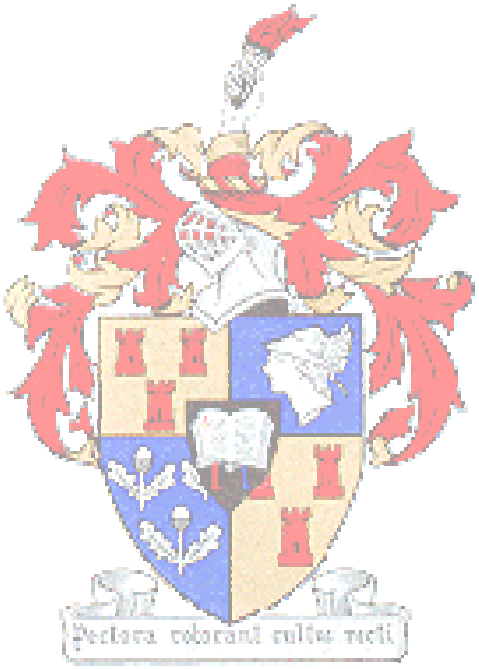
Phylogeographic variation of the Karoo Bush Rat, *Otomys unisulcatus:* a molecular and morphological perspective

Shelley Edwards



Dissertation submitted in fulfillment of the requirements for the degree of Masters of Science in the Department of Botany and Zoology, Stellenbosch University South Africa.

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DECLARATION

I, Shelley Edwards, hereby declare that the work contained in this dissertation is my own original work and has not previously been submitted for any degree or examination at any University. Specimens were collected under permits issued by Cape Nature, Western Cape Province (Permit no: AAA004-00034-0035), SANPARKS (Permit no: 2007-08-08SMAT) and the Department of Tourism, Environment and Conservation, Northern Cape Province (Permit no: 0904/07). Museum samples were obtained with the permission of the respective curators at the museums.

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Shelley Edwards

On this …… day of ……2009

ABSTRACT

Phylogeographic genetic structure has been documented for a number of southern African terrestrial taxa. Information regarding geographic population genetic structuring in multiple taxa, with differing life histories, can provide insights into abiotic processes such as vicariance. A fragment of the cytochrome *b* mitochondrial DNA gene of a plains-dwelling species, *Otomys unisulcatus*, was sequenced and analysed. Two closely related geographic assemblages were found. The first assemblage (lowland group) contains populations from both the eastern and western parts of the species range, and the second comprises populations from the Little Karoo (central group). The lowland group was shown to be in a state of population expansion after a relatively recent mitochondrial DNA (mtDNA) coalescence, while the genetic signature of the central assemblage was characterized by more genetic diversity indicative of an older lineage/genetic refuge. Areas of higher elevation (namely mountain ranges) appeared to be the main factor limiting gene flow between these two groups. Aridification cycles due to glacial maximum periods probably resulted in increased dispersal leading to the widespread distribution of common haplotypes throughout the lowland group.

Morphological variation in skull shape and size has been shown to follow environmental clines in some rodents. Geometric morphometric analyses on the ventral and dorsal views of the craniums of *O. unisulcatus* were utilised to test whether the population groupings obtained in the genetic analyses would be recovered by morphometric analyses. In addition, it was also investigated which of the environmental factors investigated influenced skull shape and size. The genetic groupings were not recovered for either the cranial shape or size. Size variation in the females correlated positively with annual rainfall, and so by proxy with habitat productivity, indicating that females which inhabited areas with lower rainfall would be larger. The significant relationship between females' centroid sizes and rainfall was thought to be as a result of the increased nutrient requirement by this gender in the production of offspring. The males did not show a significant correlation between any of the environmental variables and centroid size. There was a significant difference between the skull shapes of the genders, further verifying the sexual dimorphism in the species. Three major clusters were found (according to cranium shape) using a Two-Block Partial Least Squares Analysis (2B-PLS), which relate to the biome boundaries within the species' range. Variations in

shape were attributed to the varying needs for strong masticatory muscles resulting from differing diets. The skull shapes of specimens occurring along the escarpment were intermediate between the first two clusters. Cranial shape in the male dorsal view dataset was significantly correlated with the environmental variables block, possibly due to the much lower minimum temperature in the Sutherland population (a population which was not included in the female analyses). It was concluded that differing diets of individuals in the respective biomes influenced the shape of the cranium of both genders. The sexual dimorphism in the cranium shapes may be as a result of the females digging tunnels (using their teeth) underneath the stick nests. *Otomys unisulcatus* show high levels of phenotypic plasticity throughout the range and it thus appears that the species can adapt fast to the different environmental variables.

