Kit, Qiagen) (see Chapter 5). The authenticity of sequences was verified from multiple independent DNA extractions and PCR amplifications. The final supergene dataset used here comprised 3905 bp which included five DNA fragments. These were 951 bp for the 12S rRNA, 70 bp for the valine tRNA and 1122 bp for the 16S rRNA from the mitochondrial genome as well as exon segments of two nuclear genes: 760 bp of exon 28 of the Von Willebrand factor (vWF) and 1003 bp of the 5' region of exon 1 of the interphotoreceptor retinoid binding protein (IRBP). Given the degraded nature of the museum DNA, elephant-shrew specific primers were designed.

Successful amplification required the addition of BSA (4 ul of 0.001 g/ml to a 30 ul reaction) to lessen the effect of PCR inhibitors. Amplification was carried out in a GeneAmp PCR 2700 system (Applied Biosystems) with a thermal profile involving an initial denaturation step of 3 min at 95 C followed by 35 cycles at 95 C for 30 s, DNA fragment specific annealing for 30 s and 72 C for 60 s. Annealing varied between 46 °C to 52 °C for the mitochondrial regions, and 55 °C to 65 °C for the nuclear fragments. Sequencing reactions were performed using BigDye chemistry (v3.0; Applied Biosystems), cleaned using Centrisep spin columns (Princeton Separations), and the products sequenced on a 3100 AB (Applied Biosystems) automated sequencer. Electropherograms of the raw data were edited with Sequence Editor™ software v1.0.3a (Applied Biosystems). For polymorphic site changes within the nuclear exons the most parsimonious nucleotide was accepted.

Phylogenetic analyses

To reconstruct genealogical relationships within the Macroselidea, various phylogenetic approaches were employed. Trees were built under parsimony (MP) and maximum likelihood (ML) approaches using PAUP* (Swofford 2001) (1000 nonparametric bootstrap replicates estimated clade support) together with a Bayesian inference approach (BI) (2 x 5 x 10^6 generations) as implemented in MrBayes v3.0 (Huelsenbeck & Ronquist 2001). Where possible, more than one specimen per species was included (see Table 7.1). Under the Bayesian approach, posterior probabilities were calculated independently for the mitochondrial and nuclear matrices.

Relaxed Bayesian clock

To determine whether macroscelids are characterized by a constant rate of molecular evolution, likelihood trees constructed under the assumption of a constant rate, were compared to trees that were allowed to vary using the SH test (PAUP*). Likelihood trees were constructed for the independent nuclear and mitochondrial as well as combined multigene matrices. Given the absence of a clock-like evolution (see results), a relaxed Bayesian method (Thorne *et al.* 1998; Thorne & Kishino 2002) which allows different rates of evolution for, and across each independent region, was applied to estimate divergence times within the Macroscelidea. First, BaseML (employed in Pamlv3.14; Yang 1997) was used to calculate the maximum likelihood output files needed for the Multidivergence software. Secondly, model files were created in Paml2modelinfo independently for the five mitochondrial and nuclear gene regions so as to determine a variance-covariance matrix using Estbranches. These files were used as input to estimate the prior and posterior distribution of divergence dates as performed in Multidivtime.

Echinops telfairi (Tenrecoidea) was included because it is the closest related outgroup used to the elephant-shrews. The Markov chain Monte Carlo (MCMC) analysis involved sampling of the Markov chain 10 000 times for every 100th cycle after an initial burn-in of 100,000 cycles. Changing the Brownian motion-constant and the rate at the root did not significantly influence the clock and these values were set to the default in Multidivtime. The prior value of expected number of time units between the tip and the root was set to 100 MY (SD = 100 MY). Multidivtime has the advantage of allowing for multiple constraints on divergence times across various nodes on the evolutionary tree. To investigate the effect that different constraints have on divergence times, times of lineage separation for runs without constraints were compared to those with node constraints.

Constraints placed on various nodes were derived from two authorities. The first set incorporates the work done by Douady *et al.* (2003) following Springer *et al.* (2003) and is based on the divergence times estimated for the Paenungulate origin using fossil calibration points (a minimum of 54 MYA to maximum of 65 MYA). These represented three pairs of constraints placed for nodes A (refer to Figure 7.1 for nodes) (Macroscelidinae + Rhynchocyoninae divergence) (lower 38 and upper 48 MYA), H (*P. tetradactylus* + *E. rozeti* divergence) (lower 9 and upper 14 MYA) and I (*E. brachyrhynchus, E. rufescens, E. intufi, E. rupestris/E. myurus, Elephantulus sp. nov., E. edwardii* divergence) (lower 15 and upper 21 MYA). The second set of constraints follows Tabuce *et al.* (2001) based on a divergence time of 26 ± 3 MYA for the split between the Rhynchocyoninae and Macroscelidinae using 44 palaeontological characters and Butler (1984), who placed the divergence time of *Pronasilio* (the earliest known fossil representative of the

Macroscelidinae) at the mid-Miocene. The second set of constraints was therefore placed on node A (Macroscelidinae + Rhynchocyoninae divergence) (lower 23 and upper 20 MYA) and node D (ancestor of Macroscelidinae) (lower 10 and upper 20 MYA).

Cytogenetic, allozyme and phenotypic data mapped to the tree topology

An approach under Occam's razor (minimum number of entities necessary to explain an occurrence) was followed (see Dobigny *et al.* 2004) to map phylogenetically informative cytogenetic characters to the molecular phylogeny. Given the conserved karyotypic evolution within the Macroscelidea (Chapter 6), chromosomal changes rather than the chromosomes themselves were considered as characters and their presence/absence as character states. For this, cytogenetic characters representative of seven southern African elephant-shrew species including *Elephantulus sp. nov.*, *E. edwardii, E. rupestris, E. intufi, E. myurus, P. tetradactylus* and *M. proboscideus* are included (see Chapter 6). In addition, published allozyme allelic designations from 37 (Tolliver *et al.* 1989) and 26 (Raman & Perrin 1997) genetic loci for seven species distributed throughout southern Africa (*E. edwardii, E. rupestris, E. intufi, E. brachyrhynchus, E. myurus, P. tetradactylus* and *M. proboscideus*) are mapped to the molecular topology. The original dental, morphological and anatomical informative characters for the Macroscelidinae (Corbet & Hanks 1968 revised in Corbet 1995) as well as structural differences in penis morphology (Woodall 1995), were included.

RESULTS

Molecular phylogeny

The multigene supermatrix comprised 3905 bp from VWF, IRBP, 12S rRNA, valine tRNA and 16S rRNA (see Material and Methods above) DNA fragments. Data for both nuclear and mitochondrial fragments were generated for all elephant-shrew species with the exceptions of *E. fuscus*, *E. fuscipes*, *E. revoili*, *R.cirnei* and *R. petersi* (all museum specimens for which only mitochondrial DNA sequence data could be obtained). (In the combined gene analyses, nuclear fragments were coded as missing for these five species). The *Rhynchocyon* sp. sequence included by Douady *et al*. (2003) was verified here as *R. chrysopygus*. Although *E. edwardii* was represented in the Douady *et al.* (2003) study, this specimen was identified here as *Elephantulus sp. nov.* and consequently sequences from a correctly identified *E. edwardii* specimen were included in the present analysis. No data for the IRBP exon was obtained for *E. edwardii* due to difficulties with amplification. The models of evolution (determined using Modeltest; Posada & Crandall 2001) that best fitted the mitochondrial, nuclear and multigene combined datasets are shown in Table 7.2. Both the CI and RI indices (including uninformative characters) are shown in Table 7.3 and mitochondrial and nuclear sequence divergences between elephant-shrew species are shown in Table 7.4.

Table 7.2 Optimal models of evolution selected with Modeltest for the mitochondrial, nuclear and multigene data. Nucleotide frequencies as well as number of base pairs are provided.

	Genes	bp	Nucleotide frequencies					Molecular clock	
			A	C	G	T	Model	Diff -LnL	SH test
Mitochondrial	12S rRNA val tRNA 16S rRNA	951 70 1122	39.30	21.45	15.36	23.88	GTR + I (0.267) + G (0.4992)	6.95	p = 0.05
Nuclear	IRBP VWF	760 1003	22.35	26.76	28.73	22.15	TvM + G (0.549)	30.68	p < 0.05
Multigene	12S rRNA val tRNA 16S rRNA IRBP VWF	3905	31.32	24.11	22.34	22.23	GTR + I (0.261) + G (0.5202)	72.47	p < 0.05

Table 7.3 CI and RI indices (including uninformative characters) as well as constant, variable and parsimony informative characters are shown for the mitochondrial, nuclear and multigene data.

	Genes	bp	CI	RI	Characters		
					constant	Parsimony uninformative	Parsimony-informative
Mitochondrial	12S rRNA val tRNA 16S rRNA	951 70 1122	0.49	0.65	1094	206	842
Nuclear	IRBP VWF	760 1003	0.77	0.76	1081	280	402
Multigene	12S rRNA val tRNA 16S rRNA IRBP VWF	3905	0.58	0.52	2228	555	1122

Table 7.4 Mitochondrial (upper matrix) and nuclear (lower matrix) sequence divergences (uncorrected p-distances) between elephant-shrew species. Asteriks (*) denote comparisons for which nuclear sequences were not available for one or both of the species.

		mitochondrial															
		RCH	RPE	RCI	MPR	PTE	ERO	ERE	ERUF	EBR	EIN	ERU	EMY	EED	EPI	EFU	EFUS
nuclear	RCH		10.26	5.10	12.09	20.05	19.31	15.65	19.10	18.89	19.75	19.53	18.18	19.17	18.60	15.85	15.78
	RPE	*		5.14	19.93	20.51	19.94	17.26	21.03	20.72	21.22	21.14	20.04	20.18	20.00	12.16	13.70
	RCI	*	*		15.70	15.74	15.31	12.86	15.09	14.87	16.25	15.82	14.15	13.54	13.18	11.20	12.95
	MPR	13.94	*	*		15.47	15.27	12.17	16.13	16.38	17.38	17.36	16.44	17.30	16.94	13.64	13.91
	PTE	14.14	*	*	5.18		13.79	11.55	16.09	15.93	17.18	16.69	16.23	15.82	15.75	13.47	13.23
	ERO	14.34	*	*	5.24	3.70		10.97	14.50	14.76	16.25	15.76	15.16	16.95	16.47	12.40	12.71
	ERE	*	*	*	*	*	*		10.58	10.79	12.37	10.98	11.70	12.28	12.08	10.29	10.54
	ERUF	13.82	*	*	6.82	7.57	7.68	*		3.32	14.11	13.61	13.72	14.77	14.05	11.25	11.67
	EBR	13.42	*	*	6.09	6.74	6.94	*	2.33		16.31	13.26	13.87	14.66	14.32	11.15	11.73
	EIN	12.99	*	*	5.74	6.51	6.94	*	4.72	3.19		11.51	15.06	16.09	15.73	12.66	13.23
	ERU	13.31	*	*	6.06	6.54	7.03	*	4.67	3.27	1.56		15.00	15.83	15.68	11.94	12.45
	EMY	13.57	*	*	5.61	6.41	6.58	*	5.95	4.93	4.64	4.74		11.46	10.53	12.50	13.24
	EED	13.38	*	*	6.31	7.05	6.82	*	5.77	4.82	4.97	4.62	3.05		5.16	12.43	13.04
	EPI	13.48	*	*	5.81	6.41	6.32	*	6.15	5.18	4.78	4.67	2.87	1.53		12.55	13.10
	EFU	*	*	*	*	*	*	*	*	*	*	*	*	*	*		7.25
	EFUS	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	

R. chrysopygus = RCH; *R. petersi* = RPE; *R. cirnei* = RCI; *M. proboscideus* = MPR; *P. tetradactylus* = PTE; *E. rozeti* = ERO; *E. revoili* = ERE; *E. rufescens* = ERUF; *E. brachyrhynchus* = EBR; *E. intufi* = EIN; *E. rupestris* = ERU; *E. myurus* = EMY; *E. edwardii* = EED; *Elephantulus sp. nov.* = EPI; *E. fuscipes* = EFU; *E. fuscus* = EFUS

Irrespective method of analysis, or whether DNA fragments were considered singly or in concert, near-identical trees were recovered indicating confidence in the evolutionary relationships (see Fig. 7.1 for the representative BI tree with branch lengths; nodal support values are provided in Table 7.5). The only taxon that could not be placed with certainty was *E. revoili*, and the phylogenetic position of this species within *Elephantulus* remains unresolved. The subfamily Rhynchocyoninae (node B) forms a sister taxon to the subfamily Macroscelidinae (node D). The tree topology shows a paraphyletic origin for *Elephantulus* as currently defined, with the placement of the monotypic *Macroscelides* and *Petrodromus* within *Elephantulus*. The tree(s) support both the sister taxon grouping of *E. rozeti* + *Petrodromus* (node H), as well as the grouping of *Macroscelides* + *Petrodromus* + *E. rozeti* (node G), observations that are consistent with the results of Douady *et al.* (2003).

