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Taxonomic status of *Saccostomus campestris* (Rodentia: Cricetomyinae) from southern Africa: A multidisciplinary approach

By

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Dedication

This thesis is dedicated to my family

General abstract

The pouched mouse, *Saccostomus campestris* Peters, 1846 from southern Africa shows a high degree of karyotypic variation where up to 16 variants (2*n* = 30–50) have been reported. This has led to a systematic uncertainty that the present study attempts to assess using: 1) cytochrome *b* (cyt *b*; 1077 bp) and 16S rRNA (528 bp) partial sequences; 2) G-banding cytogenetic data; and 3) geometric morphometric data of various views of the cranium and mandible. The results from these multidisciplinary analyses are broadly similar with phylogenetic analyses of the molecular data revealing the presence of two major lineages. The first lineage comprises the high diploid numbered 2*n* = 46 cytotype from KwaZulu-Natal Province, South Africa that is considered to be ancestral. The second lineage consists of multiple inland populations that are subdivided into: 1) a sub-lineage comprising samples from a large semi-arid area in the west; and b) a sub-lineage of small distinct populations of low migrations from the east. The cytogenetic data suggest that karyotypic variation within *S. campestris* from southern Africa is due to autosomal Robertsonian fusions, with evidence of geographic structuring where cytotypes with high diploid numbers originate from the mesic east, while those with low diploid numbers originate from the arid west. The reduction in chromosome number appears to be due to adaptation to cold and dry conditions in the arid west. The X-chromosome revealed three variants that arose from a single pericentric inversion followed by the addition of genetic material, possibly heterochromatin. Variant 1 is only present in the ancestral cytotype and is found in all cytotypes throughout southern Africa, variant 2 is found in cytotypes from areas with < 600 mm of annual rainfall, while variant 3, although only found in females, is sympatric with variant 2. Geometric morphometric analysis of karyotyped specimens showed no discernible patterns of variation among karyotypic variants except for some subtle but equivocal indication of the morphological distinctiveness of the 2*n* = 46 cytotype from KwaZulu-Natal. Collation of the molecular, cytogenetic, and geometric morphometric data in the present study suggest that *S. campestris* from southern Africa is monotypic.

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Last but not least, I am sincerely indebted to thank my family, my parents, sisters and brothers for their moral support and encouragement and tolerance throughout my study life. Special thanks go to my beloved girlfriend Marloes Reinink for the wonderful support.

Disclaimer

This thesis consists of a series of chapters that have been prepared as stand-alone manuscripts for subsequent submission for publication purposes. Consequently, unavoidable overlaps and/or repetitions may occur between chapters.

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Chapter 1: General introduction

1.1. Higher-level systematics

All the generally slow-moving African pouched mice of the subfamily Cricetomyinae have cheek pouches that are used to hoard food in addition to their nocturnal, solitary and subterranean life-style (Earl 1980). However, the taxonomic affinities of the Cricetomyinae are not clear since they have previously been taxonomically allocated to both the family Cricetidae (within the subfamily Cricetinae; Smithers 1971) and to the family Muridae (Simpson 1945).

This taxonomic confusion is largely to due to the similarity in the shape of the molars between cricetid and murid rodents (Smithers 1971). Subsequently, both the subfamilies Cricetinae and Cricetomyinae were allocated to the family Muridae (Carlton & Musser 1984). It was later hypothesised that, while the cricetines and cricetomyines may be derived from a common ancestor, their cheek pouches and retractor muscles may have evolved independently (Ryan 1989).

Recently, a number of studies (Michaux & Catzeflis 2000; Michaux *et al.* 2001; Jansa & Weksler 2004) used DNA sequences of the vWF, LCAT, IRBP, GHR, BRCA1, RAG1, c-myc, and cytochrome *b* genes to define murid rodent subfamilial taxonomic boundaries, and to establish their phylogenetic relationships as well as their origins. These data suggested the partitioning of murid rodents into five major lineages, one of which comprises the subfamilies Cricetomyinae, Dendromurinae, Mystromyinae and Mesomyinae, all of which are restricted to sub-Saharan Africa and Madagascar (Michaux & Catzeflis 2000). The molecular data further suggest that the subfamily Cricetomyinae is of ancient origin, probably representing relics of an African cricetodontid from the early Miocene. Consequently, Michaux & Catzeflis (2000) and Michaux *et al.* (2001) suggested that the Cricetomyinae lineage was probably derived from the fossil subfamily Afrocricetodontinae.

However, the above studies do not agree on the monophyly of the Cricetomyinae. For example, in the IRBP gene data, *Saccostomus* grouped with dendromurines instead of cricetomyines with low bootstrap support (Jansa & Weksler 2004). In addition, the LCAT and vWF gene data revealed that the cricetomyines evolved at a high rate with *Saccostomus* evolving more quickly than the remaining cricetomyines (Michaux & Catzeflis 2000). Despite numerous attempts to clarify evolutionary relationships among murid rodents, the monophyly of the cricetomyines remains uncertain. Similarly, these taxonomic uncertainties are also prevalent at the species level, particularly in the pouched mouse, *Saccostomus campestris* that is currently allocated to the subfamily Cricetomyinae within the family Muridae.

1.2. Species-level systematics

Saccostomus campestris Peters, 1846 was originally considered a monotypic species with a distribution ranging from the Cape in South Africa north-wards to Ethiopia (Smithers 1983; Gordon 1986). Although at least four species of *Saccostomus* had been described by the end of the 19th century, these were subsequently relegated to the subspecies rank (De Graaff 1981). Based on the presence of two diploid numbers (2*n* = 40 and 42) and differences in pelage colour, ear, tail, and upper molar tooth row lengths, the species was subsequently divided into two species, one from Ethiopia and the other from southern Africa including Zambia and central Tanzania (Hubert 1978; Gordon 1986). While the specimens from southern Africa were assigned to the nominate species, those from Ethiopia were allocated to *S. mearnsi* (Hubert 1978; Gordon 1986).

However, subsequent studies in southern Africa revealed that *S. campestris* has an unusually high degree of karyotypic variation, and protein electrophoretic data suggested that *S. campestris* may represent at least two species in the subregion (Gordon 1986). One of these suggested species is characterised by a diploid number of 2*n* = 46 from the eastern parts of southern Africa (e.g., Kruger National Park), while the other included the 2*n* = 28–38 cytotypes from the south-western parts of the subregion (Gordon 1986). Gordon (1986) also reported that these geographic distributions show

divergent modes of chromosomal re-patterning that may be indicative of a complex of cryptic species.

A recent molecular study of *S. campestris* that included samples from central and northern Africa (Zambia, Tanzania, and Somalia) and a few samples from southern Africa (Kuruman, Kruger National Park, Hereroland, the Karoo, and Lalanek) suggested the recognition of 8 species in Africa that are linked to karyotypic differences among populations (Corti *et al.* 2004). These included the allocation of two forms with low diploid numbers from the south-west arid biotic zone in southern Africa and Mazoe in Zimbabwe, north of the Zambezi River to *S. anderssoni* and *S. mashonae*, respectively (Corti *et al.* 2004). However, the relationships among the forms with high diploid numbers remained unresolved.

It is clear that the number of species of *Saccostomus* in southern Africa and their associated geographic distributions are in need of a systematic assessment using a multidisciplinary approach that may allow for more robust conclusions. However, it is of fundamental importance that any attempts to define species boundaries, in general and within *S. campestris*, in particular need to be undertaken with reference to species concepts, the definitions of which represent some of the most highly debated issues in biology (Mayr & Ashlock 1991). Currently, these are partitioned into process-oriented and pattern-oriented species concepts (Wheeler & Meier 2000; Ferguson 1999)

1.3. Species Concepts

Process-oriented species concepts:

The process-oriented species concepts are mainly about the processes that give rise to new species. These include: 1) the Biological Species Concept (BSC); 2) the Recognition Species Concept (RSC); and 3) the Cohesion Species Concept, and are defined as follows:

1. The Biological Species Concept (BSC – Mayr 1942; 1963): BSC defines a species as populations reproductively isolated from other populations with emphasis on isolating mechanisms in sexually reproducing organisms (Mayr 1963). That means that the Biological species have very strong implications to gene pools. A Biological species should have the following characteristics as suggested by Mayr (1963):

a) Individuals of the same species should freely interbreed, meaning that individuals should recognise potential mating partners;
b) Individuals of a species should be reproductively isolated from other such groups; and
c) Individuals of a species form a genetic unit (King 1993), due to the existence of isolating mechanisms with respect to other species.

Following the BSC, speciation comprises the origin of a set of isolating mechanisms with respect to other closely related species (Mayr 1963). The BSC also suggests that the diagnosis of a species is clear-cut, more so where two morphologically distinct populations co-exist in sympatry without interbreeding. This means that interbreeding has to be established, or not established in the field, which has proven to be a difficult exercise. As a result, of late, this concept relies heavily on any character that might render individuals distinct from other individuals. Such characters can be anything from morphology, biochemical markers, to molecular sequence data. These can be used to determine whether there is potential for interbreeding or not.

However, the BSC is difficult to test in two or more populations that are separated in space and time (Mayr & Ashlock 1991). Another shortfall of the BSC is that it applies to sexually reproducing organisms, meaning that it cannot be applied to uniparental and asexually reproducing organisms (Simpson 1961; Paterson 1985; King 1993), a shortfall that Mayr, the main proponent of the BSC (Mayr 1982) acknowledges.

Despite its limitations, the BSC has historically been widely applied probably due to its being straight forward with uncomplicated conditions.

2. Recognition Species Concept (RSC – Paterson 1978): Because of problems associated with the BSC, Paterson (1978) suggested the RSC that is restricted to bi-parental organisms that share the same fertilisation system. According to the RSC, speciation occurs when a new fertilisation system arises. Paterson (1978) introduced the concept of the Specific Mate Recognition System (SMRS) as a subset of the fertilisation system that provide cues between mating partners. Besides members of the same species sharing the same fertilization system, the RSC is characterised by the following standards:

a) The characteristics of the SMRS are adapted to function with efficiency in a suitable habitat; and
b) When a subsection of a population is either cut-off or isolated for a long time, a new species will arise when the break-away sub-population develops a new fertilisation system.

The RSC has since been heavily criticised and the obvious one that could perhaps render the RSC unusable relates to hybridisation (e.g., between a donkey and a horse, King 1993).

3. Cohesion Species Concept (CSC – Templeton 1989): Templeton (1989) suggested the CSC that focuses on a range of cohesion mechanisms such as morphological, molecular, gene flow between populations, and hybrid infertility. Following the CSC, speciation takes place when a new set of cohesion mechanisms evolve. The CSC suggests that:

a) A species is defined by a range of cohesion mechanisms rather than gene flow;
b) The CSC applies to both sexually and asexually reproducing organisms; and
c) Genetic and demographic exchangeability are factors that drive speciation, i.e., a species should have its genetics being dependant on ecological niche that a species occupies.

However, the CSC has been criticized as being merely an extension of the BSC (King 1993) citing the following reasons:

a) The majority of characters that make a species a cohesive unit had already been covered or tacitly accepted as part of the BSC; and
b) The CSC focuses on factors that hold the species together, rather than the isolating mechanisms.

Pattern-oriented species concepts

Pattern-oriented species concepts are considered to be mainly useful for diagnosing separate species and do not make explicit reference to particular mechanisms of speciation. These include: 1) the Hennigian Species Concept (HSC), 2) the Evolutionary Species Concept (ESC), and 3) the Phylogenetic Species Concept (PSC), and are defined as follows:

1. Hennigian Species Concept (HSC – Meier & Willmann 2000): the HSC adopts the idea of isolating mechanisms, but suggests that after speciation, the ancestral species ceases to exist (Meier & Willmann 2000).

2. The Evolutionary Species Concept (ESC – Simpson 1961): The limitations of the BSC led to the development of the ESC that defines a species as a lineage with its own unitary role and tendencies separated from other such lineages (Simpson 1961).

According to the ESC, the following conditions have to be met to qualify as a species:

a) Ancestor-descendent relationship has to be established in an attempt to clarify the species status of populations that are separated through space and time;
b) Each lineage has to evolve in a different direction to other lineages;
c) The groups should be typologically distinct from other such groups; and
d) The individuals that make up the species must be subjected to similar selection pressures.

However, the ESC has been criticized because it is considered to side-step the crucial question of what a “unitary role” is and why phyletic lines do not inter-breed (Mayr & Ashlock 1991), and for being unable to quantify “range

and adaptive zone" (King 1993). In other words, the criticisms relate to the following:

a) The ESC ignores the possibility of cryptic and polytypic species (Mayr 1982); and
b) Promotes the progression of a non-branching lineage, while at the same time it restricts factors that can help explain discontinuities that may occur between species (King 1993).

These limitations led to the ESC to be redefined a number of times subsequent to Simpson's (1961) original definition. Chronologically, these redifinitions are as follows:

a) Van Valen (1976) redefined the ESC so that an ecological aspect could also be incorporated into the ESC. However, this redefinition undermines the relevance of genes in evolution but rather promotes ecology as the main driving force behind evolution and that selection was more likely to act on genotypes (King 1993);
b) Wiley (1978) redefined the ESC in an attempt to remove the complication arising from the use of phyletic evolution and suggested that while species are individuals having ontological reality, they do not evolve separately, but maintain a separate identity; and
c) Frost & Hillis (1990) redefined the ESC in an attempt to include genetically distinctive groups that could perhaps only be separated in evolutionary time (ephemerally).

3. Phylogenetic Species Concept (PSC – Cracraft 1983): The PSC focuses on differentiation of traits rather than isolation of lineages (Cracraft 1983; Nixon & Wheeler 1990).

Similar to the ESC, PSC has been redefined at least two more times following the original definition. Currently there are two main approaches: 1) a character-based approach that relies on the possession of unique diagnostic characters and 2) a history-based approach that relies on the criterion of monophyly.

a) According to Mishler & Theriot (2000) a Phylogenetic Species can be identified as long as they show some evidence of monophyly. Here the evidence of monophyly is provided by characters which must have a certain amount of support worthy of recognising them as a monophyletic group. The following conditions have to be satisfied:
    i) Replication must occur to form lineages;
    ii) Characters must show a historical sequence, meaning that they should be heritable;
    iii) There must be divergence among lineages with common shared characters, resulting in sister taxa;
    iv) Characters have to be independent to other characters; and
    v) Characters must transform at a very slow rate.

b) According to Wheeler & Platnick (2000) a species is "*the smallest aggregation of (sexual) populations or (asexual) lineages diagnosable by a unique combination of character states"*. This definition looks at patterns that bring forth differences among lineages and processes that may lead to differences among lineages. However it relies heavier on the former than the latter.

However, the PSC has been criticized because species boundaries have to be defined using either molecular or morphological differences regardless of unrestricted gene flow between lineages (King 1993; Ferguson 1999; Wheeler & Meier 2000). Other shortcomings related to the PSC are:

a) It overinflates the number of species, that could otherwise be genetically similar and capable of interbreeding, therefore questioning the biological relevance of its application in the definition of species; and
b) It is difficult to categorise closely related groups that are in the process of active speciation.

From the above, it is clear that each of the proposed species concepts has its own strengths and weaknesses, and that their development has been a result of attempts to address particular limitations in addressing the species

definition problem. It is also clear that there is a critical need for a "universal" species concept that addresses the weaknesses of the numerous proposed species concepts, as well as one that accommodates for both sexually and asexually reproducing organisms (King 1993; Ferguson 1999; Wheeler & Meier 2000). Until such a "universal" species concept has been developed, it is of fundamental importance that studies attempting to address questions of species boundaries ought to be undertaken with special reference to currently available species concepts. Consequently, the present study attempts to define species boundaries within *S. campestris* from southern Africa with reference to the currently proposed species concepts, which for more robust conclusions, is based on a multidisciplinary approach to address the specific aims as outlined below.

1.4. Aims of study

The present study is aimed at assessing the nature and extent of variation within the currently recognized *S. campestris* from southern Africa in an attempt to define the species' boundary as well as evaluate its geographic distribution. For more robust conclusions, the study is based on a multidisciplinary approach that includes molecular, cytogenetic, and geometric morphometric analyses. The present study represents the first attempt to systematically assess variation within *S. campestris* over a broader geographic area than has previously been considered for the species within the southern African subregion, and focuses on the specific research questions as outlined below:

Research questions

The following specific research questions are addressed in the present investigation:

1) What is the nature and extent of molecular, cytogenetic, and geometric morphometric variation within *S. campestris* from southern Africa?

2) Is the nature and extent of molecular, cytogenetic, and geometric morphometric variation within *S. campestris* from southern Africa associated with geographic distribution and habitat type?

3) Does the nature and extent of molecular, cytogenetic, and geometric morphometric variation warrant the recognition of a complex of cryptic species within *S. campestris* from southern Africa?

Justification of study

The nature and extent of variation within *S. campestris* from southern Africa, its associated geographic distribution, and hence its taxonomic status is uncertain. To date, there is no multidisciplinary systematic and biogeographic study of *S. campestris* from southern Africa. Apart from adding to a body of knowledge on small mammal systematics and serving as a model for other similar studies in Africa, the present study may assist nature conservation authorities in formulating conservation management strategies for *S. campestris* from the southern Africa subregion.

1.5. Thesis outline

The first part of this study (Chapter 2) is directed towards assessing molecular variation using cytochrome *b* and 16S rRNA gene sequences to elucidate phylogenetic relationships between cytotypes and to estimate the geographic origin and duration of population subdivision within *S. campestris* from southern Africa. For more robust conclusions, the series of phylogenetic analyses also included previously-published DNA sequences from East Africa.

Chapter 3 addresses questions relating to karyotypic variation within *S. campestris* using G-banding chromosome analysis, and assesses how the nature and extent of variation relates to molecular variation.

Chapter 4 assesses the nature and extent of cranial and mandibular morphological variation in *S. campestris* from southern Africa using geometric morphometric data. However, as a preliminary step, non-geographic variation was first evaluated using a series of univariate and multivariate analyses of the

cranium and mandible. This was undertaken with the primary objective of establishing whether the sexes should be treated separately or together, and which specimens have reached adult dimensions and were therefore, suitable for data recording and analysis in the subsequent assessment of variation within *S. campestris* from southern Africa.

Chapters 2–4 also provide overviews of the molecular, cytogenetic, and geometric morphometric approaches and their associated phylogenetic, univariate, and multivariate methods used in the present study. The final chapter (Chapter 5) provides a general discussion and conclusions of the major findings of this study. This includes interpretations with reference to species concepts, theoretical models of chromosomal evolution, and the taxonomic implications from the nature and extent of molecular, cytogenetic, and geometric morphometric variation.

Because each chapter was organized as an independent manuscript to facilitate submission for publication considerations, there may be some repetition in the chapters in order to enhance readability.

Chapter 2: Ancient population subdivision in the pouched mouse, *Saccostomus campestris* (Rodentia: Cricetomyinae) from southern Africa: inferences from cytochrome *b* and 16S rRNA gene sequences.

Abstract

The systematics of the pouched mouse, *Saccostomus campestris* is uncertain. Cytochrome *b* and 16S rRNA gene fragments of 34 specimens of *S. campestris* from 16 localities throughout southern Africa and from an array of different habitats were studied in order to: a) estimate the degree and duration of population subdivision in southern and eastern Africa; b) test the hypothesis that *S. campestris* from different habitats in southern Africa are genetically different; and c) test the hypothesis that *S. campestris* from southern Africa is genetically similar to *S. campestris* from eastern Africa. Maximum parsimony, maximum likelihood, Bayesian likelihood, nested clade, and gene flow analyses showed two major lineages of the pouched mouse to be present in southern Africa: 1) a KwaZulu-Natal lineage, which seems to have been isolated for a long time with a small estimated population size; and 2) a lineage that includes multiple inland populations, subdivided into western (including Namibia/Botswana) and eastern sub-lineages. The western sub-lineage covers a single large semi-arid geographical area, while the eastern population sub-lineage is divided into smaller distinct clusters, mostly with small population size estimates and smaller estimates of between-population migration. The most plausible explanation for patterns observed in the eastern inland populations is very low rates of dispersal between patches of suitable habitat, resulting in genetic isolation of newly established populations. Such colonisation of suitable habitat patches appears to have occurred about 10 MYA, resulting in ancient populations still observed at present.

Keywords: *Saccostomus campestris*, cytochrome *b*, 16S rRNA, coalescent, maximum parsimony, nested clade, migration, southern Africa.

2.1. Introduction

Pouched mice of the genus *Saccostomus* have a wide distribution in sub-Saharan Africa, ranging from the Cape Province of South Africa north-wards through East Africa to Ethiopia (De Graaff 1981; Skinner & Chimimba 2005). The genus is represented by two species: *S. campestris* occurring in southern and eastern Africa from the Cape region to south-west Tanzania and *S. mearnsi* occurring in eastern Africa from north-east Tanzania to Ethiopia (Corti *et al.* 2004). Pouched mice occur in a variety of habitats including open fields, dense bush, forests, sandy areas, rock outcrops, open grasslands near pans, dry river beds, and mopane veld (Shortridge 1934 & Smithers 1971 as cited in De Graaff 1981; Skinner & Chimimba 2005).

In addition to occurring in a diversity of habitats, *S. campestris* also exhibits extensive intra-specific karyotypic variation. To date, 16 distinct chromosome numbers have been reported (Gordon 1986; see Chapter 3). Robertsonian translocations and heterochromatic additions are considered to be the causes of this variation (Ferreira 1990). This type of variation normally does not cause reproductive isolation because it does not inhibit meiosis (Baker & Bickham 1986; King 1993).

Based on protein electrophoretic data, Gordon (1986) found populations with low diploid numbers (i.e., $2n$ = 28–38) to be different from those with high diploid numbers (i.e., $2n$ = 46), leading to a suggestion that there may be at least two species of *Saccostomus* in southern Africa. Gordon (1986) and Ferreira (1990) found that karyotypic variation in *S. campestris* is also strongly correlated with habitat and climate, with lower chromosome numbers being found in more arid habitats. In addition, Ellison (1992) reported geographic variation in morphology and physiology within *S. campestris* from the southern African sub-region. However, unlike other taxa (e.g., *Aethomys*; Breed 1997), Gordon (1986) found no difference in sperm morphology between the different karyotypic variants within *S. campestris* from southern Africa.

Genetic analysis is an important primary tool for establishing systematic relationships. Mitochondrial DNA (mtDNA) is useful for studying evolutionary

relationships among individuals, populations, and species (Bibb *et al.* 1981; Anderson *et al*. 1982; Irwin *et al*. 1991). It is maternally inherited (Lansman *et al*. 1983; Avise *et al.* 1987), is highly variable, lacks pseudogenes and has a higher evolutionary rate than nuclear DNA. Consequently, the present study uses cytochrome *b* (cyt *b*) and 16S rRNA as tools to assess phylogenetic relationships among populations of *S. campestris* from southern Africa. Specific goals of the present study are: 1) to estimate the degree and duration of population subdivisions within *S. campestris* from both southern and eastern Africa, as well as the *S. campestris*/*S. mearnsi* split; 2) test the hypothesis that *S. campestris* from different habitats in southern Africa have fixed genetic differences; and 3) assess the degree of genetic similarity/dissimilarity between *S. campestris* from southern Africa and beyond the subregion extending from Zambia through Tanzania to the Equatorial regions of Africa.

2.2. Materials and methods

Study area and samples

Tissue samples (thigh muscle, blood, and tail tissue) were obtained from 33 specimens of *S. campestris* from 13 localities in southern Africa (Fig. 2.1). Geographic coordinates for all sampled localities are summarised in Table 2.1. Animals were live-trapped using Sherman traps (H.B. Sherman Traps Inc. Florida, U.S.A.) baited with a mixture of peanut butter, syrup, oat meal and sunflower oil. After capture, during transportation and in the laboratory, animals were kept in polyurethane cages with wood shavings as bedding, with mouse pellets and water provided *ad libitum*.

Animals were maintained under the guidelines of the American Society of Mammalogists (ASM; http:///www.mammalogy.org/committees/index.asp; Animal Care and Use Committee 1998), and the research project was approved by the Animal Ethics Committee of the University of Pretoria, Pretoria, South Africa. Animals were subsequently sacrificed using halothane inhalation. Voucher specimens were prepared using standard natural history museum procedures for mammal specimens and deposited in the mammal reference collection of the Transvaal Museum (TM) of the National Flagship

Institute (NFI), Pretoria, South Africa as well as the National Museum of Namibia, Windhoek, Namibia.

DNA extraction

Approximately 100 ng/µl of genomic DNA was extracted from thigh muscle, blood and tail tissue samples. The concentration of DNA extracted was measured with a Biowave S2100 Diode Array spectrophotometer (Biochrom Ltd, Berlin, Germany). The following two extraction methods from blood and tissue samples were used:

1) Blood: Chelex DNA extraction was based on the protocol of Walsh *et al.* (1991) where 3 µl whole blood was added to 1 ml purified water (Sabax®) and incubated at room temperature for 30 minutes for cell lysis to occur. The lysate was centrifuged at 1300 rpm for 3 minutes after which the supernatant was discarded. 5% Chelex was added to make a final volume of 200 µl and incubated at 56° C for 30 minutes. The mixture was vortexed and heated on a heat-block for 8 minutes at 100° C. The sample was vortexed and centrifuged at 1300 rpm for 3 minutes after which the supernatant (DNA) was stored at -20° C.

2) Tissue: Roche's lysis buffer (200 µl) and Proteinase K (Roche Molecular kit 1993) (40 µl) were mixed with 0.2 g of homogenised tissue and incubated overnight at 55º C followed by a treatment with 100% isopropanol to isolate undissolved tissue. Roche's binding buffer was then added to the samples after which the DNA was washed twice in Roche's wash buffer and later eluted in 30 µl of pre-warmed elution buffer.

Polymerase Chain Reaction (PCR) amplification

Sequencing focused on cyt *b* and 16S rRNA mitochondrial loci. Primers for cyt *b* spanned an 1191 base pairs (bp) stretch of the locus, whereas primers for 16S rRNA spanned 615 bp as detailed below.

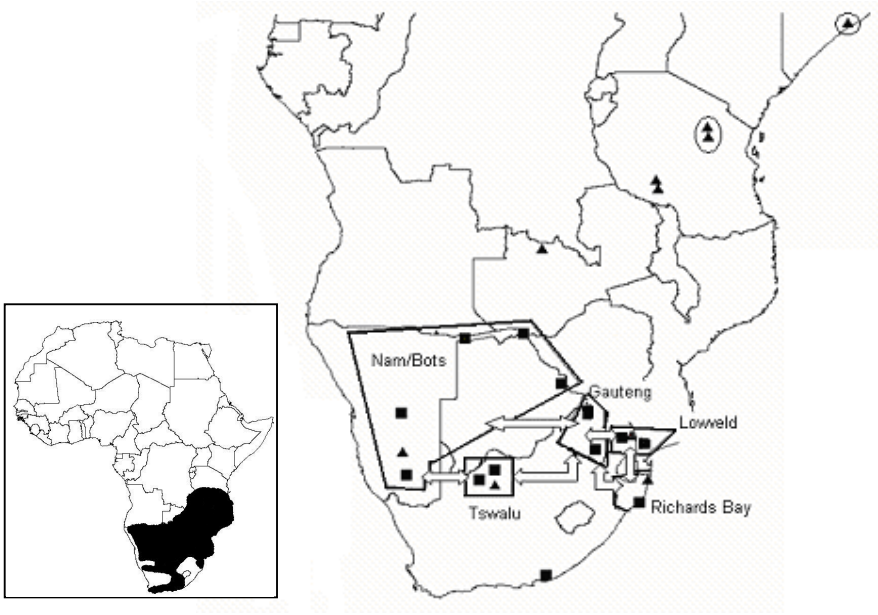


Figure 2.1. The localities of *Saccostomus campestris* and *S. mearnsi* sampled in the present study. Squares indicate localities of material collected during the present study, while triangles show localities of material analysed by Corti *et al.* (2004). Arrows indicate the postulated movement of migrants between populations following the stepping-stone model used for gene flow analysis. The insert shows the overall geographic distribution of *S. campestris* in Africa (adopted from Skinner & Smithers 1990). Sampled localities for *S. mearnsi* which is distributed from the northern parts of Tanzania towards the equator (Corti *et al.* 2004) are shown by encircled triangles.

Table 2.1. List of samples of the *Saccostomus campestris* from southern Africa including their sampled localities of origin, their associated codes that are referred to in the preset study, geographic coordinates, and sample size (*n*).

Locality	**Code**	**Geographic coordinates**	***n***
1. Richards Bay	rb	28°42'S 32°15' E	4
2. Roodeplaat	rd	25°25'S 28°05'E	4
3. Magude	mo	24º57'S 32º37' E	2
4. Tswalu	ts	27°13'S 22°26' E	3
5. Manaaneng	mn	26°35'S 23°23' E	4
6. Langjan	ln	22º51'S 29º14' E	1
7. Blouberg	bl	22º59'S 29º07' E	1
8. Blaawbank	g	25°15'S 29°34' E	3
9. Thomas Baines	tb	33º18'S 26º32' E	1
10. Dordabis	d	22°56'S 17°38'E	5
11. Hoedspruit	h	24°31'S 31°13'E	1
12. Pretoria-West	p	25°25'S 28°05'E	1
13. Francis Town	ft	13°21'S 27°30'E	1
14. Kasane	ka/ks	17°49'S 24°09'E	1
15. Keetmanshoop	kh	26°55'S 17°56'E	1
16. Caprivi	N30	17°57S 19°43E	1

To amplify the cyt *b* gene, 5 µl of mtDNA extract was mixed with 5 µl of Biotools 10 x reaction buffer, 4 µl of 2.5 mmol of Biotools dNTP's, 2.5 µl of 10 pmol/1 µl of the forward primer (L14724: 5' TGAYATGAAAAAYCATCGTTG 3' – Paäbo *et al.* 1988) and of the reverse primer (H15915-mus: 5' CATTTCAGGTTTACAAGAC 3' – Bibb *et al.* 1981), 3.5 µl of sterile distilled water (Sabax®), and 1 µl of Biotools Taq using a Geneamp® PCR System 2400 (Perkin Elmer Corporation) under the following conditions: DNA was denatured at 96° C for 45 seconds, annealed at 44.1° C for 45 seconds, extended to 72° C for 60 seconds in 34 cycles, and finally stored at 4° C until the product was purified.

To amplify the 16S rRNA gene, the following primers were used: forward primer 16S ar-L (5'CGCCTGTTTATCAAAAACAT3') and reverse primer 16S br-H (5'CCGGTCTGAACTCAGATCACGT 3') under similar conditions as above (Palumbi *et al.* 1991). The DNA was denatured at 96° C for 45 seconds, annealed at 58° C for 45 seconds, extended at 72° C for 60 seconds in 33 cycles, and stored at 4° C.

Purification

Water was added to the PCR product to make up 100 µl, followed by the addition of 500 µl of binding buffer using a High Pure™ sequencing kit. The product was run through a High Pure™ filter tube into a collection tube to bind the DNA on to the filter by centrifuging the product at 1300 rpm for 1 minute, after which the flow-through was discarded. Following this, 200 µl of wash buffer was allowed to flow through the filter tube into the collection tube after which the filter was re-centrifuged for 1 minute at 1300 rpm and the flow-through discarded.

The filter tube was then placed in a clean 1.5 ml safe-lock tube, 50 µl of sterile distilled water was added, left to stand for 10 minutes, and centrifuged for 1 minute at 1300 rpm. 2 µl of the eluted DNA in the safe-lock tube was then run for 10 minutes against 2 µl of λ DNA (Biotools) on a 1.5% agarose gel (3 g Agarose + 200 ml 1XTAE + 5 µl Ethidium Bromide) at 50 volts to quantify the

amount of DNA to be used during cycle sequencing. Quantification was performed by evaluating the brightness of the purified product against λ DNA under an ultraviolet light.

Cycle sequencing and precipitation

Approximately 2 µl – l3 µl of the purified mtDNA were used during the PCR reaction. Primers (3.2 pmol) L14724 and H15915-mus (cyt *b*), while 16S ar-L and 16S br-H (16S rRNA) were used with BigDye™ Terminator version 3.0 (Applied Biosystems) cycle sequencing according to the protocol. The purified mtDNA product was run through 25 cycles of denaturing at 96° C for 10 seconds, primer annealing at different temperatures [Cyt *b*: L14724 (46° C) and H15915-mus (48° C)] [16S rRNA: L1960 and H2575 (48° C)] for 5 seconds. This was followed by elongation at 60° C for 4 minutes, held at 4° C aided by a Geneamp® PCR System 2400 (Perkin Elmer Corporation), and finally stored at -20° C.

Sodium acetate precipitation was used where cycle sequencing products were added to 50 µl of ice cold (0° C) absolute ethanol, with 10 µl of water and 2 µl of 3 M sodium acetate solution. After precipitation, the product was washed twice in 70% ethanol, dried at 80° C on a he at-block for 3 to 5 minutes and stored at -20° C. Sequences of up to 700 (cyt *b* gene) and 550 (16S rRNA gene) base pairs from the 5' and 3' ends were obtained from an ABI377 automated sequencer (Applied Biosystems, California). The sequencing yielded a 1077 bp cyt *b* and 528 bp 16S rRNA fragments analysable across all samples. Representative sequences for both genes are presented in Appendices 1 and 2, respectively.

Phylogenetic inference and population genetic analyses

Test for saturation

To avoid the effects of saturation because of multiple substitutions in the cyt *b* data within the in-group sequences, nucleotide changes were visualised by plotting the number of transitions and transversions (for all the three codon positions analysed separately) against the Kimura 2 parameter distance

values for all pairs of specimens (Halanych *et al.* 1999; van Vuuren & Robinson 2001).

Relative rates test

The Relative-Rates test (RRTree; Robinson & Huchon 2000) was used to assess if the 348 base pairs from the 3rd positions of the cyt *b* gene of the 33 specimens of *S. campestris* from southern Africa evolved at the same rate. The following parameters were computed and compared for each lineage: a) the number of synonymous substitutions per synonymous site (Ks); b) the number of synonymous transitions per synonymous site (As); c) the number of synonymous transversions per four-fold degenerate site (B4); d) the number of non-synonymous substitutions per non-synonymous site (Ka); and e) the number of non-synonymous transversions per non-synonymous site (Ba).

DNA sequences from the house rat (*Rattus rattus)* and the house mouse (*Mus musculus)* were used to calculate divergence times as the fossil record estimates their divergence time to be 12 Mya. The origin of the Cricetomyinae (and consequently its divergence from the Murinae) is of similar age (Table 2.8). The well-known calibration based on the *Mus*/*Rattus* divergence was therefore used as a calibration for the relative-rates analysis on the cyt *b* data (Jacobs & Downs 1994), using the giant rat (*Cricetomys gambianus)* as an outgroup. As a result, a rate of 1.2% per million years was used for the relative rates analysis.

Phylogenetic interpretation

Sequences were assessed using Chromas, version 1.43 (McCarthy 1997), proof-read and adjusted by hand and aligned using Clustal X (Thompson *et al.* 1994). For a much wider geographic perspective, the data generated in the present study was combined with and analysed together with cyt *b* sequences mostly from central and east African samples analysed by Corti *et al.* (2004). Corti *et al.*'s (2004) data also included cyt *b* sequences of five specimens from Kuruman, Hereroland, Karoo, Kruger National Park and Lalanek, South Africa. No published 16S rRNA sequences were available for analysis in the present study.

Maximum likelihood analysis was performed using the program PHYML version 2.4.4 (Guindon & Gascuel 2003). Prior to the analysis, MODELTEST version 3.06 (Posada & Candrall 1998) was used to establish the evolutionary model most suited for the data (GTR+I+G).