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PREAMBLE

The challenges facing conservation biology include devising practical approaches to protect biodiversity and the processes which shape and sustain it (Moritz, 2002). The description and calculation of the spatial pattern of diversity as a field of study has grown in leaps and bounds (Avise, 2009), founded on various disciplines, including genetic and morphological studies. In a changing environment, facing perturbations by human activity, the maintenance of ecological and evolutionary processes sustaining diversity is vital (Frankel, 1974; Smith *et al.*, 1993; Balmford *et al.*, 1998; Moritz, 2002).

The conservation of biodiversity of South Africa is becoming increasingly important, and the knowledge of areas characterized by high endemicity and high species richness will aid in determining conservation priorities. A number of studies in South Africa (e.g. Gelderblom *et al.*, 1995; Gelderblom & Bronner, 1995; Mugo *et al.*, 1995; Proches *et al.*, 2003) have produced valuable data to identify hotspots (most importantly the Cape Floristic Region and the Succulent Karoo; Myers *et al.*, 2000). Such studies have stressed the importance of obtaining knowledge of the distribution of many species in an area, in order to identify regions of conservation interest and value (e.g. Happold, 2001; Tolley *et al.*, 2009). In South Africa, the Succulent Karoo has been identified as one of the least conserved biomes (Rebelo, 1997; Lombard *et al.*, 1999) and requires the largest proportion of additional reserves to protect its flora (Rebelo, 1994). The fauna are not as comprehensively studied in this biome but knowledge of past processes influencing dispersal, geneflow and possible vicariant events in this region (and how lineages responded to changes in the past) will arguably aid in the determination of areas of conservation interest. Additionally, this knowledge may provide better predictions of future responses to amongst others anthropogenic changes. In the broader context, the description of these patterns also aid in understanding evolutionary processes that gave rise to the diversity we see across the landscape today.

The present study investigated the mitochondrial DNA (mtDNA) and morphological phylogeography of *Otomys unisulcatus,* a plains-dwelling southern African rodent species. The results of this investigation will contribute to a broader phylogeographic programme at the University of Stellenbosch in which taxa with different life-history characteristics are examined for the presence of concordant genetic breaks. Investigating possible congruent phylogeographic signatures between taxa with differing life histories will enhance the understanding of past vicariant events, and their influence on the current species patterns. In turn, the approximate geographic position of historical refugia may be identified.

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AIMS AND OBJECTIVES

The focus of this project was to investigate the phylogeographic patterns of the Karoo Bush Rat *Otomys unisulcatus* using mitochondrial DNA sequence and morphological data.

This study investigated the following questions:

- Is the mtDNA variation across *O. unisulcatus* populations geographically structured?
- Can the genetic phylogeographic patterns be linked to paleoclimatic oscillations?
- Is the morphological variation across *O. unisulcatus* populations geographically structured?
- Which environmental factors most affect skull shape and size?
- Is the variation in the morphological and genetic components of the species congruent?

It is expected that, since *O. unisulcatus* is a plains-dwelling species, mountain ranges can pose barriers to gene flow within this species. More ancient intraspecific genetic divergences would most likely be reflected in the morphological divergences between phylogeographic groups. Environmental factors (such as rainfall and latitude) may affect the skull shape of *O. unisulcatus* through ontogeny via phenotypic plasticity or local adaptation. It is proposed that specimens within more arid regions (e.g. Succulent Karoo Biome) would be larger as it is a harsher environment (Armitage, 1999).