Relaxed Bayesian clock

The divergence times of posterior compared to prior runs differed (especially in the narrower credibility interval of the posteriors compared to the priors) indicating a clock that is based on the data, and not on the priors (Hassanin & Douzery 1999). In addition, there was also no difference in proportional rates of divergences between the constrained and unconstrained runs which indicates that the constraints applied did not influence the rate of divergence/evolution estimated within the macroscelids. The inclusion of multigenes provided more robust estimates of divergence times (see also Thorne & Kishino 2002; Yang & Yoder 2003). The divergence times from the first constraint based on authorities Douady-and-Springer were near-identical to those estimated by Douady *et al.* (2003). A similar divergence time was found for the split between *E. edwardii* (long-eared elephant-shrew *sensu* Springer *et al.* 2003) and *M. proboscideus* (short-eared elephant-shrew *sensu* Springer *et al.* 2003). However, these divergence times were on average 1.55 times older when compared to those estimated under the Tabuce-and-Butler (Butler 1984; Tabuce *et al*. 2001) constraints.

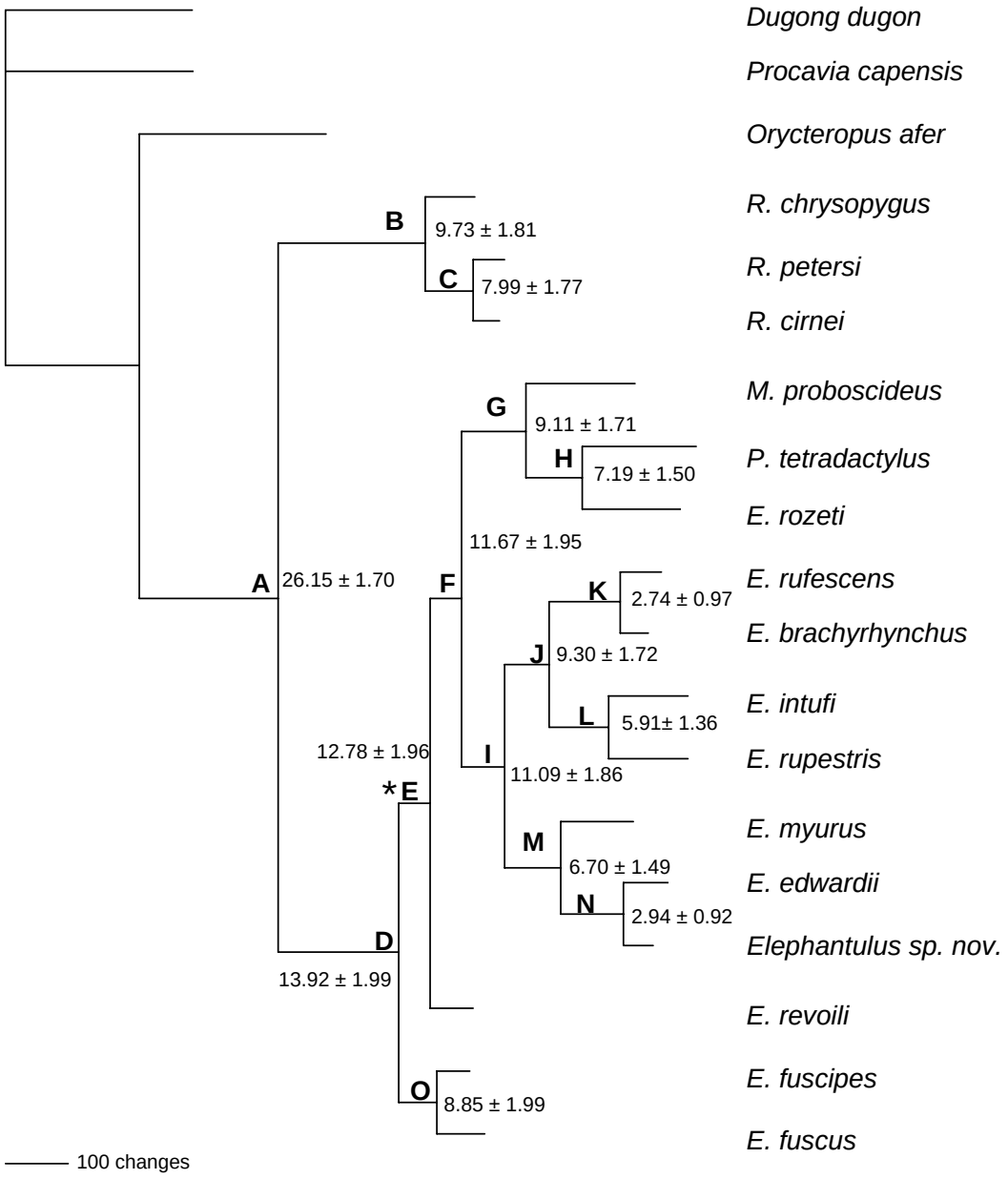

Figure 7.1 BI tree (including branch lengths) for the multigene supermatrix (3905 bp) (see text for details). The majority of nodes was supported by > 75% bootstrap (MP and/or ML) and a BI posterior probability of 0.95 with the exception of node E (*) (see Table 7.5). The relaxed Bayesian divergence times indicated on the tree are from the Tabuce-and-Butler time frame (Butler 1984; Tabuce *et al.* 2001) with nodes A and D constrained (see text).

Table 7.5 Boostrap support values and BI posterior probabilities for the multigene supermatrix (3905bp) shown in Fig. 7.1. Relaxed Bayesian divergence times with standard deviations are shown for both the Douady-and-Springer and Tabuce-and-Butler authorities.

	Node support			Douady- and-Springer		Tabuce-and-Butler	
Node	MP	ML	BI	Divergence time (MYA)	S. D.	Divergence time (MYA)	S. D.
A	100	100	1.0	42.60	2.74	26.15	1.70
B	100	100	1.0	15.34	2.87	9.73	1.81
C	*	82.4	1.0	12.59	2.78	7.99	1.77
D	98	100	1.0	23.00	2.13	13.92	1.99
E	*	*	*	21.20	1.78	12.78	1.96
F	81	93.3	1.0	19.73	1.78	11.67	1.95
G	79	98.5	1.0	15.78	1.34	9.11	1.71
H	97	100	1.0	12.48	1.45	7.19	1.50
I	79	100	1.0	18.00	1.68	11.09	1.86
J	88	100	1.0	15.09	1.69	9.30	1.72
K	100	100	1.0	4.34	1.31	2.74	0.97
L	100	100	1.0	9.58	1.55	5.91	1.36
M	100	100	1.0	10.12	0.56	6.70	1.49
N	100	100	1.0	4.37	0.88	2.94	0.92
O	100	100	1.0	14.54	2.88	8.85	1.99

* bootstrap support < 75% or posterior probability < 0.95

Chromosomal characters

The G-banded and C-banded half-karyotype comparisons of *E. edwardii*, *Elephantulus sp. nov.*, *E. intufi*, *P. tetradactylus* and *M. proboscideus*, presented in Chapter 6 (Figs 6.3 & 6.4), were used to identify character states that were mapped to the molecular supermatrix topology (see Fig. 7.2; Table 7.6 and Chapter 6). *Elephantulus myurus* was excluded from the banding comparison due to the presence of large centromeric heterochromatic blocks and poor G-banding resolution which made direct comparisons difficult. Synapomorphic chromosomal characters were represented by (I) a diploid chromosome number of 28 supporting the molecular grouping of *E. rozeti* + *P. tetradactylus* (node H) (diploid chromosome number for *E. rozeti* obtained from Matthey 1954) (no banding patterns for *E. rozeti* are available for comparison with *P. tetradactylus*); (II) three heterochromatic bands near the distal end of the long arms of EED 2 and ESN 2 and (III) the amplification of heterochromatin on EED 5 and ESN 5 which support the sister taxon grouping

of *E. edwardii* + *Elephantulus sp. nov.* (node N). Although autapomorphic changes do not constitute phylogenetically informative characters, they were included here to indicate evolutionary changes along species' branches (see Fig. 7.2; Table 7.6 and Chapter 6). Due to the absence of NOR data for both *E. brachyrhynchus* and *E. rufescens* it was impossible to polarize the character state reflecting the number of NOR pairs present in *E. edwardii* (n=2), *E. rupestris* (n=2), *E. intufi* (n=1), *E. myurus* (n=1) and *Elephantulus sp. nov.* (n=5) (also see Chapter 6) and these are therefore not indicated on the tree in support of node I (*E. brachyrhynchus* + *E. rufescens* + *E. intufi* + *E. rupestris, E. myurus* + *Elephantulus sp. nov.* + *E. edwardii* divergence).

Table 7.6 Cytogenetic characters mapped to the molecular topology (refer to Fig. 7.2).

	Symbol on tree	Node	Character state
Synapomorphic	I	H	Shared diploid number of 2n=28 in *P. tetradactylus* and *E. rozeti*
	II	N	Three distal heterochromatic bands on the long arm of EED 2 and ESN 2
	III	N	Heterochromatin amplification on EED 5 and ESN 5
Autapomorphic	i		*E. myurus*: Structural change in diploid number to 2n=30 and pronounced centromeric heterochromatin
	ii		*E. edwardii*: Morphological change of EED 3 (centromeric shift associated with presence of heterochromatin amplification on long arm of EED 3), as well as presence of a distal heterochromatic band on long arm of EED 6
	iii		*Elephantulus sp. nov.*: Heterochromatic band near distal end of long arms of both ESN 1 and ESN 4
	iv		*Macroscelides:* differs from *Elephantulus* and *Petrodromus* in the size of the heterochromatic block present on MPR 12
	v		*P. tetradactylus* and *E. intufi*: Heterochromatic differences account for the change in chromosomal morphology from subtelocentric to submetacentric in PTE 3 and EIN 3