Bayesian bootstrap analyses of cyt *b*, 16S rRNA, and the two loci combined of 47, 33, and 33 individuals respectively were run using MrBayes version 2.1 (Huelsenbeck & Ronquist 2001). This program uses a Metropolis-coupled Markov Chain Monte Carlo algorithm to explore the parameter space with models combining transition matrices (Wadell & Shelly 2003). Similar to the maximum likelihood analysis, the GTR+I+G model was used. The analysis was based on four independent runs each using a random starting tree and 5,000,000 generations. At any given point, there was one cold and three heated chains, sampling trees every 500 generations. The first 50,000 trees were excluded as "burn-in", although stable partition frequencies and good overall likelihood were achieved by generation 25,000.

Maximum parsimony and neighbour-joining analyses were performed using PAUP version 4.0b10 (Swofford 2000) and MEGA version 2.0 (Kumar *et al.* 2001). DNASp version 4 (Rozas & Rozas 2004) was used to determine the number of polymorphic sites, parsimony informative and singleton sites, GC content, haplotype diversity and divergence of haplotype diversity, nucleotide diversity, frequencies, and nucleotide divergence. GC content tests for neutrality, namely, Tajima's D, Fu and Li's D*, Fu and Li's F*, Fu's Fs statistic, and Strobeck's S.

Nested Clade Analysis

Following Templeton *et al.* (1992), haplotype trees were estimated using TCS version 1.13 (Clement *et al.* 2000) with linkage probabilities of above 95% (14 mutational steps in the case of both cyt *b* and 16S rRNA sequences) and analysed by nested clade analysis in order to infer geographic as well as historical events (Templeton 1998) using GeoDis, version 2.2 (Posada *et al.* 2000) with 10,000 Monte Carlo replications and the inference key from Templeton (1998).

Migration estimates

Sequence data were analysed using Migrate-n, version 2.0.6 (Beerli 1997). The software uses a Bayesian-based likelihood approach to assess Θ, the effective population size multiplied by mutation rate per site per generation, as well as *M*, the quotient of the immigration rate divided by mutation rate. Using this approach, the number of immigrants per generation into a particular population can be estimated as Θ•*M* for mitochondrial DNA sequence data (Beerli 1997). In the present study, migration rates were estimated from the cyt *b* and 16S rRNA sequences, which were combined to form a single locus of 1605 base pairs. The mutation model used for this analysis was the finite site model (Felsenstein 2004) and is presented in Table 2.2.

A stepping-stone model comprising a network of neighbouring populations was used to estimate migration between pairs of populations (see Fig. 2.1 and Table 2.2). The following settings were used for estimates: 30 Short chains with 10,000 trees sampled and 500 used trees, three long chains with 100,000 trees sampled and 5000 used trees, heating scheme set at four chains (with temperatures: 1.00, 1.50, 3.00, and 6.00), and swapping interval set at one. Only the individuals from southern Africa sequenced during the present study were used in the analysis because they were sequenced for both cyt *b* and 16S rRNA data.

Molecular dating

Because of unequal rates of molecular evolution in different lineages, direct means of estimating dates since divergence (Avise *et al.* 1998) could not be used alone. As a result, additional methods for estimating divergence times, which take in to account varying rates of molecular evolution were employed and these included: 1) the Bayesian method and 2) the r8s method as outlined below:

1. Bayesian molecular dating

Divergence times based on the cyt *b* data were estimated by a Bayesian approach using the software PAML version 3.14 (Yang 2004), and Multidivtime that performs Bayesian Markov Chain Monte Carlo (MCMC) analysis (Thorne & Kishino 2002).

Model parameters were estimated for 16 *S. campestris* sequences that were chosen to represent different lineages from the phylogenetic analysis. The F84 model of sequence evolution was used. The parameters estimated were: nucleotide frequencies, transition/transversion rate ratio (parameter Κ (Kappa)), and mutation rate distribution among sites (shape parameter α). By estimating the likelihood surface with a multivariate normal distribution, the results from this analysis were then used to estimate the maximum likelihood branch lengths for the rooted tree and the associated variance-covariance matrix using the software Estbranches in the multidistribute package (Thorne & Kishino 2002). This was followed by the approximation of the posterior distributions of substitution rates and divergence times using Multidivtime (Thorne & Kishino 2002).

Although the method was designed to detect correlated changes in evolutionary rates among loci, only cyt *b* was used for this study since no 16S rRNA data were available from other parts of Africa. For the analyses to be successful it is important that fossil information be used in estimating divergence times (Thorne & Kishino 2002; Thorne & Kishino 2005). The fossil record for the first appearance of the genus *Saccostomus* (10.5 Mya; Senut *et al.* 1992) and those of recognisable extant species (4 Mya, Denys 1988) were used as fossil reference points during the analysis (see Table 2.8).

The results obtained were compared with divergence times obtained when following the direct method (Avise et al. 1998) using the *Mus/Rattus* divergence time of 12 Mya as a reference (Maree 2002; Ducroz 1998). Calibration was performed using the percentage of sequence divergence obtained from distances (Kimura 2 Parameter distances) between different lineages (Michaux et al. 2003; Maree 2002) and the lower limit of 4 Mya for the fossil record of *S. major* (Denys 1988).

Table 2.2. Migration matrix depicting model used during the migration analysis of *Saccostomus campestris* using samples from various localities and provinces (Namibia, Gauteng Province, Northern Cape Province, Mozambique, and Richards Bay) in southern Africa. s = symmetric migration, 0 = migration not estimated, and * = no restriction imposed. The starting values were estimated using Fst estimates making the migration rates the same for pairs of populations and different population sizes.

	Namibia	Gauteng	Northern Cape	Mozambique	Richards Bay
1: Namibia	*	s	s	0	0
2: Gauteng	s	*	s	s	s
3: Northern Cape	s	s	*	0	0
4: Mozambique	0	s	0	*	s
5: Richards Bay	0	s	0	s	*

2. R8s molecular dating

Another method of estimating divergence times was needed in order to compare with the conventional and Thorne & Kishino's (2002) methods of molecular dating. For this, program r8s version 1.5 (M.J. Sanderson http://ginger.ucdavis.edu/r8s, Sanderson 2003) was used. R8s uses parametric, nonparametric and semiparametric methods while emphasis is placed on the latter two in order to obtain reliable results when one works with datasets of unequal rates (Sanderson 2003). These two methods estimate unknown divergence times by smoothing the rapidity of rate change among lineages (Sanderson 2003). The rooted maximum parsimony tree was estimated using PAUP version 4.0b10 (Swofford 2000). Credibility for the dates was obtained by first generating 100 random bootstrap replicates using program SeqBoot from PHYLIP version 3.6b (Felsenstein 2005). The program PAUP version 4.0b10 (Swofford 2000) was used to estimate branch lengths for the maximum parsimony topology for each of the replicates in the dataset. The ingroup root was fixed to 10.5 Mya based on the fossil record for the genus *Saccostomus* (Senut *et al.* 1992; see Table 2.8.) and the relatively young *Saccostomus campestris* fossil of 2 My (Conroy *et al.* 1992; Avery 1998; see Table 2.8.). The penalized-likelihood analysis was selected using the cross-validation test (Sanderson 2003) and confidence intervals for divergence dates followed the curvature of the likelihood surface as done in r8s.

Results

Test for Saturation

There was no evidence of cyt *b* saturation at any position for either transitions or transversions (Fig. 2.2).

Relative rates test

In general, the results of the relative rates test showed differences in the rates of evolution within the species. Most differences involved specimens from Namibia, Botswana, and Gauteng and Northern Cape Provinces of South Africa. Rate differences were statistically significant for 38 % of comparisons (479 of 1275; $P < 0.05$), and highly statistically significant for 22 % (278 of 1275; $P < 0.01$). Of all the comparisons, 35 were lower than the Bonferroni probability value of 3.92E-5, which suggests differences in the rates of evolution.

Phylogenetic inference

Results of cyt *b* analysis for southern and eastern African specimens together suggest that the number of parsimony informative sites and GC content were higher than those of southern Africa specimens alone (Tables 2.4 and 2.7.). All neutrality tests were statistically non-significant (Table 2.4).

Cladograms with bootstrap values for maximum parsimony, maximum likelihood and Bayesian likelihood for 1) cyt *b* data from southern Africa; 2) combined cyt *b* and 16S rRNA data from southern Africa; and 3) combined cyt *b* data from both southern and east Africa are shown in Figures 2.3, 2.4, and 2.5, respectively. Similar trends are evident in these analyses, all of which revealed four distinct geographic groups in the southern African subregion: 1) Inland; 2) Namibia/Botswana; 3) Lowveld; and 4) KwaZulu-Natal groups, the latter being the most distinct of all derived lineages.

Nested Clade Analysis

Association of clades and locations at 1-step and 2-step levels were not statistically significant, (Fig. 2.7a). In the 3-step clades, there was marked geographical structure although clade 3-1 (Namibia/Botswana) was the only

one that exhibited significant differentiation (P = 0.01). The haplotype sequences, with alignments showing variable sites, are presented in Table 2..10.

The haplotype network for the 26 cyt *b* haplotypes from southern Africa is shown in Fig. 2.7a. The 95 % probability connection limit between observed haplotypes was restricted to 14 or fewer single-step mutations for cyt *b*. The haplotype tree is, however, not fully resolved because the KwaZulu-Natal haplotypes as well as those from Kuruman, Karoo and Hereroland could not be linked with any degree of statistical confidence. Similar to the phylogenetic analyses, the haplotype network revealed a geographic pattern. There are three clades, which occupy three distinct geographic areas, namely: 1) a Namibia/Botswana clade (excluding Francistown); 2) a Mozambique/Lowveld clade; and 3) a clade of the rest of inland South Africa including Francistown. Kasane (ka), Keetmanshoop (kh) and Caprivi (N30) make up the tips of clade 3-1, and they all seem to radiate from the Dordabis haplotype.

For the Mozambique/Lowveld clade, the Lowveld sub-clade is possibly an ancestor to the Mozambique sub-clade. The inland clade, which ranged from arid (Northern Cape Province) to mesic regions (Gauteng and Mpumalanga Provinces) and the overall haplotype network seem to radiate from this clade (from the 1-step clade level). Permutation analysis for the total cladogram revealed significant geographic structuring (P < 0.05). The inference key indicated that the present genetic structure is the result of either isolation by distance or long distance dispersal.

Figure 2.7b shows the 11 haplotypes from the eastern African group (Corti *et al.* 2004) that could not be connected to the southern African haplotypes. There is geographic structure in that there are two main clades, which have over 14 mutational steps between them and can therefore not be connected. They are the Zambian and Tanzanian clades. For the Tanzanian clade, there are two Mbungani-Chunya haplotypes of which two were connected to the network while the other was not, and three haplotypes from Kasanga incorporated into a single network. The three haplotypes from Kasanga (KAS)

were separated by up to seven mutational steps. One haplotype from Muze could not be linked to the network. The Zambian group was represented by four haplotypes, two of which formed a network and were separated by three mutational steps. The remaining two haplotypes could not be included in the network.

A network of 12 haplotypes observed from the 528 bp fragment of the 16S rRNA gene is presented in Figure 2.6. The topology is similar to that shown in Figure 2.7a in that different haplotypes seem to radiate from the Roodeplaat Dam haplotypes. The KwaZulu-Natal haplotype, although still distant from the others, is linked to the network through haplotypes from the inland areas and not through those from Mozambique. There is significant overlap in the haplotypes when presented in terms of geographic regions. The haplotypes with variable sites are presented in Table 2.10.

Gene flow analysis

The migration values obtained are presented in Table 2.7. High migration estimates were evident between Gauteng Province, Northern Cape Province and Namibia (>>10 individuals per generation). The migration estimate between KwaZulu-Natal and Gauteng Provinces was very small (0.1 individuals per generation). Migration between KwaZulu-Natal and Mozambique populations was, however, higher than between Gauteng and KwaZulu-Natal (0.75 individuals per generation). Maximum likelihood estimates of theta suggested that the Northern Cape population is by far the largest of the populations (Table 2.7). The smallest estimates of population sizes occurred in the Mozambique and KwaZulu-Natal populations.

Table 2.3. Inferred genetic statistical parameters for two mtDNA loci in *Saccostomus campestris* from southern and eastern Africa. The first column contains information regarding cyt *b* gene for the southern African specimens. The second column contains information regarding 16S rRNA for specimens from southern Africa. Column 3 contains statistical parameters for a combined dataset of 16S rRNA and Cyt *b* for the southern African group. The last column contains statistical parameters regarding cyt *b* gene for both southern African and eastern African specimens. Sample sizes (*n*) used are also indicated.

	Cyt *b* *n* = 32	16SrRNA *n* = 33	16S+ Cyt *b* *n* = 32	Cyt *b* (southern and eastern Africa) *n* = 49
Base pairs	1077	528	1605	1077
Variable sites	85	75	104	373
Parsimony Informative sites	43	10	53	298
Singleton sites	42	65	51	75
Nucleotide diversity	0.013	0.013	0.01	0.06
Number of haplotypes	26	12	27	43
Haplotype diversity	0.98	0.84	0.98	0.99
Transition/Transversion	1.9	1.63	2.3	3.2

Table 2.4. Neutrality tests for *Saccostomus campestris* from southern and eastern Africa. Tajima's D test calculates the two-tailed test for the neutral molecular evolution (Kimura 1983) by assuming that segregating sites and the average number of nucleotide differences are correlated (Rozas & Rozas 2004). Fu and Li's tests are used to test the predictions of neutral molecular evolution (Kimura 1983), by including or excluding the outgroup taxa in certain instances. D* and F* tests are based on the neutral model prediction that estimates of η/a1 [η is the total number of mutations, a1 = Σ (1/i)], (n -1) η s/n, and of k, are unbiased estimates of theta (*n* is the total number of nucleotide sequences; k is the average number of nucleotide differences between pairs of sequences). The second column contains cyt *b* gene statistics for the southern African specimens. The third column contains 16S rRNA statistics for specimens from southern Africa. Column 4 contains statistics for a combined dataset of 16S rRNA and cyt *b* of specimens from southern Africa. The last column contains statistics for a combined cyt *b* data of specimens from both southern and eastern Africa.

	Cyt *b* *n* = 32	16S rRNA *n* = 33	16S + Cyt *b* *n* = 32	Cyt *b* (southern and eastern Africa) *n* = 49
GC Content	0.415	0.424	0.418	0.415
Tajima's D test	-1.43 (*P* > 0.10)	-0.69 (*P* > 0.10)	-1.35 (*P* > 0.10)	-0.88 (*P* > 0.10)
Fu & Li's D* test	-1.96 (*P* > 0.10)	-0.77 (*P* > 0.10)	-1.77 (*P* > 0.10)	-1.24 (*P* > 0.10)
Fu & Li's F* test	-2.11 (*P* > 0.10)	-0.87 (*P* > 0.01)	-1.93 (*P* > 0.10)	-1.32 (*P* > 0.10)
Fu's F test	-7.95	-2.61	-7.71	-4.70
Strobeck's test	1.0	0.97	1.0	1.0

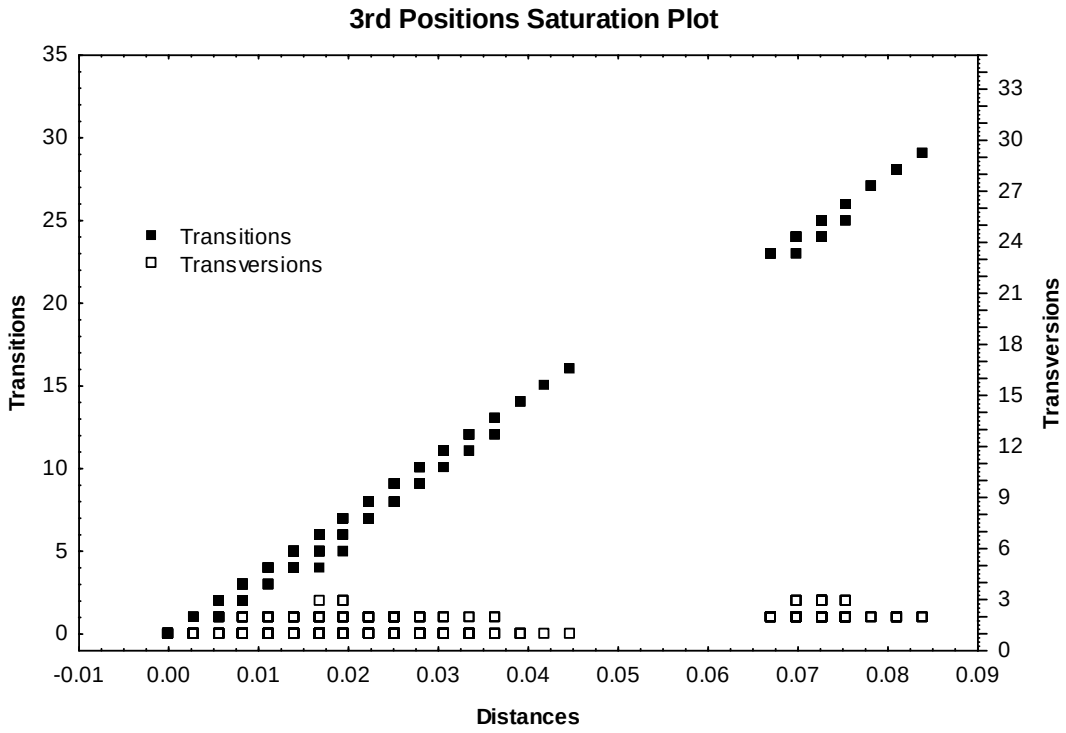


Figure 2.2. Graphs obtained from saturation analyses for 358 bp third positions from 1077bp long cyt *b* fragment of *Saccostomus campestris* from southern Africa. The graph shows both the number of transitions and transversions plotted against genetic distances (Kimura 2 Parameter distances) for all pairs of individuals.

Divergence times

Using the direct method (Avise *et al.* 1998), the highest sequence divergence (19.2 %; based on 1090 base pairs from cyt *b* sequences) was found between the Cricetomyinae and the Murinae, suggesting that the two groups diverged 16 Mya. Sequence divergence between *Saccostomus* and other cricetomyines was also relatively high at 15.7 % suggesting that the two groups diverged 13 Mya. Between *S. campestris* and *S. mearnsi*, divergence was almost half that at family level (8.7 %) suggesting a split 7 Mya. Between the South African/Namibian and Zambian populations it was 4.1 %, suggesting the divergence time of 4 Mya. Lastly, KwaZulu-Natal and inland groups had the lowest (1.1 %) sequence divergence, suggesting a split approximately 1 Mya. However, since the relative rates test demonstrated heterogeneity in molecular evolutionary rates within the Cricetomyinae, a considerable degree of scepticism exists with respect to the above estimates and more sophisticated methods of estimation are required.

Estimates from the Bayesian analysis suggested a longer time duration (15 Mya; 95 % probability interval of 11–23 Mya) since divergence between *S. campestris* and *S. mearnsi* than the direct method. The divergence time between the southern and eastern African (including Zambia) *S. campestris* was estimated at 11 Mya (95 % confidence interval of 6.7–15 Mya). KwaZulu-Natal and inland populations were estimated to have diverged 6.8 Mya (95 % confidence interval of 1.5–14 Mya). The divergence times for the rest of the inland clades were not biologically meaningful since these populations are subject to continuous gene flow.

The R8s method estimated the divergence between *S. campestris* and *S. mearnsi* to have occurred 5 Mya. The divergence time between the southern and eastern African (including Zambia) *S. campestris* was estimated at 2 Mya. KwaZulu-Natal and inland populations were estimated to have diverged 2.07 Mya. Similar to the Bayesian estimates, the divergence times for the rest of the inland clades were not biologically meaningful (Table 2.9).

Table 2.5. Alignment of *Saccostomus campestris* haplotypes from southern Africa showing only the variable sites on cyt *b* (a) and 16S rRNA (b). Dots (.) indicate the similarity with the first sequences on the alignments. The grey indicates shared characters. Number of individuals with a given haplotype is indicated by *n*. KZN denotes KwaZulu-Natal Province.

(a) Locality, Region	Haplotype	*n*	Cyt *b* Nucleotide sites 1 10 20 30 40 50 60 70 80 85
Dordabis, Namibia/Botswana	d1	1	GGATAGGAAATCCTATCGAAACCTATAGGCTTATATACAATATCTCCCCGGGGTGATCTCAACACATCTCTTGTTTTATACACCC
Dordabis, Namibia/Botswana	d2	2	.A.....A.....T.....C.....G...T.....C.
Dordabis, Namibia/Botswana	d4	1	.A.....A.....T.....C.....A.....T.....C.
Dordabis, Namibia/Botswana	d5	1	.A.....G....A.....T.....C.....T.....C.....C.
Keetmanshoop, Namibia/Botswana	kh1	1	.A.....C.....A.....T.G.....C.....T.....C.
Caprivi, Namibia/Botswana	N30	1	TA..G.TG.....A.....A.....TC.....CA..C.T...A..G.T.T.....C.
Kasane, Namibia/Botswana	ka1	1	.A.....C.....T.A..A..T.....T.....C.....T.....C.
Mozambique, Lowveld	mo1	1	CA.....G.....C.....T....G.T..CT.....A...CTG....G.....G.G...T.
Mozambique, Lowveld	mo2	1	.A.....G.....C.....C.....A...CTG....G.....G.G...T.
Hoedspruit, Lowveld	h1	1	.A.....G.....C.....C.....A.A...CT.....G.....C.....G.G...T.
Pretoria, Gauteng	p1	1	.A.....G.....C.....T.....C..T.....G.....T.
Roodeplaat, Gauteng	rd1	2	.A.....G...G.....C.....T.....C..T.....G.....T.
Groblersdal, Gauteng	g1	1	.A.....T..G.....C.....T.....C..T.....G.....T.
Groblersdal, Gauteng	g10	1	.A.....T.....A.....C.....G.....T..CA.....T.....G.T.
Groblersdal, Gauteng	g12	1	.A.....G.....C.....TT....A.....T.....T..T.
Manaaneng, Northern Cape	mn1	1	.A.C.....C.....G.....C.....T.....C..T.....C.....G.T.
Manaaneng, Northern Cape	mn2	1	.A.....G.....C....C.....T....A.....CT.....C.....T.
Manaaneng, Northern Cape	mn4	1	.A.....G.....C.....T.....T.....TC.....T.
Roodeplaat, Gauteng	rd3	2	.A.....G.....C.....T.....T.....T.
Roodeplaat, Gauteng	rd8	1	.A.....G.....G.....C.....T.....T.....T.
Tswalu, Northern Cape	ts1	1	.A.....C.....GC....C.....C.T....A.....CT.....T.
Tswalu, Northern Cape	ts2	1	.A.....C.....G.....C.....GG.....T.....T.G.G.....C.....T.
Tswalu, Northern Cape	ts3	1	.A.....TA.....A.....C..C.....T.....T.....TT
Francistown, Gauteng	f1	1	.A.....T.....A.....C.....T.....G...T.....T.
Richards Bay, KZN	rb3	4	.AG..A..GG....GC..G....CAAG.ATT...G.....C.....T.....AC..C.CT.GT.T....T.GTC...GC...AT.
Langjan, Gauteng	ln1	1	.A.....G...G.....C.....T.....T.....T.

(b) Locality	Haplotype	*n*	16S rRNA Nucleotide sites GATATCAGATATTACC
Tswalu, Northern Cape	ts1	12	
Tswalu, Northern Cape	ts2	1	.....T.
Dordabis, Namibia/Botswana	d2	1	.CC....A.....
Kasane, Namibia/Botswana	ka1	6	..C....A.....
Caprivi, Namibia/Botswana	N30	1	..C....A....A...
Richards Bay, KZN	rb19	5	....CT....TC.T.T
Roodeplaat, Gauteng	rd8	1	..C.....
Francistown, Gauteng	f1	2	..CG.....
Thomas Baines, Eastern Cape	tb1	1	..C.....G.....
Mozambique, Lowveld	mo2	2	C.....
Groblersdal, Gauteng	g10	1	....C.....
Hoedspruit, Lowveld	h1	1	C.....GA.C.....

Table 2.6. Genetic distance matrix (K2P method) for 1077 base pairs for cyt *b* of selected *Saccostomus campestris* specimens indicated in Fig. 2.5. Values above the diagonal indicate the standard error of estimates.

	rb3	mo1	h1	d1	KAR	kh1	rd3	ts1	g12	f1	KU1	HER	KAS	ZM9	MBU3	NDA
rb3		[0.006]	[0.005]	[0.005]	[0.007]	[0.005]	[0.005]	[0.005]	[0.005]	[0.005]	[0.008]	[0.009]	[0.013]	[0.013]	[0.013]	[0.015]
mo1	0.036		[0.003]	[0.004]	[0.006]	[0.004]	[0.003]	[0.004]	[0.004]	[0.004]	[0.007]	[0.008]	[0.013]	[0.014]	[0.013]	[0.015]
h1	0.030	0.007		[0.003]	[0.005]	[0.003]	[0.003]	[0.003]	[0.003]	[0.003]	[0.006]	[0.008]	[0.012]	[0.013]	[0.013]	[0.014]
d1	0.031	0.015	0.011		[0.005]	[0.002]	[0.002]	[0.003]	[0.003]	[0.002]	[0.007]	[0.007]	[0.012]	[0.013]	[0.013]	[0.014]
KAR	0.048	0.036	0.033	0.026		[0.005]	[0.005]	[0.005]	[0.005]	[0.005]	[0.008]	[0.009]	[0.012]	[0.013]	[0.013]	[0.015]
kh1	0.029	0.015	0.011	0.004	0.026		[0.002]	[0.003]	[0.003]	[0.002]	[0.007]	[0.007]	[0.012]	[0.013]	[0.013]	[0.014]
rd3	0.029	0.011	0.007	0.006	0.027	0.006		[0.002]	[0.002]	[0.001]	[0.006]	[0.007]	[0.012]	[0.013]	[0.013]	[0.014]
ts1	0.029	0.014	0.010	0.010	0.032	0.008	0.005		[0.002]	[0.002]	[0.006]	[0.007]	[0.012]	[0.013]	[0.013]	[0.014]
g12	0.032	0.014	0.010	0.008	0.028	0.008	0.003	0.006		[0.002]	[0.006]	[0.007]	[0.013]	[0.013]	[0.013]	[0.015]
f1	0.030	0.014	0.010	0.007	0.026	0.007	0.003	0.007	0.006		[0.006]	[0.007]	[0.012]	[0.013]	[0.013]	[0.014]
KU1	0.063	0.048	0.044	0.047	0.066	0.047	0.041	0.043	0.043	0.044		[0.006]	[0.014]	[0.014]	[0.015]	[0.016]
HER	0.080	0.066	0.061	0.059	0.080	0.059	0.055	0.059	0.057	0.054	0.051		[0.015]	[0.015]	[0.015]	[0.017]
KAS	0.129	0.127	0.119	0.116	0.130	0.118	0.116	0.118	0.117	0.119	0.148	0.165		[0.006]	[0.006]	[0.017]
ZM9	0.136	0.133	0.125	0.121	0.137	0.124	0.124	0.126	0.125	0.127	0.153	0.172	0.030		[0.007]	[0.017]
MBU3	0.144	0.138	0.130	0.127	0.136	0.130	0.128	0.131	0.130	0.132	0.166	0.182	0.037	0.050		[0.016]
NDA	0.200	0.195	0.187	0.190	0.214	0.190	0.190	0.187	0.191	0.191	0.223	0.239	0.195	0.190	0.202	

Table 2.7. Maximum likelihood estimates of migration between *Saccostomus campestris* populations from South Africa, Namibia, Botswana, and Mozambique together with ɵ (Nμ, N = population size, μ = mutation rate) for each population from the combined sequences of cyt b and 16S rRNA. ɵ was estimated with migrate-n (Beerli & Felsenstein 1999, 2001). Both M, the relative contribution of migration versus mutation in causing genetic diversity within a population (above the diagonal), and Nm, the effective number of migrants (below the diagonal) are presented. Maximum likelihood estimates of thetas (ɵ) are shown on the diagonal. Columns represent the receiving populations. L = lower 95 % percentile and U = upper 95 % percentile. Cells with asterisks indicate values that were not estimated because of the stepping-stone model employed in this analysis.

		Namibia	Gauteng	Northern Cape	Mozambique	KwaZulu-Natal
1: Namibia	*MLE*	0.008	42.336	33.008	*	*
	L	0.005	37.044	28.882		
	U	0.042	162.149	120.9		
2: Gauteng		3.65 *MLE*	0.001	581.193	37.750	0.000
		L	0.001	358.984	3.979	0.000
		U	0.004	2207.42	279.139	61.485
3: Northern Cape		>>10	>>10 *MLE*	4700000	*	*
			L	0.056		
			U	5.88E+09		
4: Mozambique		*	2.25	* *MLE*	0.004	33.222
				L	0.002	3.507
				U	0.012	121.486
5: KwaZulu-Natal		*	0.10	*	0.75 *MLE*	0.0000033
					L	0.000
					U	0.000

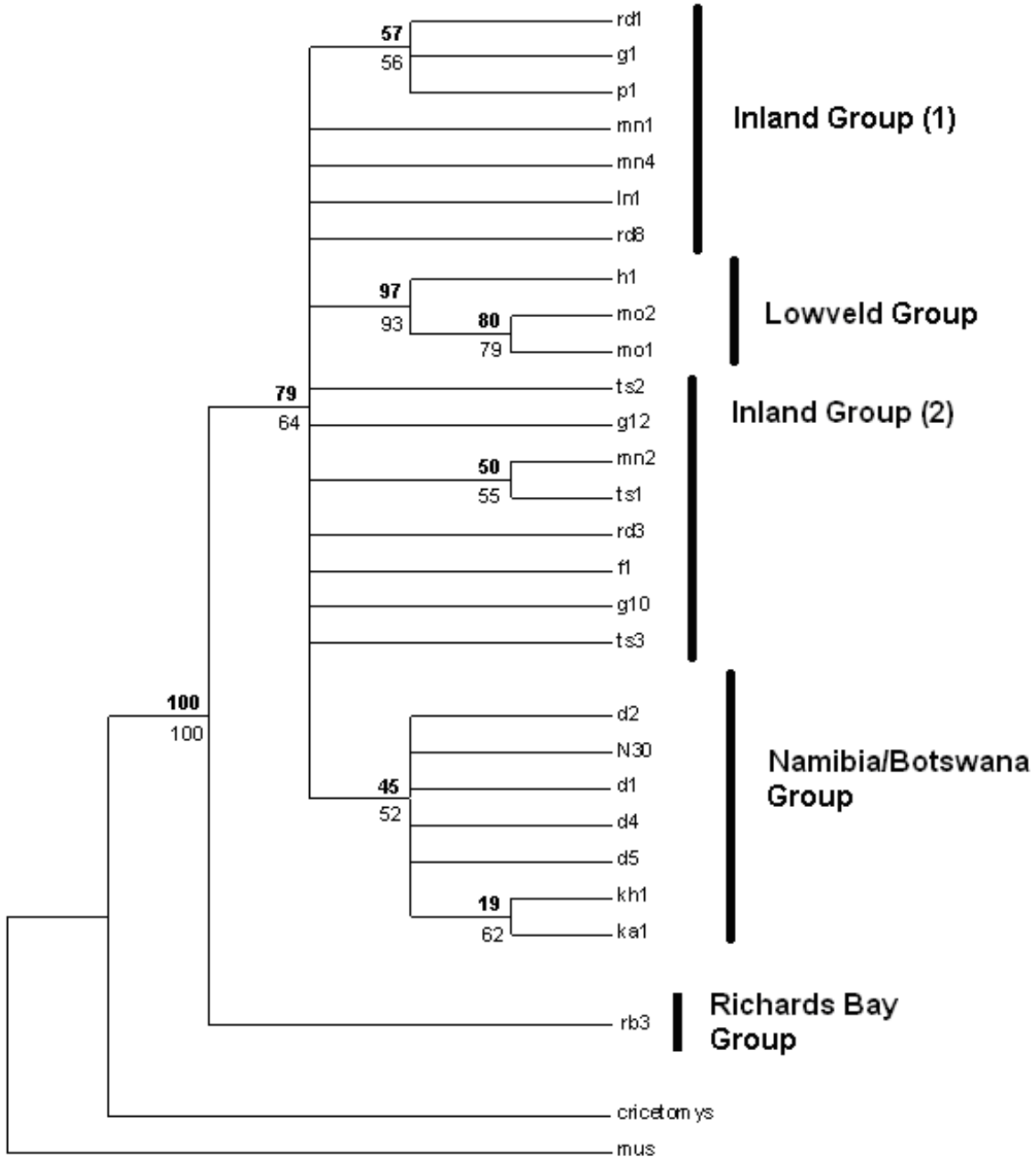


Figure 2.3. A cladogram with maximum parsimony and maximum likelihood bootstrap (bold) values for all positions of 1077 base-pair long fragment of cyt *b* sequences of individuals of *Saccostomus campestris* from southern Africa.

Table 2.8. Fossil records and important dates from molecular data of *Saccostomus* spp. and its nearest relatives through time in millions of years. The latest fossil record is 0.26 million years while the appearance of the genus *Saccostomus* is estimated to be around 10.5 million years.