CHAPTER 1
GENERAL INTRODUCTION

Taxonomy of the otomyine rodents

The order Rodentia is the most speciose of the mammal orders, comprising almost half of the extant mammalian species (Roberts, 1951). The placement of the otomyine group (laminate-toothed rats) within this order has ranged from being ranked as a family (Roberts, 1951), as a subfamily within Muridae, defined as either *sensu lato* (Thomas, 1896; Ellerman, 1941; Roberts, 1951), or *sensu stricto* (Tullberg, 1899; Miller & Gidley, 1918; Simpson, 1945; Reig, 1981), as a subfamily within Cricetidae (Misonne, 1974) or Nesomyidae (Chaline *et al.*, 1977; Lavocat, 1978), and more recently as a tribe within the Murinae (Otomyini; Watts & Baverstock, 1995; Ducroz *et al.*, 2001; Micheaux *et al.*, 2001; Sénégas, 2001; Bronner *et al.*, 2003; Jansa & Weksler, 2004; Steppan *et al.,* 2004). Paleontological and morphological evidences (Pocock, 1976; Carleton & Musser, 1984; Bernad *et al.*, 1991; Sènègas, 2001) support a murine origin for the otomyine group. At the generic level, the laminate-toothed rats have been variously classified as a single genus (Bohmann, 1952), but more commonly as the two genera *Otomys* and *Parotomys* (Ellerman, 1941; Ellerman *et al.*, 1953; Misonne, 1974; De Graaff, 1981; Smithers, 1983; Meester *et al.*, 1986; Corbet & Hill, 1991; Musser & Carleton, 1993). Some even suggest three (*Myotomys*, *Otomys* and *Parotomys*; Thomas, 1918; Pocock, 1976) or five (*Myotomys*, *Otomys, Lamotomys, Liotomys* and *Parotomys*; Roberts, 1951) genera. Bohmann (1952), using dental morphology, concluded that *Parotomys* is ancestral to *Otomys*. Since *Parotomys* is endemic to southern Africa, he inferred a southern African origin for the otomyine rodents. The divergence of the ancestral lineage of the *Otomys* genus most likely occurred as a result of fragmentation of the South African range (possibly due to, amongst others, eustatically induced transgressions (higher sea levels) and regressions (lower sea levels) of the sea level; Deacon, 1985). During the cooling and warming periods in the Pleistocene, further radiation of the resultant *Otomys* lineages occurred (Bohmann, 1952). It has been hypothesised that colonization of the rest of the African continent followed a single dispersal event from the south (Taylor *et al.*, 2004a). In contrast, Denys (2003) suggested multiple south-north invasions using fossil evidence. Both of these authors maintained the monophyly of *Otomys*.

Myotomys sensu, as defined by Roberts (1951) and consisting of *O. unisulcatus* and *O. sloggetti*, has been shown to be more closely related to *Parotomys* than to *Otomys* (allozyme data: Taylor *et al.,* 1989; Meester *et al.,* 1992; sperm morphology analyses: Bernard *et al.,* 1990; immunoblot analyses: Contrafatto *et al.,* 1997; mtDNA sequencing data: Maree, 2002; and morphology: Taylor *et al.* 2004a); a relationship which led the authors to suggest that *Otomys* is a polyphyletic genus. Pocock (1976) suggested a 'diphyly hypothesis' using paleontological evidence, which is supported by Taylor *et al.* (1989) and Contrafatto *et al.* (1997) based on allozyme and immunoblot data. This hypothesis suggests that an arid clade (*Parotomys* and *O. unisulcatus*, and sometimes *O. sloggetti*) and a mesic clade (all other *Otomys* species) make up the otomyine rodent group.