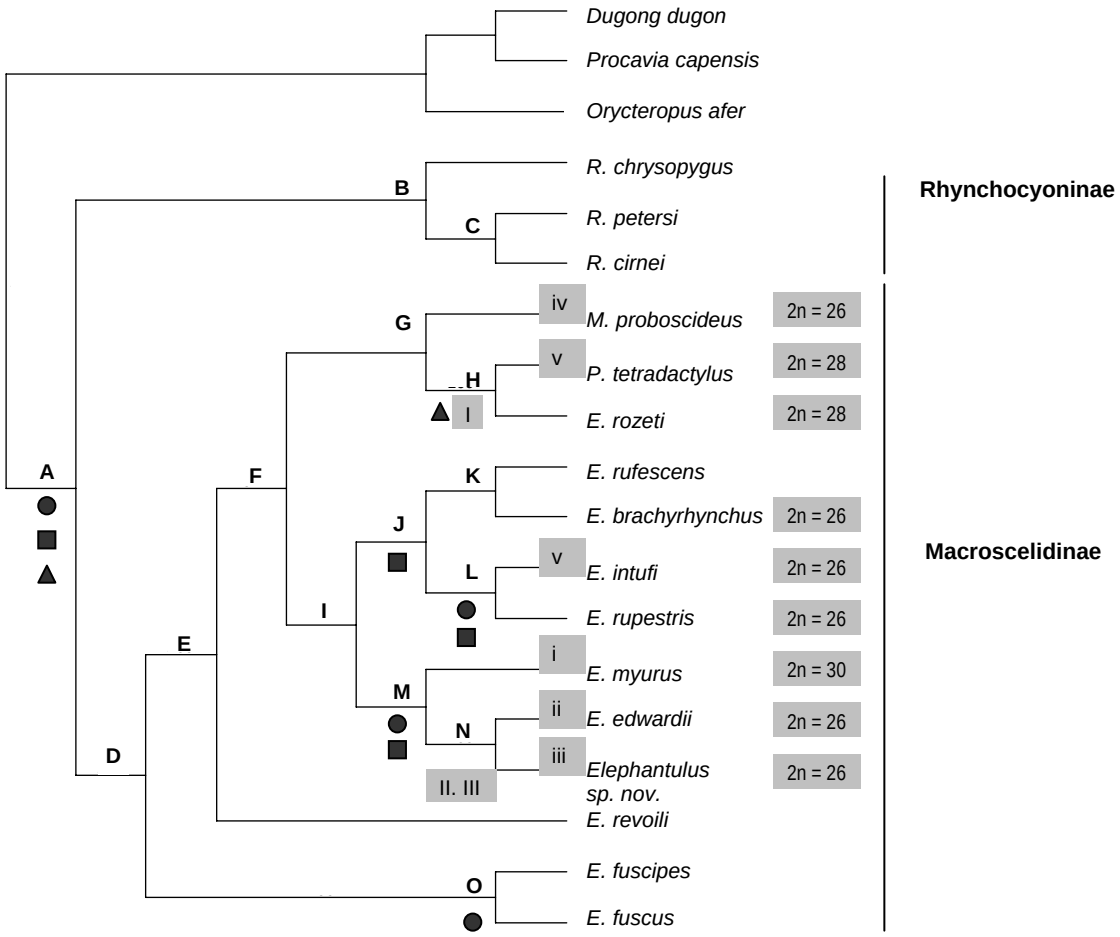

Figure 7.2 Non-molecular character support mapped to the molecular topology. Synapomorphic (I - III) and autapomorphic (i – v) cytogenetic characters (described in Table 7.6) in grey blocks as well as known diploid numbers are indicated. Morphological, dental or anatomical support (Corbet 1995) is shown as filled circles, that from allozymes (Tolliver *et al.* 1989; Raman & Perrin 1997) as filled squares, and support from penis morphology (Woodall 1995) as filled triangles (see text for details).

Phenetic characters – Corbet (1995)

The cladistic approach used by Corbet (1995) based on the morphological, dental and anatomical classification of the Macroscelidinae by Corbet & Hanks (1968) supported some of the groupings retrieved by the molecular analysis. These were the sister taxon groupings of *E. edwardii* + *E. myurus* (node M; swollen ectotympanic bullae), *E. intufi* + *E. rupestris* (node L; P^1 with lingual cups) as well as that of (*E. fuscus* + *E. fuscipes* (node O; supratragus twisted) (see Fig. 7.2). In contrast to the molecular findings, the phenetic results do not support the placement of *M. proboscideus* and *P. tetradactylus* within *Elephantulus*

(node B), but rather they support a monophyletic *Elephantulus*. The trichotomy/polytomy of the placement of the three species' groups (*E. edwardii* + *E. myurus*), (*E. rupestris* + *E. intufi*) and (*E. brachyrhynchus* + *E. fuscus* + *E. fuscipes*; I^3 with posterior cusp) was unresolved by the phenetic study. Additionally the sister grouping of (*E. brachyrhynchus* + *E. fuscus* + *E. fuscipes*; presence of third molar (M^3) absent in other Macroscelidea species) is not supported by the molecular data. Lastly, the inclusion of *E. rozeti* (I^2 with posterior cusp) within a monophyletic *Elephantulus* and the sister taxon grouping of *E. revoili* + *E. rufescens* (rhinarium hairy) in the phenetic cladogram were not supported by the molecular findings of this study.

Allozyme data – Tolliver et al. 1989 and Raman & Perrin 1997

Nei's genetic identity values for allozyme allelic designations from 37 (Tolliver *et al.* 1989) and 26 genetic loci (Raman & Perrin 1997) for seven southern African species supported a monophyletic origin of *Elephantulus*. The placement of *E. myurus, E. intufi* and *E. rupestris* on allelic designations is consistent with the molecular findings (see Fig. 7.2). However, some controversy surrounds the placement of *E. edwardii* that grouped with either *E. myurus* (Nei's genetic identity values) or *E. brachyrhynchus* (cladistic analyses) in Tolliver *et al.*'s study (1989), as well as the placement of *E. brachyrhynchus* which was unresolved (Nei's D and I values) or grouped with *E. intufi* + *E. rupestris* (Roger's genetic distance) in that of Raman & Perrin (1997).

Penis morphology – Woodall 1995

The structure of the penis in *R. chrysopygus* supported the division of the subfamilies Rhynchocyoninae and Macroscelidinae (node A; see Fig. 7.2). Characters include the bulk of connective tissue found in the body of the penis compared to a more vascular penis in all Macroscelidinae examined (*E. edwardii, E. brachyrhynchus, E. intufi, E. myurus, E. rupestris, E. rozeti*, *P. tetradactylus* and *M. proboscideus*), as well as the tip of the penis which ends in a spatulate shape with a small urethral process and a row of spines not found in the Macroscelidinae. *Macroscelides* is distinctly separated by the lobes fused into a collar near the end of the glans, which ends in an expanded and flattened form. In five of the six *Elephantulus* species examined (listed above) there was limited variation in male reproductive organ morphology. However, the penis morphology of *E. rozeti* resembled that of *Petrodromus* (with two lateral lobes and a narrowing distal spear-like tip) and supports the molecular and cytogenetic grouping of these two taxa (node H; see Fig. 7.2) (also see the discussion of Douady *et al.* 2003).

DISCUSSION

This study provides the first molecular phylogeny that includes all extant elephant-shrew species. Several important conclusions are drawn from this comprehensive dataset which is based on molecular and cytogenetic characters (this study), morphological, dental and anatomical studies (Corbet 1995), allozyme variation (Tolliver *et al.* 1989) as well as penis morphology (Woodall 1995). Arguably the most important finding is that *Elephantulus*, as currently described, is not monophyletic or alternatively that both *Petrodromus* and *Macroscelides* should be subsumed in *Elephantulus* (*Elephantulus* includes 11 of the 13 species recognized within the Macroscelidinae). This begs a more detailed taxonomic revision that may benefit from the inclusion of additional characters such as the distribution and function of scent glands (see for example the work already done by Faurie & Perrin 1995) as well as data on placental and fetal membrane evolution (see work done by de Lange 1949; Starck 1949; van der Horst 1950; Oduor-Okelo *et al.* 1980; Oduor-Okelo 1984; Oduor-Okelo 1985; Oduor-Okelo *et al.* 2004).

Evolutionary biogeography

Two evolutionary time frames have been put forward in the literature for the divergences of lineages within the Macroscelidea. These are based on paenungulate fossil calibration points (herein referred to as the Douady-and-Springer time frame [Douady *et al*. 2003; Springer *et al.* 2003]), as well as a set of calibration points based on Macroscelidea fossils termed the Tabuce-and-Butler time frame (Butler 1984; Tabuce *et al*. 2001). Divergence times derived from relaxed Bayesian analyses are, on average, 1.5 times more recent when the Tabuce-and-Butler constraints are implemented. Although neither set of divergence times are unrealistic, the Tabuce-and-Butler time frame is followed here given that it is based on macroscelid fossils rather than the more distant paenungulate fossils and may therefore reflect more accurate divergence dates for elephant-shrews.

The ancestral macroscelid lineage diverged in east Africa at ~26 ± 1.7 MYA and gave rise to two daughter lineages: the first, which occurs mainly in east and central Africa is adapted to a forest habitat (subfamily Rhynchocyoninae) and the other, with a largely east and southern African distribution is adapted to open savanna grasslands and arid habitat (subfamily Macroscelidinae). Within the forest adapted Rhynchocyoninae, speciation events would have been largely driven by fragmentation and the concomitant disruption of gene flow in response to aridification which reduced forest biomes during the late Miocene (see e.g. Hamilton 1982; Boaz 1985; Lovett 1993). The timing of speciation within this group, dated at 8 - 10 MYA, is coincidental with speciation events in several other forest specialists such as duikers (*Cephalophus*; Jansen van Vuuren & Robinson 2001) and also the divergence between duikers and klipspringers (*Oreatragus*; Hassanin & Douzery 1999).

Aridification would not affect savanna and arid adapted lineages in the same way as forest lineages in that habitat may not have been as fragmented (Bobe 2006). Rather, aridification caused shifts and expansions in ranges of species. In addition climatic changes during this period gave rise to arid corridors that connected the Horn of Africa via the mountains of eastern Africa extending to the Namib Desert in south-western Africa (Bobe 2006; Wilfert *et al.* 2006; Jürgens 2007); these corridors repeatedly opened and closed allowing exchanges between eastern and south-western Africa (see e.g. Balinsky 1962; Clancey 1986; Coe & Skinner 1993; Simmons *et al.* 1998; Barnes 2000; Herron *et al.* 2005; Simmons *et al.* 2005). Opening of these routes would have provided ideal opportunities for east Africa savanna / arid adapted elephant-shrews to colonize southern Africa. Indeed, at ~11.5 MYA there was a dispersal event from eastern Africa to south-western Africa giving rise to the modern lineages inhabiting southern Africa (*M. proboscideus*, *E. intufi*, *E. rupestris*, *E. myurus*, *E. edwardii and Elephantulus sp. nov.*) (see Fig. 7.3). The current distributions of *E. brachyrhynchus* (a savanna species) and *P. tetradactylus* (though adapted to a thicker "forest" vegetation type) closely follows these arid corridors as do a number of plant species (see Coleman *et al.* 2003 and references therein). Speciation events within southern Africa fit closely with periods of aridification and an increase in C4 grasslands in the Late Miocene at ~6 MYA (*E. intufi* + *E. rupestris*; node L; Fig. 7.1 as well as *E. myurus* and the ancestor of *E. edwardii* + *Elephantulus sp. nov.;* node M; Fig. 7.1), after 3 MYA in the Pliocene (*E. edwardii* + *Elephantulus sp. nov.;* node N; Fig. 7.1) and after 2 MYA in the Plio-Pleistocene (divergences among species; see Chapters 2 & 4) (deMenocal 1995; deMenocal 2004; Bobe 2006; Ségalen *et al*. 2006; Sepulchre *et al.* 2006).

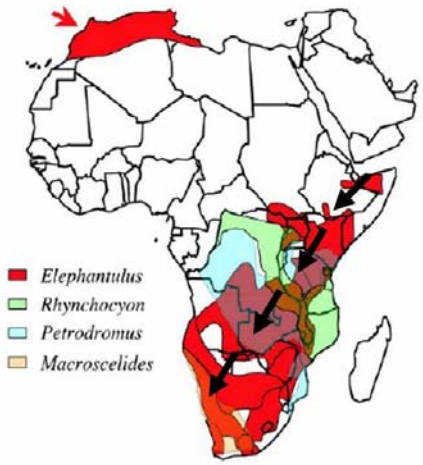

Figure 7.3 Current distribution of the macroscelid genera in Africa (taken from Corbet & Hanks 1968). The black arrows indicate the migration route from eastern Africa to southwestern Africa at ~11.5 MYA.