Time in Million years	15 (Michaux *et al.* 2001; Steppan *et al.* 2004) Molecular data	12.5 (Michaux & Catzeflis 2000) Molecular Data	10.5 (Senut *et al.* 1992) Fossil record	4 (Denys 1988) Fossil record – cranium/teeth	2 (Conroy *et al.* 1992, Avery 1998) Fossil record	0.6 (Denys 1988) Fossil record – cranium /teeth	0.26 (Denys 1988) Fossil record – cranium /teeth
	Dendromurinae			*Dendromus (5 Mya)*			
	Steatomurinae		*Steatomys (8.5 Mya)*				
	Nesomyinae						
		Beamys					
	Cricetomyinae		***Saccostomus***	*S. major*			
				S. mearnsi			
				S. campestris	***S. campestris***	***S. campestris***	***S. campestris***
		Cricetomys					*Cricetomys* (0.4Mya)

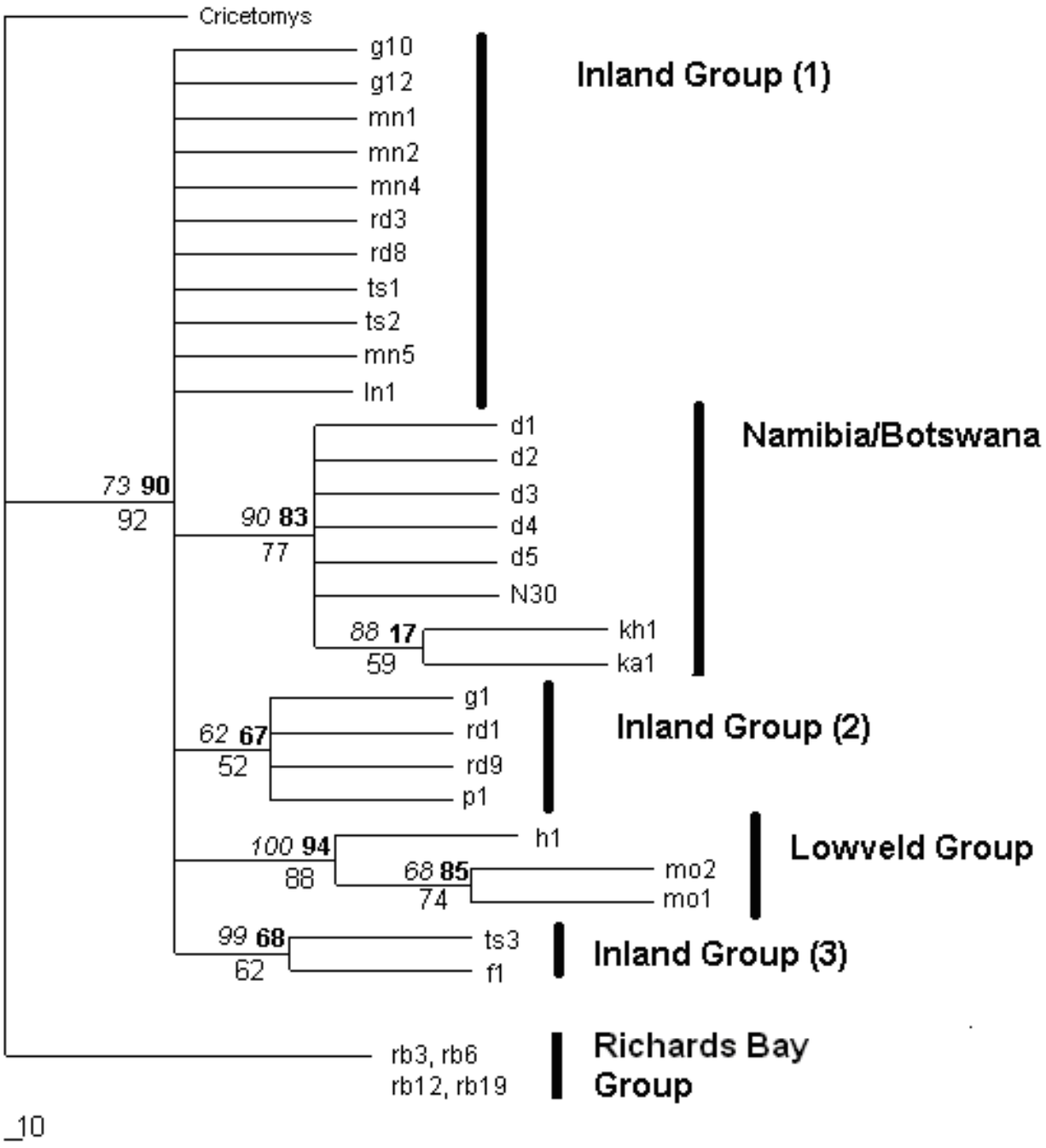


Figure 2.4. A phylogram with bootstrap values of a) Maximum parsimony (bottom), b) maximum likelihood (bold) and c) Bayesian likelihood (italics) from a combined sequence of 1607 base pairs (Cyt *b* and 16S rRNA) for coding and non-coding positions from 33 *Saccostomus campestris* individuals from southern Africa. All the bootstrap values were obtained from 1000 replicates.

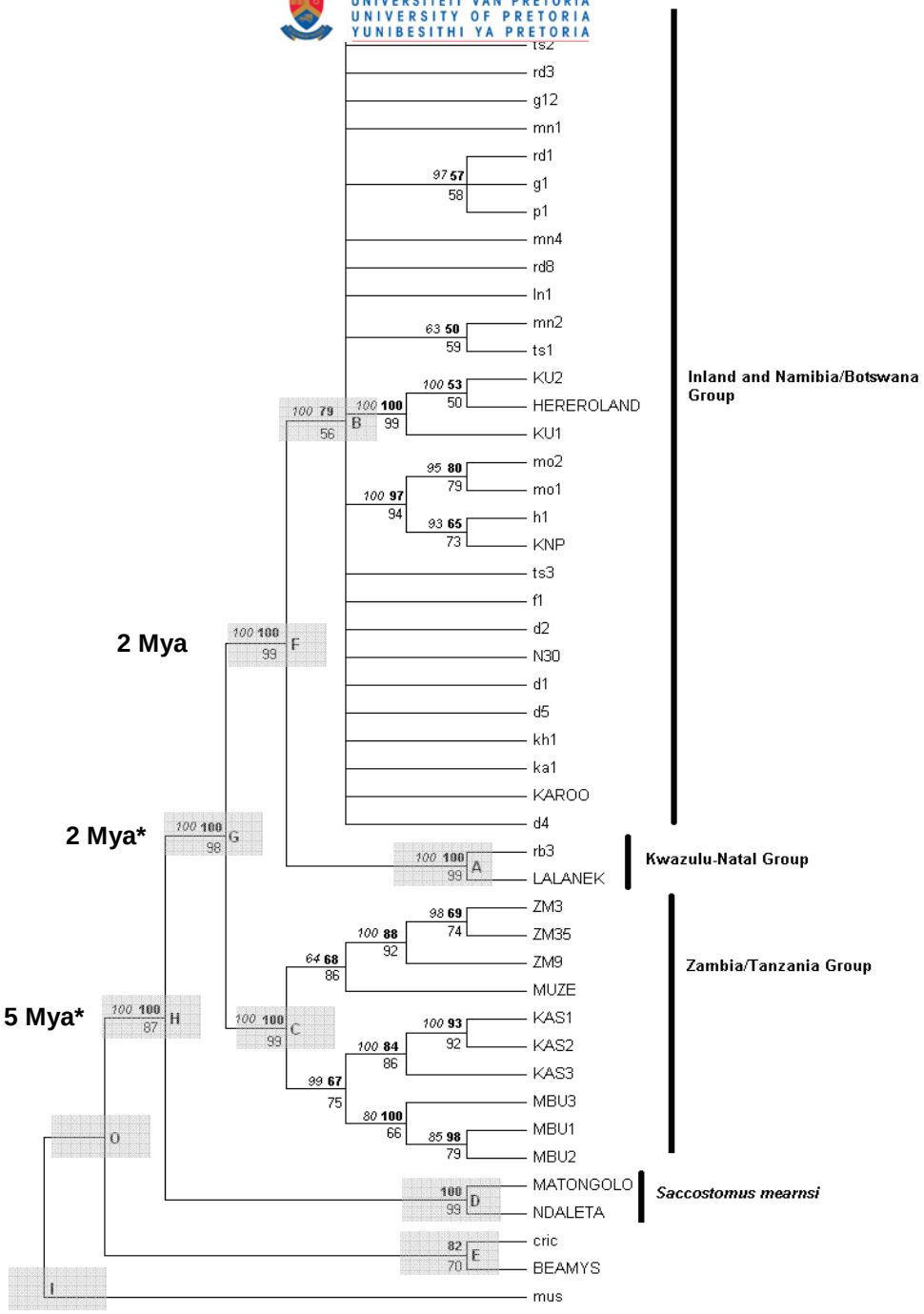


Figure 2.5. A phylogram with bootstrap values (Bottom: Parsimony; Bold: Maximum likelihood; Italic: Bayesian likelihood) from 1077 bp cyt *b* sequences for *Saccostomus campestris* from sub-Saharan Africa. The values for the highlighted nodes are presented in Table 2.9 below. The 19 capitalised specimens were used in a study by Corti *et al.* (2004). Analysis was based on all coding positions excluding non-coding regions. The tree also illustrates the three major splits in the evolution of the genus, with Bayesian estimated dates as described in the text. The asterisk (*) symbolizes calibration points.

Table 2.9. Results from r8s divergence estimates for each node on the phylogenetic analysis (Fig. 2.5), with the range, mean standard deviation, standard error and 95 % confidence interval.

Node	Range	Mean	Std. Dev.	Std. Err.	95% Conf. Int.
A	1.27-11.52	1.68	-	-	-
B	1.72-1.96	1.85	0.059 (1.79 - 1.91)	0.01	1.84 - 1.87
C	1.68-13.60	1.98	-	-	-
D	1.42-14.39	4.35	4.264 (0.09 - 8.62)	0.46	3.43 - 5.27
E	12.50-12.50	12.50	-	-	-
F	1.79-14.59	2.07	-	-	-
G	2.00-2.00	2.00	-	-	-
H	2.05-15.11	4.97	4.95 (0.02 - 9.91)	0.55	3.87 - 6.06
I	20.08-43.41	30.03	4.29 (25.74 - 34.31)	0.47	29.10 - 30.95
O	13.24–16.87	15.02	0.73 (14.29 – 15.74)	0.08	14.86 – 15.17

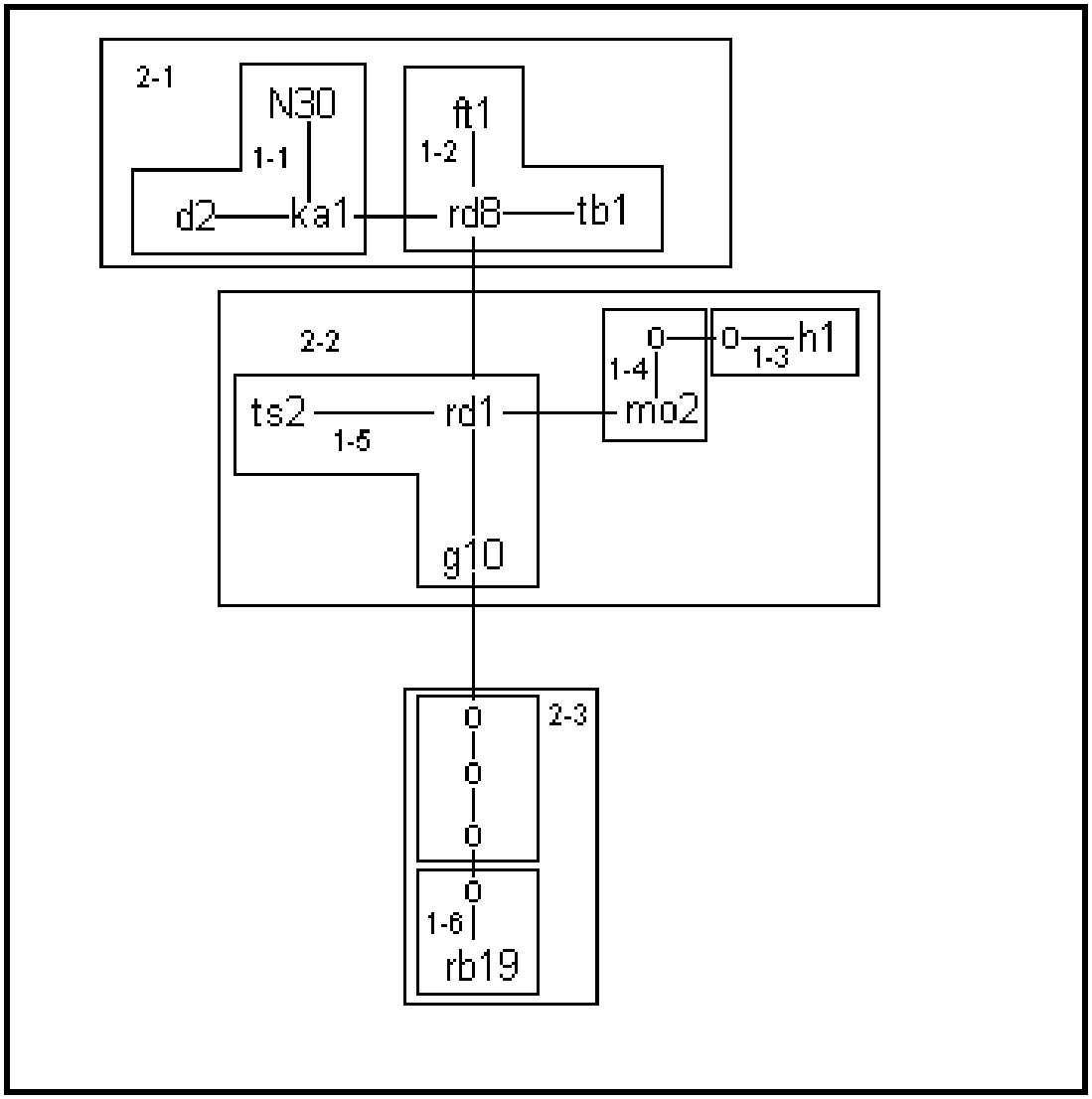
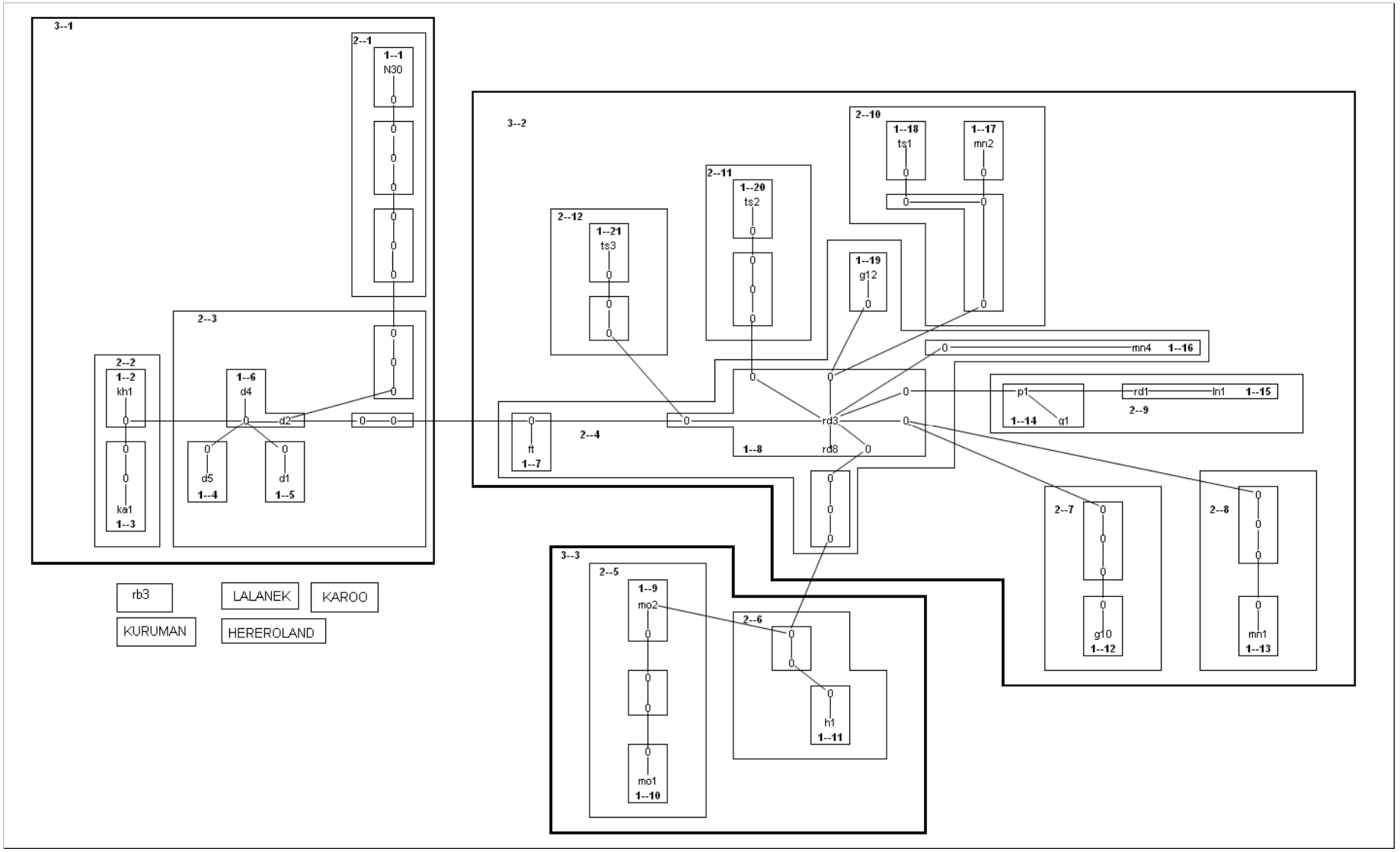


Figure 2.6. A *Saccostomus campestris* haplotype network for 528 bp 16SrRNA sequence fragments of 12 haplotypes observed in 13 localities from southern Africa. The network was estimated under 95 % connection limit at 14 steps. Haplotypes are named according to the first individual representing each haplotype.


3--1
2--1
1--1
N30
2--3
2--2
1--2
kh1
ka1
1--3
1--6
d4
d2
d5
1--4
d1
1--5
3--2
2--10
1--18
ts1
1--17
mn2
2--11
1--20
ts2
2--12
1--21
ts3
1--19
g12
mn4
1--16
p1
rd1
ln1
1--15
1--14
g1
2--9
ft
1--7
2--4
1--8
rd3
rd8
2--7
g10
1--12
2--8
mn1
1--13
3--3
2--5
1--9
mo2
mo1
1--10
2--6
h1
1--11
rb3
KURUMAN
LALANEK
HERERO LAND
KAROO

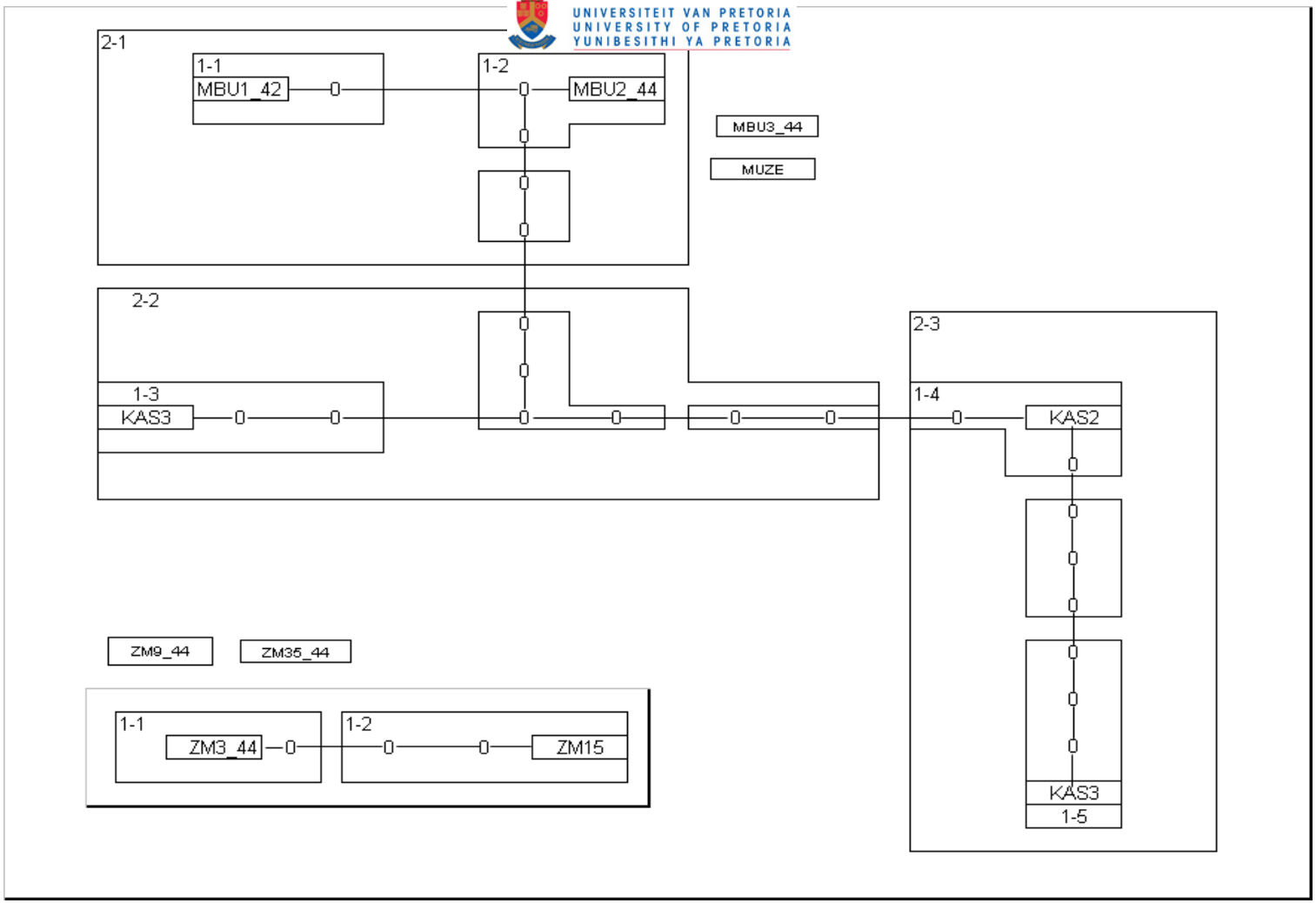


Figure 2.7. A haplotype network for a 1077 bp long fragment of cyt *b* gene from *Saccostomus campestris* from (a) southern Africa and (b) Zambia/Tanzania. Each line in the network symbolises a single mutational step, 0 indicates missing haplotypes within the network. Instead of using numbers for haplotypes, the identity of the first individual of the group was used.

Discussion

Divergence times

Some of the present patterns in species diversity (mostly North American birds and mammals) are attributed to the events of the Pleistocene (2 Mya), in that speciation happened in part or completely within this epoch (Zink 1993; Avise *et al.* 1998; Avise & Walker 1998). While some of the approaches used to estimate divergence times in the present study have similar conclusions, others show different conclusions (Table 2.10.).

Due to rate heterogeneity the conventional method (Avise *et al.* 1998) was not used in the present study. R8s estimate that the split between *S. campestris* and *S. mearnsi* occurred during the Early Pliocene, approximately 5 Mya, with the split between the eastern and southern African *S. campestris* occurring during the Late Pliocene approximately 2 Mya. The split between KwaZulu-Natal *S. campestris* and the rest of the inland *S. campestris* was estimated at a similar time of 2 Mya. The results correlate with the fossil records from Early Pliocene to Late Pleistocene and the Holocene reported by Denys (1998). On the other hand, the Bayesian analysis suggests that different populations of *S. campestris* were separated before the Pleistocene and survived severe climatic oscillations reported to have occurred during that period. Divergence between *S. campestris* from southern and eastern Africa occurred during the Miocene (11 Mya). Furthermore, the KwaZulu-Natal population diverged from the inland populations during the Late Miocene (6 Mya). This time period coincides with the differentiation that took place in South Africa in the south-western region during Early Pliocene (Denys 1998). The use of fossil record from 10 Mya to calibrate the clock is justifiable because fossils provide concrete evidence that members of the genus occupied the southern African subregion more than 10 Mya. However, the confidence intervals of the estimated phylogenetic divergence dates from the three methods overlap markedly (Table 2.9), with the estimates from the direct method and r8s being within the 95 % probability intervals of the Bayesian estimates, albeit close to the lower limits (for the direct method). This suggests that stronger inferences regarding the major splits within the genus *Saccostomus* involving the two existing species and within *S. campestris* can be made.

Collating the results from the three methods used in estimating divergence times, some suggestions can be made, i.e., that: 1) divergence between different *Saccostomus* groups observed at present did not occur over a short period of time; 2) the date of 10 Mya is the first appearance date (FAD), suggesting that the genus occupied the region during that time period if not for a much longer time duration; and 3) the suggested times are just estimates and as a result are open to correction. However, it needs to be emphasized that these suggestions are made while recognizing the well-known *Mus/Rattus* dichotomy that is believed to have occurred 12 Mya.

The tree topologies from maximum parsimony, maximum likelihood and Bayesian analyses with very good bootstrap supports also suggest that the KwaZulu-Natal population may be the remnant of an ancestral stock as a result of the historical events during the Miocene that gave rise to the present geographic pattern within *S. campestris* (Figs. 2.3; 2.4; 2.5). This pattern may be linked to the events (contraction/expansion of the savannah biome, volcanism and tectonic activity) that led to the patterns observed in the speciation of the bushveld gerbil, *Tatera* in East Africa (Colangelo *et al.* 2005).

Previous studies suggested that divergence between *Saccostomus* species and among *S. campestris* populations occurred more recently. Corti *et al.* (2004) estimated that the divergence between *S. campestris* and *S. mearnsi* occurred 7 Mya, whereas in the present study, the divergence between these two species is estimated at around 11 Mya. Similarly, Corti *et al*. (2004) estimated the time of divergence between the southern and eastern African populations to be 3.7 Mya while the estimated time in the present study is 6 Mya. This discrepancy may be due differences in calibration points used in the two studies. Corti *et al.* (2004) used *Mus*/*Rattus* and *Tatera*/*Gerbillurus* in their calibration, while the present study was exclusively based on fossil calibration.

Phylogenetic inferences for southern African populations

The neutrality tests did not reveal significant differences between loci in departure from assumptions of neutrality (Table 2.4). The analyses based on either cyt *b* and 16S rRNA or the two loci combined (see Table 2.3) show similar trends of genetic affinities of the different populations. Maximum parsimony, maximum likelihood, Bayesian as well as neighbour-joining analyses showed clear patterns of geographical organisation in the two main lineages from the KwaZulu-Natal and inland populations. The KwaZulu-Natal populations from Richards Bay and Lalanek form a distinct basal group, well-supported by high bootstrap values (Figs. 2.3, 2.4 and 2.5).

This is despite genetic differences between the two populations with the Lalanek sample clustering outside the Richards Bay samples, supported by high bootstrap values for maximum parsimony, maximum likelihood and Bayesian likelihood analyses (Fig. 2.5). The isolation of the KwaZulu-Natal lineage suggests a relatively long period of separation between pouched mice from KwaZulu-Natal and the rest of southern Africa. Furthermore, the KwaZulu-Natal lineage forms an ancestral lineage to the rest of *S. campestris* lineages in southern Africa. Although the sub-divisions within the large inland clade are not clearly resolved, they can be sub-divided into the Mozambique/Hoedspruit lineage (supported by high bootstrap values; Figs. 2.3, 2.4, and 2.5), the Namibia/northern Botswana lineage (Figs. 2.3 and 2.4), and the other inland populations.

Table 2.10. Duration since major splits (Mya) estimated for the main groups within *S. campestris* using the three methods including the *campestris/mearnsi* split. For the conventional method sequence divergences are also shown. For the Bayesian and r8s analyses, presented are the ranges obtained from the analyses.

Dichotomies	Bayesian	R8s
KZN – Inland	6.8	2.0
	1.5-14	1.8 -14.6
Southern – Eastern Africa	11.0	2.0
	6.7-15	2.0-2.0
S. mearnsi – S. campestris	15	5.0
	11-23	2.1-15.1

Gene flow between regions

The maximum likelihood estimate of relative population size for the Northern Cape Province population was higher than the rest of the populations. This reflects a high level of genetic diversity (cyt *b* and 16S rRNA) within this population, together with the availability of suitable habitats (sandy soil and *Acacia* sp. trees) over a large area. The Northern Cape population also appears to have high rates of gene flow with its immediate neighbours, Gauteng and Namibia/Botswana populations, reflected by high migration estimates among the three populations (Table 2.8.). This is expected because the absence of a prominent barrier between these large populations provides an opportunity for high migration rates between pairs of populations.

On the other hand, the estimates of population size for KwaZulu-Natal and the lowveld were small compared to the other populations (Table 2.8.). Bittner & King (2003), in their study on garter snakes (*Thamnophis sirtalis sirtalis),* found that island populations may have experienced fluctuations in population size over time as a result of their less stable ecology in comparison with mainland populations. Although the KwaZulu-Natal population is not on an island, present results suggest that it is isolated from other populations; therefore the garter snake example may also apply to this population.

In addition, migration between the Gauteng Province and KwaZulu-Natal populations was very low, suggesting the presence of a barrier between the two populations (Table 2.8.). The possible route of gene flow from Gauteng Province to KwaZulu-Natal may be via Mozambique as is suggested by the measurable but relatively low migration rate between KwaZulu-Natal and Mozambique. These results indicate that the Drakensberg Mountains may be a physical barrier between the inland populations and those from KwaZulu-Natal.

Historical events giving rise to the present pattern of molecular variation

The nested clade analysis (NCA) for cyt *b* is consistent with the results from the phylogenetic and gene flow analyses. Although there is no geographic structure within clades at restricted geographic scales, the higher levels (3-step clades) reveal a strong geographic structure. Clade 3-1 consists of

populations from Namibia and Botswana, clade 3-2 contains populations from inland South Africa (i.e., from Groblersdal in Mpumalanga Province to the Kalahari in the North West and Northern Cape Provinces), and clade 3-3 consists of the lowveld and Mozambique clade (Fig. 2.7a).

The three clades represent different habitat types in southern Africa (Gordon 1986): the lowveld clade (Hoedspruit and Mozambique) comprises mesic or coastal woodland, the inland clade is a combination of specimens from the south-west arid zone (Northern Cape Province) and savannah woodland (Pretoria, Langjan, Francistown, and Groblersdal), while the Namibia/Botswana clade consists of specimens from the northerly arid zone. At a finer scale, the haplotype tree for cyt *b* suggests that the Dordabis (d4) lineage is an ancestral haplotype in the south-west arid zone (i.e., the Namibia/Botswana group), and Roodeplaat (Rd3) is an ancestral haplotype in the north-eastern group which is predominantly savannah woodland.

For the geographical area and number of specimens (*n* = 32) sampled, 26 haplotypes is very high. When six additional southern African sequences from Corti *et al.* (2004) were included, this increased to 29 haplotypes. With the whole dataset of *S. campestris* (including Corti *et al.* 2004 data for eastern Africa), the haplotype number increased to 43 from 49 specimens. KwaZulu-Natal, Kuruman, Hereroland and Karoo haplotypes could not be joined with the rest in the haplotype network (cyt *b* gene as well as combined sequences), resulting in the exclusion of these localities (Fig. 2.7a) from the nested clade analysis.

In a similar study on fox sparrows, *Passerella iliaca* Zink (1993) concluded that vicariant events rather than isolation by distance could best explain diversity within mtDNA in fox sparrows. The results from the present analysis show that there is robust genetic structure within the species in southern Africa, but because of the sparse locations where samples were found, the present genetic structure may be explained by isolation by distance or long distance dispersal.

Nested clade analysis (NCA) of the 16S rRNA also adds another perspective to the interpretation of the results obtained in the present study. First, all the haplotypes in the network are joined, and second, there are only 12 haplotypes from 34 sequences for specimens from southern Africa (Fig. 2.6). Although the KwaZulu-Natal population would be expected to link with the Mozambique population, the analysis is not consistent with gene flow analysis, in that it suggests that the KwaZulu-Natal population is more closely related to the Gauteng Province populations than to the Lowveld (Mozambique) population. Since 16S rRNA evolves at a slower rate than cyt *b* (Anderson *et al.* 1982), the affinities evident in the 16S haplotype tree probably reflect more ancient relationships than is evident in the gene flow analysis and NCA using cyt *b*.

Inferences on the origin of *Saccostomus campestris*

Maximum parsimony, maximum likelihood, and Bayesian analyses using combined cyt *b* data from both southern Africa (this study) and eastern Africa (Corti *et al.* 2004) revealed two distinct lineages (southern and east Africa) supported by high bootstrap values. These results also suggest that the East African populations are ancestral to southern African populations, supported by the fossil record. Denys (1988) found that *S. mearnsi* from northern Tanzania shared more primitive characters with the 3.7 million year old fossil of *S. major* from Tanzania than with *S. campestris*. In Tanzania, *S. campestris* and *S. mearnsi* occur in the southern and northern parts of the country, respectively.

Palaeoecological conditions were probably suitable for *Saccostomus* since caesalpinioid legumes (which included *Acacia mahangensis*) already occupied northern Tanzania (Jacobs & Herendeen 2004; Jacobs 2004) during the Eocene (46 Mya). The discovery of fruit-bearing *Grewia* fossils together with some woodland tree species from the Kenyan Miocene (24 Mya; reviewed by Collinson *et al.* 1991), may also suggest that the suitable habitat for *Saccostomus* has been present for a long time.

The discovery of a 10.5 million year old fossil of *Saccostomus* in Namibia (Senut *et al.* 1992) indicates that the genus was present before 3.7 Mya in southern Africa and that there were suitable habitats in the western parts of South Africa. Tankard & Rogers (1978) reported that during the pre-Miocene (25 Mya), the vegetation in southern Africa was sclerophyllous, with some grassland and xerophyllous flora. This suggests the presence of *Acacia* trees during that period, as several species of this genus are xeric in nature. This may have happened before the origin of the relatively young Namib Desert, which dates back to the Miocene (23-25 Mya) (Tankard & Rogers 1978, Bobe in press.).

Pouched mice prefer open woodland habitats where they can burrow easily and hoard seeds and fruit (Kingdon 1974). However, there is no clear indication of the direction of legume colonization in southern Africa except the contemporary report by Acocks (1975) that *Acacia erioloba* spread into South Africa from the north. The phylogenetic analysis suggests that the genus *Saccostomus* has a central African origin, with *S. campestris* expanding its range south-wards. A similar trend in the phylogeography and speciation of African mole-rats of the genus *Cryptomys* suggests an east African origin, and further suggests that their divergence was influenced by the formation of the Great East African Rift Valley and consequent vegetation, as well as by climate change during the Miocene (Faulkes *et al.* 2004). Speciation within *Cryptomys* (12–17 Mya) coincides with the estimated time (15 Mya) of divergence between *S. campestris* and *S. mearnsi* suggesting that speciation within the burrowing *Saccostomus* and *Cryptomys* may have been influenced by similar environmental changes. A possible explanation is that *Saccostomus* migrated into southern Africa from eastern Africa through Mozambique. Before migrating into KwaZulu-Natal, it moved back north towards Mozambique and branched around the eastern escarpment and thereafter colonized the rest of southern Africa (Fig. 2.8).

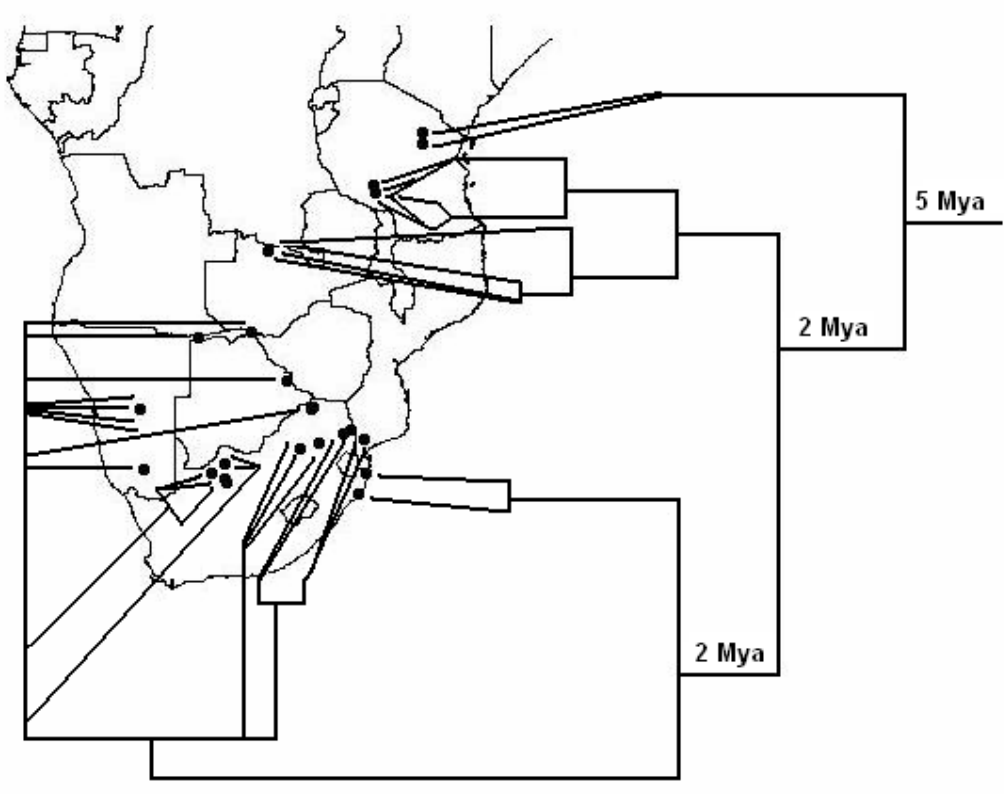


Figure 2.8. Maximum parsimony tree for 1077 bp long cytochrome *b* sequences indicating the geographic origin of specimen of *Saccostomus campestris* and *S. mearnsi* with time in millions of years indicating the major splits in the phylogeny.