Fossil record pertaining to the otomyine rodents

The fossil record for the otomyine rodents is sparse (Sènègas & Avery, 1998). Nevertheless, two fossil species of *Euryotomys* from South Africa, *E. pelymoides* (3.7 – 5.0 Mya, Mio-Pliocene, Langebaanweg, South Africa; Pocock, 1976) and *E. bolti* (4.0 – 5.0 Mya, Early Pliocene, Bolt's Farm, South Africa; Sènègas and Avery, 1998) provide evidence for the murine origins of the Otomyini. *Euryotomys bolti* is considered as the likely ancestor of all otomyine rodents, as it has been unequivocally shown to possess dental features that were more derived than that of *E. pelymoides*, but less so than the first true otomyine fossil *Otomys* cf. *gracilis* (approximately 3.7 Mya, Mid-Pliocene, Makapansgat, South Africa; Pocock, 1987). Due to these characters, it was suggested that the otomyine rodents should not be categorized as a subfamily of the Muridae, but rather considered as a tribe of the Murinae subfamily, which is supported by recent molecular, morphological and paleontological studies (Chevret *et al.*, 1993; Sènègas & Avery, 1998; Sènègas, 2001; Ducroz *et al.*, 2001; Taylor *et al.*, 2004a). A cooling and a drying phase began during the Late Miocene (Kennet, 1995; Cerling *et al.*, 1997). C_4 plants progressively replaced C_3 plants during this time (Cerling *et al.*, 1997), and the properties of the grasses caused vast savannahs to open up (Bond, 2008). These changes in climate and environment may have induced this group of rodents to adapt to a more abrasive diet that pose strong selection on their dental patterns (Sènègas, 2001). The grasslands which opened up during the aridification of the African continent enabled more arid-adapted species, as well as plains-dwelling species, to proliferate in concert with the spread of the grasslands (McCarthy & Rubidge, 2005; Bond, 2008).

The use of molecular and protein molecular clocks have been employed in order to determine the ages of the nodes in the Otomyini phylogeny. According to a molecular calibration done using allozymes (Taylor *et al.*, 2004b), the Arvicanthini and Otomyini tribes split from the Murinae during the Late Miocene between 7.0 – 9.0 Mya, an estimate supporting the findings of Chevret *et al.* (1993), Ducroz *et al.* (1998), and Sènègas (2001). Steppan *et al.* (2004) estimated the split of the otomyines from other murines to have occurred at 5.4 – 6.6 Mya, supporting the date of Sènègas and Avery in 1998 (4.5 – 6.6 Mya). The mitochondrial cytochrome *b* (cyt *b*) molecular clock estimates the split in the Otomyini to be around 6.3 Mya (Maree, 2002), which is close to the dates estimated for the protein molecular clock (Taylor *et al.*, 1989). However, both these estimates predate the earliest fossil records (3.7 – 5.0 Mya) of *Euryotomys* (Pocock, 1976; Denys *et al.*, 1989; Sènègas & Avery 1998; Sènègas, 2001). Fossils of *Parotomys* and *O. unisulcatus* have not yet been described (Taylor *et al.*, 2004a), which may provide a clearer picture with regards to the ancestry of the arid-occurring otomyines.

Background on *Otomys unisulcatus*

The Karoo Bush Rat is a terrestrial species endemic to South Africa, which is restricted to the semi-arid regions of the Eastern, Western and Northern Cape Provinces (Figure 1.1). It has been described as either diurnal (Du Plessis, 1989; Du Plessis & Kerley, 1991) or more recently as crepuscular (Skinner & Chimimba, 2005). Though this species has historically been found to occur only marginally in the Western Cape Province (Davis, 1962; Davis, 1974; De Graaff, 1981; Skinner & Chimimba, 2005), Avery *et al.* (2005) found that *O. unisulcatus* could range as far south as the De Hoop Nature Reserve in the Western Cape Province. In part of its range, *O. unisulcatus* occurs sympatrically with the other arid-occurring Otomyini species, *Parotomys brantsii* and *P. littledalei* within the Nama Karoo and Succulent Karoo biomes (Jackson *et al.*, 2004). The Karoo Bush Rat prefers habitats with high plant cover and dense foliage near to rocky outcrops, and has been seen to be associated with woody vegetation occurring alongside ephemeral streams and rivers (Shortridge, 1934; Dieckmann, 1979).