E. rozeti *dispersal*

Douady *et al.* (2003) placed the divergence of *P. tetradactylus* + *E. rozeti* at 11.2 ± 2.2 MYA and postulated the Sahara as a fundamental vicariance event in the speciation of these taxa. This vicariance hypothesis was proposed as an alternative to the dispersal hypothesis summarized by Corbet & Hanks (1968) which predicted that *E. rozeti* originated from an eastern species of *Elephantulus* that travelled along the Nile valley.

Using the Tabuce-and-Butler time frame, the divergence of *P. tetradactylus* + *E. rozeti* is 7.2 ± 1.5 MYA. At this time (between 7 - 8 MYA) the Asian monsoon evolved in association with the uplifting of Tibet and the displacement of the North African desert zone northwards. As a consequence of the development of the monsoon/desert system, the "Zeit Wet Phase" was initiated and central and eastern North Africa changed from dry to seasonally humid with the formation of an evaporitic couplet in the Gulf of Suez/Red Sea (Griffin 2002). This would support the dispersal hypothesis (following Corbet & Hanks 1968) and the formation of a corridor through a humid north-eastern Africa to north-western Africa. Subsequent climatic and biogeographical changes, supported by pollen data show that increased aridity of the Sahara at 3 - 2 MYA (Dupont & Leroy 1995) could have restricted/isolated *E. rozeti* to north-western Africa.

Chromosomal evolution

In spite of the slow pace of chromosomal evolution within most Afroinsectiphillia (Robinson *et al.* 2004; Gilbert *et al.* 2006), interspecific chromosomal changes in the macroscelids (see Chapters 3 & 6) provided powerful evidence of evolutionary relatedness. Differences in diploid numbers were limited to a change from 2n=26 (*E. edwardii, E. rupestris, Elephantulus sp. nov., E. intufi, E. brachyrhynchus* and *M. proboscideus*) to 2n=28 in *P. tetradactylus* and *E. rozeti,* and 2n=30 in *E. myurus*. In terms of the rates of change, the rock elephant-shrew lineage (*E. myurus* + *E. edwardii* + *Elephantulus sp. nov.*; node M), and specifically the divergence of the lineage leading from the rock elephant-shrew ancestor to the ancestor of *E. edwardii* + *Elephantulus sp. nov.* (node N; Fig. 7.2) are accelerated compared to the more constrained karyotypic evolution detected within the remaining macroscelids examined herein (see Chapter 3). In conclusion, information on diploid numbers and banding patterns from cross-species comparison are absent for a number of *Elephantulus* species and for the Rhynchocyoninae, all data that could improve the resolution of macroscelid phylogenetic relationships.

SUMMARY

Southern Africa is rich in elephant-shrew diversity with nine of the 15 species found in this region. In addition to these, a new species, which does not correspond to any previously described and/or currently recognized taxonomic entity is described from the central Nama Karoo (Chapter 3). The newly proposed species (*Elephantulus sp. nov.*) is phenotypically very similar to its sister species *E. edwardii*. *Elephantulus sp. nov.* was initially recognized in a phylogeography study that included seven specimens from the Transvaal Museum that failed to cluster with *E. edwardii sensu stricto* (Chapter 2). The taxonomic affinities of these museum vouchers were examined by comparing their mitochondrial sequences to corresponding gene regions from the type specimens of *E. capensis* and *E. karoensis*, both of which are presently synonimized within *E. edwardii*. Molecular sequencing supported the placement of *E. capensis* within *E. edwardii*; however, the *E. karoensis* type was genetically verified as *E. rupestris* emphasizing the difficulties associated with morphological classification (Chapter 2).

The formal description of *Elephantulus sp. nov.* (Chapter 3) presented here is based on 14 museum specimens and three live trapped individuals bringing the known number of vouchered specimens to 17. Genetic evidence in support of the recognition of the new species comprised mitochondrial and nuclear sequences as well as chromosomal differences. In addition, subtle phenotypic characters were identified that underpin the delimitation of *Elephantulus sp. nov.* from other rock elephant-shrew species with overlapping distributions (*E. edwardii* and *E. rupestris*) (see Chapter 3; Table 3.5 & Fig. 3.3). *Elephantulus sp. nov.* can be distinguished from its sister species *E. edwardii* by a suite of characters which include the tuft of hair at the tip of the tail, the colour of the flanks and ventral pelage. Although the species' distribution is thought to be restricted to the central Nama Karoo of South Africa (it is currently known from localities Calvinia, Beaufort-West, Carnarvon, Loxton and Williston), the extent of its range and its conservation status needs to be determined.

This study further emphasizes the importance of the upkeep of museum collections which, as demonstrated by the identification of *Elephantulus sp. nov.*, provide a wealth of biological information (see Chapter 5). More than half the material included in the phylogeographic analysis was from museum vouchers housed in different South African museums. Tissue was preferentially taken from within the skull cavity to minimise damage to vouchers and to limit possibilities of contamination. Refinement of extraction and PCR protocols as well as the design of species-specific primers (Chapter 5; Table 5.1) ensured successful amplification, even for tissue taken from specimens 100 years post mortem. The inclusion of museum material is particularly beneficial where trapping success is low and population densities fluctuate, and provides a temporal perspective that is not possible in studies that rely exclusively on fresh material.

The description and comparison of phylogeographic patterns in three southern African endemic elephant-shrew species emphasized the link between genetic structure and the effects of vicariance, geographical and climatological events. The geographic delimitation of the northern Namaqua and central Fynbos clades within *E. edwardii sensu stricto* corresponded closely to patterns described for other rock dwelling vertebrate species such as the red rock rabbit (*P. rupestris*) and a number of reptile species (*A. atra*, *Goggia* and *Pachydactylus* species) indicating a shared biogeographic history (Chapter 2). Importantly, these rupicolous (rock-dwelling) species have distributions that span the Knersvlakte bioregion which is located on the border of the Fynbos and Succulent Karoo vegetation biomes along the western seaboard of South Africa. Coalescent modelling revealed that these Namaqua and Fynbos populations diverged at ~1.7 MYA. This estimate closely corresponds to major evolutionary changes in other vertebrates that are associated with a period of aridification of the African continent and a series of marine transgressions that inundated the Knersvlakte bioregion presumably leading to the isolation of suitable rocky habitats.

The phylogeographic study highlighted the role of habitat use in shaping the genetic profiles of two elephant-shrew species that are distributed in the interior of south-western Africa. *Macroscelides p. proboscideus* is confined to a more continuous habitat of gravel plains and is characterized by a genetic pattern of isolation-by-distance compared to a structured genetic pattern in *E. rupestris* which prefers a patchier habitat comprising rocky outcrops (Chapter 4). It is further shown that physical barriers to gene flow tend to have more pronounced effects in species with naturally clustered distributions (i.e. *E. rupestris*) compared to species with continuous distributions (i.e. *M. proboscideus*). Whereas the rocky outcrops can be described as partially isolated “islands in an open sea of plains”, increasing agricultural activities (disrupters to gene flow caused by human interference) could impact on the distribution of plain dwelling species resulting in fragmentation with potential conservation consequences. This emphasizes the need for more detailed spatial genetic population studies that can inform future conservation plans.

This study presents an evolutionary phylogeny that includes all the extant elephant-shrew species. The analysis of a topology, based on the multigene supermatrix comprising 3905 bp of mitochondrial and nuclear markers from all recognized Macroscelidea resulted in a tree that was well supported; only the placement of *E. revoili* remains problematic (Chapter 7; Fig. 7.1). Complete taxon representation and the use of multiple phylogenetic markers proved valuable in elucidating evolutionary relationships between taxa and in testing the monophyly of higher-order relationships. Chromosomal, morphological, dental and anatomical characters as well as allozyme data and penis morphology were evaluated for informativeness and mapped to the tree (Chapter 7; Fig. 7.2). These data show, among others, that the grouping of *E. rozeti* and *P. tetradactylus* as sister taxa on sequences is similarly supported by a shared diploid chromosome number

and the morphology of the glans penis (two lateral lobes and a narrowing distal spear-like tip). The multigene results do not support a monophyletic origin for *Elephantulus* but rather suggest that *Petrodromus* and *Macroscelides* should be included in the genus *Elephantulus*. The different gene regions (mitochondrial *vs* nuclear) evolved at different evolutionary rates and the use of a relaxed Bayesian analysis was useful in estimating estimate divergence times within the order. An arid-adapted Macroscelidinae lineage dispersed from east Africa at ~11.5 MYA via the African arid corridor to south-western Africa, and subsequently colonized southern Africa and gave rise to the southern African elephant-shrew species. The timing of speciation within the Rhynchocyoninae at 8 - 10 MYA is coincidental with the diversification of several other forest specialists. In turn, divergence within the Macroscelidinae coincides with three major events of aridification of the African continent.

Karyotype evolution is conserved within elephant-shrews with known diploid numbers ranging from 2n=26-30. The cytogenetic dataset was based on G- and C-banding comparisons, the identification of NORs (nucleolar organizer regions) and the chromosome painting of five smaller sized autosomes and the X chromosomes from six southern African elephant-shrews, including the newly proposed *Elephantulus sp. nov.* (Chapter 3 & Chapter 6). The use of chromosome painting (zoo-FISH) entailed the hybridization of selected *E. edwardii* flow-sorted probes to metaphase chromosomes of *Elephantulus sp. nov., E. intufi, P. tetradactylus, M. proboscideus* and *E. myurus* and confirmed the G-banding results by showing no disruption of synteny in the small autosomes. Therefore their involvement can be excluded from structural changes which account for the difference in diploid number from the 2n=26 species group to the 2n=28 observed in *P. tetradactylus* and the 2n=30 in *E. myurus*. Three chromosomal synapomorphies were identified that support the sister associations suggested by sequence analysis: (i) the derived 2n=28 which supports the grouping of *P. tetradactylus* + *E. rozeti*; (ii) the shared presence of three heterochromatic bands near the distal end of the long arms of the homologues EED 2 and ESN 2 and (iii) the amplification of heterochromatin on the homologous EED 5 and ESN 5 which collectively support the evolutionary relatedness of *E. edwardii* + *Elephantulus sp. nov.* (Chapter 7; Table 7.6 & Fig. 7.2). These findings clearly indicate that in spite of the macroscelids relatively conserved karyotypic evolution, these data are extremely useful for substantiating phylogenetic associations suggested by nucleotide sequences. Cytogenetic data are currently missing for a number of *Elephantulus* species (Macroscelidinae) and the Rhynchocyoninae and future studies that include these species may be crucial in testing the monophyly of *Elephantulus*.