Habitat as a factor causing genetic variation in *Saccostomus campestris*

The vegetation of southern Africa comprises coastal thicket on the eastern coast, semi-arid woodland covering large parts of Namibia and Botswana, mesic woodland in the central north, and grassland on the central plateau (Low & Rebelo 1998). While *S. campestris* is not common in the plateau grassland, the clusters of samples from the parsimony analyses (Figs. 2.3–2.5) closely reflect vegetation associations. The KwaZulu-Natal and the Lowveld populations associate with the coastal bush, the Namibia-Botswana populations with semi-arid woodland, and the inland populations with mesic woodland.

These results are consistent with those of Ellison (1992) who found that populations from similar ecological areas showed similar physiological characteristics. He found that body sizes of individuals from different localities were positively correlated with rainfall and were heritable. Ellison (1992) also found that pouched mice from colder regions showed the capacity to produce more body heat than animals from warmer regions and speculated that the observed correlation between local temperature and the extent of torpor was probably genetic.

What does this mean in terms of habitat diversity observed in southern Africa? It is possible that there are few physical barriers that fragment the extant habitats. As a result, the inland group seems to be panmictic, with numerous populations experiencing relatively high rates of gene flow. The association between *Saccostomus* and open woodlands dominated by legumes and having soft soils (e.g., sand and cultivated areas) has long been documented (Kingdon 1974). This may explain the strong association between *S. campestris* and *Acacia* trees. Pouched mice are known to dig their burrows next to a food source and move in the direction of food availability when their source has been depleted (Kingdon 1974).

In South Africa, the geographic range of *S. campestris* seems to overlap with the yellow golden mole (*Calcochloris obtusirotris*), the narrow headed golden mole (genus: *Amblysomus*), and the Cape golden mole (genus: *Chrysochloris*) because they are all limited by the availability of soft soils to dig

their burrows (Kingdon 1997; S. Maree pers. comm.). In terms of vegetation, it overlaps with *Acacia* rats of the genus *Thallomys*, which seem to be confined to dry woodlands dominated by *Acacia* and savannas from South Africa to Ethiopia (Kingdon 1997).

Although *S. campestris* occurs mainly in the savannah biome, the different populations have two factors in common, namely the presence of soft soils (preferably sand) and *Acacia* trees. While soft soils may be patchy in distribution, *Acacia* trees are prevalent throughout the distribution of *Saccostomus*, which stretches from the South African Northern Cape Province, through North West Province, into Gauteng Province, Mpumalanga Province, and some parts of KwaZulu-Natal and Limpopo Provinces (Low and Rebelo 1998). The soft soil and the availability of *Acacia* trees seem to be a limiting factor in the distribution of the pouched mouse that may have led to the genetically diverse lineages observed in the present study.

Conclusion

If the sandy habitat with appropriate seed-bearing food trees (e.g., *Acacia erioloba*) were a habitat constraint, one would expect a large homogenous population of *S. campestris* in central and western southern Africa with more isolated populations in the east. This is consistent with the gene flow estimates (NCA, phylogenetic analyses) in which the Namibia/Botswana populations formed a cluster from a very large geographical area, with large estimated population sizes for some populations within this area. On the other hand, the eastern populations formed smaller, distinct clusters, mostly with small population size estimates and smaller estimates of between-population migration. The most parsimonious explanation is low rates of colonisation in the east between patches of suitable habitat, resulting in genetic isolation of these newly established populations.

Such colonisation of suitable habitat appears to have happened very long ago (about 5-10 Mya), resulting in ancient populations still observed at present. The results of mtDNA analysis do not necessarily suggest the possibility of different species. This is because population isolation does not automatically result in speciation, even when there are long-standing genetic differences between populations. Other aspects that need to be investigated further include reproductive isolation as well as behavioural and ecological changes that may have occurred during that time period (Ferguson 2002).

With regard to the suggestion that *S. campestris* comprises two species in southern Africa represented by 2*n* = 46 and 2*n* = 30-38 cytotypes (Gordon 1986), and the additional inferences of Corti *et al.* (2004) that the species comprises different species and subspecies, there is no unequivocal evidence in the present study to suggest that *S. campestris* may be a species complex. Apart from the molecular data used in the present study, other aspects of the biology of *Saccostomus* need to be investigated further before robust taxonomic conclusions can be reached.

Chapter 3: Systematic implications of karyotypic variation in the pouched mouse, *Saccostomus campestris* from southern Africa

Abstract

The nature and extent of chromosomal variation within the karyotypically variant pouched mouse, *Saccostomus campestris* from southern Africa is assessed in an attempt to address questions relating to its taxonomic status. Ten cytotypes ranging in diploid number ranging from 2*n* = 30 to 2*n* = 46 are reported involving autosomal Robertsonian fusions. There is evidence of a geographic structuring in the distribution of cytotypes in southern Africa with cytotypes with high diploid numbers originating from the more mesic eastern part of the subregion while cytotypes with low diploid numbers originate from the more arid west. A phylogenetic analysis of partial cytochrome *b* (1077 bp) and 16S rRNA (528 bp) sequences suggest that the high diploid numbered 2*n* = 46 cytotype from the North-East Dune Forest in KwaZulu-Natal represents the ancestral cytotype. Cytotypes with low chromosomal numbers appear to have arisen several times independently in southern Africa. There are three X-chromosome variants showing evidence of geographic structuring caused by a single pericentric inversion followed by the addition of genetic material, possibly heterochromatin. X-chromosome Variant 1, the only variant present in the ancestral cytotype is found in all cytotypes throughout the distributional range of the species in southern Africa, variant 2 is found in cytotypes from areas that receive < 600 mm of rainfall per annum, while variant 3, although only found in females, has the same geographic distribution as that of variant 2. The nature and extent of karyotypic variation in *S. campestris* is compared and interpreted with published karyotypes of *Saccostomus* from East (Tanzania) and Central (Zambia) Africa. Published physiological data suggest a strong mesic-to-arid adaptation to climatic variation in southern Africa. The karyotypic variation in *S. campestris* from southern Africa probably reflects a monotypic taxon and that the reduction in chromosome numbers is possibly due to its adaptation to cold and dry conditions in the more arid south-western Africa.

Key words: *Saccostomus campestris*, karyotypic variants, autosomes, Robertsonian fusion, X-chromosomes, biotic zones, systematics, distribution, southern Africa

3.1. Introduction

Pouched mice of the genus *Saccostomus* (Peters 1896) are endemic to Africa, south of the Sahara with a wide distribution ranging from the Western Cape Province in South Africa north-ward through East Africa to Ethiopia (De Graaff 1981). The genus is represented by two species, *S. campestris* Peters, 1846 from the Western Cape Province to south-west Tanzania and *S. mearnsi* (Heller 1910) from north-east Tanzania to Ethiopia (Corti *et al.* 2004). *Saccostomus campestris* shows an unusual degree of karyotypic variation where up to 16 chromosomal variants ranging from diploid numbers 2*n* = 28 to 2*n* = 50 have been reported (Gordon 1986). There is therefore, a critical need to gain an insight into the evolutionary and taxonomic implications of this unusually high degree of karyotypic variation within *S. campestris*.

In many rodent genera, closely related species sometimes have different karyotypes. In blind mole rats, *Spalax ehrenbergi*, with a diploid number that ranges from 2*n* = 52 to 2*n* = 60, Robertsonian translocations and chromosomal micro-changes, pericentric inversions as well as centromeric shifting have been regarded as the leading causes of chromosomal variation (Wharman *et al.* 1985). These changes in the four well-established karyotypic races in this species are considered to be actively involved in the process of speciation (Wharman *et al.* 1985; Nevo *et al.* 1993; Nevo *et al.* 1995; Nevo *et al.* 2000).

Several reasons are considered to account for the karyotypic diversity in different taxa. A classical example is the house mouse, *Mus musculus domesticus* from western Europe where up to 40 chromosomal races encompassing 89 different combinations of centric fusions (see Nachman & Searle 1995 for a review) that have arisen over the last 10,000 years have been reported. The driving forces behind this pattern have been attributed to mutation, drift, selection, and meiotic processes (Nachman & Searle 1995).

Similarly, in indigenous humans from Italy and Greece, the Aegean Sea has been implicated as a barrier to gene flow (Di Giacomo *et al.* 2003) and that local founder events as well as genetic drift may have been the main determinants of the Y chromosomal diversity at small spatial scales.

Many, if not most karyotypic changes reflect Robertsonian fusions, which become fixed within local populations (King 1993; Nachman & Searle 2002). These fusions, however, are not known to create reproductive barriers between founder populations and the parental stock because normal meiotic processes are not affected (King 1993). This is illustrated by Pialek *et al.* (2001) who devised a simulation model which predicted that hybrid geographic karyotypic races of *M. musculus* in the Alps can arise at a considerable frequency resulting in recombinant homozygous karyotypes.

Sometimes, however, chromosomal variation can lead to reproductive isolation if isolated populations fixed for monobrachial centric fusions were to hybridise (Baker & Bickham 1986) resulting in meiotic impairment and reproductive isolation. In sub-Saharan Africa, mammalian chromosomal differentiation that may have led to speciation in mole-rats (Faulkes *et al.* 2004) and gerbils (Colangelo *et al.* 2005) for example, has been attributed to the formation of the Great East African Rift Valley and the subsequent climatic changes that led to the expansion and contraction of the savannah biome in this region.

Taylor (2000) reviewed patterns of chromosomal variation among southern African rodents, focussing on hybridization among chromosomal races and suggested that: 1) vlei rats (*Otomys* spp.) and four-striped grass mice (*Rhabdomys pumilio*) had freely inter-breeding chromosomal races which led to reproductive isolation of hybrids due to tandem fusions in certain races; 2) some sibling species which were morphologically conserved but genetically divergent such as multi-mammate mice (*Mastomys natalensis* and *M. coucha*), tree rats (*Thallomys paedulcus* and *T. nigricauda*) as well as veld rats (*Aethomys chrysophilus* and *A. ineptus*) were reproductively isolated, showing signs of a correlation between genetic distance and the onset of reproductive isolation; and 3) gerbils (*Tatera brantsii* and *T. afra*) showed little to no pre-

zygotic isolation but had a well-developed male biased post-zygotic isolation linked to the Y chromosome.

While patterns within other southern African taxa have been explored and defined, there is also a need to gain an insight into the patterns of karyotypic variation in *S. campestris*. The available data suggest some geographic structuring in the distribution of cytotypes within the subregion where the cytotypes with high diploid numbers ranging from $2n = 42$ to $2n = 46$ have been recorded to occur in the north-eastern savannah woodland, while the cytotypes with low diploid numbers ranging from $2n = 28$ to $2n = 38$ are associated with the south-west arid zone (Fig. 3.1). Although there was no reported polymorphism in sperm morphology associated with the karyotypic variants within *S. campestris*, Gordon (1986) using protein electrophoretic data suggested the presence of two sibling species in southern Africa. Gordon (1986) associated one of the species to the $2n = 46$ cytotype from the north-eastern parts of southern Africa, and the other to $2n = 28–38$ cytotypes from the south-western parts of the subregion. Subsequently, Corti *et al.* (2004) went further to suggest that *Saccostomus* from the southern African subregion represented a species-complex whose nomenclature was in critical need of a revision.

Consequently, in the present study, chromosomal variation within *S. campestris* from southern Africa is assessed in order to address the following questions: 1) Is there any congruence in geographic patterns of variation between cytogenetic and DNA sequence data?; 2) Given the high degree of karyotypic variation and based on DNA analysis, which karyotype is ancestral?; 3) Are the karyotypes with low diploid numbers monophyletic as suggested by Corti *et al.* (2004)?; 4) Is there any support for the recognition of two karyotypically-based species within *S. campestris* as suggested by Gordon (1986)?; and 5) Is the pattern of chromosomal centric fusions in the pouched mouse random or do the chromosomes follow a sequential pattern of fusion or fission?

3.2. Materials and methods

Study area and samples

The present study is based on 57 animals collected from 13 localities in southern Africa (Table 3.1; Fig. 3.1). Animals were live-trapped using Sherman traps (H.B. Sherman Traps Inc., Florida, U.S.A.) baited with a mixture of peanut butter, syrup, oat meal and sunflower oil. After capture, during transportation, and in the laboratory, animals were kept in polyurethane cages with wood shavings as bedding, and with mouse pellets and water provided *ad libitum*. Animals were subsequently sacrificed using halothane inhalation. Animals were maintained under the guidelines of the American Society of Mammalogists (ASM; http:///www.mammalogy.org/committees/index.asp; Animal Care and Use Committee 1998), and under the approval of the Animal Ethics Committee of the University of Pretoria, Pretoria, South Africa. Six karyotypes from southern Africa studied by Corti et al (2004), as well as their karyotypes from central Africa and for *S. mearnsi* were used as comparative material.

Blood sampling and culturing

The collection of blood was based on the protocol of Barch *et al.* (1997) as follows: 1) 0.3 ml of blood was obtained from anaesthetised animals and transferred into bone marrow transport medium with heparin; 2) the blood was then cultured in growth medium with fetal bovine serum and incubated at 37° C in 5 % CO_2 for 68 hours; 3) 0.2 ml Colcemid was used to arrest metaphases before harvest; 4) the mix was centrifuged at 1200 rpm for 10 minutes after which the supernatant was discarded; and 5) 10 ml Potassium Chloride (KCl) was used for hypotonic treatment after which the mix was fixed in 10 ml of 3:1 ratio of a Methanol/Acetic acid fix at –20° C. The mix was then centrifuged and the supernatant removed. This was repeated four times until the culture was clear. The mix was then stored at –20° C.

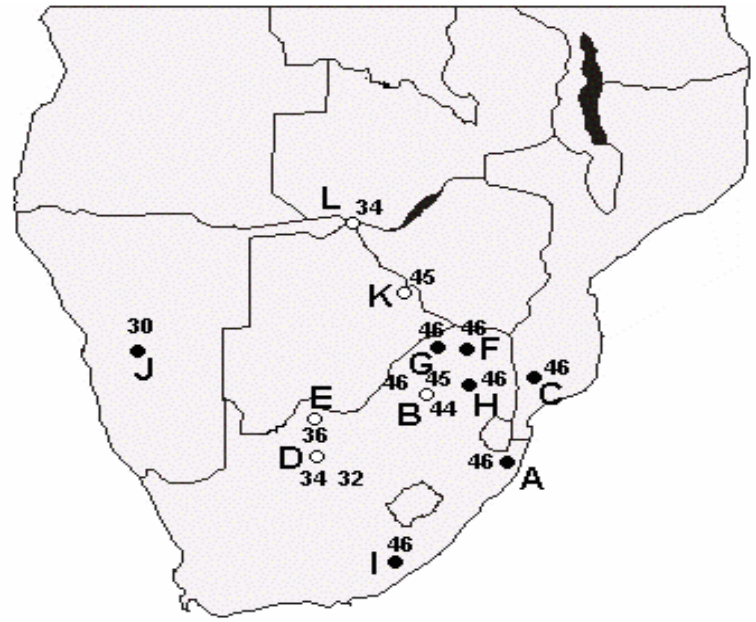


Figure 3.1. A map showing collecting localities (A–L) of the pouched mouse, *Saccostomus campestris* from southern Africa examined in the present study. The geographic coordinates of these sampled localities are presented in Table 3.1. The numbers indicate the diploid (2*n*) chromosome number associated with each sampled locality. Closed circles indicate homozygous populations with or without fixed Robertsonian centric fusions, while open circles denote populations where heterozygotes with respect to chromosomal translocations were found.

Slide preparation

Slides were cleaned in 99 % ethanol for up to 18 hours and then stored at –20° C. The cell suspension was washed with fresh methanol/acetic acid fixative by centrifuging and removing the supernatant after which cells were re-suspended in 0.5 ml fixative. 5–6 drops of re-suspended cells were then dripped onto the slides. The slides were allowed to dry and viewed under inverted microscope to examine the mitotic index and the quality of the metaphase spreads. The slides were then aged at 60° C for up to 48 hours before Giemsa-staining.

Banding and staining

Before staining, slides were treated in trypsin (0.014 g trypsin dissolved in 250 ml PBS) solution for a few seconds (10–30 seconds) at 30° C, rinsed in a stop bath (1 ml FBS in 250 ml PBS) and lastly rinsed in rinsing bath (250 ml PBS). The slides were then stained with Giemsa stain (30 ml Giemsa; 60 ml Gurr Buffer; 160 ml d H_2O) for 5–15 minutes and examined under a light microscope to establish whether adjustments were needed for the exposure of metaphases to trypsin and Giemsa.

Karyotyping

Metaphases were viewed using a light microscope at 1000 x magnification and under oil immersion. Metaphase photos were taken with a built-in camera and karyotyped using Quips® Karyotyping System computer programme (Vysis, Bergisch – Gladbach, Germany). Chromosome pairs were aligned according to their banding patterns and arranged according to decreasing length. Within- and between-population diploid number (2*n*) differences and number of autosomal chromosomal arms (NFa) were compared. Where possible, 10 metaphases per specimen were analysed. Differences within populations were first assessed in order to establish whether the populations exhibited chromosomal polymorphism before comparisons between cytotypes were made. Representative karyotypes from each cytotype were used for between-cytotype comparisons. The karyotypes in the present study were also compared with published karyotypes of *Saccostomus* from East (Tanzania) and Central (Zambia) Africa reported by Corti *et al.* (2004).

Molecular analyses

Total mitochondrial genome was extracted from blood and muscle tissue of karyotyped individuals and cytochrome *b* (cyt *b*; 1077 bp) and 16S rRNA (528 bp) mitochondrial loci sequenced (Chapter 2). The resulting sequence alignments were analysed using maximum parsimony, maximum likelihood, and Bayesian analyses and haplotype genealogies. Maximum parsimony analysis was based on MEGA version 2.0 (Kumar *et al.* 2001). DNASp version 4 (Rozas & Rozas 2004) was used to determine the number of polymorphic, parsimony informative, and singleton sites. Maximum likelihood was based on program PHYML version 2.4.4 (Guindon & Gascuel 2003) while Bayesian analysis was based on MrBayes version 2.1 (Huelsenbeck & Ronquist 2001). Haplotype trees were estimated using TCS version 1.13 (Clement *et al.* 2000) with linkage probabilities of above 95% (14 mutational steps for both cyt *b* and 16S rRNA sequences).

3.3. Results

Cytogenetic analysis

Ten karyotypes ranging from 2*n* = 30 to 46 with the number of chromosomal arms for the autosomes (NFa) ranging from 44 to 54 were identified (Table 3.1). The X- and Y-chromosomes were identified by comparing karyotypes from male and female individuals. The following karyotypes in decreasing diploid numbers were identified where particular combinations of chromosomes refer to the codes indicated in Fig. 3.3 and Appendix 3.1:

1) **2*n* = 46** (*n* = 32).–This karyotype was characterised by three pairs of large sub-telocentric chromosomes and occasionally a pair of small metacentric chromosomes. The remaining chromosome pairs were all acrocentric (Table 3.1; Fig. 3.2a). The karyotype was found in individuals from the Eastern Cape, KwaZulu-Natal, Mpumalanga, and Limpopo Provinces of South Africa and Mozambique. The NFa number ranged from 48 to 50. This karyotype is considered the standard with which other karyotypes are compared.

2) **2*n* = 45; NFa = 48–50** (*n* = 4).–This karyotype was characterised by centric fusion of the long arm of one chromosome 6 and the long arm of one chromosome 9 of the 2*n* = 46 karyotype. The cytotype occurred in sympatry with the 2*n* = 44 and 46 cytotypes from Roodeplaat Dam, Gauteng Province, South Africa (Fig. 3.3).

3) **2*n* = 44** (*n* = 4).–These four specimens from Roodeplaat Dam, Gauteng Province, South Africa occurred in sympatry with the 2*n* = 45 and 46 cytotypes. In this karyotype, the fused chromosomes described in the 2*n* = 45 karyotype form a homologous pair (6.9) of metacentric chromosomes (Fig. 3.3; combination a). The NFa number was 50.

4) **2*n* = 36** (*n* = 4).–This cytotype was characterised by the following two sympatric variants from Manaaneng, Northern Cape Province, South Africa:

   a. **2*n* = 36a** (*n* = 1).–The autosomal chromosomes consisted of a pair of metacentric chromosomes (6.9) and an unpaired metacentric chromosome (8.12; combination f), a large unpaired sub-metacentric chromosome (3.5; combination d), three pairs of sub-telocentric chromosomes (1.17; 2.18; 4.13; combinations b, c, e), two unpaired acrocentric chromosomes and 11 pairs of acrocentric chromosomes. The NFa number was 46.

   b. **2*n* = 36b** (*n* = 3).–The large sub-metacentric chromosome (3.5; combination d) identified in the 2*n* = 36a variant was absent from these individuals. However, there was an unpaired metacentric chromosome (8.12; combination f), an unpaired sub-metacentric chromosome (7.16; combination g), three pairs of sub-telocentric chromosomes (1.17; 2.18; 4.13; combinations b, c, e), four unpaired and 12 paired acrocentric chromosomes. The NFa number was 46.

5) **2*n* = 34** (*n* = 3).–This cytotype was characterised by the following two allopatric variants:

a. **2*n* = 34a** (*n* = 1).–The autosomal chromosomes of this karyotype from Kasane, Botswana consisted of two pairs of metacentric chromosomes (6.9; 8.12; combinations a, f), one pair of sub-metacentric chromosomes (7.16), three pairs of sub-telocentric chromosomes (1.17; 2.18; 4.13), and 10 pairs of acrocentric chromosomes.

b. **2*n* = 34b** (*n* = 2).–This karyotype from Tswalu, Northern Cape Province, South Africa was characterised by two pairs of metacentric chromosomes (6.9; 8.12), three pairs of sub-telocentric chromosomes (1.17; 2.18; 4.13) and 12 pairs of acrocentric chromosomes. The NFa number was 46.

6) **2*n* = 32** (*n* = 1).–This karyotype from Tswalu, Northern Cape Province, South Africa was characterised by two pairs of metacentric chromosomes (6.9; 8.12), two pairs of sub-metacentric chromosomes (3.5; 7.16), three pairs of sub-telocentric chromosomes (1.17; 2.18; 4.13), a pair of small metacentrics and six pairs of small acrocentric chromosomes (Fig. 3.2b). The NFa number was 46.

7) **2*n* = 30** (*n* = 5).–This karyotype from Dordabis, Namibia was characterised by three pairs of metacentric chromosomes (6.9; 8.12; 14.19), two pairs of sub-metacentric chromosomes (3.5; 7.16), three pairs of sub-telocentric chromosomes (1.17; 2.18; 4.13) and seven pairs of acrocentric chromosomes. The NFa number was 44.

Sex chromosomes

X-chromosome.–The X-chromosome was characterised by the following three variants designated Variants 1, 2, and 3 (Fig. 3.4 & 3.8; Appendix 3.2).

1) **Variant 1**.–This X-chromosome was characterised by a large sub-metacentric with three dark bands on the long (q) arm just after the half-way mark from the centromere and two dark bands on the short (p) arm. The difference in arm lengths is distinct. This variant was found at all localities except Francistown in Botswana.

2) **Variant 2**.–This X-chromosome is metacentric with two dark bands on the q arm, one close to the centromere, and the second, half-way on the q-arm. The p-arm has three bands. This variant was found in areas of less than 600 ml per annum, i.e. away from the coastal dune forest on the east coast of South Africa.

3) **Variant 3**.–This X-chromosome is a large sub-metacentric with banding patterns as in Variant 2 except that the p-arm is longer than the q-arm. This was only found in two areas sampled, Tswalu and Manaaneng.

The sequence of events that may have led to this polymorphism in the X-chromosome in the pouched mouse from southern Africa is illustrated in Fig, 3.4). While a pericentric inversion of X-chromosome Variant 1 led to the formation of Variant 2, Variant 3 arose from additional material to Variant 2.

Y-chromosome.–The Y-chromosome was characterised by a single small sub-telocentric chromosome. No polymorphism was found between the Y-chromosomes of different cytotypes.

Comparisons between karyotypes from East, Central and southern Africa

A comparison of chromosomal re-arrangements and the morphology of the gonosomes of published karyotypes of *S. mearnsi* with a diploid number of 2*n* = 32 and an NFa = 44 from Ndaleta, Tanzania (Corti *et al.* 2004) and that of *S. campestris* from Tswalu, South Africa with the same diploid and NFa numbers showed marked chromosomal differences. Consequently, the karyotype of *S.*

mearnsi from Ndaleta was compared with both the low diploid numbered 2*n* = 32 cytotype and the high diploid numbered 2*n* = 46 cytotype of *S. campestris* from southern Africa. Similar to Corti *et al.* (2004), chromosome 1 of *S. mearnsi* corresponds with a fusion of acrocentric chromosomes 2 and 8 from the 2*n* = 46 *S. campestris* cytotype, while in the 2*n* = 32 cytotype from southern Africa, chromosome 8 fused with chromosome 12 (combination f; Fig. 3.3) and chromosome 2 fused with chromosome 18 (combination c; Fig. 3.3).

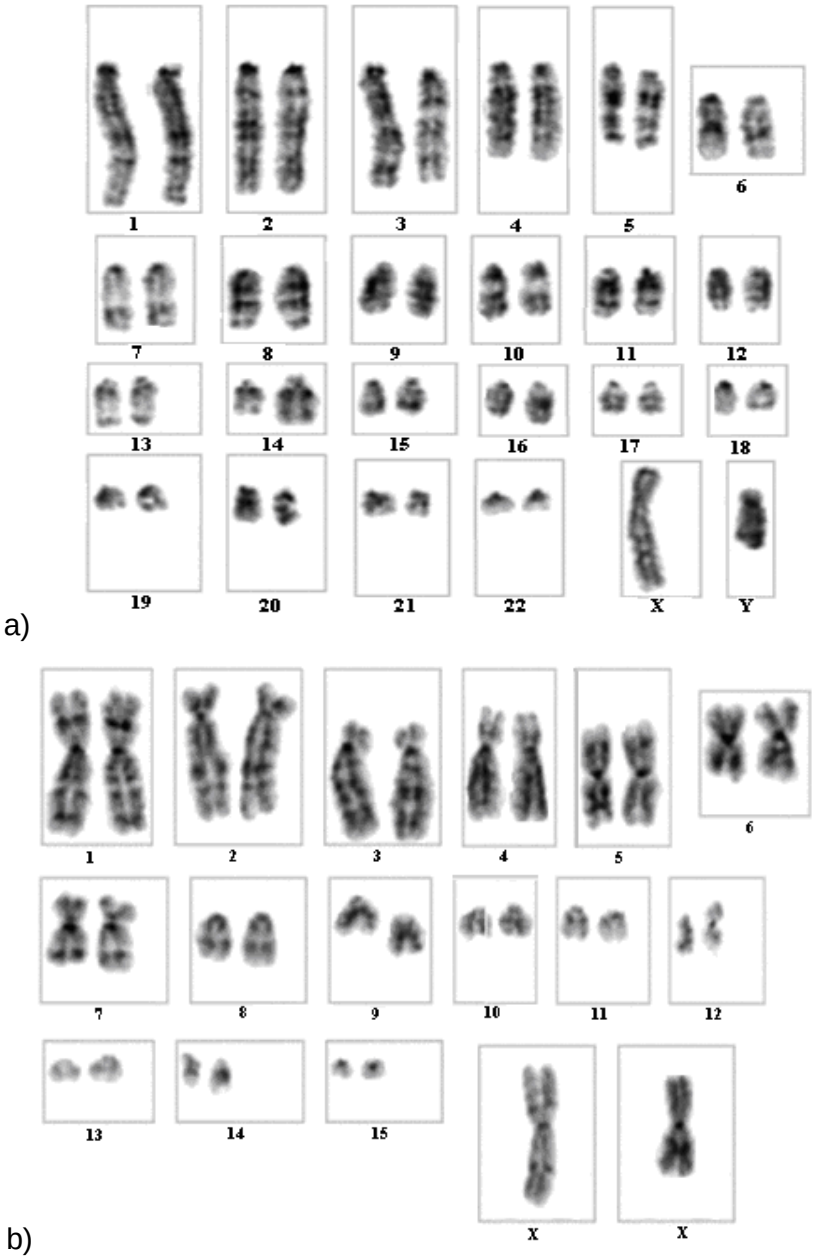
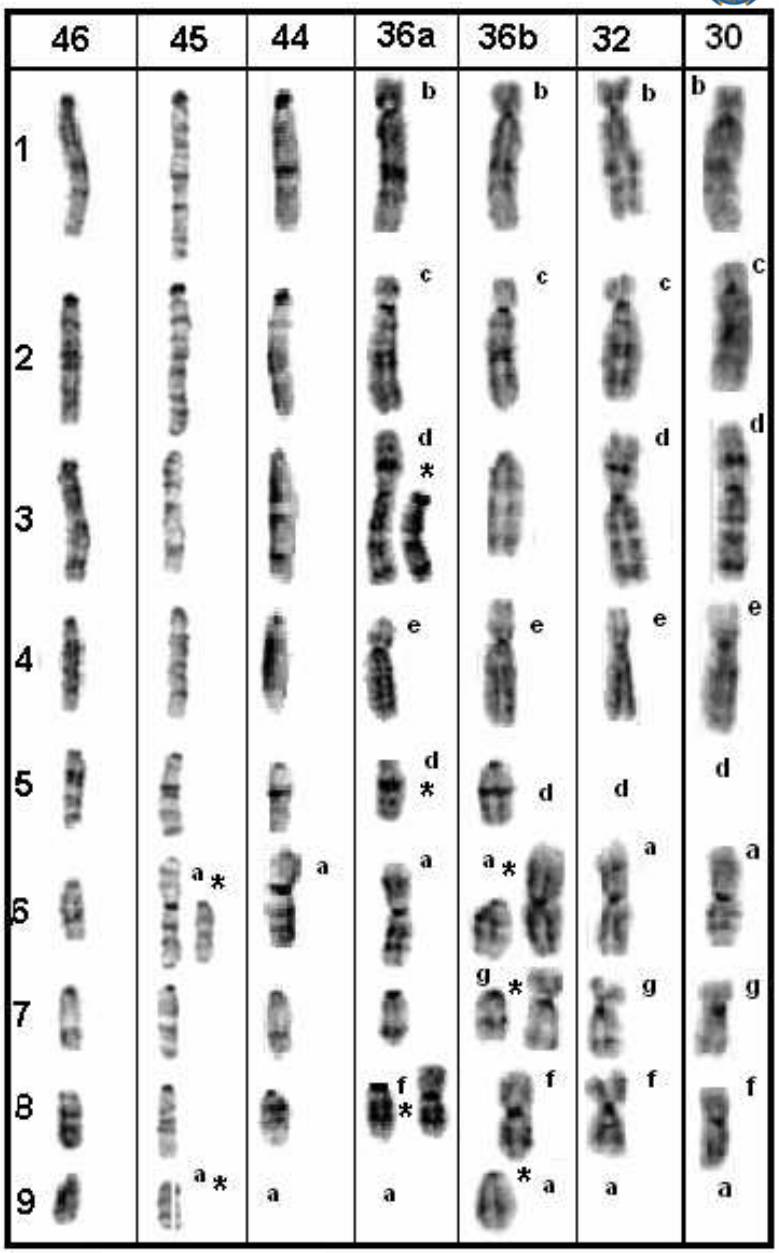


Figure 3.2 G-banded karyotype of the pouched mouse, *Saccostomus campestris* from southern Africa. The karyotypes ranged from a diploid number of 2*n* = 30 to 2*n* = 46. The karyotypes represent: a) a karyotype of a male specimen from KwaZulu-Natal Province, South Africa with a diploid number of 2*n* = 46 and an NFa = 50, and b) a karyotype of a female individual from Tswalu, Northern Cape Province, South Africa with a diploid number of 2*n* = 32 and an NFa = 46.

Table 3.1. Geographic coordinates and collecting localities, sample size, sexes, diploid (2*n*) numbers and autosomal numbers of chromosomal arms (NFa) of the pouched mouse, *Saccostomus campestris* from southern Africa examined in the present study. The locality codes (A–L) correspond to those in Fig. 3.1.

Locality	Geographic coordinates	*n*	Males	Females	Diploid number	NFa number
A) Richards Bay	28°42' S; 32°15' E	16	13	5	46	50, 52
B) Roodeplaat	25°25' S; 28°05' E	12	7	5	44, 45, 46	50, 52
C) Magude	24º57' S; 32º37' E	2	1	1	46	50, 52
D) Tswalu	27°13' S; 22°26' E	4	2	2	32/34	46
E) Manaaneng	26°35' S; 23°23' E	5	2	3	36	46
F) Langjan	22º51' S; 29º14' E	3	2	1	46	52
G) Blouberg	22º59' S; 29º07' E	1	1	-	46	52
H) Blaawbank	25°15' S; 29°34' E	5	3	2	46	50, 52
I) Thomas Baines	33º18' S; 26º32' E	1	-	1	46	50
J) Dordabis	22°56' S; 17°38' E	5	1	4	30	44
K) Francistown	13°21' S; 27°30' E	1	1	-	45	46
L) Kasane	17°49' S; 24°09' E	1	1	-	34	44


46
45
44
36a
36b
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30
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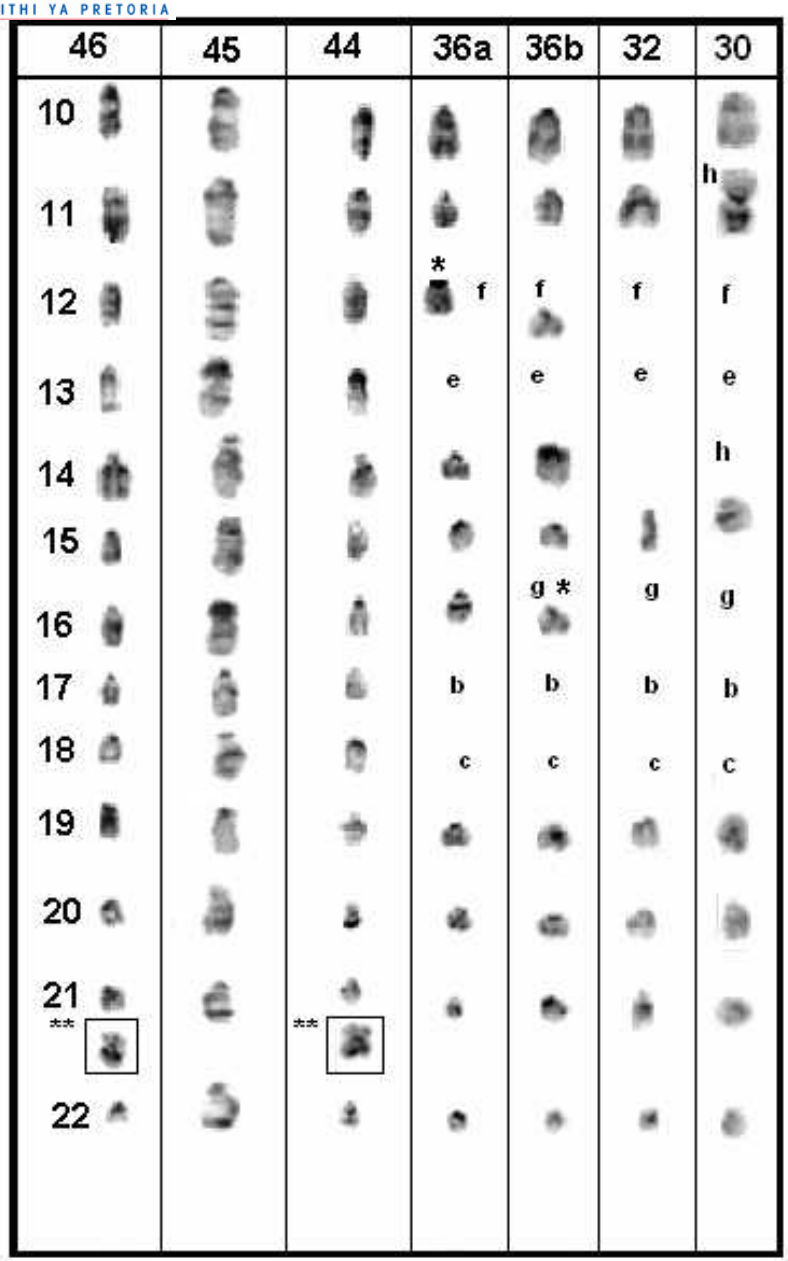
8
9


46
45
44
36a
36b
32
30
10
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12
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21
22

Figure 3.3. Comparisons between autosomes in the high diploid numbered 2*n* = 46 and lower diploid numbered individuals in the pouched mouse, *Saccostomus campestris* from southern Africa examined in the present investigation. The figure illustrates centric fusions of different chromosomes that resulted in the reduction of the diploid number (2*n*) of some individuals. The chromosomes are arranged in decreasing length. Letters a – h (referred to in the text) represent the different centric fusions to illustrate the consistency among cytotypes if present. A single asterisk depicts hybrid chromosomes for single centric fusions, while a double asterisk represents small metacentric chromosomes.