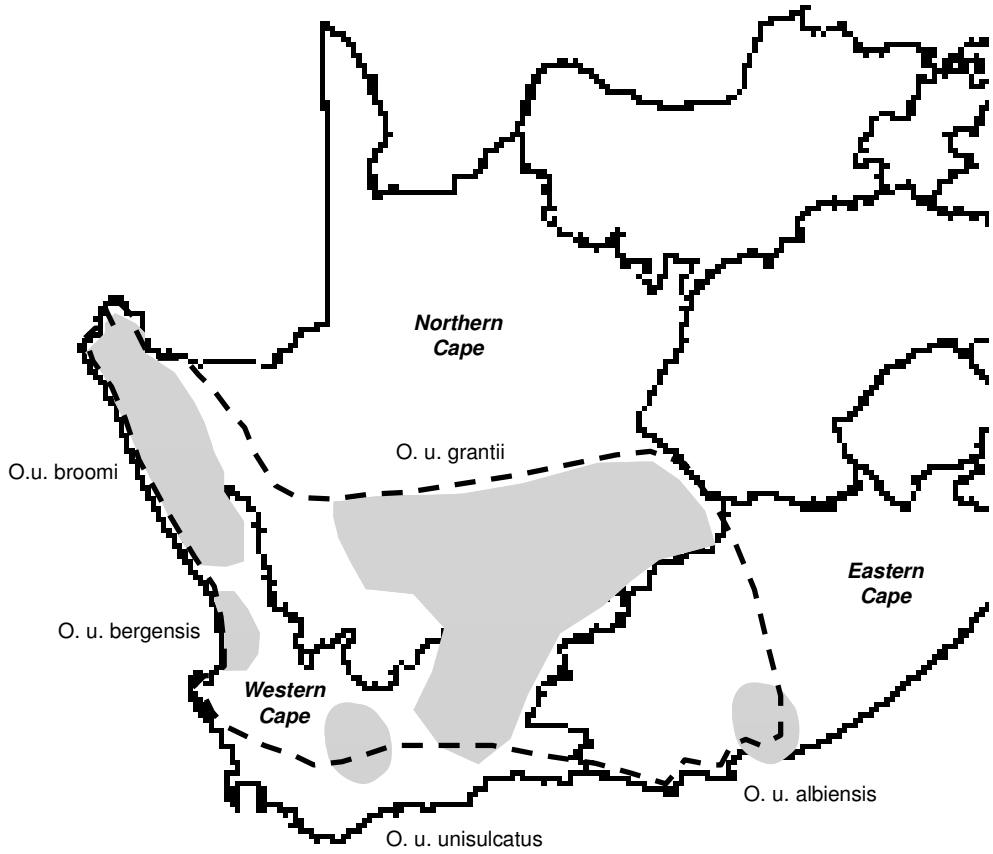

Figure 1.1: Distribution map of *O. unisulcatus*, showing the species' range within South Africa (dashed line; adapted from Skinner & Chimimba, 2005), the five previously described subspecies boundaries (shaded grey; described by Roberts, 1951), and the provinces (solid line).

Otomys unisulcatus is a medium sized rodent (adult mass range 70 – 135g; Pillay, 2001), with the males having a greater mass than the females (Skinner & Chimimba, 2005). The dimensions of the body (the head-body, tail, hind-foot and ear measurements) also reflect the sexual dimorphism within this species (De Graaff, 1981; Skinner & Chimimba, 2005). The Karoo Bush Rat has a shaggy pelage, ash-grey dorsally and buff-white ventrally, which is interspersed dorsally with black hairs (Figure 1.2; Pillay, 2001; Skinner &