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APPENDIX

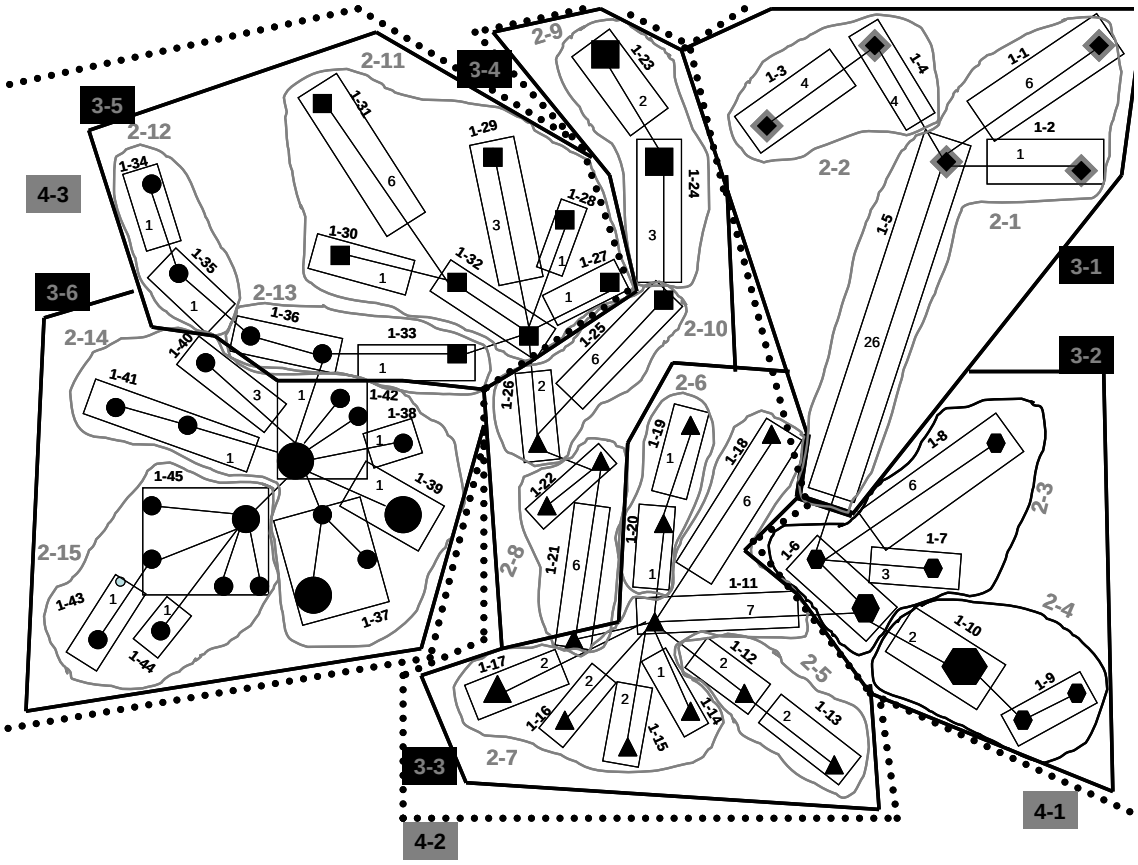

Figure 1 Mitochondrial DNA haplotype tree (for 1389 bp) constructed by hand with the associated nested design used for the statistical analyses and inferred using the latest inferece key. Haplotypes are shape coded and representative of the five depicted evolutionary lineages (see Fig. 2.1 & 2.3). The northern Namaqua clade is randomly connected to the central Fynbos clade since it falls outside the parsimony 95% confidence interval of 16 mutational steps. Intermediate haplotypes are indicated by number; 1-step clades (narrow black lines); 2-step clades (grey free-hand circles); 3-step clades (broad black lines) and 4-step clades (broad black dotted lines).

Table 1 Additional information on the voucher material analysed in the combined dataset. Museum locations, geographic localities and GenBank accession numbers are shown. (Transvaal Museum = TM and Iziko Museum = IM).

Voucher nr	South African Museum	Locality	GenBank accession nr	
			cytochrome *b*	control region
TM2312 - type	TM	Klawer, Van Rhynsdorp	*	DQ901249
TM 688 - type	TM	Deelfontein, Richmond	*	DQ901238
TM27279	TM	Prieska	DQ901016	DQ901092
TM41922	TM	Calvinia	DQ901019	DQ901095
TM2313	TM	Klawer, Van Rhynsdorp	DQ901020	DQ901096
TM41921	TM	Calvinia	DQ901021	DQ901097
TM4737	TM	Klawer, Van Rhynsdorp	DQ901022	DQ901098
TM5123	TM	Klawer, Van Rhynsdorp	DQ901023	DQ901100
TM5125	TM	Klawer, Van Rhynsdorp	DQ901024	DQ901102
TM40757	TM	Vredenburg	DQ901037	DQ901117
TM41440	TM	Hopefield	DQ901038	DQ901118
TM41441	TM	Hopefield	DQ901039	DQ901119
TM41442	TM	Hopefield	DQ901040	DQ901120
TM38136	TM	Strand	DQ901047	DQ901129
TM38137	TM	Strand	DQ901048	DQ901130
TM38138	TM	Strand	DQ901049	DQ901131
GMNR/M/127	IM	Gamkaberg, Calitzdorp	DQ901062	DQ901146
GMNR/M/181	IM	Gamkaberg, Calitzdorp	DQ901063	DQ901148
SC40	IM	Cherrydouw, Oudtshoorn	DQ901064	DQ901149
SC84	IM	Cherrydouw, Oudtshoorn	DQ901065	DQ901152
GMNR/M76	IM	Gamkaberg, Calitzdorp	DQ901066	DQ901153
GMNR/M96	IM	Gamkaberg, Calitzdorp	DQ901067	DQ901154
SC10	IM	Cherrydouw, Oudtshoorn	DQ901068	DQ901155
SC40	IM	Cherrydouw, Oudtshoorn	DQ901069	DQ901156
SC105	IM	Cherrydouw, Oudtshoorn	DQ901070	DQ901157
SC109	IM	Cherrydouw, Oudtshoorn	DQ901071	DQ901158
TOWE/M/22	IM	Besemfontein, Ladismith	DQ901072	DQ901162
TOWE/M/31	IM	Besemfontein, Ladismith	DQ901073	DQ901163
TOWE/M/46	IM	Besemfontein, Ladismith	DQ901075	DQ901164
OUTE/M/401	IM	Doringrivier, Oudtshoorn	DQ901076	DQ901165
OUTE/M/405	IM	Doringrivier, Oudtshoorn	DQ901077	DQ901166
OUTE/M/415	IM	Doringrivier, Oudtshoorn	DQ901078	DQ901167
OUTE/M/419	IM	Doringrivier, Oudtshoorn	DQ901079	DQ901168
OUTE/M/426	IM	Doringrivier, Oudtshoorn	DQ901080	DQ901169
M/501	IM	Ou Tol, Oudtshoorn	DQ901081	DQ901171
TM32032	TM	Mossel Bay	DQ901082	DQ901172
TM32036	TM	Mossel Bay	DQ901083	DQ901173

Voucher nr	South African Museum	Locality	GenBank accession nr	
			cytochrome *b*	control region
TM32058	TM	Mossel Bay	DQ901084	DQ901174
TM29678	TM	Uniondale	DQ901085	DQ901177
TM29682	TM	Uniondale	DQ901086	DQ901178
TM29692	TM	Uniondale	DQ901087	DQ901179
TM29699	TM	Uniondale	DQ901088	DQ901180
TM29700	TM	Uniondale	DQ901089	DQ901181
TM29708	TM	Uniondale	DQ901090	DQ901182
TM30785	TM	Uniondale	DQ901091	DQ901183
TM29496	TM	Beaufort-West	DQ901212	DQ901250
TM29497	TM	Beaufort-West	DQ901213	DQ901251
TM29498	TM	Beaufort-West	DQ901214	DQ901252
TM29528	TM	Beaufort-West	DQ901215	DQ901253
TM29529	TM	Beaufort-West	DQ901216	DQ901254
TM27303	TM	Williston	DQ901217	DQ901255
TM27304	TM	Williston	DQ901218	DQ901256

* not sequenced

Table 2 Interpretation of the results of appendix Fig. 1 using the latest Inference Key (http://darwin.uvigo.es/software/geodis.html). Only those clades that resulted in a rejection of the null hypothesis of panmixia are included in this table.

Clade	Chi-square probability	Chain of inference	Demographic event inferred
3-4	p = 0.015	1No; 11No; 17Yes; 4No	Restricted gene flow with Isolation by distance
3-5	p = 0.011	1No; 2Yes; 3Yes; 5No; 6No; 7Yes	Restricted gene flow and/or long distance dispersal
4-1	p < 0.001	1Yes; 19Yes; 20Yes; 2Yes; 3Yes; 15No	Past fragmentation
4-2	p = 0.012	1No; 2Tip/Interior status cannot be determined	Inconclusive outcome
4-3	p < 0.001	1No; 2Yes; 3Yes; 5No; 6No; 7Yes	Restricted gene flow and/or long distance dispersal

Table 3 Specimens included in the dissertation. Stellenbosch University (US) and museum voucher numbers, geographic localities and coordinates as well as GenBank accession numbers are provided (Museum abbreviations: CAS = California Academy of Sciences, USA; CN = Cape Nature, South Africa; DM = Durban Museum, South Africa; IM = Iziko Museum, South Africa; MMK/M = McGregor Museum, South Africa; NHM = Natural History Museum, England; NMB = National Museum Bloemfontein, South Africa and TM = Transvaal Museum, South Africa); (Country abbreviations: Namibia = Nam and SA = South Africa); (South African province abbreviations: EC = Eastern Cape; KZN = Kwa-Zulu Natal; MP = Mpumalanga; NC = Northern Cape; NW =Northwest and WC = Western Cape).

Species	US	Museum voucher	Mu-seum	Locality	Coun-try	Grid ref	GenBank accession nr.					
							cytochrome *b*	control region	Fibrino-gen 7	12S rRNA, val tRNA, 16S rRNA	IRBP exon 1	VWF exon 28
R. chrysopygus	HS462	1928.12.7.2	NHM	Sokoke Forest, East Africa	Kenya					EU136152		
R. petersi	HS463	1975.857	NHM	Kivumoni Forest, Kwale	Kenya					EU136153		
R. cirnei	HS464	1897.10.1.26	NHM	Fort Hill, N. Nyasaland	Malawi					EU136154		
M. proboscideus	HS51	NMB12599	NMB	Steytlerville, EC	SA	33.30S 24.30E	EF141821	EF141755				
M. proboscideus	HS52	NMB12596	NMB	Steytlerville, EC	SA	33.30S 24.30E	EF141822	EF141756				
M. proboscideus	HS53			Tankwa NP, NC	SA	32.02S 20.05E	EF141808	EF141742				
M. proboscideus	HS87			Victoria-West, NC	SA	31.30S 23.00E	EF141809	EF141743				
M. proboscideus	HS130			Goegap NR, NC	SA	29.22S 17.50E	EF141782	EF141716				
M. proboscideus	HS131			Goegap NR, NC	SA	29.22S 17.50E	EF141783	EF141717				
M. proboscideus	HS132			Goegap NR, NC	SA	29.22S 17.50E	EF141784	EF141718				
M. proboscideus	HS133			Goegap NR, NC	SA	29.22S 17.50E	EF141785	EF141719				
M. proboscideus	HS134			Goegap NR, NC	SA	29.22S 17.50E	EF141786	EF141720				
M. proboscideus	HS135			Goegap NR, NC	SA	29.22S 17.50E	EF141787	EF141721				
M. proboscideus	HS136			Goegap NR, NC	SA	29.22S 17.50E	EF141788					
M. proboscideus	HS141			Paulshoek, Kamieskroon, NC	SA	30.38S 18.28E		EF141722				
M. proboscideus	HS142			Paulshoek, Kamieskroon, NC	SA	30.38S 18.28E	EF141789	EF141723				
M. proboscideus	HS143			Paulshoek, Kamieskroon, NC	SA	30.38S 18.28E	EF141790	EF141724				
M. proboscideus	HS145			Paulshoek, Kamieskroon, NC	SA	30.38S 18.28E	EF141791	EF141725		EU136155		
M. proboscideus	HS167			Paulshoek, Kamieskroon, NC	SA	30.38S 18.28E	EF141792	EF141726				
M. proboscideus	HS170			Paulshoek, Kamieskroon, NC	SA	30.38S 18.28E	EF141793	EF141727				
M. proboscideus	HS312	TM4988	TM	Vredendal, WC	SA	31.65S 18.50E	EF141804	EF141738				
M. proboscideus	HS314	TM5127	TM	Klawer, Van Rhynsdorp, WC	SA	31.37S 18.43E	EF141806	EF141740				
M. proboscideus	HS315	TM5129	TM	Klawer, Van Rhynsdorp, WC	SA	31.37S 18.43E	EF141807	EF141741				
M. proboscideus	HS318	TM7390	TM	Vredendal, WC	SA	31.65S 18.50E	EF141805	EF141739				