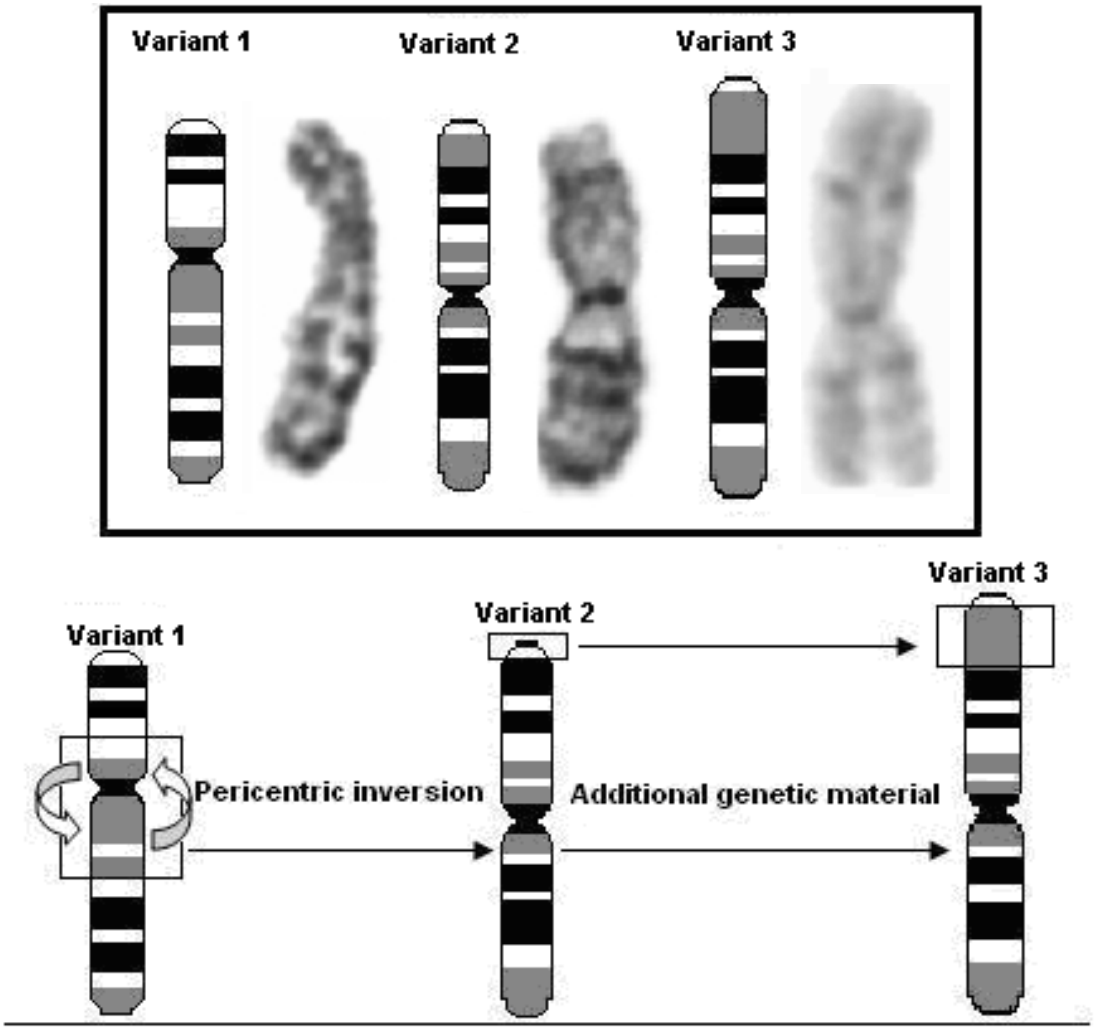

Figure 3.4. Ideograms of X-chromosome variants (Variant 1–3) in the pouched mouse, *Saccostomus campestris* from southern Africa examined in the present study showing chromosomal polymorphism. The bottom part of the figure illustrates the possible sequence of events that led to this polymorphism.

In addition, chromosome 3 of *S. mearnsi* is homologous with chromosome 4 of *S. campestris* from the southern African 2*n* = 32 karyotype while the q-arms of the other sub-metacentrics: 4, 5, 6, and 7 from *S. mearnsi* (Corti *et al.* 2004) correspond with acrocentric chromosomes 4, 6, 7, and 8 of the karyotype of *S. campestris* from KwaZulu-Natal Province, South Africa. The difference between the sub-telocentric chromosome 1 from KwaZulu-Natal and Mbungani-Chunya, Zambia and the sub-metacentric chromosome 2 from Ndaleta (Corti *et al.* 2004) is considered to be a monobrachial homology. The gonosomes in the karyotypes of *S. mearnsi* from Ndaleta and *S. campestris* cytotypes from southern Africa differ. For *S. mearnsi*, the X-chromosome is a large acrocentric chromosome, while it is represented by a large sub-metacentric, and/or metacentric chromosome in *S. campestris* from southern Africa. The Y-chromosome in the cytotype of *S. mearnsi* from Ndaleta is represented by a small acrocentric chromosome, while that of *S. campestris* from southern Africa is represented by a medium-sized sub-metacentric chromosome.

Within *S. campestris*, there are no high diploid numbered 2*n* = 46 cytotypes reported from Zambia and Tanzania, except for the 2*n* = 42 and 2*n* = 44 cytotype (Corti *et al.* 2004). A comparison of the chromosome morphology of the 2*n* = 44 cytotypes from Mbunganyi-Chunya, Zambia (NFa = 48) and that from Roodeplaat Dam, Gauteng Province, South Africa (NFa = 50) showed a conserved banding pattern. The numbering of chromosomes between the two karyotypes was similar except for the small chromosomes which have ambiguous banding patterns. Direct comparison revealed that while the karyotype of the Zambian *S. campestris* showed no Robertsonian fusions, chromosome 9 is absent from this karyotype, which explains the lower NFa. The southern African 2*n* = 44 karyotype on the other hand, is characterized by the centric fusion of chromosomes 6 and 9 (Fig. 3.3) suggesting further investigation on the systematic status of *S. campestris* from Zambia and southern Africa. While the Y-chromosomes of these two karyotypes were homologous, the X-chromosome of the Zambian karyotype is morphologically similar to the X-chromosome variant 1 from southern Africa.

Molecular analysis

Phylogenetic analyses

The results of the phylogentic analyses (Maximum parsiomony, maximum likelihood and Bayesian analyses) were similar (Chapter 2) and are exemplified by the results of maximum parsimony analysis. There were 1077 base pairs of cyt *b* data generated, and included 85 variable sites of which 42 constituted point mutations and 43 were parsimony informative. Parsimony analysis showed that the 2*n* = 46 cytotype with X-chromosome variant 1 from KwaZulu-Natal's North-East Dune Forest forms the basal group to all the inland cytotypes, well-supported by a high bootstrap value (Fig. 3.5). However, although it formed part of a larger inland clade, the geographically proximal 2*n* = 46 cytotype with X-chromosome variants 1, 2, and 3 from the Lowveld North-Eastern Biotic Zone covering Mozambique and South Africa clustered separately, and was well-supported by a high bootstrap value. Other cytotypes forming a separate clade included the 2*n* = 30 and 2*n* = 34 cytotypes with X-chromosome variants 1 and 2 from the South-West Arid Zone covering northern Botswana and Namibia.

Phylogenetic analysis of 16S rRNA data yielded 16 variable sites from 528 base pairs. There were six point mutations and 10 parsimony informative sites. The analysis yielded limited information about the association between cytotype and geographic structuring. There were only two main groups, the first comprising individuals of the 2*n* = 46 cytotype with X-chromosome variant 1 from KwaZulu-Natal's North-East Dune Forest and the other a polytomy of different cytotypes from the rest of the southern African subregion that ranged from a diploid number of 2*n* = 32 to 2*n* = 46.

Haplotype networks

For the Cyt *b* data, there was a marked geographic structuring in the haplotype network (Fig. 3.6). The haplotype network geographically partitioned cytotypes and X-chromosome variants into the following three biotic regions:

a) Individuals of 2*n* = 30–34 cytotypes with X-chromosome variants 1 and 2 from the South-Western arid zone covering Namibia and Botswana;

b) Individuals of 2*n* = 32–46 cytotypes with X-chromosome variants 1, 2, and 3 from the North-Eastern, Savannah Woodland and the South-Western Biotic Zones covering Botswana and inland South Africa; and
c) Individuals of the 2*n* = 46 cytotype with X-chromosome variant 1 from the Lowveld North-Eastern, Savannah Woodland and the South-Western Biotic Zones covering Mozambique and South Africa.

Individuals of the 2*n* = 46 cytotype with X-chromosome variant 1 from the KwaZulu-Natal's North-East Dune Forest could not be linked to the rest of the haplotypes because there were more than 14 missing haplotypes between them and the rest of the southern African haplotypes.

For 16S rRNA data, all the populations were included in the network of 12 haplotypes (Fig. 3.7). The haplotypes tend to radiate from the 2*n* = 44–46 cytotype from Roodeplaat Dam, Gauteng Province, South Africa. The haplotype of the 2*n* = 46 cytotype with X-chromosome variant 1 from KwaZulu-Natal's North-East Dune Forest, although geographically isolated, is linked to the network through haplotypes from the inland populations. The haplotype groupings in the two geographically extreme samples represented by the 2*n* = 30–34 cytotypes from Namibia and the 2*n* = 46 cytotype from KwaZulu-Natal, are also located on the tips of the haplotype tree, while the middle of the network shows a significant overlap in the haplotypes with regard to cytotypes and geographic regions (Fig. 3.7). The Lowveld/Mozambique group also clustered separately from the KwaZulu-Natal group.

3.4. Discussion

Karyotype configuration

From the karyotype analysis, it is evident that karyotypes observed in *S. mearnsi* are different from those of *S. campestris* as is evident from different chromosomal fusions and differences in gonosomes. However, the primary objective of the present study was to assess the nature and extent of chromosomal variation within the karyotypically variant *S. campestris* from southern Africa in an attempt to address questions relating to its taxonomic status. The analyses identified 10 cytotypes ranging in diploid number from 2*n*

= 30 to 2*n* = 46 that involve Robertsonian fusions. However, these centric fusions only involve autosomes (Fig. 3.3). Although there is a general trend for a west-ward reduction in diploid number, the centric fusions appear to be spatially and genealogically random in inland areas.

From about the centre of the geographic distribution of *S. campestris* (e.g., around Pretoria, Gauteng Province, South Africa) towards the west, there are numerous chromosomal heterozygotes that suggest inter-breeding between cytotypes in this part of the southern African subregion (Fig. 3.1). More importantly, the centric fusions observed in *S. campestris* involve similar chromosomes across all karyotypic variants.

In addition, the results of the phylogenetic analysis (Fig. 3.5) suggest that the numerous homologous cytotypes appear to have arisen independently of each other. These include the 2*n* = 34 and the 2*n* = 36 cytotypes that arose independently on three different occasions each and the 2*n* = 45 that arose independently on two different occasions (Fig. 3.5). In addition, the haplotype network indicates eight independent reductions and two increases in chromosome number (Fig. 3.6 & 3.7). This suggests a high degree of plasticity in karyotypic configuration within *S. campestris* from southern Africa.

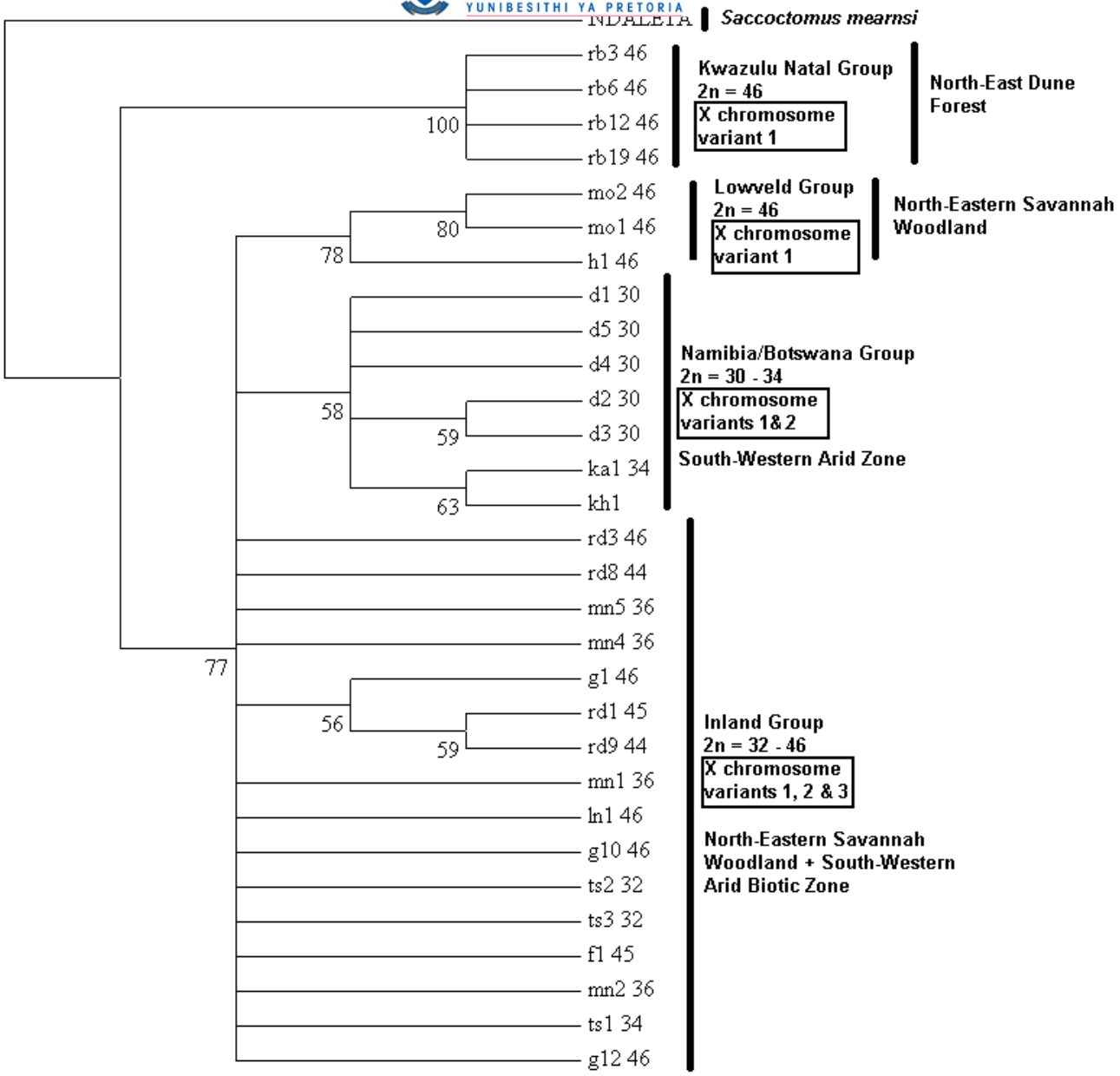

Figure 3.5. Maximum parsimony tree with bootstrap values (1000 replicates) for coding and non-coding positions of 1077 base-pair long fragment of cytchrome *b* sequences of 33 individuals of the pouched mouse, *Saccostomus campestris* from southern Africa examined in the present study (also see Chapter 2). The tree was rooted with *S. mearnsi* from Ndaleta (sequence taken from Corti *et al.* 2004). Karyotyped individuals are followed by a space and a double digit number which represents the diploid (2*n*) number of the individual. X-chromosome variants and the biotic zone associated with each clade are superimposed on the tree.

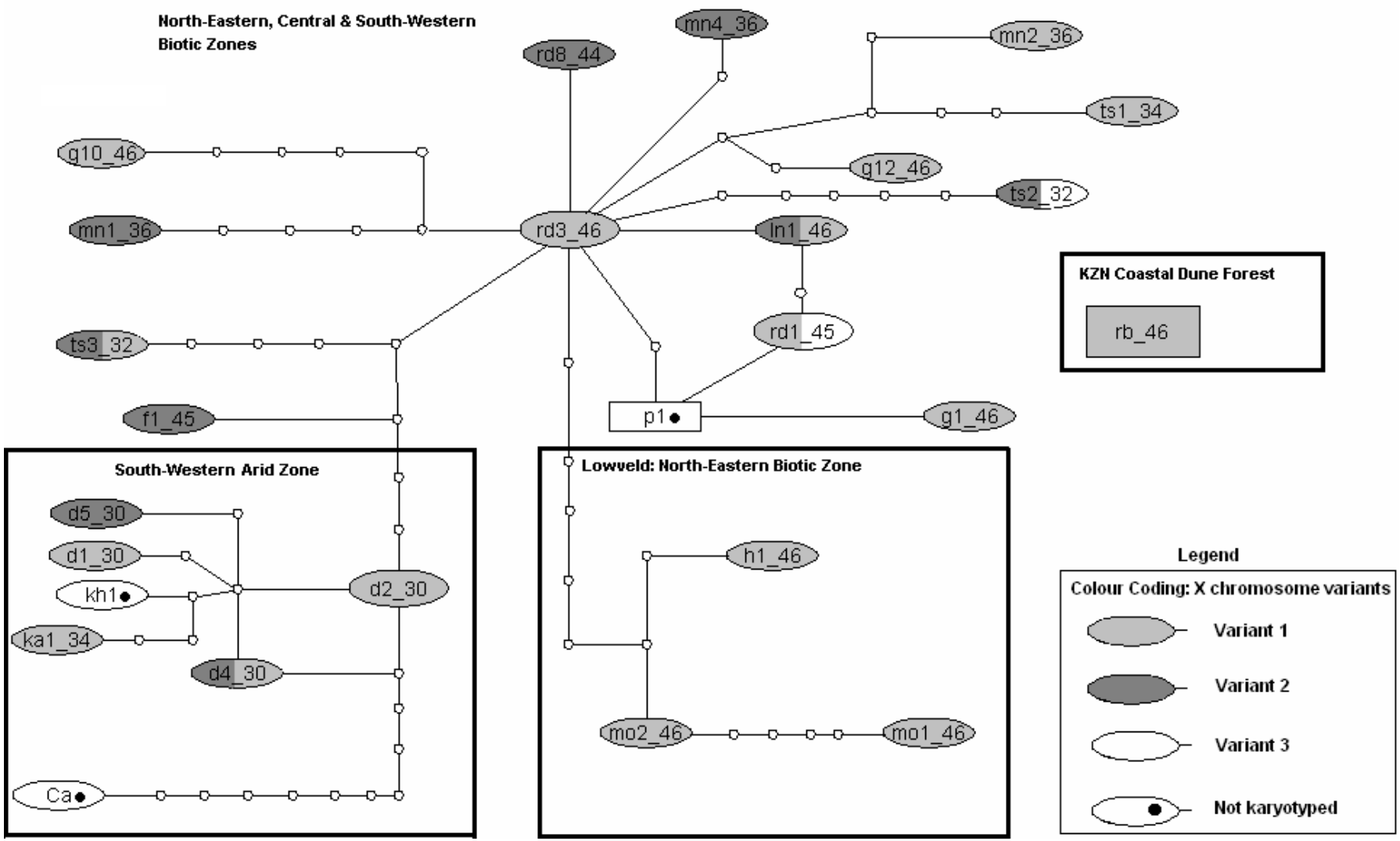
North-Eastern, Central & South-Western Biotic Zones
mn4_36
mn2_36
rd8_44
ts1_34
g10_46
g12_46
ts2_32
mn1_36
rd3_46
ln1_46
KZN Coastal Dune Forest
rb_46
rd1_45
ts3_32
f1_45
p1
g1_46
South-Western Arid Zone
Lowveld: North-Eastern Biotic Zone
d5_30
h1_46
d1_30
d2_30
kh1
ka1_34
d4_30
mo2_46
mo1_46
Ca
Legend
Colour Coding: X chromosome variants
Variant 1
Variant 2
Variant 3
Not karyotyped

Figure 3.6. A haplotype network for a 1077 bp long fragment of cytochrome *b* gene in the pouched mouse, *Saccostomus campestris* from southern Africa examined in the present study. Each line in the network symbolises a single mutational step while a “0” indicates missing haplotypes within the network. Instead of using numbers for haplotypes, the identity of the first individual of the group followed by a space and double-digit number which represents the diploid (2*n*) chromosome number was used because many of the haplotypes are represented by a single specimen. X-chromosome variants found in each haplotype are indicated by either: Light grey (Variant 1), Dark grey (Variant 2), or white (Variant 3). Haplotypes with two shades have two X-chromosome variants. Haplotypes with black dots were not karyotyped. The biotic zones associated with each haplotype grouping are superimposed on the haplotype network.

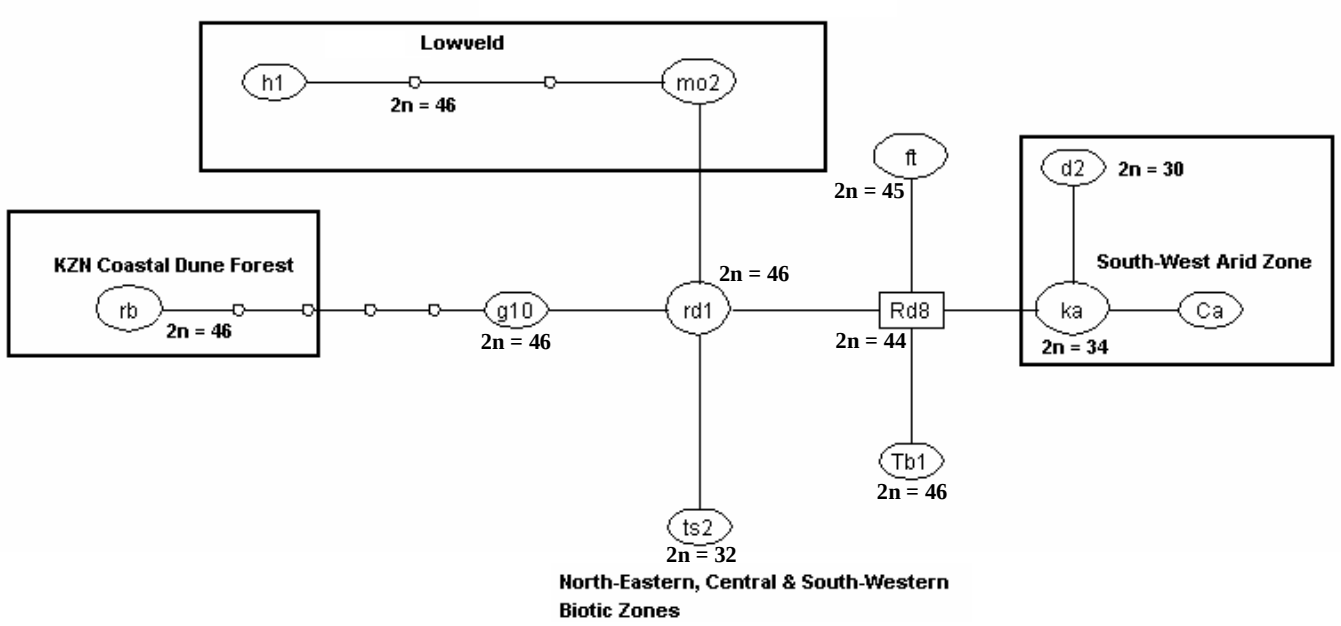

Figure 3.7. A haplotype network for a 528 bp long fragment of 12 haplotypes of the 16S rRNA gene in the pouched mouse, *Saccostomus campestris* from 13 localities in southern Africa examined in the present study. The network was estimated under 95 % connection limit at 14 steps. Haplotypes are named according to the first individual representing each haplotype. The diploid (2*n*) number and the biotic zone associated with each grouping are superimposed on the figure.

Karyotypic variation, geographic structuring and climatic variables

The results in the present study suggest a climate-related geographic structuring of karyotypic variants within the pouched mouse from southern Africa (Fig. 3.1). Both maximum parsimony analysis and a haplotype network of mitochondrial DNA data revealed two main groupings of karyotypic variants. This included: 1) a population of 2*n* = 46 cytotype from the North-East Dune Forest in KwaZulu-Natal Province, South Africa; and 2) a grouping of all the remaining cytotypes (2*n* = 32–46) from the southern African subregion. The latter grouping was further sub-divided into three groupings of karyotypic variants that included:

1) a grouping of western populations of individuals 2*n* = 30 and 2n = 34 cytotypes from a large area in the South-Western arid zone covering Namibia and Botswana;

2) a polytomy of inland groups of individuals of seven karyotypic variants encompassing 2*n* = 32–46 cytotypes that consisted of numerous small groups found over a number of isolated central inland areas in the North-Eastern, Savannah Woodland and the South-Western Biotic Zones covering Botswana and inland South Africa; and

3) Individuals of the 2*n* = 46 cytotype from the Lowveld North-Eastern, Savannah Woodland and the South-Western Biotic Zones covering Mozambique and South Africa.

A collation of maximum parsimony, maximum likelihood, and Bayesian analyses and a haplotype network of mitochondrial DNA data as summarised in Fig. 3.8 suggest a gradient of cytotypes with diploid numbers increasing with increasing rainfall suggesting a possible adaptation to rainfall and climate. However, in sibling species of the multimammate mice of the genus *Mastomys* from southern Africa, high diploid numbers are associated with areas of low rainfall where *M. coucha* (2*n* = 36) occurs in the more arid areas in the west and *M. natalensis* (2*n* = 32) occurs in the high rainfall areas in the eastern parts of the subregion (Venturi *et al.* 2003).

A similar pattern of the occurrence of high diploid numbers in areas of increasing aridity has also been reported in blind mole-rats, *Spalax ehrenbergi* from Turkey and Israel based on cytogenetic and haplotype networks (Nevo *et al.* 1995). In the case of *S. campestris* from southern Africa, however, there is perhaps a need for a further investigation over a small spatial scale using Geographic Information System (GIS) analysis involving environmental and/or climatic variables in order to gain a better insight into the environmental factors that may explain the distribution of karyotypic variants within the pouched mouse in the subregion.

Ancestral cytotype

The results in the present study support previous reports that suggest that *S. mearnsi* may be ancestral to *S. campestris.* They include:

1) The results from maximum parsimony, maximum likelihood, and Bayesian analyses of both cyt *b* and 16S rRNA data that showed *S. mearnsi* to be basal to *S. campestris* (Chapter 2).
2) On morphological grounds, Denys (1988) suggested that *S. mearnsi* has more primitive characters in common with the fossil *S. major* than with *S. campestris.*

In addition, given that karyotypic variants have also been reported in *S. mearnsi*, it is possibile that the species as currently recognized may actually represent a complex of cryptic species (Corti *et al.* 2004) suggesting it is evolving differently from *S. campestris* from southern Africa. Furthermore, it is possible that the ancestral cytotype for *S. mearnsi* may be represented by the cytotype from Ndaleta (Corti *et al.* 2004). Moreover, the chromosomal fusions in *S. mearnsi* are different to those in *S. campestris* suggesting that they may not have been involved in the split between the two species, although they may have fascilitated reproductive isolation (Corti *et al.* 2004). However, the karyotypic differences between *S. mearnsi* and *S. campestris* are so many and so large that, based on karyotypic characters alone, it is not possible to make a statement on which of the karyotypes may be ancestral. Consequently, DNA analysis is a more reliable source of inference on the ancestral and descendant karyotypic states. Phylogenetic analysis of the mtDNA using *Cricetomys gambia*e as an outgroup suggests that *S. mearnsii* is

ancestral to *S. campestris* (Maputla 2008 Chapter 2). This infers that the *S. mearnsi* karyotype is ancestral to that of *S. campestris*.

The results of the phylogenetic analysis of *S. campestris* from southern Africa based on cyt *b* and 16S rRNA data suggest that the 2*n* = 46 cytotype from the North-East Dune Forest in KwaZulu-Natal Province, South Africa represents the ancestral cytotype (Fig. 3.5). Apart from a good boot-strap support, the basal division between this population and those from the rest of southern Africa is further supported by a haplotype network where the 2*n* = 46 cytotype from the North-East Dune Forest in KwaZulu-Natal forms an isolated haplotype that is not linked to the rest of the haplotype network. This suggests that this ancestral 2*n* = 46 cytotype from the North-East Dune Forest in KwaZulu-Natal and those from the rest of southern Africa diverged a long time ago. Of particular relevance is that the 2*n* = 46 cytotype from the inland Gauteng Province, South Africa is close to the ancestral form from which all other inland cytotypes originated from (Fig. 3.6) suggesting an evolutionary trend towards a reduction in diploid number within *S. campestris* from southern Africa over time.

Only a few other studies that include *Graomys griseoflavus* (Zambelli *et al.* 2003), *Lemniscomys* and *Arvicanthis dembeensis* (Castiglia *et al.* 2002) support an evolutionary trend towards a reduction rather than an increase in diploid number over time. It has been suggested from studies in *Mus musculus* that alleles from centromeric loci on different acrocentric chromosomes arising from centric fusions may be advantageous by playing a role in local adaptation (Nachman & Searle 1995). On the other hand, other studies support an evolutionary trend towards an increase rather than a reduction in diploid number over time. For example, it has been suggested from studies in the South American sub-terranean rodent of the family Octodontidae, *Ctenomys magellanicus*, that a 2*n* = 36 cytotype was derived from a 2*n* = 34 cytotype through chromosomal fission (Lizarralde *et al.* 2003). A similar speciation scenario has also been suggested for *Spalax* (Wahrman *et al.* 1985). However, the cytogenetic and molecular evidence in the present study suggests that all the karyotypic variants within *S. campestris* from southern Africa arose from centric fusions rooted in an ancestral 2*n* = 46 cytotype from

the North-East Dune Forest in KwaZulu-Natal. This further suggests a recent and rapid chromosomal evolution and differentiation within the species in the subregion.

Taxonomic implications

From the evidence in the present study, it is unlikely that the karyotypic variants within *S. campestris* from southern Africa represent a species complex. The rationales behind this suggestion include:

1) All chromosomal translocations within *S. campestris* from southern Africa in the present study involved centric fusions that do not normally result in problems during meiosis (King 1993), and there was no evidence of monobrachial combinations of centric fusions (Baker & Bickham 1986).

2) Evidence from cyt *b* and 16S rRNA sequence data suggest high levels of gene flow between populations that differ in karyotype within *S. campestris* from inland southern Africa (Chapter 2). Population size and gene flow estimates of populations of the 2*n* = 32–36 cytotypes from the Northern Cape/North-West Provinces of South Africa showed the highest population size and a very high rate of gene flow with its immediate neighbouring populations of the 2*n* = 44–46 cytotype from Gauteng and the 2*n* = 30–34 cytotype from Namibia and Botswana (Chapter 2). The high population size and the high degree of gene flow between karyotypic variants are also consistent with the high degree of polymorphism in the X-chromosome (Fig. 3.8). In addition, the high degree of gene flow between karyotypic variants is further supported by successful laboratory crossings and back-crossing of two extreme karyotypic variants of the 2*n* = 46 cytoptype from the Lowveld and the 2*n* = 32 cytotype from the South-West arid zone for a number of generations, with the F_1 off-spring having a diploid number of 2*n* = 39 (E. Dempster *pers. comm*.).

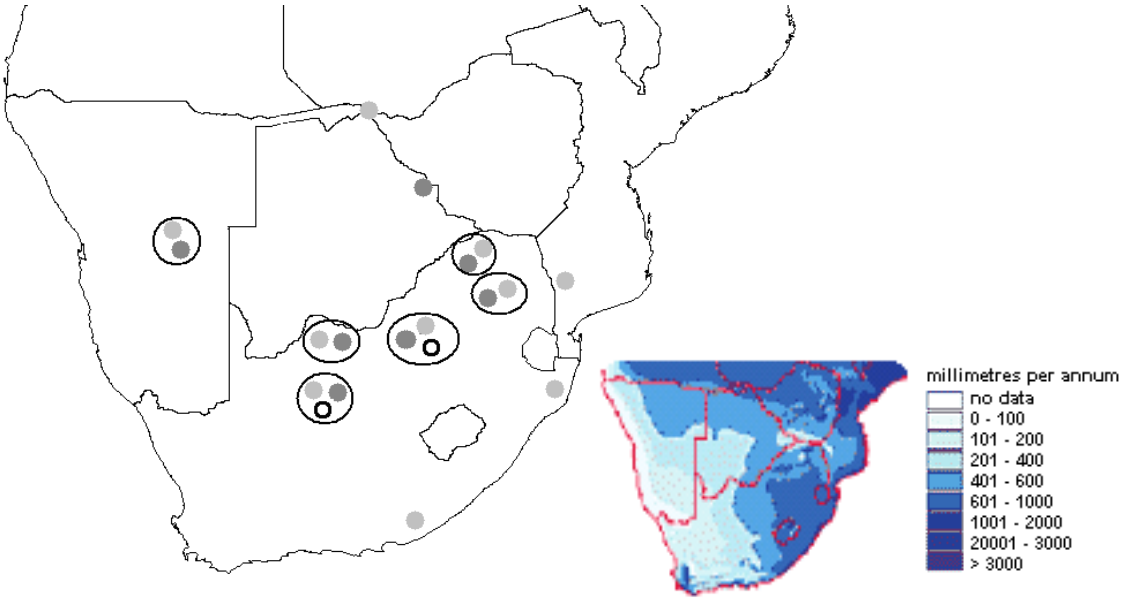

Figure 3.8 The distribution of the X-chromosome variants in the pouched mouse, *Saccostomus campestris* from southern Africa as illustrated in Fig. 3.6. Light grey dots, dark grey dots, and open dots denote X-chromosome variants 1, 2, and 3, respectively. The insert shows an annual rainfall (pattern in mm per annum) in southern Africa derived from data spanning from the early-1900s to the mid-1980s as adapted from (UNEP 1985; http://www.grida.no/aeo/032.htm).