Chimimba, 2005). The tail is longer, relative to body length, when compared to the other *Otomys* species (Skinner & Chimimba, 2005). The skull morphology of the Karoo Bush Rat is similar to the other otomyine rodents, however, the petrotympanic foramen appears as a round hole in the tympanic bullae (Skinner & Chimimba, 2005). Both *Parotomys* and *Otomys* are semi-hypsodont, and these otomyine rodents possess jugal teeth with plane occlusal surfaces, which are characterized by transverse laminae. The dental formula for the species' in the genus *Otomys* is $I\,^{1}_{1}, C\,^{0}_{0}, P\,^{0}_{0}, M\,^{3}_{3} = 16$ (Skinner & Chimimba, 2005). The upper third molar (M^3) has a small circular posterior portion, which is often worn-down in adults, with four distinct laminae (De Graaff, 1981). The first lower molar (M_1) has a distinct kidney-shaped anterior circular portion and these teeth have two laminae (De Graaff, 1981). The upper incisors may exhibit shallow grooves, whilst the lower incisors in the majority of specimens are not usually grooved (De Graaff, 1981). In fact, the name of the species (Latin *uni* = single, *sulcus* = groove) refers to the single faint groove which may persist on the lower incisor (De Graaff, 1981). The tympanic bullae of *O. unisulcatus* are small relative to those of *Parotomys*. Pocock (1976) suggested that the enlargement of the bullae in the *Parotomys* species was an adaptation to the arid environment, and Taylor *et al.* (2004a) argued that the enlargement is an ancestral trait of the Otomyini, due to the basal position of the *Parotomys* genus.

Figure 1.2: Photograph of *O. unisulcatus* (photograph taken by J. Visser).

The Karoo Bush Rat has been described as a generalist herbivore (Du Plessis & Kerley, 1991; Coetzee & Jackson, 1999; Pillay, 2001; Skinner & Chimimba, 2005), whose main diet consists of succulents and leaves from shrubs rather than annuals or trees (Du Plessis & Kerley, 1991). This kind of diet provides the water which is essential to survival (Brown & Willan, 1991). They collect large quantities of food while foraging, which is consumed later at the nest (Kerley & Erasmus, 1992). Much of the material is fermented in the large intestine (caecum) due to its low nutritive quality (Du Plessis, 1989).

Reproduction information for this species is scanty at best. The available literature indicates that *O. unisulcatus* young are born as late as May (Skinner & Chimimba, 2005) after a gestation period of 37 – 39 days (Pillay, 2001), with a mean litter size of between 2.07 and 2.09 (Pillay *et al.*, 1993; Pillay, 2001). The neonates are semiprecocial and this species retrieves its young by nipple-clinging (Pillay, 2001) and mouth-carrying (which is poorly developed; Skinner & Chimimba, 2005). Unlike the other congeners, neonates of this species are born with dark extremities (Pillay, 2001), which might be correlated with the high thermal conductance of the species (Du Plessis *et al.*, 1989). Most newborn mammals have poor thermoregulatory abilities resulting from large surface to volume ratios (Millar, 1977; Wilson, 1979). The males reach sexual maturity at six weeks of age, a week earlier than *O. angioniensis* and *O. irroratus*, but the females reach sexual maturity a week later than the latter two species, at five weeks of age (Pillay, 2001).

In the Karoo, this species faces a number of difficulties rarely encountered by other mesic members of the genus such as poor quality of the vegetation and low plant cover. These difficulties also include higher temperatures and less freely available water (Kerley & Erasmus, 1992). The Karoo consists of two biomes, namely the Succulent Karoo in the west and the Nama-Karoo in the east (Cowling *et al.*, 1999). Mean annual rainfall in these biomes is between 50 and 500mm with regular droughts (Kerley & Erasmus, 1992). The temperatures of this region range from a maximum > 35^{o}C to a minimum < 0^{o}C (Kerley & Erasmus, 1992). The vegetation is different between the biomes, with succulents dominating the Succulent Karoo, and dwarf deciduous shrubs forming the majority of the plants in the Nama-Karoo, while grass becomes more abundant in the eastern parts of this biome (Mucina & Rutherford, 2006).