Table 3 continued

Species	US	Museum voucher	Museum	Locality	Country	Grid ref	GenBank accession nr.					
							cytochrome *b*	control region	Fibrinogen 7	12S rRNA, val tRNA, 16S rRNA	IRBP exon 1	VWF exon 28
M. proboscideus	HS319	TM8257	TM	Brospan, Carnarvon, WC	SA	30.90S 22.10E	EF141799	EF141733				
M. proboscideus	HS320	TM8259	TM	Brandvlei/Williston, NC	SA	30.25S 20.25E	EF141798	EF141732				
M. proboscideus	HS323	TM8264	TM	Komaggas, NC	SA	29.80S 17.45E	EF141774	EF141708				
M. proboscideus	HS327	TM9011	TM	Laingsburg, WC	SA	33.25S 20.80E	EF141815	EF141749				
M. proboscideus	HS328	TM9012	TM	Laingsburg, WC	SA	33.25S 20.80E	EF141816	EF141750				
M. proboscideus	HS332	TM10958	TM	Rietfontein, Kenhardt, NC	SA	29.40S 21.15E	EF141795	EF141729				
M. proboscideus	HS334	TM12907	TM	Rietfontein, Kenhardt, NC	SA	29.40S 21.15E	EF141800	EF141734				
M. proboscideus	HS335	TM12908	TM	Carnarvon, NC	SA	30.90S 22.10E	EF141801	EF141735				
M. proboscideus	HS336	TM16421	TM	Carnarvon, NC	SA	30.90S 22.10E	EF141778	EF141712				
M. proboscideus	HS337	TM16422	TM	Twee Rivieren, NW	SA	26.45S 20.60E	EF141779	EF141713				
M. proboscideus	HS338	TM27397	TM	Williston, NC	SA	31.20S 20.80E	EF141802	EF141736				
M. proboscideus	HS339	TM27398	TM	Williston, NC	SA	31.20S 20.80E	EF141803	EF141737				
M. proboscideus	HS340	TM29596	TM	Beaufort-West, WC	SA	32.40S 22.60E	EF141817	EF141751				
M. proboscideus	HS341	TM29597	TM	Beaufort-West, WC	SA	32.40S 22.60E	EF141818	EF141752				
M. proboscideus	HS343	TM29608	TM	Beaufort-West, WC	SA	32.40S 22.60E	EF141819	EF141753				
M. proboscideus	HS344	TM30799	TM	Aberdeen, EC	SA	32.45S 24.10E	EF141820	EF141754				
M. proboscideus	HS346	TM32460	TM	Kenhardt, NC	SA	29.40S 21.15E	EF141796	EF141730				
M. proboscideus	HS347	TM32461	TM	Kenhardt, NC	SA	29.40S 21.15E	EF141797	EF141731				
M. proboscideus	HS348	TM39303	TM	Augrabies, NC	SA	28.60S 20.30E	EF141780	EF141714				
M. proboscideus	HS349	TM39330	TM	Augrabies, NC	SA	28.60S 20.30E	EF141781	EF141715				
M. proboscideus	HS350	TM39355	TM	Tankwa NP, NC	SA	32.02S 20.05E	EF141810	EF141744				
M. proboscideus	HS352	TM39362	TM	Tankwa NP, NC	SA	32.02S 20.05E	EF141811	EF141745				
M. proboscideus	HS353	TM39363	TM	Tankwa NP, NC	SA	32.02S 20.05E	EF141812	EF141746				
M. proboscideus	HS354	TM39372	TM	Tankwa NP, NC	SA	32.02S 20.05E	EF141813	EF141747				
M. proboscideus	HS355	TM39373	TM	Tankwa NP, NC	SA	32.02S 20.05E	EF141814	EF141748				
M. proboscideus	HS356	TM43640	TM	Richtersveld, NC	SA	28.80S 17.30E	EF141775	EF141709				
M. proboscideus	HS357	TM43642	TM	Richtersveld, NC	SA	28.80S 17.30E	EF141776	EF141710				
M. proboscideus	HS358	TM43643	TM	Richtersveld, NC	SA	28.80S 17.30E	EF141777	EF141711				
M. proboscideus	HS360	TM32634	TM	Oranjemund, NC	SA	28.60S 16.40E	EF141773	EF141707				

Table 3 continued

Species	US	Museum voucher	Museum	Locality	Country	Grid ref	GenBank accession nr.					
							cytochrome *b*	control region	Fibrinogen 7	12S rRNA, val tRNA, 16S rRNA	IRBP exon 1	VWF exon 28
M. proboscideus	HS401	TM360	TM	Keetmanshoop	Nam	26.60S 18.10E	EF141772	EF141706				
M. proboscideus	HS403	TM10213	TM	Okahandja	Nam	21.95S 16.95E	EF141767	EF141701				
M. proboscideus	HS405	TM28898	TM	Ganab, Namib Park	Nam	23.20S 15.70E	EF141764	EF141698				
M. proboscideus	HS406	TM28899	TM	Ganab, Namib Park	Nam	23.20S 15.70E	EF141765	EF141699				
M. proboscideus	HS407	TM28906	TM	Ganab, Namib Park	Nam	23.20S 15.70E	EF141766	EF141700				
M. proboscideus	HS409	TM32693	TM	Bethanie	Nam	26.50S 17.20E	EF141771	EF141705				
M. proboscideus	HS410	TM37607	TM	Maltahohe	Nam	24.80S 17.00E	EF141769	EF141703				
M. proboscideus	HS411	TM37625	TM	Maltahohe	Nam	24.80S 17.00E	EF141770	EF141704				
M. proboscideus	HS423	TM2358	TM	Cradock, EC	SA	32.20S 25.60E		EF141757				
M. proboscideus	HS424	TM5128	TM	Van Rhynsdorp, WC	SA	31.37S 18.43E		EF141758				
M. proboscideus	HS425	TM6924	TM	Upington, NC	SA	28.40S 21.30E		EF141762				
M. proboscideus	HS426	TM8255	TM	Aus	Nam	26.60S 16.20E		EF141763				
M. proboscideus	HS427	TM8256	TM	Brandvlei/Van Wyksvlei, NC	SA	30.25S 20.25E		EF141761				
M. proboscideus	HS428	TM8258	TM	Brandvlei, NC	SA	30.25S 20.25E		EF141760				
M. proboscideus	HS429	TM8262	TM	Calvinia, NC	SA	31.25S 19.71E		EF141759				
M. proboscideus	HS430	TM10499	TM	North of Omaruru river	Nam	23.20S 15.70E		EF141697				
P. tetradactylus	HS85	NMB12603	NMB	Bonamanzi Game Park, KZN	SA	28.00S 31.10E	DQ901190	DQ901226		EU136156	EU136145	EU136138
E. brachyrhynchus	HS88	NMB12604	NMB	Spitskop, MP	SA	24.82S 30.12E	DQ901191	DQ901227		EU136160	EU136146	EU136139
E. brachyrhynchus	HS89	NMB12605	NMB	Spitskop, MP	SA	24.82S 30.12E	DQ901192	DQ901228				
E. brachyrhynchus	HS138	NMB12606	NMB	Kgaswane Nature Reserve, NW	SA	25.70S 27.18E	DQ901193	DQ901229		EU136161	EU136147	
E. edwardii	HS5			Namaqua NP, NC	SA	29.42S 17.55E	DQ901017	DQ901093	EU076267			
E. edwardii	HS7			West Coast NP, NC	SA	33.08S 18.06E	DQ901036	DQ901116				
E. edwardii	HS11			Cederberg, WC	SA	32.23S 19.05E	DQ901032	DQ901112				
E. edwardii	HS12			Cederberg, WC	SA	32.23S 19.05E	DQ901033	DQ901113				
E. edwardii	HS13			Algeria, Cederberg, WC	SA	32.23S 19.05E	DQ901034	DQ901114				
E. edwardii	HS14			Algeria, Cederberg, WC	SA	32.23S 19.05E	DQ901035	DQ901115				
E. edwardii	HS18			Jonaskop, Villiersdorp, WC	SA	33.59S 19.14E	DQ901052	DQ901136				

Table 3 continued

Species	US	Museum voucher	Museum	Locality	Country	Grid ref	GenBank accession nr.					
							cytochrome *b*	control region	Fibrinogen 7	12S rRNA, val tRNA, 16S rRNA	IRBP exon 1	VWF exon 28
E. edwardii	HS20			Elandsberg, Wellington, WC	SA	32.80S 18.50E	DQ901042	DQ901124				
E. edwardii	HS21			Elandsberg, Wellington, WC	SA	32.80S 18.50E	DQ901043	DQ901125	EU076271			
E. edwardii	HS22			Travellers Rest, Clanwilliam, WC	SA	32.10S 18.49E	DQ901025	DQ901103				
E. edwardii	HS23			Travellers Rest, Clanwilliam, WC	SA	32.10S 18.49E		DQ901104				
E. edwardii	HS24			Travellers Rest, Clanwilliam, WC	SA	32.10S 18.49E	DQ901026	DQ901105				
E. edwardii	HS25			Travellers Rest, Clanwilliam, WC	SA	32.10S 18.49E	DQ901027	DQ901106				
E. edwardii	HS26			Travellers Rest, Clanwilliam, WC	SA	32.10S 18.49E		DQ901107				
E. edwardii	HS27			Travellers Rest, Clanwilliam, WC	SA	32.10S 18.49E	DQ901028	DQ901108				
E. edwardii	HS28			Travellers Rest, Clanwilliam, WC	SA	32.10S 18.49E	DQ901029	DQ901109				
E. edwardii	HS29			Travellers Rest, Clanwilliam, WC	SA	32.10S 18.49E	DQ901030	DQ901110				
E. edwardii	HS30			Travellers Rest, Clanwilliam, WC	SA	32.10S 18.49E	DQ901031	DQ901111				
E. edwardii	HS32	NMB12588		Fairfield, Napier, WC	SA	33.49S 18.29E	DQ901055	DQ901139				
E. edwardii	HS33	NMB12589		Fairfield, Napier, WC	SA	33.49S 18.29E	DQ901056	DQ901140				
E. edwardii	HS34	NMB12590		Fairfield, Napier, WC	SA	33.49S 18.29E	DQ901057	DQ901141				
E. edwardii	HS35	NMB12591		Fairfield, Napier, WC	SA	33.49S 18.29E	DQ901058	DQ901142	EU076268	EU136166	EU136144	
E. edwardii	HS36	NMB12592		Fairfield, Napier, WC	SA	33.49S 18.29E	DQ901059	DQ901143	EU076269			
E. edwardii	HS37	NMB12593		Fairfield, Napier, WC	SA	33.49S 18.29E	DQ901060	DQ901144				
E. edwardii	HS38	NMB12594		Fairfield, Napier, WC	SA	33.49S 18.29E	DQ901061	DQ901145	EU076270			
E. edwardii	HS44	GMNR/M/165	IM	Gamkaberg, Calitzdorp, WC	SA	33.25S 21.55E	DQ901062	DQ901146				
E. edwardii	HS45	GMNR/M/171		Gamkaberg, Calitzdorp, WC	SA	33.25S 21.55E		DQ901147				