However, the estimates of population size and gene flow among populations of karyotypic variants from the eastern part of the subregion were low (Maputla 2008, Chapter 2). It is possible that this may be due to the Great Eastern Escarpment (represented by the Drakensberg) acting as a potential barrier to the KwaZulu-Natal populations. This suggestion may be supported by the relatively low migration rate between karyotypic variants of the 2*n* = 46 cytotype from the ancestral cytotype from Richards Bay, North-East Dune Forest, KwaZulu-Natal and that from the Lowveld North-Eastern, Savannah Woodland and the South-Western Biotic Zones covering Mozambique and South Africa (Chapter 2).

3) Cytogenetic results in the present study revealed the presence of three X-chromosome variants (Figs. 3.4 & 3.8). While Variant 1 associated with the ancestral 2*n* = 46 cytotype is distributed throughout southern Africa, Variants 2 and 3 associated with the remaining karyotypic variants on the other hand, are predominantly found in the more arid and semi-arid western parts of the subregion. These results together with the phylogenetic analysis of cyt *b* and 16S rRNA sequence data (Fig. 3.5) suggest that Variant 1 may represent the ancestral form.

From these data, the following sequences of karyotypic events for the X-chromosome are hypothesized:

a) there was a single pericentric inversion on variant 1, giving rise to variant 2; and

b) there was added genetic material (possibly heterochromatin) to variant 2 leading to the formation of variant 3 (Fig. 3.4).

In addition, the pattern of X-chromosome variation does not associate with autosomal cytotypes. Because of the relatively high frequency of Variant 2 in different karyotypic variants in inland populations, it is unlikely that the pericentric inversion in this X-chromosome variant would create a

reproductive barrier between karyotypic variants. More importantly, there was no evidence in the present study that this X-chromosome variant leads to abnormal phenotypes in wild-caught animals. King (1993) noted that although the gene order is altered in inversions, in the heterozygote, an inversion loop is expected during meiosis in order to maximise pairing.

As was evident in the present study, the additional genetic material of variant 3 is probably heterochromatin that would only be present in females and would not lead to reproductive isolation in females. In contrast, unlike in *S. campestris* from southern Africa in the present study, numerous highly deleterious autosome/sex chromosome translocations with no less than four X autosome translocations as well as either partial or complete deletions of the X-chromosomes have been reported in *Mus minutoides* (Castiglia *et al.* 2002; Veyrunes *et al.* 2004).

4) Although short arms on chromosome 21 were occasionally found in the 2*n* = 46 cytotype (Fig. 3.3), this is unlikely to be due to translocations because the diploid number remained unchanged. It is possible that within *S. campestris* from southern Africa, heterochromatic additions resulted in chromosomal polymorphism within the same population and occasionally within cells of the same individual. In contrast and unlike in vlei rats of the genus *Otomys* from southern Africa (Taylor *et al.* 2003), there was no evidence in *S. campestris* from southern Africa in the present study of any tandem fusions that may lead to reproductive isolation particularly in first generation hybrids.

5) Except for some equivocal indications of cranial morphological distinctness of the ancestral 2*n* = 46 cytotype from a geometric morphometric analysis, there were no discernible morphological differences between karyotypic variants within *S. campestris* from southern Africa (Maputla, 2008 Chapter 4). In addition, Gordon (1986) also found no differences in sperm morphology between karyotypic variants within the pouched mouse from the southern African subregion.

3.5. Conclusion

Ellison (1992) and subsequently, Ellison *et al.* (1994) found significant geographic differences in several physiological traits within *S. campestris* from southern Africa. Firstly, there was evidence of heritable differences in body mass between pouched mice from mesic areas with more food resources than those from unpredictable arid habitats. Secondly, heat production and non-shivering thermogenesis (NST), an important form of heat production in small rodents during nocturnal foraging, animals from cooler environments showed a higher capacity for NST than those from warmer areas, taking their cue from the decline in photoperiod.

Thirdly, there was evidence of a differential expression of torpor in that animals from energetically stressful environments experienced longer bouts of torpor than animals from warmer localities even after laboratory acclimation. It is possible that the nature and extent of karyotypic variation found in the *S. campestris* from southern Africa together with these physiological traits may be associated with adaptation to strong environmental gradients within the subregion.

Collating all the results in the present study together with results from previous studies strongly suggests that *S. campestris* from southern Africa is a monotypic, but polymorphic species and is contrary to suggestions by Gordon (1986) and subsequently, Corti *et al.* (2004) who suggested the presence of either two or more species within *S. campestris* from southern Africa. From the results in the present study, it is postulated that the suggested recent reduction of diploid numbers within *S. campestris* from southern Africa may be an incidental result of an adaptation to a cold, dry environment in the arid south-western parts of the southern African subregion.

Chapter 4: Geometric morphometric variation in the karyotypically variable pouched mouse, *Saccostomus campestris* (Rodentia: Cricetomyinae) from southern Africa

Abstract

The nature and extent of morphological variation in positively-identified (karyotyped) individuals of the cytogenetically variable pouched mouse, *Saccostomus campestris* Peters, 1846 from southern Africa was assessed using cranial geometric morphometric data. The dorsal, ventral, and lateral views of the cranium as well as the lateral view of the mandible of nine cytotypes that ranged from 2*n* = 32 to 2*n* = 46 (2*n* = 32–35, 37, 42, and 44–46) were examined. Multivariate analyses of the different views of the cranium and the mandible showed no discernible cranial geometric morphometric patterns of variation between the nine karyotypic variants except for some subtle but equivocal indication of the morphological distinctiveness of the 2*n* = 46 cytotype. The equivocal indications of the morphological distinctiveness of the 2*n* = 46 cytotype is also supported by mitochondrial cytochrome *b* and 16S rRNA sequence data that also formed part of a multidisciplinary characterization of *S. campestris* from southern Africa.

Key words: *Saccostomus campestris*, infraspecific variation, cytotypes, cranial morphology, geometric morphometrics, systematics, southern Africa

4.1 Introduction

Two species are currently recognized within the genus *Saccostomus* (Musser & Carleton 2005). These include *S. mearnsi* (taxonomic authority) that occurs in the more northern parts of the distribution in Africa and *S. campestris* Peters, 1846 that occurs in the more southern parts of the distribution (Musser & Carleton 2005). The two were recognized as separate species after Hubert (1978) found differences in pelage colouration, length of ear, tail and maxillary tooth row as well as in karyotypes. The present study focusses on *S. campestris* from southern Africa.

Up to 16 chromosomal variants ranging from 2*n* = 28 to 2*n* = 50 have been reported in the pouched mouse, *S. campestris* from southern Africa (Gordon 1986). In attempting to collate these data, Gordon (1986) suggested the presence of at least two species within *S. campestris* from southern Africa, with one characterised by a diploid number of 2*n* = 46, and the other encompassing the cytotypes with diploid numbers of between 2*n* = 28 to 2*n* = 38. Such a high level of cytogenetic variation necessitated investigations into the cytogenetic, ecological, physiological, reproductive and behavioural variation within *S. campestris*.

For example, results from karyotypic studies suggested that the variation within *S. campestris* from southern Africa is a result of centric fusions, heterochromatic additions, and the presence of a single pericentric inversion in the X-chromosome (Ferreira 1990; Maputla 2008, Chapter 3). Ellison (1992) found geographic differences in body mass and some physiological characteristics in adult pouched mice. Furthermore, although Ellison *et al.* (1994) found *S. campestris* to enter torpor, they found that the effect of temperature and photoperiod differed between two populations from different habitats, but with no statistically significant differences in body mass. All these results suggest a critical need for a multidisciplinary characterisation of the species in southern Africa.

Consequently, as part of a multidisciplinary characterization of *S. campestris* that includes molecular (Chapter 2) and cytogenetic (Chapter 3)

analyses, the present study attempts to assess the nature and extent of morphological variation within the species using cranial geometric morphometric data. Morphometrics is useful for quantifying morphological differences both *within* and *among* Operational Taxonomic Units (OTUs; Sneath & Sokal 1973), where joint relationships in character complexes are assessed simultaneously through the reduction of large character sets to a few dimensions (James & McCulloch 1990). This is achieved by either linear/orthogonal measurement-based traditional morphometrics and/or unit-free landmark/outline-based geometric morphometrics (Marcus 1990; Rohlf & Marcus 1993), where the generated data are in turn subjected to univariate and multivariate statistical analyses.

Geometric morphometrics is a relatively new approach that is effective in capturing information about the shape of an organism and results in scientifically accurate conclusions about shape and size differences (Bookstein 1989, 1991; Rohlf & Bookstein 1990; Marcus *et al.* 1993; Rohlf 1996; Small 1996; Dryden & Mardia 1998). This method is arguably considered to be analytically superior in the partitioning of shape variation than traditional morphometrics (Corti *et al*. 1998; Marcus & Corti 1996). The digitized landmarks, captured either in 2- or 3-dimensions, are analysed using techniques such as the thin-plate spline (TPS) (Rohlf & Marcus 1993) that allow the visualization of differences in morphological shape.

These analyses also generate data such as procrustes distances, relative warps, and relative weights that can be subjected to multivariate analyses such as the *a priori* cluster analysis and principal components analysis (PCA) and the *a posteriori* canonical variates (discriminant) analysis (CVA) (Rohlf & Marcus 1993), respectively. Put simply, these methods are a collection of approaches for multivariate statistical analyses of Cartesian co-ordinate data, mostly limited to landmark point locations.

Mammalian morphometric studies are usually based on the cranium, mandible and teeth for assessing the nature and extent of morphological variation and have been useful for addressing systematic questions in other southern African small mammals. These include studies on southern African

species of rodents within the genera *Acomys* (Dippenaar & Rautenbach 1986), *Otomys* (Robinson & Elder 1987; Contrafatto *et al.* 1992; Meester *et al.* 1992; Taylor *et al.* 1992) and *Aethomys* (Chimimba & Dippenaar 1994; Chimimba *et al.* 1999; Chimimba 2000, 2001) that have been subjected to rigorous analyses of morphological variation based on traditional morphometric data. Geometric morphometrics on the other hand, have been successfully applied to numerous taxonomic and phylogenetic problems in a wide range of small mammals (e.g., Taylor & Contrafatto 1996; Courant *et al.* 1997; Fadda & Corti 1998; Corti *et al.* 2001; Mullin 2003; Abdel-Rahman 2005).

The aim of the present study therefore, is to determine whether individuals with different karyotypes differ in cranial morphological shape. The study is based on geometric morphometric analyses of the dorsal, ventral, and lateral views of the cranium, and the lateral view of the mandible and is unique because the analyses are restricted to positively-identified (karyotyped) individuals representing nine karyotypic variants within *S. campestris* from southern Africa (2*n* = 32–35, 37, 42, and 44–46). The present study therefore represents the first attempt to assess cranial morphological variation within *S. campestris* over a broader geographic area than has previously been considered for the species.

4.2. Materials and methods

While ideally, inter- and intra-specific comparisons for both *S. campestris* and *S. mearnsi* would have been appropriate, museum-preserved and/or positively-identified (karyotyped) specimens for the latter species were not available for analysis in the present study. Consequently, conclusions on the morphological differences between *S. campestris* and *S. mearnsi* in the present study were based on the widely accepted taxonomic authority of Musser & Carleton (2005) and earlier taxonomic review by Hubert (1978) who consider the two to be valid species based on karyology, belly pelage couration, and ear, tail, and maxillary toothrow lengths.

Sixty-two positively-identified (karyotyped) wild-caught specimens of *S. campestris* from 27 localities encompassing a wide range of habitats that

represent an adequate geographic coverage of the species in southern Africa were examined (Fig. 4.1; Appendix 3). The specimens examined included 60 karyotyped museum-preserved specimens obtained on loan from the mammal reference collections of the Transvaal Museum (TM) of the National Flagship Institute (NFI), Pretoria and two from the Durban Natural Science Museum (DM), Durban, South Africa.

Animals were live-trapped in the field using Sherman traps (H.B. Sherman Traps Inc. Florida, U.S.A.) baited with a mixture of peanut butter, syrup, oat meal, and fish oil. After capture, during transportation and in the laboratory, animals were kept in polyurethane cages with wood shavings as bedding, with mouse pellets and water provided *ad libitum*. Animals were maintained under the guidelines of the American Society of Mammalogists (ASM; http://www.mammalogy.org/committees/index.asp; Animal Care and Use Committee 1998) and as approved by the Animal Ethics Committee of the University of Pretoria, Pretoria, South Africa. Animals were prepared for karyotype extraction and subsequently sacrificed using halothane inhalation. Voucher specimens were prepared using standard natural history museum procedures for mammal specimens and will be deposited in the mammal reference collection of the Transvaal Museum (TM) of the National Flagship Institute (NFI), Pretoria, South Africa.

Prior to the assessment of the nature and extent of cranial morphological variation within *S. campestris* from southern Africa, non-geographic cranial morphological variation due to age and sexual dimorphism were first assessed. These analyses were undertaken with the objective of establishing criteria for the selection of adult specimens for subsequent image capturing and analysis, and whether to analyse the sexes separately or together during the assessment of cranial morphological variation within *S. campestris*.

Specimens were first classified into four relative age classes based on the degree of maxillary tooth-wear (Dippenaar & Rautenbach 1986; Chimimba & Dippenaar 1994). Relative age of specimens was based on the degree of molar eruption and wear (Fig. 4.2) and were defined as follows: 1) age class I – cheek-teeth were not fully-erupted to cases where cheek-teeth were fully

erupted but with minimal tooth-wear; 2) age class II – cheek-teeth with obvious, but not extensive tooth-wear; 3) age class III – cheek-teeth with extensive tooth-wear, but with cusps still distinguishable; and 4) age class IV – extensive tooth-wear, but with cusps not completely worn to cases where tooth-wear was severe, with occlusal surfaces worn smooth with no traces of cusps.

Two-dimensional images of the dorsal, ventral, and lateral views of the cranium, as well as the lateral view of the mandible of each specimen were captured using a Canon® PowerShot A20 digital camera. The camera was attached to a tripod stand. In order to standardize the image-capturing procedure, each specimen was placed on a surface with a background of a marked graph paper in a fixed position, with all images being captured by one observer (NM).

A Thin Plate Spline (TPS; Rohlf 2004a) sub-routine, TPSDig, was used to digitize landmarks considered to represent homologous points between specimens, each with an (x, y) 2-dimensional coordinates, on each of the four views for each specimen (Fig. 4.3). These included 21, 19, and 12 landmarks of the dorsal, ventral, and lateral views of the cranium, respectively, as well as eight landmarks on the lateral view of the mandible (Fig. 4.3; Appendix 4).

In order to assess the magnitude of landmark digitizing error (DE), the degree of error was expressed as a percentage (%DE) of the total variability due to *within*-individual variation (Pankakoski *et al.* 1987; Bailey & Byrnes 1990). The analysis of %DE was based on three independent data sets of repeated digitized landmarks derived by one observer (NM) on three separate occasions. Landmarks were collected from three skulls ($2n$ = 46), belonging to three male individuals of age class III from the Lower-Umfolozi. Because these analyses revealed very low %DE values that ranged from 0.01% to 1.49%, averages of landmarks were computed and used in all subsequent geometric morphometric analyses in the present study.

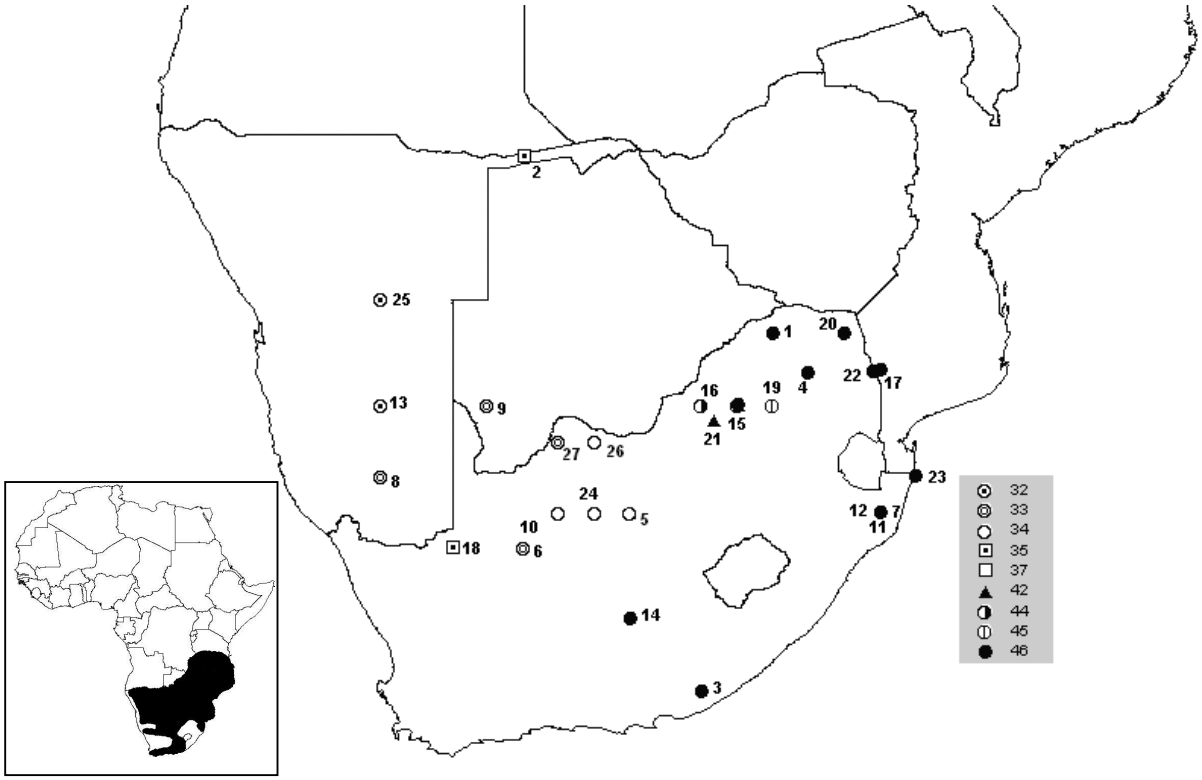

Figure 4.1. A map of southern Africa showing the distribution of nine cytotypes (2*n* = 32–35, 37, 42, and 44–46) of the pouched mouse, *Saccostomus campestris* and their collecting localities examined in the present study: 1 = Alldays; 2 = Caprivi; 3 = Grahamstown; 4 = Groblersdal; 5 = Hartswater; 6 = Hay; 7 = Hluhluwe; 8 = Keetmanshoop; 9 = Kgalagadi National Park (KGNP); 10 = Kuruman; 11 = Lower-Umfolozi; 12 = Hlabisa; 13 = Namibia; 14 = Noupoort; 15 = Rustenburg; 16 = Thabazimbi; 17 = Pilgrim's Rest; 18 = Pofadder; 19 = Pretoria; 20 = Punda Maria; 21 = Settlers; 22 = Satara; 23 = Lalanek; 24 = Griekwast; 25 = Windhoek; 26 = Morokweng; and 27 = Vryburg. The legend shows the corresponding karyotype for each locality. The locality numbering corresponds to the numbering of the collecting localities and their associated geographic coordinates in Appendix 3. The insert shows the overall geographic distribution of *S. campestris* in Africa as adapted from Skinner & Chimimba (2005) and the symbols used for the corresponding cytotypes.

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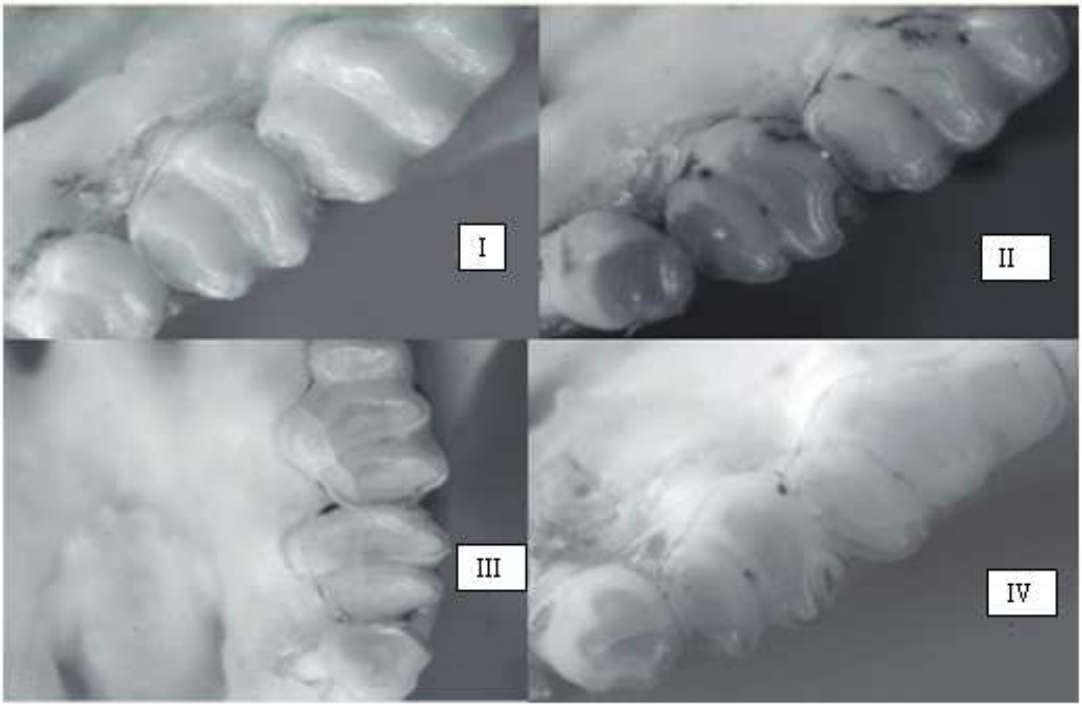


Figure 4.2. An illustration of four relative toothwear classes assigned to specimens of the pouched mouse, *Saccostomus campestris* from southern Africa used for the analysis of age variation: I = age class I; II = age class II; III = age class III; and IV = age class IV. Age classes are defined in the "Materials and methods section" above.

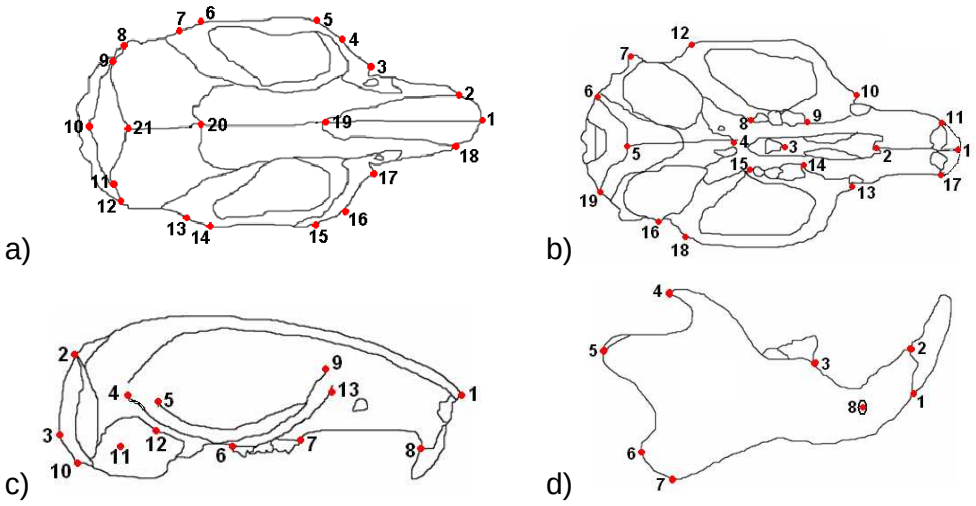


Figure 4.3. Landmarks of the dorsal (a), ventral (b), and lateral (c) views of the cranium, and the lateral view of the mandible (d) used in the geometric morphometric analyses of the pouched mouse, *Saccostomus campestris* in the present study. The specific positions of these landmarks are described in Appendix 4.

Specimens were aligned with respect to the average configuration using generalized procrustes analysis (GPA: the orthogonal GLS procedure of Rohlf & Slice 1990). The closeness of the approximation of the tangent space to shape space was estimated using the program TPS-Small (Rohlf, 1998). It is considered that the higher the correlation between shape space and tangent space, the more accurate the non-linear shape space can be approximated from the mathematically simpler and linear tangent space (Marcus & Corti 1996).

Samples from Lower Umfolozi, Hluhluwe, and Tembe, KwaZulu-Natal Province, South Africa were pooled due to their ecological and geographical proximity following Rautenbach (1970), Dippenaar & Rautenbach (1986) and Chimimba & Dippenaar (1994) because the sample size from single localities were too small to allow meaningful statistical analyses of non-geographic variation (Appendix 3). The initial analysis of cranial geometric morphometric age variation and sexual dimorphism within *S. campestris* comprised an independent univariate analysis of variance (ANOVA; Zar 1996) of age variation using specimens from Tembe, Hluhluwe, and Lower Umfolozi and sexual dimorphism using individuals from age class III in the pooled sample from Lower Umfolozi and Hluhluwe, KwaZulu-Natal Province, South Africa.

Where statistically significant age differences were detected by the ANOVA, non-significant subsets ($P > 0.05$) were identified by Tukey's HSD (Gabriel & Sokal 1969; Sokal & Rohlf 1981) *post hoc* tests of ranked means. All ANOVAs were undertaken after tests for normality and homogeneity of variances showed that the data satisfied the assumptions of ANOVA (Zar 1996). The univariate analyses that included ANOVA and Tukey's HSD *post hoc* tests were always followed by multivariate analyses of the different views of the cranium and mandible in order to assess age variation, sexual dimorphism, and morphological differences between the nine chromosomal groups within *S. campestris*.

The software TPSspline (Rohlf 2004b), was used to compute splines for each view and were used to compare each view of a specimen with its associated consensus configuration in order to detect any differences in

cranial and mandibular shape morphology (Marcus & Corti 1996). This procedure is used to visualise shape changes as deviations from the consensus configuration and is based on deformation of a generated grid (Bookstein 1989). The generated geometric morphometric data were subjected to a series of multivariate morphometric analyses in order to identify phenetic groupings with reference to age variation, sexual dimorphism, and morphological differences between chromosomal groups in which no *a priori* sub-divisions of samples were presumed (Sneath & Sokal 1973).

The *a priori* multivariate morphometric analyses included unweighted pair-group arithmetic average (UPGMA) analysis and principal components analysis of normalised variables (Sneath & Sokal 1973). Cluster analysis is a multivariate method used to group entities *a priori* based on distances with sets arranged hierarchically and represented in a one-dimensional summary in the form of a phenogram (or dendrogram) in which similar entities are clustered together. Among various clustering methods, UPGMA cluster analysis is recommended in systematics (Sneath & Sokal 1973) because being a cross-averaging algorithm, it conserves space by minimizing input and output distances leading to the distribution of OTUs (Sneath & Sokal 1973) into a reasonable number of groups (James & McCulloch 1990). The UPGMA cluster analysis of the geometric morphometric data was based on procrustes distances of landmarks generated from the TPS sub-routine, TPSsmall (Rohlf 2004d).

PCA is also an *a priori* data reduction method in which variables or components of linear combinations of original data responsible for much of the variation in the data set are shown (Jolliffee 1986). PCA projects points from the original data on 2-dimensions with axes corresponding to two informative components. Minimum information is lost during its computation and is recommended for analyzing morphometric data. The PCA of the geometric morphometric data was based on a weighted matrix generated from the TPS subroutine TPSRelw (Rohlf 2004c) to perform a relative warp analysis (RWA; Rohlf 2004c) for assessing shape variation with reference to age variation, sexual dimorphism, and morphological variation among chromosomal groups within *S. campestris*. RWA is essentially a PCA of a weighted matrix of non-

uniform components of Generalised Least Squares (GLS; Rohlf & Slice 1990) as well as of uniform components (U1 and U2) of shape change. Partial weight scores were computed from a Generalised Procrustes Analysis (GPA; Rohlf & Slice 1990). The GPA residuals in both non-uniform (non-affine) and uniform (affine) shape components were partitioned where affine and non-affine represent total shape change.

Age, sex, and chromosomal groups were also subjected to *a posteriori* analyses that assess the integrity of designated groups by maximizing the variation *between* groups while minimizing variation *within* groups, and for the classification of unknown specimens (Sneath & Sokal 1973). This included canonical variates (discriminant) analysis (CVA; Sneath & Sokal 1973). The CVA was based on partial weight matrices of landmarks generated from the TPS subroutine TPSRegr (Rohlf 2004c), and was always followed by a multivariate analysis of variance (MANOVA; Zar 1996) to test for statistically significant differences between the designated group centroids.

All geometric morphometric analyses in the present study were accomplished using sub-routines in the TPS (Rohlf 2004) series of programmes and in Statistica version 7.0 (StatSoft 2004).

4.3. Results

The correlation (*r*) between Procrustes and Euclidean distances was 0.99 suggesting subtle variation (Rohlf & Slice, 1990) between landmarks within the context of total shape space, therefore justifying the use of Euclidean statistics on coordinate data in the present study.

Age variation

The results of the geometric morphometric analyses of the dorsal, lateral and ventral views of the cranium, and the lateral view of the mandible were similar, and are exemplified by those of the dorsal view of the cranium. *F*-values from a one-way ANOVA of the sample showed statistically significant differences between the age classes ($F = 10.49$; $P < 0.05$). However, *post hoc* tests of ranked means using Tukey's HSD tests to detect non-significant subsets ($P >$

0.05) of age classes showed that the significant differences were between comparisons of age groups III and IV with age group I ($P < 0.05$).

The PCA scatterplot (Fig. 4.4) of the first relative warp (RW) explained 33.94% of the total variance and the second RW accounts for 24.30% of the total variance in the geometric morphometric data of individuals of age classes I–IV. The PCA of age variation showed the separation of the individual of age class I and overlaps between individuals of the age classes II, III and IV on the second RW axis (Fig. 4.4). Similarly, the procrustes distance phenogram from the UPGMA cluster analysis (Fig. 4.5) showed two distinct clusters, designated A and B. Cluster A comprised individuals age classes II, III and IV and cluster B consisted of the single individual of age class I.

The morphological differences between the individual of age class I and age classes II, III and IV are shown by the changes in the position of landmarks as shown by splines and their associated vectors as shown by arrows for the younger and older age classes with reference to a consensus configuration of the dorsal view of the cranium derived from TPSSpline (Fig. 4.6 a & b). Differences in the dorsal view of the cranium between the younger (Fig. 4.6 c & d) and the older (Fig. 4.6 e & f) age classes suggest that older age classes are much broader in dorsal configuration of the cranium than younger individuals. The general broadness of older individuals was also apparent in the ventral and lateral views of the cranium, as well as in the lateral view of the mandible (not illustrated).

While ideally, a canonical variates analysis (CVA, Sneath & Sokal 1973) that maximizes variation *among* and minimizes variation *within* specified groups should also have been undertaken to assess age variation, the analysis was not possible due to *within*-cell sample size limitations. However, collation of the results of all univariate and multivariate analyses of the geometric morphometric data despite the unavoidable sample size limitation that is typical of small mammal non-geographic variation data suggest the general lack of age variation between age groups II, III, and IV in pouched mice in the present sample, and justifies the pooling of these age classes in all subsequent data recording and analyses of variation among cytotypes.

Sexual dimorphism

Geometric morphometric analyses of the dorsal, lateral, and ventral views of the cranium, and the lateral view of the mandible indicate that males and females are generally similar. This similarity is best demonstrated by the results of the dorsal view of the cranium. *F*-values from a one-way ANOVA of the sample showed no statistically significant differences in the centroid sizes between the sexes ($F = 1.61$; $P > 0.05$).

Similarly, the procrustes distance phenogram from the UPGMA cluster analysis (Fig. 4.8) showed no discrete groupings of the sexes. The lack of sexual dimorphism is also shown by minimal changes in the position of landmarks as shown by splines and their associated vectors as shown by arrows for males and females with reference to a consensus configuration of the dorsal view of the cranium derived from TPSSpline (Fig. 4.9). The splines for the dorsal view of the consensus configuration for males and females were broadly similar (Fig. 4.9) further suggesting the general lack of sexual dimorphism.

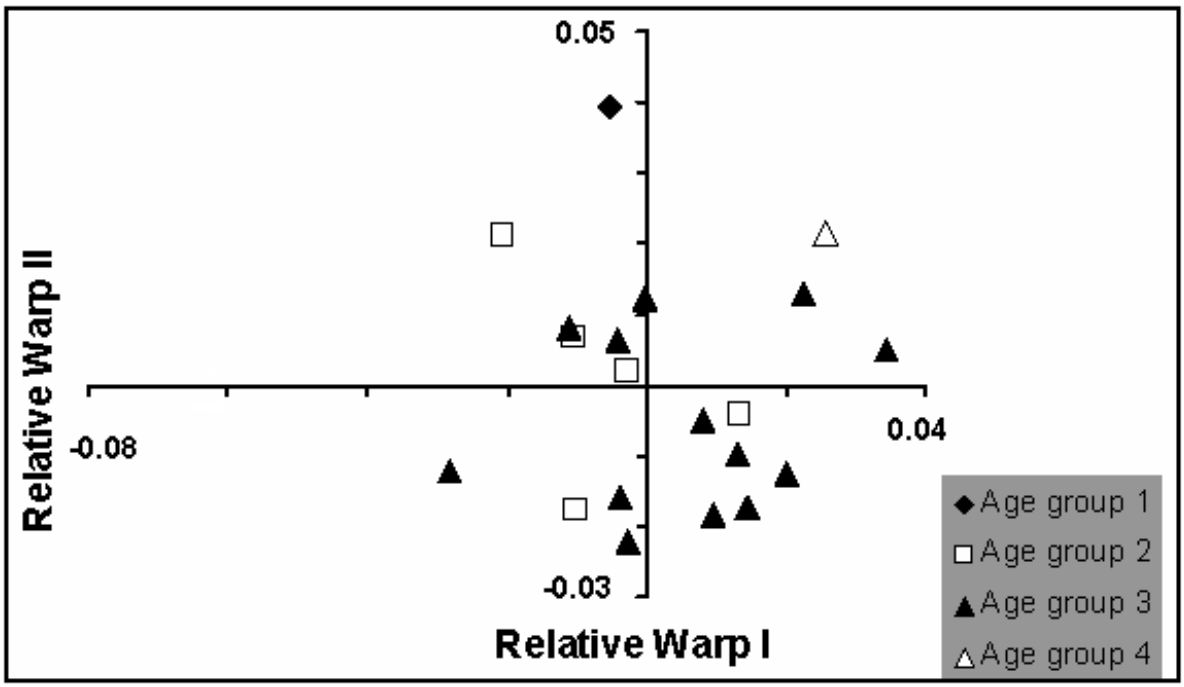


Figure 4.4. A scatterplot of relative warps (RW) I and II from a principal components analysis (PCA) of geometric morphometric data of the dorsal view of the cranium used to assess variation in age classes I, II, III, and IV in the pouched mouse, *Saccostomus campestris* from Tembe, Lower Umfolozi and Hluhluwe, KwaZulu-Natal Province, South Africa.