The Karoo Bush Rat also inhabits two other biomes in South Africa, namely the Thicket Bushveld biome and the Fynbos biome. Low & Rebelo (1995) classified the semi-succulent thorny scrub of the river valleys of the eastern seaboard of South Africa as the Thicket Bushveld biome, and it is proposed that this biome is less harsh with more precipitation and milder temperatures during both the summer and winter seasons (Mucina & Rutherford, 2006). The Fynbos biome is a winter-rainfall area with relatively high levels of precipitation and larger temperature fluctuations (Mucina & Rutherford, 2006). Apart from natural fluctuations in habitat, the distribution of this species may be limited by fire causing permanent damage to their stick nests (see below, Kerley & Erasmus, 1992), and the Karoo biome characterized by a low incidence of natural fire damage (Manry & Knight, 1986) may thus provide a more stable environment for the species. The Orange River, forming the natural border between South Africa and Namibia, appears to be a barrier for *O. unisulcatus*, as the distribution of this species extends up to, but not across this river into Namibia.

The presence of this species is often indicated by extensive stick lodges surrounding bushes, usually one nest per bush (De Graaff, 1981; Kerley & Erasmus, 1992; Brown & Willan, 1991; Jackson *et al.*, 2004). A similar behaviour is also exhibited by the Desert Woodrat, *Neotoma lepida* in America (Cameron & Rainey, 1972), and the Australian Stick Rat *Leporillus* spp (Vermeulen & Nel, 1988). Unlike some of the other Otomyini species, *O. unisulcatus* does not require suitable habitat for burrowing. The Karoo Bush Rat may dig one or two tunnels under the nest (presumably to escape predators; Vermeulen & Nel, 1988),

however, time is mostly spent above ground, within the nest (Jackson *et al*., 2002). The species prefer open plains but is able to also inhabit areas with soft soils, such as dry riverbeds (De Graaff, 1981), coastal dunes (Vermeulen & Nel, 1988; Du Plessis & Kerley, 1991) or rocky outcrops (Roberts, 1951), assuming that these areas possess a relatively high percentage of plant cover (Jackson *et al.*, 2004). Runways are created by the rats between nests and/or possible food sources, a characteristic also exhibited by *Parotomys* (Jackson, 1999 and personal observation)*, Rhabdomys* (Jackson, 1999), and *Elephantulus* (Walker, 1964), to name a few. The refuge strategies of the arid-adapted otomyines differ, with *P. brantsii* constructing extensive burrow systems, and *P. littledalei* seeming to be intermediate between that of the other two species (Jackson, 2000). Due to the sympatry of the three arid-adapted otomyines, *O. unisulcatus* may be in competition for resources with *P. littledalei,* and to a lesser degree with *P. brantsii*, because of its dependency on dense shrubs for its nesting requirements, and because the three species mostly feed on the succulent vegetation (Jackson, 2000).

Roberts (1951) and Meester *et al.* (1986) listed five subspecies of the Karoo Bush Rat whose distributions range from Port Nolloth (*Otomys unisulcatus broomi*), down the West Coast to Lamberts Bay (*O. u. bergensis*), inland to Matjiesfontein (*O. u. unisulcatus*), the Central Karoo (*O. u. grantii*) and east to the Albany district (*O. u. albiensis*) (Figure 1.1). From the north-west to the eastern parts of South Africa, the morphological differences between the subspecies show a clinal decrease in overall body size, variation in colouration, and a decrease in tympanic bullae size (Roberts, 1951). Phenotypic plasticity may thus be playing a larger role in morphological characteristics, should the morphological differences not be reflected in the genetic phylogeographic structuring of the species. Whilst Van Dyk *et al.* (1991) consider the recognition of these subspecies to be unwarranted, the use of neutral molecular markers and morphological analyses may contribute towards clarifying this.

Background on genetic phylogeography

Ongoing evolutionary processes, such as gene flow (long distance migration), can be distinguished from historical events, such as vicariance and range expansion, by analyzing the relative ages and historical relationships of alleles in a geographic context (Hare, 2001). These processes leave their imprints in the distribution of intra- and inter-population variation (Tajima, 1983; Slatkin, 1987; Avise, 2000; Hewitt, 2000). Population genetic structure is formed through environmental events (such as vicariance) and/or through active or passive dispersal (Avise, 2000). Phylogeography as a field has been set apart from classical population genetics by dealing explicitly with a species' history and the spatial distributions of gene lineages (Knowles & Maddison, 2002). Phylogeography has grown as a discipline because it allows historical scenarios which were the cause of present-day spatial arrangements of organisms to be assessed, as well as the processes which formed the spatial arrangements, such as vicariance, dispersal, population expansions and bottlenecks or migration to be inferred (Hare, 2001; Knowles & Maddison, 2002).