Table 3 continued

Species	US	Museum voucher	Museum	Locality	Country	Grid ref	GenBank accession nr.					
							cytochrome *b*	control region	Fibrinogen 7	12S rRNA, val tRNA, 16S rRNA	IRBP exon 1	VWF exon 28
E. edwardii	HS46	GMNR/M/181	IM	Gamkaberg, Calitzdorp, WC	SA	33.25S 21.55E	DQ901063	DQ901148				
E. edwardii	HS49	NMB12598	NMB	Villiersdorp, WC	SA	33.59S 19.14E	DQ901053	DQ901137				
E. edwardii	HS50			Villiersdorp, WC	SA	33.59S 19.14E	DQ901054	DQ901138				
E. edwardii	HS54			Victoria-West, NC	SA	31.30S 23.00E	DQ901041	DQ901122				
E. edwardii	HS55	SC40	IM	Cherrydouw, Oudtshoorn, WC	SA	33.25S 22.50E	DQ901064	DQ901149				
E. edwardii	HS57	SC50	IM	Cherrydouw, Oudtshoorn, WC	SA	33.25S 22.50E		DQ901150				
E. edwardii	HS59	SC59	IM	Cherrydouw, Oudtshoorn, WC	SA	33.25S 22.50E		DQ901151				
E. edwardii	HS60	SC84	IM	Cherrydouw, Oudtshoorn, WC	SA	33.25S 22.50E	DQ901065	DQ901152				
E. edwardii	HS61	GMNR/M76	IM	Gamkaberg, Calitzdorp, WC	SA	33.25S 21.55E	DQ901066	DQ901153				
E. edwardii	HS62	GMNR/M96	IM	Gamkaberg, Calitzdorp, WC	SA	33.25S 21.55E	DQ901067	DQ901154				
E. edwardii	HS65	SC10	IM	Cherrydouw, Oudtshoorn, WC	SA	33.25S 22.50E	DQ901068	DQ901155				
E. edwardii	HS66	SC40	IM	Cherrydouw, Oudtshoorn, WC	SA	33.25S 22.50E	DQ901069	DQ901156				
E. edwardii	HS67	SC105	IM	Cherrydouw, Oudtshoorn, WC	SA	33.25S 22.50E	DQ901070	DQ901157				
E. edwardii	HS68	SC109	IM	Cherrydouw, Oudtshoorn, WC	SA	33.25S 22.50E	DQ901071	DQ901158				
E. edwardii	HS69	TOWE/M/34	IM	Besemfontein, Ladismith, WC	SA	33.23S 20.27E		DQ901159				
E. edwardii	HS70	TOWE/M/35	IM	Besemfontein, Ladismith, WC	SA	33.23S 20.27E		DQ901160				
E. edwardii	HS71	TOWE/M/37	IM	Besemfontein, Ladismith, WC	SA	33.23S 20.27E		DQ901161				
E. edwardii	HS72	TOWE/M/22	IM	Besemfontein, Ladismith, WC	SA	33.23S 20.27E	DQ901072	DQ901162				
E. edwardii	HS73	TOWE/M/31	IM	Besemfontein, Ladismith, WC	SA	33.23S 20.27E	DQ901073	DQ901163				
E. edwardii	HS75	TOWE/M/46	IM	Besemfontein, Ladismith, WC	SA	33.23S 20.27E	DQ901075	DQ901164				
E. edwardii	HS76	OUTE/M/401	IM	Doringrivier, Oudtshoorn, WC	SA	33.15S 22.10E	DQ901076	DQ901165				
E. edwardii	HS77	OUTE/M/405	IM	Doringrivier, Oudtshoorn, WC	SA	33.15S 22.10E	DQ901077	DQ901166				
E. edwardii	HS78	OUTE/M/415	IM	Doringrivier, Oudtshoorn, WC	SA	33.15S 22.10E	DQ901078	DQ901167				
E. edwardii	HS80	OUTE/M/426	IM	Doringrivier, Oudtshoorn, WC	SA	33.15S 22.10E	DQ901080	DQ901169				
E. edwardii	HS81			Elandsberg, Wellington, WC	SA	32.80S 18.50E	DQ901044	DQ901126	EU076272			
E. edwardii	HS82			Elandsberg, Wellington, WC	SA	32.80S 18.50E	DQ901045	DQ901127	EU076273			
E. edwardii	HS83	NMB12601	NMB	Mizpah, Grabouw, WC	SA	33.49S 18.40E		DQ901132				

Table 3 continued

Species	US	Museum voucher	Museum	Locality	Country	Grid ref	GenBank accession nr.					
							cytochrome *b*	control region	Fibrinogen 7	12S rRNA, val tRNA, 16S rRNA	IRBP exon 1	VWF exon 28
E. edwardii	HS84	NMB12602	NMB	Mizpah, Grabouw, WC	SA	33.49S 18.40E	DQ901050	DQ901133				
E. edwardii	HS86	M/570	CN	Ou Tol, Oudtshoorn, WC	SA	33.30S 22.20E		DQ901170				
E. edwardii	HS90			Mizpah, Grabouw, WC	SA	33.49S 18.40E	DQ901051	DQ901134				
E. edwardii	HS91	M/501	CN	Ou Tol, Oudtshoorn, WC	SA	33.30S 22.20E	DQ901081	DQ901171				
E. edwardii	HS116			Paulshoek, Kamieskroon, NC	SA	30.38S 18.28E	DQ901018	DQ901094				
E. edwardii	HS213	TM2313	TM	Klawer, Van Rhynsdorp, WC	SA	31.37S 18.43E	DQ901020	DQ901096				
E. edwardii	HS215	TM4737	TM	Klawer, Van Rhynsdorp, WC	SA	31.37S 18.43E	DQ901022	DQ901098				
E. edwardii	HS216	TM5122	TM	Klawer, Van Rhynsdorp, WC	SA	31.37S 18.43E		DQ901099				
E. edwardii	HS217	TM5123	TM	Klawer, Van Rhynsdorp, WC	SA	31.37S 18.43E	DQ901023	DQ901100				
E. edwardii	HS218	TM5124	TM	Klawer, Van Rhynsdorp, WC	SA	31.37S 18.43E		DQ901101				
E. edwardii	HS219	TM5125	TM	Klawer, Van Rhynsdorp, WC	SA	31.37S 18.43E	DQ901024	DQ901102				
E. edwardii	HS242	TM29678	TM	Uniondale, EC	SA	33.40S 23.20E	DQ901085	DQ901177				
E. edwardii	HS243	TM29682	TM	Uniondale, EC	SA	33.40S 23.20E	DQ901086	DQ901178				
E. edwardii	HS244	TM29692	TM	Uniondale, EC	SA	33.40S 23.20E	DQ901087	DQ901179				
E. edwardii	HS245	TM29699	TM	Uniondale, EC	SA	33.40S 23.20E	DQ901088	DQ901180				
E. edwardii	HS246	TM29700	TM	Uniondale, EC	SA	33.40S 23.20E	DQ901089	DQ901181				
E. edwardii	HS247	TM29708	TM	Uniondale, EC	SA	33.40S 23.20E	DQ901090	DQ901182				
E. edwardii	HS248	TM30785	TM	Uniondale, EC	SA	33.40S 23.20E	DQ901091	DQ901183				
E. edwardii	HS253	TM32032	TM	Mossel Bay, WC	SA	34.20S 22.15E	DQ901082	DQ901172				
E. edwardii	HS254	TM32036	TM	Mossel Bay, WC	SA	34.20S 22.15E	DQ901083	DQ901173				
E. edwardii	HS255	TM32058	TM	Mossel Bay, WC	SA	34.20S 22.15E	DQ901084	DQ901174				
E. edwardii	HS256	TM38136	TM	Strand, WC	SA	34.10S 18.55E	DQ901047	DQ901129				
E. edwardii	HS257	TM38137	TM	Strand, WC	SA	34.10S 18.55E	DQ901048	DQ901130				
E. edwardii	HS260	TM40757	TM	Vredenburg, WC	SA	32.54S 18.00E	DQ901037	DQ901117				
E. edwardii	HS261	TM41440	TM	Hopefield, WC	SA	33.02S 18.21E	DQ901038	DQ901118				
E. edwardii	HS262	TM41441	TM	Hopefield, WC	SA	33.02S 18.21E	DQ901039	DQ901119				
E. edwardii	HS263	TM41442	TM	Hopefield, WC	SA	33.02S 18.21E	DQ901040	DQ901120				

Table 3 continued

Species	US	Museum voucher	Museum	Locality	Country	Grid ref	GenBank accession nr.					
							cytochrome *b*	control region	Fibrinogen 7	12S rRNA, val tRNA, 16S rRNA	IRBP exon 1	VWF exon 28
E. edwardii	HS264	TM41463	TM	Hopefield, WC	SA	33.02S 18.21E		DQ901121				
E. edwardii	HS265	DM4165	DM	Nantes Dam, Paarl	SA	33.42S 19.02E	DQ901046	DQ901128				
E. edwardii	HS273			Cherrydouw, Oudtshoorn, WC	SA	33.25S 22.50E		DQ901135				
E. edwardii	HS293	TM27279	TM	Prieska, NC	SA	29.65S 22.70E	DQ901016	DQ901092				
E. edwardii	HS306	TM41921	TM	Calvinia, NC	SA	31.25S 19.71E	DQ901021	DQ901097				
E. edwardii	HS307	TM41922	TM	Calvinia, NC	SA	31.25S 19.71E	DQ901019	DQ901095				
E. edwardii	HS363	NGP/MR3	IM	Matjiesrivier, Cederberg, WC	SA	32.23S 19.05E		DQ901123				
E. edwardii	HS445	MMK/M/7308	MMK	Kraaifontein,Nieuwoudtville, WC	SA	31.20S 19.06E						
E. edwardii	HS446	MMK/M/7309	MMK	Kliprivier, Nieuwoudtville, WC	SA	31.19S 19.07E						
E. edwardii	HS447	MMK/M/7310	MMK	Kliprivier, Nieuwoudtville, WC	SA	31.19S 19.07E						
E. edwardii	HS448	MMK/M/7311	MMK	Papkuilsfontein,Nieuwoudtville, WC	SA	31.33S 19.09E						
E. edwardii	HS449	MMK/M/7312	MMK	Kraaifontein,Nieuwoudtville, WC	SA	31.20S 19.06E						
E. edwardii	HS450	MMK/M/7313	MMK	Kraaifontein,Nieuwoudtville, WC	SA	31.20S 19.06E						
E. fuscipes	HS459	1935.3.22.1	NHM	Awach, Paicho Country, Gulu	Uganda					EU136157		
E. fuscus	HS461	1907.1.11.11	NHM	Mterize River, E. Loangwa	Zambia					EU136158		
E. intufi	HS40	NMB12595	NMB	Molopo Nature Reserve, NW	SA	25.78S 22.90E	DQ901202	DQ901239				
E. intufi	HS122			Southern Namibia	Nam	26.38S 17.98E	DQ901203	DQ901240		EU136162	EU136148	EU136140
E. intufi	HS123			Southern Namibia	Nam	26.38S 17.98E	DQ901204	DQ901241				
E. intufi	HS124			Otjiwarongo	Nam	21.58S 16.93E	DQ901205	DQ901242				
E. intufi	HS125			Otjiwarongo	Nam	21.58S 16.93E	DQ901206	DQ901243				
E. myurus	HS41	NMB12596	NMB	Kgaswane Nature Reserve, NW	SA	25.70S 27.18E	DQ901207	DQ901244	EU076260			
E. myurus	HS42	NMB12597	NMB	Kgaswane Nature Reserve, NW	SA	25.70S 27.18E	DQ901208	DQ901245	EU076261			
E. myurus	HS157			Lapalala Game Reserve, Limpopo	SA	23.80S 28.25E			EU076262			
E. myurus	HS158			Hopetown, NC	SA	29.50S 24.13E	DQ901209	DQ901246	EU076263	EU136165	EU136151	EU136143