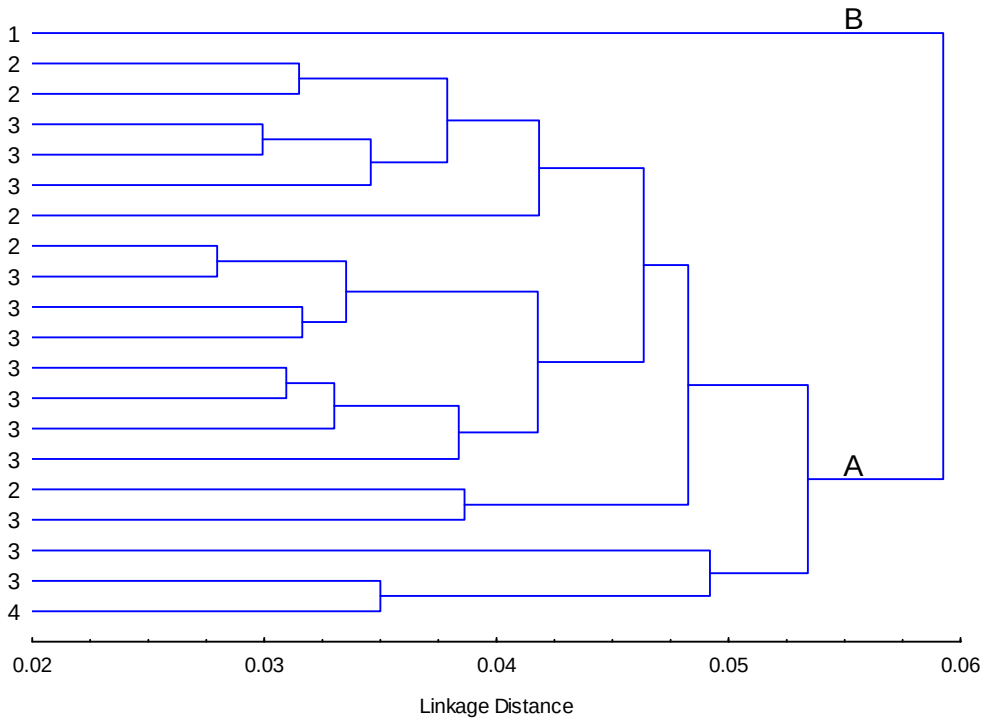


Figure 4.5. A procrustes distance phenogram from an Unweighted pair–group arithmetic averages (UPGMA) cluster analysis of the dorsal view of the cranium used to assess age variation in individuals of age classes I, II, III and IV in the pouched mouse, *Saccostomus campestris* from Lower Umfolozi and Hluhluwe, KwaZulu-Natal Province, South Africa.

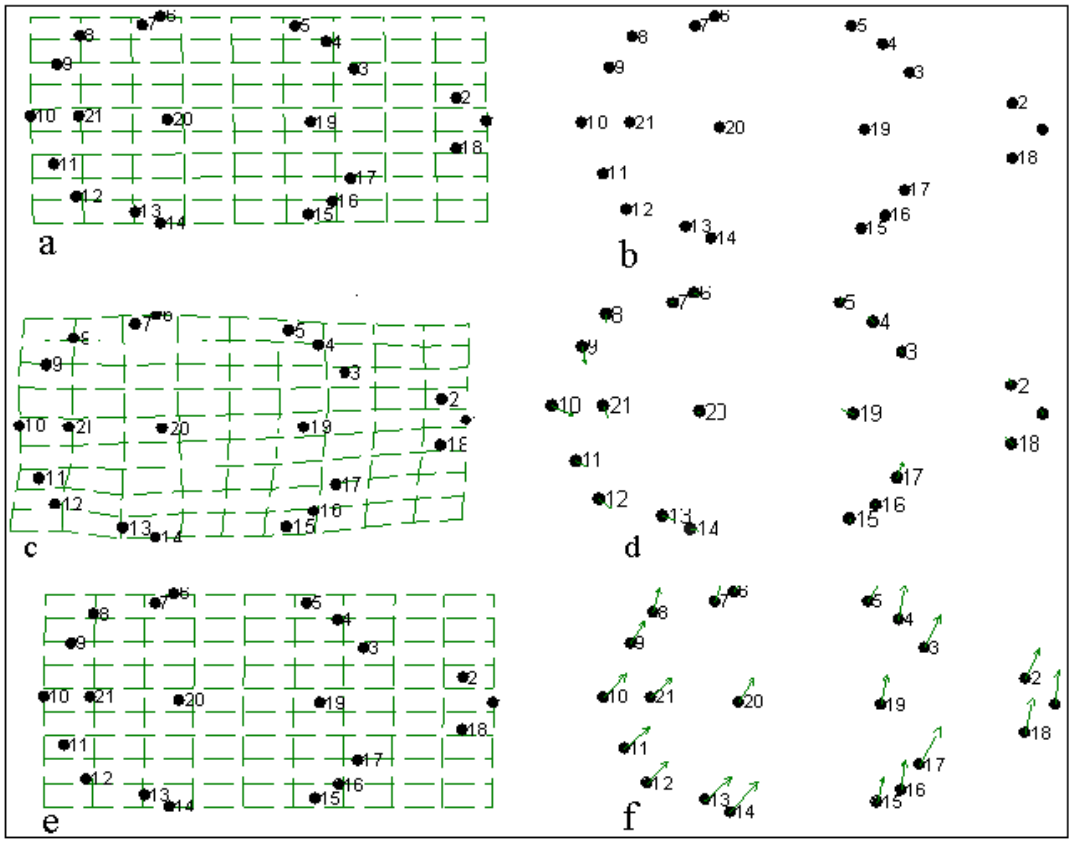


Figure 4.6. Changes in the position of landmarks with reference to a consensus configuration (splines and their associated vectors as shown by arrows) of the dorsal view of the cranium of the pouched mouse, *Saccostomus campestris* from southern Africa derived from TPSSpline (Rohlf 2004b): a and b represent the consensus configuration, c and d represents the individual of age class I, while e and f represent individuals of age classes II, III and IV.

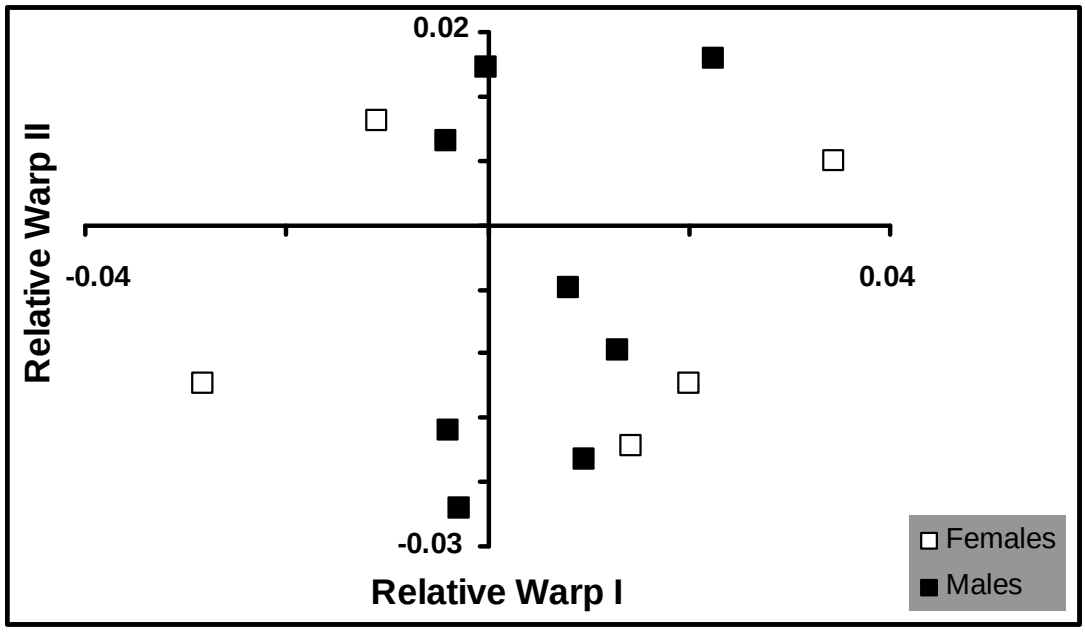


Figure 4.7. A scatterplot of relative warps (RW) I and II from a principal components analysis (PCA) of geometric morphometric data for age class III of the dorsal view of the cranium used to assess sexual dimorphism in the pouched mouse, *Saccostomus campestris* from Lower Umfolozi and Hluhluwe, KwaZulu-Natal Province, South Africa.

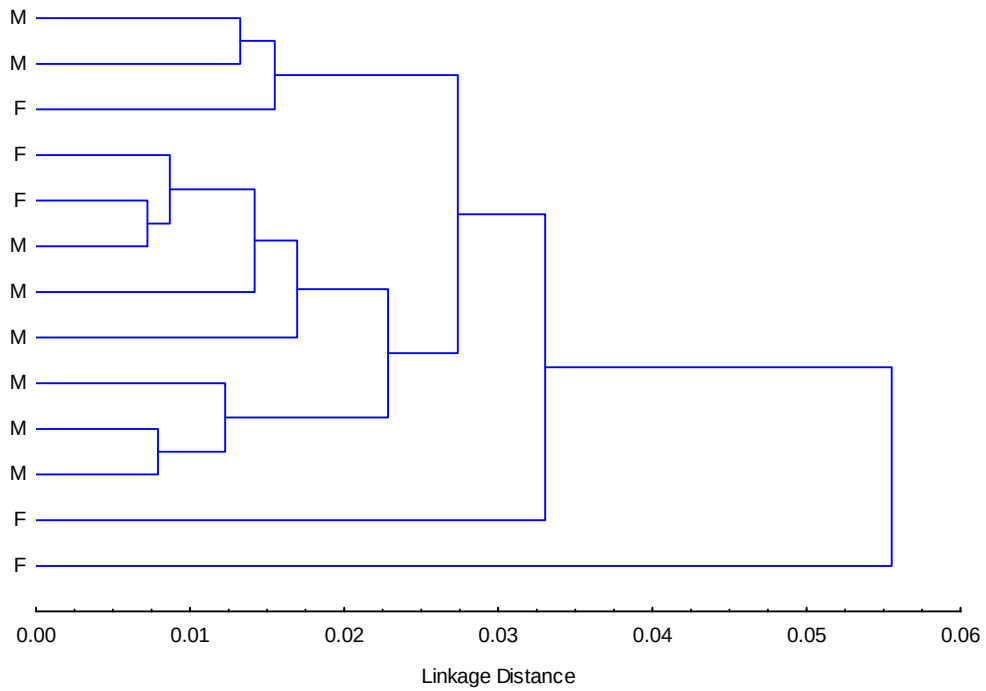


Figure 4.8. A procrustes distance phenogram from an Unweighted-pair group arithmetic average (UPGMA) cluster analysis of geometric morphometric data for age class III of the dorsal view of the cranium used to assess sexual dimorphism in the pouched mouse, *Saccostomus campestris* from Lower Umfolozi and Hluhluwe, KwaZulu-Natal Province, South Africa.

Collation of the results of all univariate and multivariate analyses of the geometric morphometric data despite the unavoidable sample size limitation that is typical of small mammal non-geographic variation data suggest the general lack of sexual dimorphism in pouched mice from southern Africa. Consequently, these results justified the pooling of sexes in all subsequent analyses of variation among cytotypes.

Geometric morphometric variation among cytotypes

Geometric morphometric analyses of the dorsal, lateral and ventral views of the cranium and the lateral view of the mandible reveal little variation among cytotypes. This similarity is best demonstrated by data from the dorsal view of the cranium. *F*-values from a one-way ANOVA of the sample did not show statistically significant differences between cytotypes. However, similar to the ANOVA of age variation above, *post hoc* tests of ranked means using Tukey's HSD tests to detect non-significant subsets ($P > 0.05$) among cytotypes were not possible due to *within*-cell sample size limitations.

Nevertheless, the PCA scatterplot (Fig. 4.10) of the first relative warp (RW) explained 25.39% of the total variance and the second RW accounts for 15.42% of the total variance in the geometric morphometric data. The PCA of morphological variation among cytotypes showed no geographically discernible pattern (Fig. 4.10). This pattern of variation is also evident in analyses based on locality mean values of the first relative warp (RW) that explained 19.88% of the total variance and the second RW that accounts for 15.96% of the total variance in the geometric morphometric data (not illustrated). An examination of the generated RW axes subsequent to the first two did not similarly show any geographically discernible patterns of geometric morphometric variation within *S. campestris* from southern Africa.

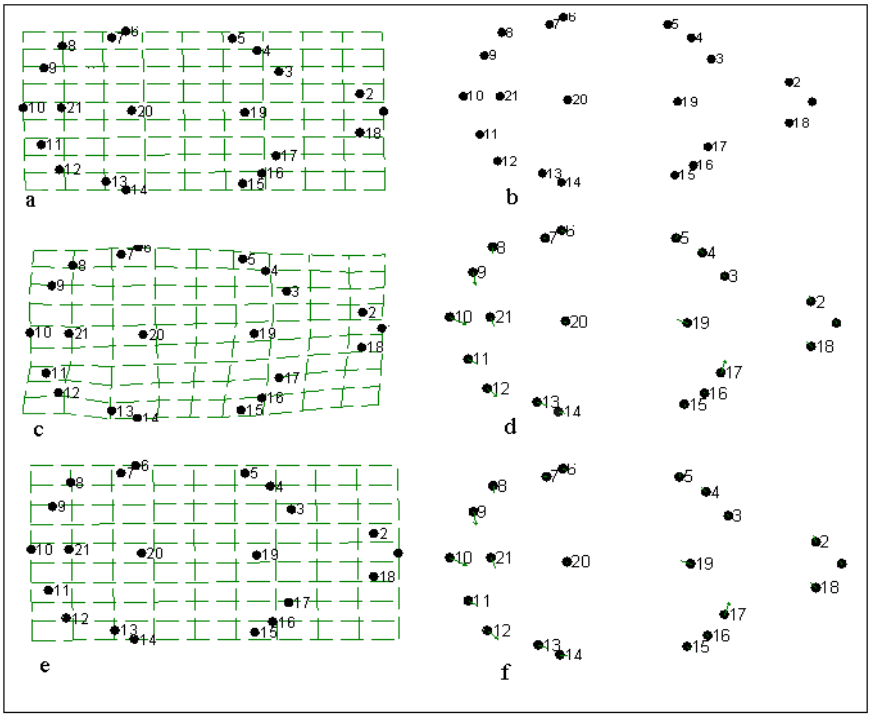


Figure 4.9. Changes in the position of landmarks with reference to a consensus configuration (splines and their associated vectors as shown by arrows) of the dorsal view of the cranium used to assess sexual dimorphism in the pouched mouse, *Saccostomus campestris* from Lower Umfolozi and Hluhluwe, KwaZulu-Natal Province, South Africa. Splines are indicated for the consensus configuration (a & b), males (c & d) and females (e & f).

However, although the procrustes distance phenogram from the UPGMA cluster analysis (Fig. 4.11) showed no geographically discernible pattern of geometric morphometric variation among cytotypes within the pouched mouse from southern African, there is a statistically nonsignificant tendency for individuals of the 2*n* = 46 cytotype from KwaZulu-Natal to cluster together. All other cytotypes from the southern African subregion were randomly placed in the phenogram. A similar pattern of variation among cytotypes within *S. campestris* from southern Africa was also evident in UPGMA cluster analysis based on locality mean values. This suggests that the morphology of the 2*n* = 46 cytotype from KwaZulu-Natal may differ subtly from other cytotypes.

A CVA of four cytotypes (2*n* = 33–34, 42, and 46) that had adequate samples for analysis was not possible due to singularity in the data matrix. Collation of all the results of the multivariate analyses to assess morphological variation among cytotypes within the pouched mouse from southern Africa suggest some subtle but equivocal morphological distinctiveness of the 2*n* = 46 cytotype from KwaZulu-Natal. This is reflected in the changes in the position of landmarks as shown by splines and their associated vectors as shown by arrows and with reference to a consensus configuration of the dorsal view of the cranium (Fig. 4.11). Although the dorsal configurations of the cytotypes generally differ from that of the consensus configuration, these differences are much more prominent in the 2*n* = 46 cytotype from KwaZulu-Natal that is much broader than all other cytotypes. The broadness of the 2*n* = 46 cytotype from KwaZulu-Natal is also reflected in the geometric morphometric results of the lateral and ventral views of the cranium, and the lateral view of the mandible (not illustrated).

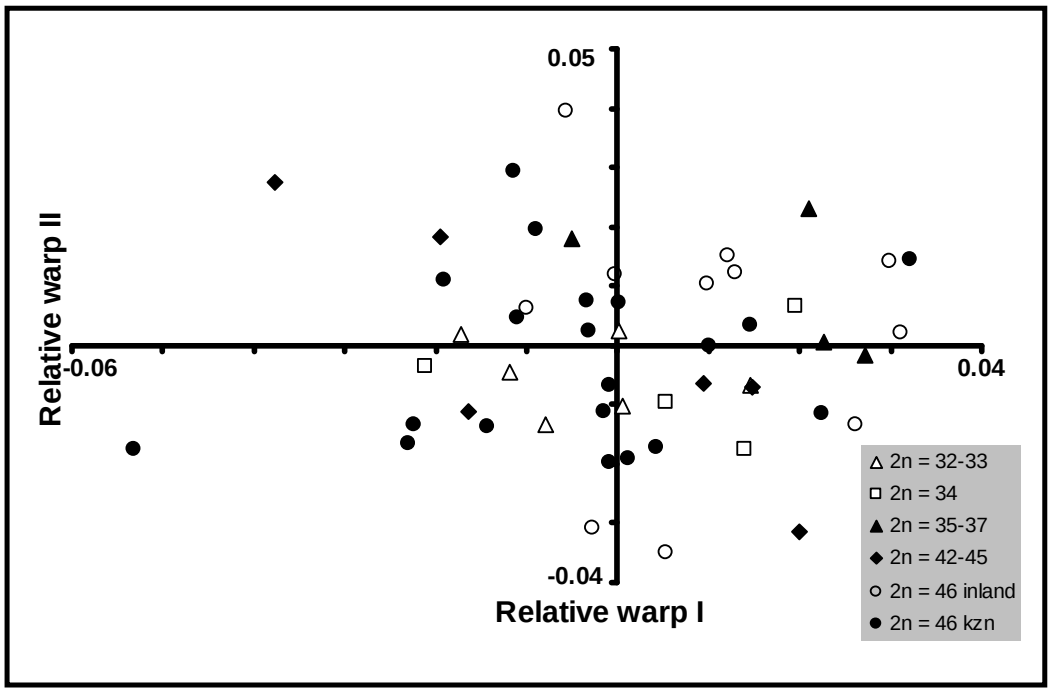
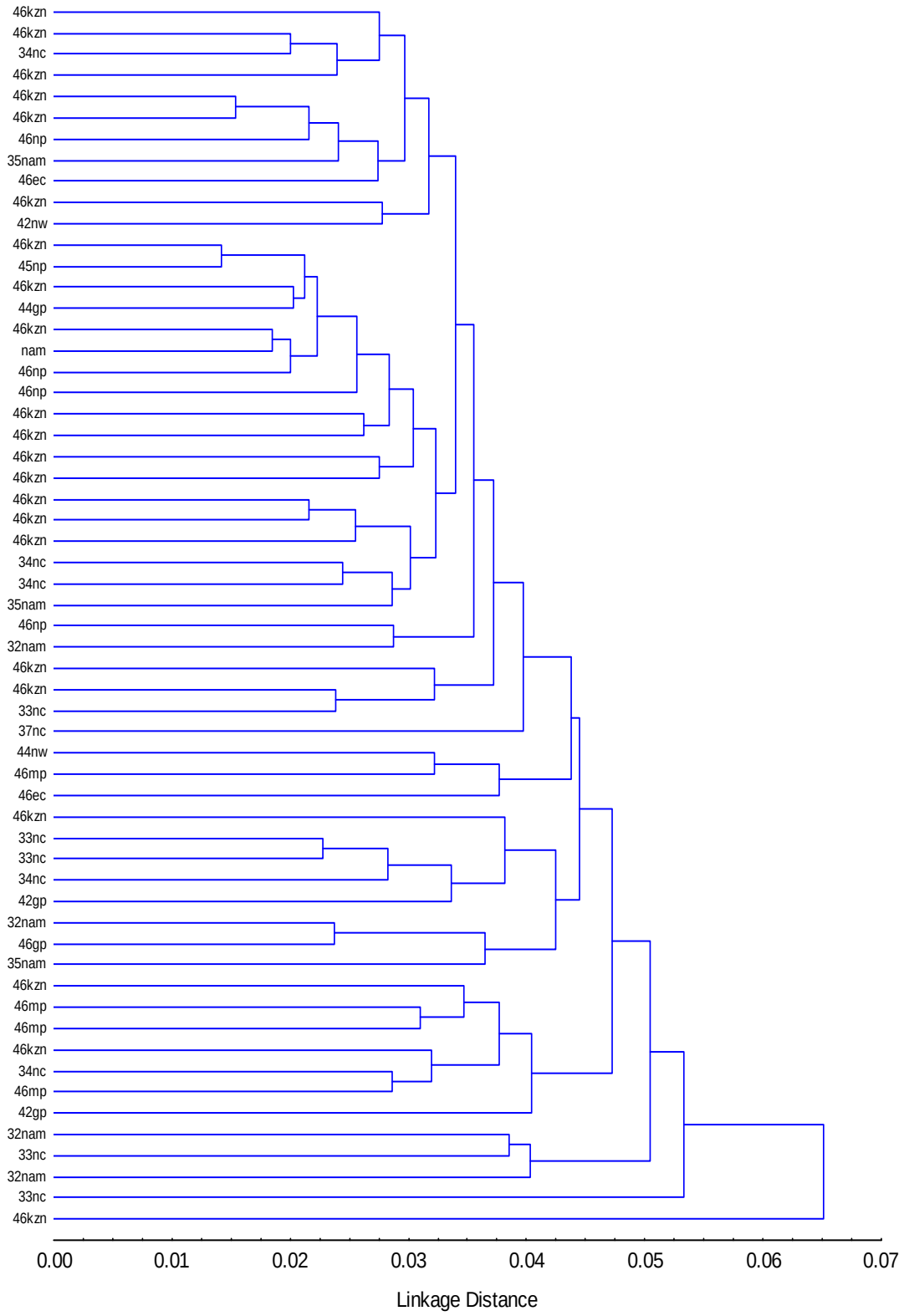


Figure 4.10. A scatterplot of relative warps (RW) I and II from a principal components analysis (PCA) of individuals used to assess morphological variation among karyotypes within the pouched mouse, *Saccostomus campestris* from southern Africa based on geometric morphometric data of the dorsal view of the cranium. The two 2*n* = 46 groups represent the inland group (2*n* = 46 inland) and the KwaZulu-Natal (2*n* = 46 kzn) group.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46mp
42gp
32nam
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32nam
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0.05
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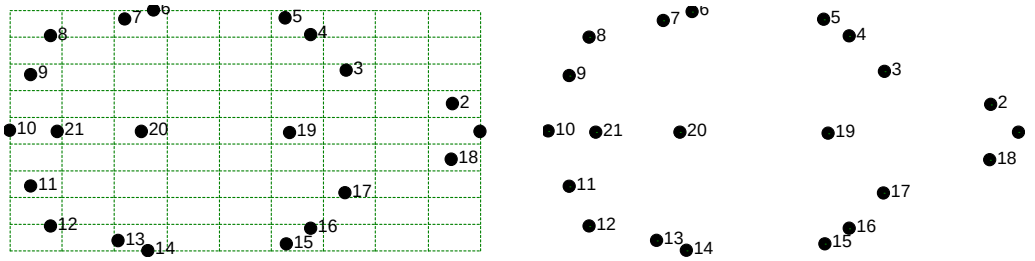
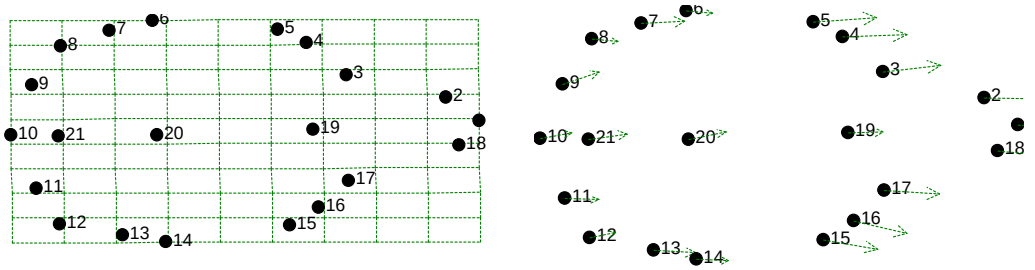
0.07
Linkage Distance

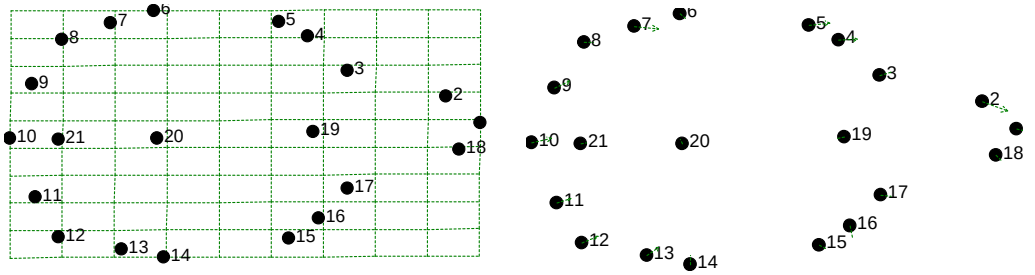
Figure 4.11. A procrustes distance phenogram from an Unweighted-pair group arithmetic average (UPGMA) cluster analysis of individuals used to assess morphological variation among cytoypes within the pouched mouse, *Saccostomus campestris* from southern Africa based on the dorsal view of the cranium. The numbers correspond to cytotypes. Origin of specimens is indicated as follows: kzn = KwaZulu-Natal Province; nc = Northern Cape Province; mp = Mpumalanga Province; ec = Eastern Cape Province; nw = North-West Province; gp = Gauteng Province; np = Limpopo Province; and nam = Namibia.



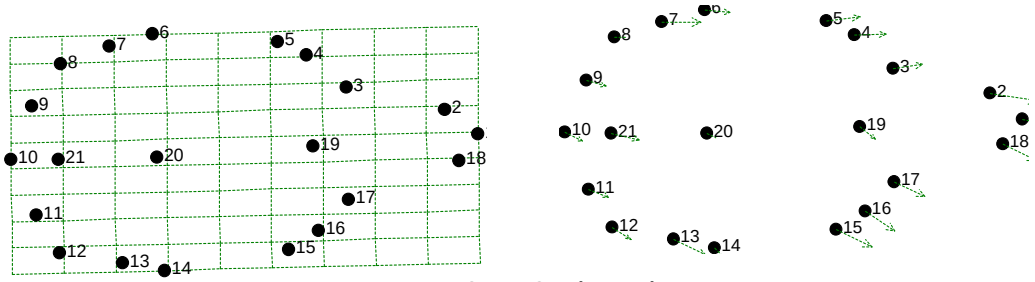
Consensus



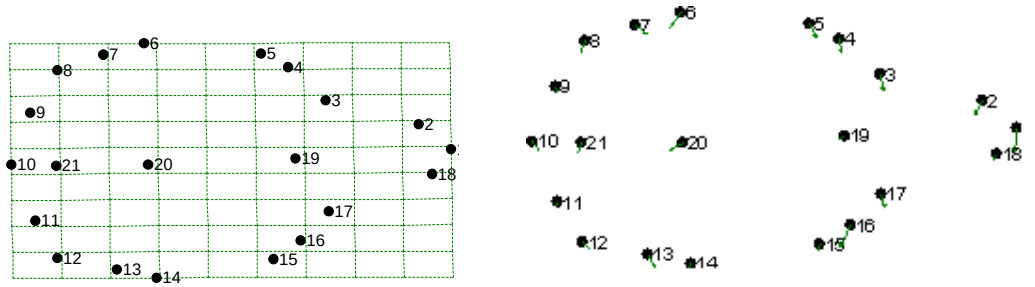
$2n = 32$ ($n = 2$)



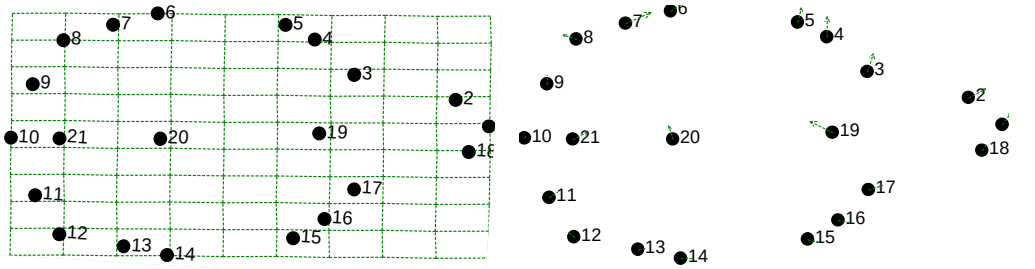
$2n = 33$ ($n = 4$)



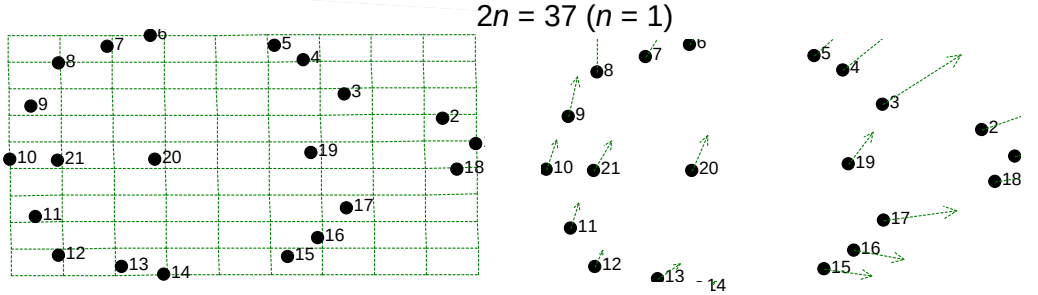
$2n = 34$ ($n = 4$)



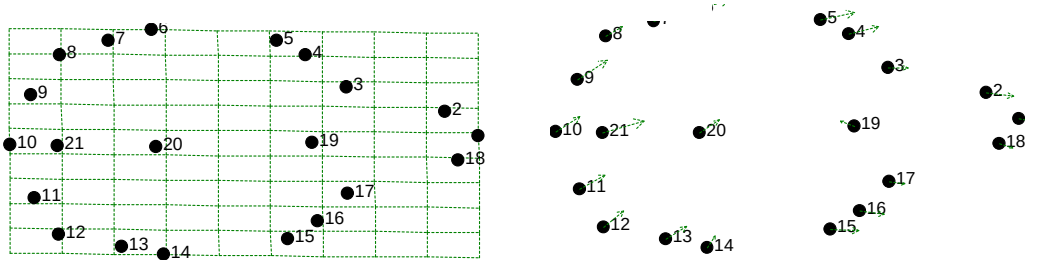
$2n = 35$ ($n = 3$)



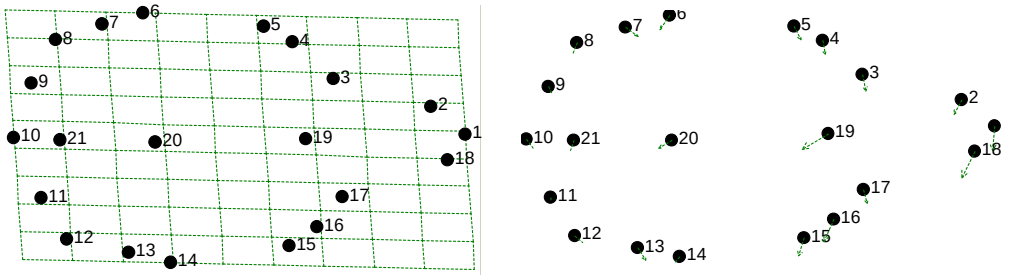
$2n = 37$ ($n = 1$)



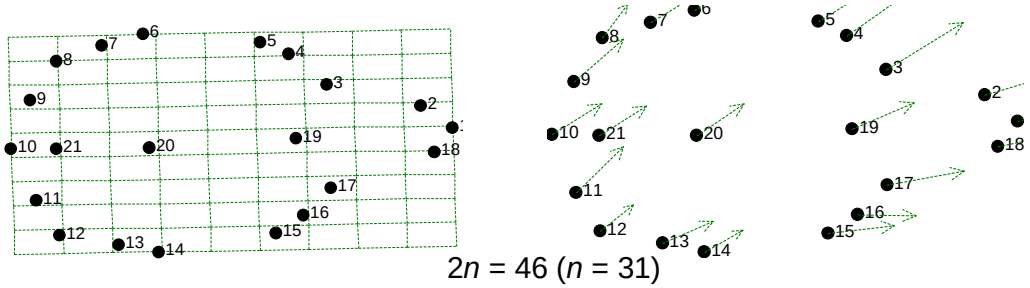
$2n = 42$ ($n = 3$)



$2n = 44$ ($n = 2$)



$2n = 45$ ($n = 1$)



$2n = 46$ ($n = 31$)

Figure 4.12. Changes in the position of landmarks with reference to a consensus configuration (splines and their associated vectors as shown by arrows) of the dorsal view of the cranium derived from TPSSpline (Rohlf 2004a) are indicated for the specimens of the pouched mouse, *Saccostomus campestris* from southern Africa.

4.4. Discussion

Despite limited sample size, the analysis of non-geographic variation in *S. campestris* from southern Africa suggest the lack of sexual dimorphism, but marked variation between age class I and age classes II–IV. Sample size limitations in the analysis of non-geographic variation is unavoidable in small mammals as in most cases, younger and/or very old individuals in some instances are rarely captured in nature. Consequently, instead of making subjective decisions, the very few individuals (particularly of the younger age) that are available may allow an objecyive insight into the nature and extent of non-geographic variation in small mammals. Consequently, it could be argued that the analyses in the present study justified the pooling of sexes and individuals of age class II-IV that are considered to have reached adult dimensions in all subsequent analyses of morphological variation among cytotypes.

The geometric morphometric analyses in the present study indicate that karyotypic variants within *S. campestris* from southern Africa do not differ morphologically. However, the 2*n* = 46 cytotype from KwaZulu-Natal exhibits subtle, although non-significant morphological differences relative to the other cytotypes. However, the possibility that *S. campestris* from the more mesic areas within the southern African subregion may be larger than those from more arid regions needs further investigation.

This pattern of morphological variation seems consistent with the results based on 16S rRNA and cytochrome *b* sequence data (Chapter 2), in which individuals with a diploid number of 2*n* = 46 from KwaZulu-Natal tended to form a basal lineage. Ellison (1992) also found statistically significant geographic differences in body mass and size within *S. campestris* from southern Africa with animals from the more mesic regions having larger body sizes and mass than those from the more arid regions. Ellison (1992) suggested that these differences may be innate since they were also evident in some laboratory-reared generations. It is also possible that these body size and mass differences may reflect differences in food availability in these regions.

The question that may be posed is what makes the KwaZulu-Natal cytotype unique from the other karyotypic variants within *S. campestris* from southern Africa? The vegetation of southern Africa comprises coastal thicket on the east coast, semi-arid woodland covering large parts of Namibia and Botswana, mesic woodland in the central-north, and grassland on the central plateau (Low & Rebelo 1998). While *S. campestris* is not common in the plateau grassland, there is a tendency for the KwaZulu-Natal cytotype to occupy the coastal thicket, while the inland southern African populations tend to occupy savannah woodland, suggesting that the distribution of cytotypes may be linked to vegetation types. However, in the case of *S. campestris*, this suggested link between its distribution and vegetation needs to be investigated further using a range of systematic and biogeographic techniques, as well as predictive Geographic Information System (GIS) analysis.

Apart from vegetation, there is also a need to assess other environmental and climatic variables that may allow insight into the distribution of the various karyotypic variants within *S. campestris* from southern Africa. For example, Ellison (1992) suggested that populations of *S. campestris* from ecologically similar areas also have similar physiological characteristics especially with reference to the innate and heritable body size that was shown to be positively correlated with rainfall. Moreover, Ellison (1992) found animals from colder regions had the capacity to produce more body heat than those from warmer regions. He speculated that this correlation between local temperature and extent of torpor was probably genetic.

Currently, there are a number of physical barriers that fragment the extant habitats of *S. campestris* in southern Africa. The largest of these few barriers include the Drakensberg mountain range and the Kalahari Desert that separate the populations in the west from those in the east including those from KwaZulu-Natal. The results of the molecular analyses (Chapter 2) showed indications that *S. campestris* from southern Africa may be a panmictic species that is sub-divided into numerous populations with relatively high rates of gene flow in inland southern Africa compared to the KwaZulu-Natal populations.

Based on cytochrome *b* gene data (Corti *et al.* 2004; Chapter 2), there are indications that the genus *Saccostomus* may have originated from East Africa. This seems to be supported by the fossil record where Denys (1988) reported that *S. mearnsi* shared more primitive characteristics with a 3.7 million year old fossil of *S. major* than with *S. campestris*. The most plausible explanation for the movement of the species may be a south-ward dispersal towards southern Africa into KwaZulu-Natal, followed by a north-ward dispersal around the eastern escarpment, and a later colonization of the rest of southern Africa.

Faulkes *et al.* (2004) also reported on a similar dispersal scenario in mole-rats of the genus *Cryptomys*. If the suggested dispersal scenario for *S. campestris* is valid, then it could be argued that the KwaZulu-Natal population may represent remnants of the ancestral population that occupied southern Africa. Consequently, the KwaZulu-Natal population has, based on molecular data (Chapter 2), been isolated since the Late Miocene, at least 2 million years ago.

The next question that may be posed is whether the results of the geometric morphometric analyses, together with the molecular (Chapter 2) and cytogenetic (Chapter 3) data, suggest that the currently recognized *S. campestris* from southern Africa represents a species complex? In hares of the genus *Lepus*, the presence of viable hybrids of well-defined biological species has been attributed to the lack of morphological and karyological barriers (Angermann 1983). In the cryptic murid rodents, *Aethomys chrysophilus* (2*n* = 50) and *A. ineptus* (2*n* = 44), on the other hand, the species not only show genetic differences (Russo 2003), but also well marked differences in sperm morphology (Breed 1997). No hybrids have been reported, with the difference in sperm being a more likely barrier to inter-breeding than the genetic difference. However, a lack of hybridization has similarly been reported in the cryptic murid rodents *Mastomys natalensis* (2*n* = 32) and *M. coucha* (2*n* = 36) (Skinner & Chimimba 2005), with the difference in chromosome number being the only known barrier.

In the case of *S. campestris* from southern Africa, there have neither been any reported differences in sperm morphology (Gordon 1986), nor of any

known deleterious chromosomal rearrangements (Gordon 1986; Ferreira 1990; Chapter 3). However, the extent of morphological overlaps shown by the geometric morphometric data in the present study as evidence of gene flow between karyotypic variants shown by the molecular data (Chapter 2) suggest that *S. campestris* from southern Africa is monotypic.

4.5. Conclusion

Evidence from the geometric morphometric analysis in the present study suggests that *S. campestris* from southern Africa is morphologically conservative. If the suggested pattern of dispersal for the species, its habitat preferences, and evidence of gene flow from the molecular data (Chapter 2) are the driving forces behind the cranial shape variation within *S. campestris*, then a more morphologically homogenous population would be expected in central and western southern Africa but with more isolated and morphlogically distinct populations in the east.

This seems to correspond with the results of a wide range of phylogenetic analyses (Chapter 2). In the geometric morphometric analyses in the present study, samples from a large geographic area in the west of the eastern escarpment tended to be morphologically similar. Similarly, some populations from this large geographic area showed large population size estimates in the molecular analyses (Chapter 2). The eastern populations, particularly those from KwaZulu-Natal on the other hand, although not statistically significant, tended to show some evidence of morphological distinctiveness in the geometric morphometric analyses, while the molecular analysis (Chapter 2) showed small population size estimates as well as evidence of limited gene flow with populations of other karyotypic variants. From the molecular evidence, it is possible that the colonisation of suitable habitats in southern Africa may have happened as long as 10 million years ago. The estimated time of divergence between the inland and KwaZulu-Natal populations estimated at 6 Mya may possibly explain the subtle morphological distinctiveness of the KwaZulu-Natal populations.

However, the results of the geometric morphometric analysis only indicate some subtle and statistically non-significant phenetic distinctiveness of the 2*n* = 46 cytotype from KwaZulu-Natal but do not suggest the presence of more than one species within the currently recognized *S. campestris* from southern Africa. Similarly, the estimated time of divergence is not a requirement that would automatically lead to speciation events (Ferguson 2002). Gordon (1986) and subsequently, Corti *et al.* (2004) suggested that *S. campestris* from southern Africa comprises two species, namely one with a diploid number of 2*n* = 46 and the other with a diploid number of between 2*n* = 30–38 that could be split further into subspecies. However, evidence from molecular (Chapter 2), cytogenetic (Chapter 3) and geometric morphometric data (present study) suggests that *S. campestris* from southern Africa is monotypic.

Chapter 5: General discussion

5.1. Theoretical considerations

For a species that inhabits a large geographic area, local populations may be subjected to biological and physical constraints that are specific to the local environment, and that may give rise to site-specific traits. This scenario may apply to *Saccostomus campestris* from southern Africa because: 1) it occurs over a large geographic area, with contrasting climatic conditions and habitats, 2) it is geographically variable in numerous physiological traits (Ellison 1992); and 3) it shows a high degree of chromosomal variation geographically. While this degree of variation may generally be difficult to interpret, the development of molecular techniques has made it increasingly possible to interpret, including making inferences of historical events from patterns of genetic variation (Templeton 1998; Barrowclough *et al.* 1999).

The present chapter is therefore aimed at interpreting molecular, karyotypic, and geometric morphometric variation in *S. campestris* within the context of developments in evolutionary theory over the last 15 years. These include the theoretical underpinnings relating to: 1) the role of chromosomes in speciation; 2) the question of whether chromosomal re-arrangements play a role in morphological, physiological, and behavioural changes; 3) the interpretation of the results obtained in the present study within the context of species concepts; and 4) taxonomic considerations.

5.2. Chromosomal re-arrangements and speciation

The role of chromosomal re-arrangements in speciation is controversial and a subject of considerable debate in evolutionary biology. Numerous models of the role of chromosomes in speciation have been proposed (for reviews, see Mayr 1970; Futuyma & Mayer 1980; King 1993). These models that appear to have been developed within the framework of the Biological Species Concept (BSC; Mayr 1970) include: 1) Allopatric speciation; 2) Stasipatric speciation (White 1978); 3) Chain process concept (White 1978); and 4) Speciation by monobrachial centric fusions (Baker & Bickham 1986). Accordingly, the involvement of chromosomal re-arrangements in speciation is dependent on

meiotic constraints acting on chromosomal heterozygotes (White 1978; Baker & Bickham 1980; Baker & Bickham 1986). While White (1978) suggested that the chain process of speciation is compatible with the stasipatric model, this was criticized by Baker & Bickham (1980), but subsequently supported by King (1993). Mayr (1970) reported on a number of reasons why chromosomal speciation was unlikely, and subsequently, Futuyma & Mayer (1980) argued that there was not enough evidence to suggest that karyotypic divergence drives genetic divergence and speciation. Futuyma & Mayer (1980) went further to cite a number of cases where multiple re-arrangements did not correlate with genetic divergence. However, these views were later countered by King (1993) who argued that their associated inferences were erroneous. Furthermore, Baker & Bickham (1986) suggested that chromosomal variation can lead to reproductive isolation if isolated populations fixed for monobrachial centric fusions were to hybridize resulting in meiotic impairment and reproductive isolation. However, reproductive isolation by itself does not automatically imply a speciation event. Coyne (1994) criticized speciation by chromosomal re-arrangements, and argued that the process is likely to be rare, and called for the need of rigorous experiments to determine whether chromosomal re-arrangements were an important cause of reproductive isolation. The generally accepted model of speciation is the allopatric model of speciation.

Given the above background and interpreting it with reference to the data generated for *S. campestris* from southern Africa, there seems to be no evidence of reproductive isolation among karyotypic variants within the species that might have resulted from karyotypic events. This is because: a) there are many heterozygote karyotypes, b) similar heterozygote cytotypes arose in different DNA lineages, c) there is lack of monobrachial centric fusions, and d) there are relatively high migration rates among populations resulting in high gene flow estimates (Chapter 2) despite many chromosomal re-arrangements within the species.

5.3. The relationship between chromosomal re-arrangements, morphology, physiology and behaviour

In inferring a phylogeny for *S. campestris*, the question that may be posed relates to whether there is a link between chromosomal re-arrangements, morphology and behaviour in the species. Robertsonian translocations have been attributed to differences in the behaviour and skull morphology of certain chromosomal races of the house mouse, *Mus musculus domesticus* (Gérard *et al.* 1994; Corti & Rohlf 2001). Gerard *et al.* (1994) also found that heterozygotes of *Mus musculus* were less likely to be attracted to traps than homozygotes. Other differences in behaviour ranged from aggression, sexual selection, learning behaviour, to exploration (Gerard *et al.* 1994). Corti & Rohlf (2001) found a correlation between chromosomal races of the house mouse with differences in skull morphology and suggested that these differences may be responsible for the variable degrees of aggressive behaviour in some chromosomal races.

Numerous studies have used behavioural traits to elucidate phylogenetic relationships between taxa. For example, Delport (2000) used behavioural, morphological, and molecular analyses to gain an insight into the taxonomic status of the red-billed hornbill from southern Africa. Paterson *et al.* (1995) used behavioural and life history data to reconstruct the phylogeny of some sea birds, the results of which were similar to those based on molecular data that suggested that sister taxa had similar behavioural and life-history traits. Similar results were found in a study on Pelicaniformes by Kennedy *et al.* (1996) who reiterated that behavioural characters were no more homoplasious than both morphological and genetic characters.

Despite the challenge being the identification of synapomorphies in behaviour, the use of behavioural traits as phylogenetic characters is further supported by De Queiroz & Wimberger (1993), and could be valuable in the elucidation of phylogenetic relationships in *S. campestris*. Results in the present study (Chapter 3) indicated that chromosomal re-arrangements in *S. campestris* may be correlated with climatic conditions in southern Africa. In addition, Ellison (1992) and subsequently Ellison *et al.* (1994) found geographic differences in a range of physiological traits in *S. campestris* that

included differences in the nature and extent of torpor and morphological differences in terms of body mass. For example, animals from arid and energetically stressful environments experienced longer bouts of torpor than those from warmer mesic areas suggesting that karyotypic variation in *S. campestris* from southern Africa be correlated with an adaptation to environmental gradients. However, although the low diploid numbered populations occupy unpredictable environments, the distribution of cytotypes is random (i.e., certain cytotypes of similar karyotypes evolved on separate occasions independent of each other according to cyt *b* and 16S rRNA data). This therefore, suggests that karyotypic characters cannot be useful in delineating the phylogeny for *S. campestris*. According to the geometric morphometric analysis (Chapter 4), there was no discernable variation that was observed among different populations. Furthermore Gordon (1986) found no variation in sperm morphology among cytotypes. This suggests that *S. campestris* from southern Africa is morphologically conserved, meaning that morphology would cannot be useful in constructing a phylogeny for *S. campestris*. In the light of this discussion, chromosomal re-arrangements, morphology, physiology and behaviour in *S. campestris* from southern Africa appear to be highly plastic or homoplasious. As a result these traits would not be appropriate for constructing a phylogeny for *S. campestris* from southern Africa.

5.4. Species concepts and taxonomic considerations

The taxomomic relevance of the present study is dependent on the interpretation of the multidisciplinary results (Chapters 2–4) with reference to the species concepts alluded to in Chapter 1. This will be argued in seven parts. Firstly, I will discuss the low diploid number populations in the western arid parts only. Second I will look at high diploid number populations in the east excluding Kwazulu-Natal population. Fourth I will include Kwazulu Natal. Fifth I will attempt to discuss the status of *S. campestris* in southern Africa. Sixth I will shortly discuss the east African populations. Lastly I will attempt to discuss *S. campestris* throughout the distribution, meaning that I will attempt to discuss east African populations as well.

1. Low diploid number populations. Peterson & Navarr-Sigüenza (1999) outlined the problems of making biodiversity estimates when more than one species concept is applied to the same data set. When they applied the Biological Species Concept (BSC) and the Phylogenetic Species Concept (PSC), they found there were either 101 or 249 endemic bird species in Mexico. They argued that in comparison to other species concepts, the PSC may over-estimate the number of species.

In the present study, an attempt is similarly made to interpret the multidisciplinary data with reference to species concepts. The 2*n* = 30–36 cytotypes in *S. campestris* are represented by four populations that occupy a large semi-arid to arid area in the south-western parts of southern Africa. Within these cytotypes, the 2*n* = 34 and 2*n* = 36 karyotypes are represented by more than one cytotype each that arose on three independent occasions suggesting large scale plasticity in karyotypic configuration (Chapter 3). If the BSC were to be applied, then there may be two reasons to suggest that the 2*n* = 30–36 cytotypes represent a single biological species.

Firstly, the number of independent karyohybrids was high suggesting that there is a lot of inter-breeding between cytotypes in the large semi-arid to arid area in the south-western parts of southern Africa. Secondly, the high gene flow estimates from cyt *b* data further suggest that there is a lot of gene flow among these populations (Chapter 2). Therefore, there seems to be no isolating mechanisms between the cytotypes, suggesting the presence of a single biological species. The evidence of significant gene flow between populations implies that pouched mice of different cytotypes are able to recognize each other in the wild and are therefore, able to freely inter-breed, further suggesting a single recognition species (RSC) within the low diploid numbered *S. campestris* from southern Africa.

The Cohesion Species Concept (CSC) is based on testable hypotheses of genetic exchangeability (involving factors of successful gene flow or isolation) and demographic exchangeability (concerned with adaptation) (Templeton 1989). If CSC were to be applied, a case for the recognition of a single species within *S. campestris* from the arid western parts of southern Africa

could be developed. Based on the maximum likelihood estimates of migration between these low diploid numbered populations, the genetic exchange between the pouched mice of Namibia and of the Northern Cape was very high (>> 10 effective number of migrants, Table 2.7., Maputla 2008 Chapter 2). This therefore suggests that the conditions for genetic exchangeability are satisfied. When assesing the factors relating to adaptation, both populations are from the arid areas and are limited by sandy soils and *Acacia sp.*, therefore, demonstrating demographic exchangeability and satisfying the conditions for a single cohesion species.

Similarly, following the Evolutionary Species Concept (ESC; Simpson 1961) that defines a species as having independent evolutionary fates and separate identities, a case for the recognition of one species within *S. campestris* from the south-western part of the distribution could also be developed. The cyt *b* genealogy suggests that for the low diploid numbered populations, there are several populations (Fig. 3.6, Chapter 3). In all these low diploid numbered populations, a case of separate identities cannot be argued, because there is very high gene flow estimated between the Namibia/Botswana and the Northern Cape groups. The high gene flow estimate eliminates questions relating to separate identities and as a results argues for a single evolutionary species in the region (>> 10 effective number of migrants, Table 2.7., Chapter 2).

If the Phylogenetic Species Concept (PSC) *sensu* Nixon & Wheeler (1990) that defines species as being characterised by fixed character state differences, a case for the recognition of more than one species within *S. campestris* from southern Africa could be developed. The phylogenetic analyses of the cyt *b* gene data for animals from the arid western parts of South Africa suggest that these are separated from the Namibia/Botswana animals, which form their own cluster. This means that for the low diploid numbered populations, there are 2 phylogenetic species based on the cyt *b* gene fragment. By comparing sequences from Namibia/Botswana and the Northern Cape, there are four base pair differences that are consistent between the two populations. These are at positions 25, 31, 47 and 84 on the variable site alignments table in Chapter 2 (Table 2.5a; Chapter 2). The

analysis of the 16S rRNA haplotype sequences also suggests the possibility of two phylogenetic species as there are two consistent base pair differences for pouched mice from the two localities, which can be used as character states (positions 3 and 8, Table 2.5b; Chapter 2). The two arguments therefore, justify the argument for two phylogenetic species within the low diploid numbered populations in the south-west arid region, the Namibia/Botswana and the Northern Cape species.

2. High diploid number populations. In so far as the remaining cytotypes (2*n* = 44–46) from Lowveld/Mozambique, Gauteng, Mpumalanga, Limpopo, and Eastern Cape Provinces of South Africa are concerned, it was not possible to test for isolating mechanisms (BSC) and species recognition (RSC) in the present study. However, given that independent hybrids of the 2*n* = 45 cytotype were found in Pretoria, Gauteng Province, South Africa and Francistown, Botswana, as outlined below there may be evidence for a single biological species (BSC) and an inference can be made for a single recognition species (RSC).

Gene genealogies for the two mitochondrial loci also show that there is genetic exchangeability between many populations, while the individual from the Karoo (Eastern Cape Province, South Africa) cannot be connected with any degree of statistical confidence, however, the animals are still limited by the same key ecological combination of *Acacia sp.* and sandy soils, therefore, suggesting a single cohesion species (CSC). High gene flow estimates also argue for a single cohesion species (>> 10 effective number of migrants: Table 2.7; Chapter 2). For the PSC, a case for two phylogenetic species (the Lowveld/Mozambique and Gauteng populations [including the Karoo]) from the current dataset can be argued based on the variable sites (positions 55, 59, 66, 78, and 86; Table 2.5a; Chapter 2) that are consistent in haplotype sequence alignment and also as presented on the cyt *b* gene genealogy (Fig. 2.7a; Chapter 2). The ESC on the other hand, suggests that there is one evolutionary species because of the extremely high migration rate estimates (>> 10 effective number of migrants, Table 2.5; Chapter 2).

3. Combining the east with the west. Generally in the present study, the number of chromosomal heterozygotes in the 2*n* = 30–46 cytotypes was very high (Chapter 3), and mostly found in the large central parts of the subregion. In addition, there has been a successful laboratory crossing and back-crossing of the two extreme karyotypic variants of the 2*n* = 46 cytoptype from the Lowveld and the 2*n* = 32 cytotype from the South-West arid zone for a number of generations, with the F_1 off-spring having a diploid number of 2*n* = 39 (E. Dempster *pers. comm*.). This suggests that the two cytotypes share a similar fertilization system that would lead to a single biological species (BSC) and a single recognition species (RSC).

Gene genealogies suggest that there are three haplotypes that cannot be connected to the main haplotype tree for inland South Africa (Fig. 2.7a, Chapter 2). However, the high gene flow estimate between the east and the west overrides the visualised genealogy. Furthermore, it agrees with the ecological factors that are paramount to the survival of the species. Based on these arguments, there is a single cohesion species (CSC) of *S. campestris* from inland South Africa including Namibia, Botswana and southern Mozambique. Based on single-nucleotide DNA differences, the PSC suggests the presence of three phylogenetic species as discussed above. These are: 1) Namibia/Botswana, 2) the Lowveld, and 3) the rest of the populations west of the Lowveld (See Table 2.5a; Chapter 2). In so far as the ESC is concerned, a single species can still be argued as there is no evidence of separate identities between populations of *S. campestris* from inland southern Africa.

4. Kwazulu-Natal. For the 2*n* = 30–46 cytotypes from inland southern Africa and the 2*n* = 46 cytotype from KwaZulu-Natal, there is probably a single biological species (BSC) and a single recognition species (RSC). This is based on chromosome morphology as the KwaZulu-Natal population has the 2*n* = 46 karyotype which is also found in the Lowveld and inland South Africa. Furthermore, Gordon (1986) found no differences in sperm morphology between the southern African *Saccostomus* suggesting similar fertilization systems that characterise both Biological (BSC) and Recognition (RSC) species. However, this conclusion is contradicted by the phylogenetic

analyses that suggest that the KwaZulu-Natal populations differ from the inland populations (Chapter 2).

According to the PSC, there are four species of *S. campestris* in southern Africa namely: 1) KwaZulu-Natal (21 distinct single-basepair characters for cyt *b* and 5 characters for 16S rRNA); 2) Lowveld (3 distinct characters for cyt *b*, and 1 character for 16S rRNA); 3) Namibia/Botswana (1 distinct character for cyt *b*, and 1 character for 16S rRNA); and 4) the rest of southern African inland populations (characters not defined). Haplotype sequence alignments of variable sites show these groups as having several characters that are consistent for each group as shown in parentheses next to each group mentioned above (Table 2.5a & b).

The conditions for the CSC relate to genetic and ecological compatibility. In the case of *S. campestris* from southern Africa, the KwaZulu-Natal population has been separated from the rest of the inland populations for a long time (approximately 2 Mya; Chapter 2), probably due to the eastern escarpment. As a result, the cyt *b* gene shows many differences between the KwaZulu-Natal population and the rest of southern Africa. Gene flow analysis also shows close to zero migration estimates between KwaZulu-Natal and other populations (0.1 [between KwaZulu-Natal and Gauteng] & 0.75 [between KwaZulu-Natal and the Lowveld]; Table 2.7; Chapter 2). In terms of ecological compatibility, the KwaZulu-Natal population is adapted to the dune forests where the humidity is high and the inland populations are restricted to drier environments. As a result, a case for two cohesion (CSC) species can be argued for *S. campestris* from southern Africa.

In terms of the ESC, one condition can be used to argue for more than one species. As was mentioned in the previous paragraph, the presence of the eastern escarpment between KwaZulu-Natal and inland populations has made it possible that the two groups evolve independently of each other. As the two groups are evolving separately, the variation in cyt *b* sequence alignments presently is the result of a long period of restricted gene flow. As a result, the two groups have separate identities suggesting two evolutionary (ESC) species.

5. Southern Africa. Overall, based on the phylogenetic analyses in the present study, the ESC, CSC and PSC suggest the presence of more than one species of *S. campestris* in southern Africa. The main problem with interpretation of the results in the present study relates to the allopatric KwaZulu-Natal population that has been separated from the other populations for as long as 2 million years (Chapter 2). Any two allopatric populations with little or no gene flow and fixed phenotypic and genetic differences are likely to be treated as more than one species under the ESC, CSC, and PSC. These species concepts do not take into account genetic drift and local geographic genetic divergence in rapidly evolving taxa such as the genus *Saccostomus*.

The question that may be posed is whether genetic divergence or even large genetic differences imply the presence of more than one species. Under the BSC and the RSC, the taxonomic status of allopatric populations with little or no gene flow would be ambiguous and would be dependent on further observations and/or experiments. In addition, Ferguson (2002) argued that large genetic distances do not automatically imply reproductive isolation. However, even if more than one species exists within *S. campestris* in southern Africa, these do not correspond to those suggested by Gordon (1986) and Corti *et al.* (2004) because the 2*n* = 46 karyotype falls in each of the two allopatric populations (i.e., the KwaZulu-Natal and inland populations).

In so far as the rest of southern African subregion is concerned, there appears to be high degrees of gene flow and labile karyotypic events that renders the recognition of new species meaningless, given the evidence in the present study. The 2*n* = 46 karyotype may therefore, not constitute a separate species because the KwaZulu-Natal population only represents a small part of the populations with a 2*n* = 46 cytotype.

6. East African populations. Similar to the southern African populations, the east African populations pose practical problems when the BSC and RSC are applied. The haplotype network for specimens from east Africa (Fig. 2.7. b, Maputla 2008, Chapter 2) shows a lot of missing haplotypes. On other occations more than 14 missing haplotypes were indicated, preventing the

linkage of haplotypes in a single haplotype tree and making it difficult to establish the presence or absence of gene flow. Similarly CSC poses challenges with regards to gene exchange and therefore argues for more than one cohesive species in east Africa. The PSC and ESC also argue for more than one species in the east,.

7. Southern and eastern African animals. Looking at Zambia and Tanzania, there is little evidence of gene-flow with other southern African populations because Zimbabwe (Mashonaland), Malawi and Angola were not sampled. An in-depth look at the low diploid number populations from Zimbabwe (Gordon 1986), would give a clear indication of the relatedness between Zambian, Zimbabwean and the rest of southern African pouched mouse populations. If the BSC were to be applied for the southern African and eastern African populations the following reasons would pose two challenges. Firstly, the presence or lack of gene flow cannot be easily tested because of the distance. Secondly, similar to the Kwazulu Natal population and other southern African populations, the east African populations have been separated to the southern African populations more than 2 Mya. As a result, further investigations are necessary, such as behavioural experiments and tests to see if these animals can recognise each other in the wild and interbreed freely. Similarly, following the RSC one would have to establish if the fertilization systems of the two goups are compatible. This means that certain behavioural (e.g. potential mate selection) and morphological (e.g. sperm morphology) aspects should be investigated.

Under CSC, ESC, and PSC there are more than one species of *S. campestris*. For CSC a case of ecological and genetic connectivity has to be proven, but the present phylogenetic analyses do not show that there is genetic connectivity between the southern African and eastern African populations. Based on this reason alone a case of more than one cohesion species can be argued. For the ESC, the two population groups have more tha 2 My since divergence meaning that the two groups are probably evolving in different directions and therefore suggesting more than one species. As mentioned earlier, with respect to PSC the number of species sometimes tend to be overinflated when this concept is enforced. Phylogenetic analyses alone

provide enough evidence for one to argue that because there are so many haplotypes all with fixed genetic differences, it is clear that eastern and southern African *S. campestris* are separate species..

To sum up, further studies that would incorporate fresh material including KwaZulu-Natal, Zimbabwe, Angola and Malawi, and covering aspects of ecology and behaviour would be necessary in order to fully resolve the taxonomic status of *S. campestris* in sub-Saharan Africa. The present study concludes that karyotype, morphology and behaviour cannot be used to delineate the phylogeny of *S. campestris*, but with the use of molecular markers such as mitochondrial DNA, it was possible to get an insight into the evolutionary history of the pouched mouse in the subregion.

5.5. Conclusion

The present study attempted to test different hypotheses within the framework of a range of concepts. While the CSC, PSC, and ESC suggest the presence of more than one species, the BSC and RSC suggest that *S. campestris* from southern Africa is monotypic. In conclusion, even if there were more than one species of *S. campestris* in southern Africa, this is not the same as the species suggested by Gordon (1986) and Corti *et al.* (2004) as was demonstrated by the analyses of mitochondrial DNA.

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Appendices

Appendix 1

A reference sequence (d1) of 1077 base pairs from the cyt *b* haplotypes for *Saccostomus campestris* from southern Africa starting at position 26 to 1103.

GAAAGACACACCCTCTACTCAAAATTATTAACAACACATTTATTGATCTCCCAACCCCCTCAAATATCTC
TTCATGGTGAAACTTCGGCTCCCTCCTAGGAATCTGTCTAATTATCCAAATTATCACAGGCTTATTTCTA
GCCATACACTACACATCAGACACAACAACAGCATTTTCATCAGTCACCCATATCTGCCGAGACGTCAATT
ACGGTTGACTAATTCGATATCTACATGCAAACGGAGCCTCAATATTCTTCATTTGCCTCTTCATCCACGT
AGGACGAGGTATCTACTACGGCTCCTACACCTCCACAGAAACATGAAACATTGGCATTATCCTCCTACTT
ACAGTAATAGCAACAGCCTTCATAGGATATGTTCTTCCATGAGGACAAATATCATTCTGAGGGGCAACAG
TCATCACAAACCTCCTATCAGCCATCCCATATATTGGCCCCACTTTAGTAGAGTGGATCTGAGGCGGATT
CTCAGTAGATAAAGCCACTCTTACACGATTTTTCGCCTTTCACTTTATCCTACCCTTCATTATTGCAGCC
CTAGCAGCAGTCCACCTATTATTTTTACACGAAAAAGGATCCAATAACCCCACAGGACTAAATTCAAATG
CTGATAAAATCCCCTTCCACCCTTATTACACAATCAAAGACCTCCTAGGCATTCTCCTCCTCATCTTAAC
CCTTGTTTCCCTAGTCTTATTTACCCCAGATCTGCTAGGAGACCCGGACAACTACACCCCCGCCAACCCA
CTAAACACGCCCCCACATATTAAACCAGAGTGATATTTCCTATTCGCTTATGCCATCTTACGCTCAGTAC
CCAATAAACTTGGAGGAGTCCTAGCACTAATTATATCCATTTTAATCCTATCTGTACTACCCCATTTATA
TAAATCAAAAATACAAAGTCTCATGTTCCGCCCAATCACCCAATTTTGTTTCTGATTACTAGTCGCTGAT
ATTCTTATCCTTACTTGAATTGGAGGTCAACCTGTCGAATACCCATTCATCATAATTGGTCAACTCGCCT
CTATCCTCTACTTTAGTGTTCTCATT

Appendix 2

A reference sequence of 528 base pairs of the 3' end of the mitochondrial DNA 16S rRNA gene (ts1) in *Saccostomus campestris* from southern Africa used to produce 12 haplotypes.

TATTAGAGGCACTGCCTGCCCAGTGACTTACGTTCAACGGCCGCGGTACCCTGACCGTGCAAAGGTAGCA
TAATCACTTGTTCCTTAATTGGGGACTAGCATGAAAGGCAAGACGAGGGCTAAACTGTCTCTTATCTCCA
ATCAGTGAAATTGACCTCCCTGTGAAGAGGCAGGGATATATAAATAAGACGAGAAGACCCTATGGAGCTT
TAATTTACTAATTTAATCTACCAAATCCCCACCCTATTGGCACAAACCCCGTGGTAATAAATTAGAAATT
TTGATTGGGGTGATCTCGGAGCACAAACAAACCTCCGAGTGTTTTTTACCAAGACATACAAGTCAAAGTT
ATTACACAACACAATTGACCCAATTACTTTGATCAACGGACCAAGTTACCCTAGGGATAACAGCGCAATC
CTATTCAAGAGTTCATATCGACAATTAGGGTTTACGACCTCGATGTTGGATCAGGACATCCCAATGGTGC
AGAAGCTATTAAAGGTTCGTTTGTTCAACGATTAAA

Appendix 3

A gazetteer and geographic coordinates of sampled localities and specimens of the pouched mouse, *Saccostomus campestris* from southern Africa examined in the present study. TM denotes the Transvaal Museum of the Northern Flagship Institute (NFI), Pretoria, South Africa. Specimens from the highlighted localities were used to test for sexual dimorphism and age variation. The locality numbering corresponds to the numbering of the collecting localities and their associated cytotypes indicated in Figure 4.1.

Locality	Geograp		Transvaal Museum (TM) number
	Latitude (S)	**Longitude (E)**	
1 Alldays	22° 42’	29° 04’	34305
2 Caprivi	18° 07’	21° 33’	38580; 38581
3 Grahamstown	33° 19’	26° 32’	34306
4 Groblersdal	23° 03’	29° 04’	38779
5 Hartswater	27° 46’	24° 45’	35564; 35911; 35912
6 Hay	28° 32’	22° 29’	38576
7 Hluhluwe	28° 05’	32° 04’	35559; 35561; 35562; 35563; 35566; 35560 35578; 35579
8 Jan Kempdorp	27° 56’	24° 57’	37190
9 Keetmanshoop	26° 57’	17° 56’	32613
10 KGNP	25° 23’	20° 36’	35915
11 Kuruman	27° 34’	23° 22’	35560; 35578; 35579
12 Lower-Umfolozi	28° 16’	31° 44’	35552; 35558; 35568; 35569; 35570;35573; 35574; 35575;35576
13 Namibia	25° 26’	17° 44’	38574; 38575; 38594
14 Noupoort	31° 07’	25° 12’	34304
15 Rustenburg	25° 42’	27° 10’	34307
16 Satara	24° 23’	31° 47’	38583
18 Pilgrim’s Rest, KNP	24° 24’	31° 46’	35949; 35950; 35951; 35952; 35953; 36650; 36653; 36659
19 Pofadder	28° 51’	19° 42’	41391
20 Pretoria	25° 25’	28° 15’	39257; 39258; 39259
21 Punda Maria	22° 43’	31° 02’	37045
22 Roodeplaat	25° 25’	28° 15’	41036
23 Nylsvlei	24° 39’	28° 40’	38968
24 Tembe	27° 02’	23° 35’	40442
25 Vryburg	25° 44’	23° 40‘	35917; 35919
26 Morokweng	26° 03’	22° 73’	38577
27 Windhoek	21° 55’	17° 37’	38573

Appendix 4

A list and description of landmarks of the dorsal (a), ventral (b), and lateral (c) views of the cranium, and the lateral (d) view of the mandible in the pouched mouse, *Saccostomus campestris* from southern Africa used in the present study. Landmark numbering corresponds to that in Fig. 4.3.

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a) Landmarks of the dorsal view of the cranium:

1. Tip of the rostrum;
2. Left rostrum at the point of maximum curvature;
3. Left tip of maxilla at the point of maximum curvature
4. Left tip of Maxilla and zygomatic arch anterior point attachment;
5. Left tip of Maxilla and zygomatic arch posterior point attachment;
6. Left posterior superior tip of squamosal root of the zygomatic bar;
7. Left posterior inferior tip of squamosal root of the zygomatic bar;
8. Left maximum point of the tympanic bulla at the point of maximum curvature ;
9. Left joining point of the parietals and inter parietal;
10. Edge of the occipital condyle;
11. Right joining point of the parietals and inter parietal;
12. Right maximum point of the tympanic bulla at the point of maximum curvature
13. Right posterior inferior tip of squamosal root of the zygomatic bar;
14. Right posterior superior tip of squamosal root of the zygomatic bar;
15. Right tip of Maxilla and zygomatic arch posterior point attachment;
16. Right tip of Maxilla and zygomatic arch anterior point attachment;
17. Right tip of maxilla at the point of maximum curvature;
18. Right rostrum at the point of maximum curvature;
19. Maximum width of the rostrum at the point of maximum curvature;
20. Mid-point between frontals and parietals; and
21. Mid-point between the interparietal and parietals

b) Landmarks of the ventral view of the cranium:

1. Tip of nasals at their anterior suture;
2. Anterior end of incisive foramen at the anterior end of the suture of the palatines;
3. Posterior end of incisive foramen;
4. Anterior end of the prephenoid;
5. Posterior end of the suture of the palatines at the anterior end of the basilar part of the occipital bone;
6. Left hypoglysal canal;
7. Left preoccipital process;
8. Left lateral tip of occipital crest at the posterior margin of M^3 alveolus;
9. Left anterior margin of PM^4 alveolus;
10. Left tip of maxilla at the point of maximum curvature;

11. Left rostrum at the point of maximum curvature;
12. Left posterior inferior tip of squamosal root of the zygomatic bar;
13. Right tip of maxilla the point of maximum curvature;
14. Right anterior margin of PM^4 alveolus;
15. Right lateral tip of occipital crest at the posterior margin of M^3 alveolus;
16. Right preoccipital process;
17. Right rostrum at the point of maximum curvature;
18. Right posterior inferior tip of squamosal root of the zygomatic bar; and
19. Right hypoglysal canal.

c) Landmarks of the lateral view of the cranium:

1. Tip of nasals;
2. Edge of the interparietal and supraoccipital;
3. Tip of the foramen magnum;
4. Posterior incision of the jugal to the squamosal;
5. Anterior incision of the jugal to the squamosal;
6. Posterior margin of M^3 alveolus;
7. Anterior margin of PM^4 alveolus;
8. Interior margin of I^1 alveolus;
9. Upper margin of the root of the zygomatic process;
10. Edge of the occipital condyle;
11. External auditory meatus;
12. Post-glenoid foramen; and
13. Lower margin of the root of the zygomatic process.

d) Landmarks of the lateral view of the mandible:

1. Exterior margin of I_1 alveolus;
2. Interior margin of I_1 alveolus;
3. Anterior margin of PM_4 alveolus;
4. Tip of the coronoid process;
5. Tip of the condylar process;
6. Upper tip of the angular process; and
7. Lower tip of the angular process.