Table 3 continued

Species	US	Museum voucher	Museum	Locality	Country	Grid ref	GenBank accession nr.					
							cytochrome *b*	control region	Fibrinogen 7	12S rRNA, val tRNA, 16S rRNA	IRBP exon 1	VWF exon 28
E. myurus	HS159			Vryburg, NW	SA	27.00S 24.71E	DQ901210	DQ901247	EU076264			
E. myurus	HS160			Vryburg, NW	SA	27.00S 24.71E	DQ901211	DQ901248				
E. myurus	HS195	NMB12607	NMB	Ellisras, Limpopo	SA	23.50S 27.50E			EU076265			
E. myurus	HS196	NMB12608	NMB	Ellisras, Limpopo	SA	23.50S 27.50E			EU076266			
E. revoili	HS460	1897.8.9.6	NHM	Somalia	Somalia					EU136159		
E. rupestris	HS1			Namaqua NP, NC	SA	29.42S 17.55E	EF141664	EF141594		EU136163	EU136149	EU136141
E. rupestris	HS2			Namaqua NP, NC	SA	29.42S 17.55E	EF141665	EF141595				
E. rupestris	HS3			Namaqua NP, NC	SA	29.42S 17.55E	EF141666	EF141596				
E. rupestris	HS4			Namaqua NP, NC	SA	29.42S 17.55E	EF141667	EF141597				
E. rupestris	HS6			Namaqua NP, NC	SA	29.42S 17.55E	EF141668	EF141598				
E. rupestris	HS56	SC42	IM	Cherrydouw, Oudtshoorn, WC	SA	33.25S 22.50E	EF141684	EF141614				
E. rupestris	HS63			Windhoek	Nam	22.50S 17.10E	EF141636	EF141566		EU136164	EU136150	EU136142
E. rupestris	HS112			Paulshoek, Kamieskroon, NC	SA	30.25S 17.90E	EF141669	EF141599				
E. rupestris	HS113			Paulshoek, Kamieskroon, NC	SA	30.25S 17.90E	EF141670	EF141600				
E. rupestris	HS114			Paulshoek, Kamieskroon, NC	SA	30.25S 17.90E	EF141671	EF141601	EU076252			
E. rupestris	HS115			Paulshoek, Kamieskroon, NC	SA	30.25S 17.90E	EF141672	EF141602				
E. rupestris	HS117			Paulshoek, Kamieskroon, NC	SA	30.25S 17.90E	EF141673	EF141603	EU076253			
E. rupestris	HS118			Paulshoek, Kamieskroon, NC	SA	30.25S 17.90E	EF141674	EF141604				
E. rupestris	HS120			Paulshoek, Kamieskroon, NC	SA	30.25S 17.90E	EF141675	EF141605	EU076254			
E. rupestris	HS129			Goegap NR, NC	SA	29.22S 17.50E	EF141662	EF141592	EU076255			
E. rupestris	HS137			Goegap NR, NC	SA	29.22S 17.50E	EF141663	EF141593	EU076256			
E. rupestris	HS156			Oudtshoorn, WC	SA	33.30S 22.20E	EF141686	EF141616	EU076257			
E. rupestris	HS161			Springbok, NC	SA	29.42S 17.55E	EF141659	EF141589				
E. rupestris	HS162			Springbok, NC	SA	29.42S 17.55E	EF141660	EF141590	EU076258			
E. rupestris	HS163			Springbok, NC	SA	29.42S 17.55E	EF141661	EF141591	EU076259			
E. rupestris	HS266	GMNR/M/150	IM	Gamkaberg, Calitzdorp, WC	SA	33.25S 21.55E	EF141687	EF141617				
E. rupestris	HS267	GMNR/M/173	IM	Gamkaberg, Calitzdorp, WC	SA	33.25S 21.55E	EF141688	EF141618				

Table 3 continued

Species	US	Museum voucher	Museum	Locality	Country	Grid ref	GenBank accession nr.					
							cytochrome *b*	control region	Fibrinogen 7	12S rRNA, val tRNA, 16S rRNA	IRBP exon 1	VWF exon 28
E. rupestris	HS268	GMNR/M/161	IM	Elandskloof, Calitzdorp, WC	SA	33.30S 21.30E	EF141689	EF141619				
E. rupestris	HS269	GMNR/M/169	IM	Elandskloof, Calitzdorp, WC	SA	33.30S 21.30E	EF141690	EF141620				
E. rupestris	HS275	TM2360	TM	Cradock, EC	SA	32.20S 25.60E	EF141695	EF141625				
E. rupestris	HS276	TM5121	TM	Port Elizabeth, EC	SA	33.90S 25.60E	EF141691	EF141621				
E. rupestris	HS277	TM6665	TM	Albany, Grahamstown, EC	SA	33.30S 26.60E	EF141692	EF141622				
E. rupestris	HS278	TM6666	TM	Albany, Grahamstown, EC	SA	33.30S 26.60E	EF141693	EF141623				
E. rupestris	HS279	TM6667	TM	Albany, Grahamstown, EC	SA	33.30S 26.60E	EF141694	EF141624				
E. rupestris	HS282	TM8278	TM	Albany, Grahamstown, EC	SA	33.30S 26.60E	EF141655	EF141585				
E. rupestris	HS283	TM8280	TM	Komaggas, NC	SA	29.80S 17.45E	EF141677	EF141607				
E. rupestris	HS284	TM94320	TM	Van Wyksvlei/Carnarvon, NC	SA	30.90S 22.10E	EF141654	EF141584				
E. rupestris	HS290	TM15508	TM	Nababeep, NC	SA	29.60S 17.80E	EF141656	EF141586				
E. rupestris	HS294	TM27283	TM	Prieska, NC	SA	29.65S 22.70E	EF141653	EF141583				
E. rupestris	HS295	TM29530	TM	Beaufort-West, WC	SA	32.40S 22.60E	EF141681	EF141611				
E. rupestris	HS296	TM29569	TM	Beaufort-West, WC	SA	32.40S 22.60E	EF141682	EF141612				
E. rupestris	HS297	TM29607	TM	Beaufort-West, WC	SA	32.40S 22.60E	EF141683	EF141613				
E. rupestris	HS298	TM30787	TM	Louisvale, NC	SA	28.60S 21.20E	EF141651	EF141581				
E. rupestris	HS299	TM30791	TM	Louisvale, NC	SA	28.60S 21.20E	EF141652	EF141582				
E. rupestris	HS301	TM32459	TM	Kenhardt, NC	SA	29.40S 21.15E	EF141676	EF141606				
E. rupestris	HS302	TM32483	TM	Lutzputs, NC	SA	28.35S 20.60E	EF141647	EF141577				
E. rupestris	HS303	TM33762	TM	Kangnas, Springbok, NC	SA	29.42S 17.55E	EF141657	EF141587				
E. rupestris	HS304	TM33763	TM	Kangnas, Springbok, NC	SA	29.42S 17.55E	EF141658	EF141588				
E. rupestris	HS361	NGP1940	IM	Akkerendam, Calvinia, NC	SA	31.25S 19.71E	EF141678	EF141608				
E. rupestris	HS362	NGP1941	IM	Akkerendam, Calvinia, NC	SA	31.25S 19.71E	EF141679	EF141609				
E. rupestris	HS375	ZM17059	IM	Upington, NC	SA	28.40S 21.30E	EF141648	EF141578				
E. rupestris	HS376	ZM17060	IM	Upington, NC	SA	28.40S 21.30E	EF141649	EF141579				
E. rupestris	HS378	ZM13358	IM	Touwsriver, WC	SA	33.40S 20.10E		EF141610				
E. rupestris	HS382	TM8266	TM	Aus	Nam	26.60S 16.20E	EF141644	EF141574				
E. rupestris	HS383	TM8276	TM	Helmeringhausen	Nam	25.80S 16.80E	EF141640	EF141570				

Table 3 continued

Species	US	Museum voucher	Museum	Locality	Country	Grid ref	GenBank accession nr.					
							cytochrome *b*	control region	Fibrinogen 7	12S rRNA, val tRNA, 16S rRNA	IRBP exon 1	VWF exon 28
E. rupestris	HS384	TM8276a	TM	Berseba	Nam	26.00S 17.60E	EF141642	EF141572				
E. rupestris	HS386	TM9435	TM	Karibib-Omaruru	Nam	21.90S 15.80E	EF141634	EF141564				
E. rupestris	HS388	TM10216	TM	Rehoboth	Nam	23.40S 17.10E	EF141637	EF141567				
E. rupestris	HS389	TM10218	TM	Rehoboth	Nam	23.40S 17.10E	EF141638	EF141568				
E. rupestris	HS390	TM10954	TM	Kaokoveld	Nam	18.20S 12.60E	EF141628	EF141558				
E. rupestris	HS392	TM12927	TM	Walvis Bay	Nam	22.90S 14.50E	EF141632	EF141562				
E. rupestris	HS393	TM16235	TM	Karibib	Nam	21.90S 15.80E	EF141635	EF141565				
E. rupestris	HS396	TM16240	TM	Kamanjab	Nam	19.60S 14.80E	EF141631	EF141561				
E. rupestris	HS397	TM30789	TM	Aus	Nam	26.60S 16.20E	EF141645	EF141575				
E. rupestris	HS399	TM40991	TM	Kaokoveld	Nam	18.20S 12.60E	EF141629	EF141559				
E. rupestris	HS400	TM41924	TM	Brukkaros	Nam	25.70S 18.10E	EF141643	EF141573				
E. rupestris	HS416	TM2361	TM	Cradock, EC	SA	32.20S 25.60E	EF141696	EF141626				
E. rupestris	HS417	TM8264	TM	Aus	Nam	26.60S 16.20E	EF141646	EF141576				
E. rupestris	HS418	TM8268	TM	Rehoboth	Nam	23.40S 17.10E	EF141639	EF141569				
E. rupestris	HS419	TM8275	TM	Helmeringhausen	Nam	25.80S 16.80E	EF141641	EF141571				
E. rupestris	HS420	TM9431	TM	Upington, NC	SA	28.40S 21.30E	EF141650	EF141580				
E. rupestris	HS421	TM9433	TM	Damaraland	Nam	21.00S 14.50E	EF141630	EF141560				
E. rupestris	HS422	TM10502	TM	Kaokoveld	Nam	18.20S 12.60E	EF141627	EF141558				
Elephantulus sp. nov.	HS237	TM29496	TM	Beaufort-West, WC	SA	32.20S 22.33E	DQ901212	DQ901250	EU076275			
Elephantulus sp. nov.	HS238	TM29497	TM	Beaufort-West, WC	SA	32.20S 22.33E	DQ901213	DQ901251	EU076276			
Elephantulus sp. nov.	HS239	TM29498	TM	Beaufort-West, WC	SA	32.20S 22.33E	DQ901214	DQ901252				
Elephantulus sp. nov.	HS240	TM29528	TM	Beaufort-West, WC	SA	32.20S 22.33E	DQ901215	DQ901253				
Elephantulus sp. nov.	HS241	TM29529	TM	Beaufort-West, WC	SA	32.20S 22.33E	DQ901216	DQ901254	EU076277			
Elephantulus sp. nov.	HS235	TM27303	TM	Williston, NC	SA	31.10S 21.35E	DQ901217	DQ901255				
Elephantulus sp. nov.	HS236	TM27304	TM	Williston, NC	SA	31.10S 21.35E	DQ901218	DQ901256				
Elephantulus sp. nov.	HS451	MMk/M	MMK	Vondelingsfontein, Calvinia, NC	SA	31.80S 19.82E			EU076274	EU136167		
Elephantulus sp. nov.	HS452		MMK	Vondelingsfontein, Calvinia, NC	SA	31.80S 19.82E	EU076251	EU076245	EU076283			
Elephantulus sp. nov.	HS453		MMK	Vondelingsfontein, Calvinia, NC	SA	31.80S 19.82E			EU076279			
Elephantulus sp. nov.	HS454	MMK/M/2167	MMK	Carnarvon, NC	SA	30.50S 22.10E	EU076246	EU076240				

Table 3 continued

Species	US	Museum voucher	Museum	Locality	Country	Grid ref	GenBank accession nr.					
							cytochrome *b*	control region	Fibrinogen 7	12S rRNA, val tRNA, 16S rRNA	IRBP exon 1	VWF exon 28
Elephantulus sp. nov.	HS455	MMK/M/2168	MMK	Carnarvon, NC	SA	30.50S 22.10E	EU076247	EU076241				
Elephantulus sp. nov.	HS456	MMK/M/2169	MMK	Carnarvon, NC	SA	30.50S 22.10E	EU076248	EU076242	EU076280			
Elephantulus sp. nov.	HS457	MMK/M/2170	MMK	Carnarvon, NC	SA	30.50S 22.10E	EU076249	EU076243	EU076281			
Elephantulus sp. nov.	HS458	MMK/M/2171	MMK	Carnarvon, NC	SA	30.50S 22.10E	EU076250	EU076244	EU076282			
Elephantulus sp. nov.	HS465	GBR624	CAS	Slytfontein, Loxton, NC	SA	31.47S 22.37E						
Elephantulus sp. nov.	HS466	GBR631	CAS	Slytfontein, Loxton, NC	SA	31.47S 22.37E						