The methods used in phylogeographic analyses examine the phylogenetic relationships among geographically distinct populations, and make inferences about evolutionary diversification of the populations (Polly, 2003; Avise, 2009). Statistical phylogeography is used to estimate population

parameters, such as genetic diversity, divergence times, growth rates and gene flow between populations (Knowles & Maddison, 2002). Though traditional phylogeography has used gene trees of non-recombining uniparentally inherited loci (e.g. mtDNA; Avise, 2000; 2009), statistical phylogeography is more concerned with population parameters than gene trees (Brumfield *et al.*, 2003). MtDNA has the phylogenetic advantages of maternal transmission, extensive intraspecific variation and usually exhibits an absence of genetic recombination (Avise, 2000), which have made it the historical marker of choice.

Phylogeographic structure has been described for various taxa in South Africa. Significant partitioning of genetic variation across the landscape have been described for some of these (e.g. invertebrates: Daniels *et al.*, 2001; Gouws *et al.*, 2003; reptiles: Branch *et al.*, 1995, 1996; Lamb & Bauer, 2000; Matthee & Flemming, 2002; Daniels *et al.*, 2004; Tolley *et al.*, 2004, 2006; Makokha, 2006; Swart *et al.*, 2009; birds: Bowie *et al.*, 2005; mammals: Prinsloo & Robinson, 1992; Matthee & Robinson, 1996; Rambau *et al.*, 2003; Kryger *et al.*, 2004; Smit *et al.*, 2007). Other species have shown shallow genetic structuring (Jansen van Vuuren & Robinson, 1997; Matthee & Robinson, 1997; Russo *et al.,* 2006). Of the above mentioned studies, the majority of the vertebrate species investigated are rock-dwelling (saxicolous). *Otomys unisulcatus* prefers more open habitats (Roberts, 1951; Vermeulen & Nel, 1988; Du Plessis & Kerley, 1991; Jackson *et al.*, 2004), and so its habitat requirements are different to rock-dwelling species, as well as to those species which require sandy areas for burrowing, such as *P. brantsii*.

Background on geometric morphometrics

Historically, the taxonomic classification of organisms and the description of patterns of variation, as well as the inference of the underlying processes involved in the formation of these patterns, were based on morphological characters (Monteiro *et al.*, 2003; Adams *et al.*, 2004). Comparisons of the anatomical shape of organisms is a fundamental part of biological studies and the use of morphometrics (the analysis of the variation in shape and its covariation with other variables; Bookstein, 1991; Dryden & Mardia, 1998) is thus an important complement to molecular gene trees (Cardini, 2003).

In the 1960's and 1970's, traditional morphometrics (Marcus, 1990; Reyment, 1991) or multivariate morphometrics (Blackith & Reyment, 1971), which deals with applying multivariate analyses of sets of quantitative morphological variables, was the preferred method of shape analysis. There were many shortcomings associated with traditional morphometrics, mainly that aspects of shape variation was lost in the use of linear distance measurements, and the proposed size corrections at the time produced conflicting results (Adams *et al.*, 2004). It was only in the late 1980's that the field of geometric morphometrics was developed in earnest, and Rohlf & Marcus (1993), in an introductory overview of geometric morphometrics, described this procedure as a "revolution in morphometrics".

Geometric morphometrics (Bookstein, 1991; Rohlf & Marcus, 1993; Corti *et al.*, 2000; O'Higgins, 2000) involves the comparisons of the geometry (shape) of objects, such as the cranium or the mandible of an organism, in which landmarks (corresponding points on the individual structures) are used in the description and analysis of shape variation. The use of landmarks, employed in a Cartesian system (Dryden

& Mardia, 1998), converts the shape of the individual structure into a function of the relative positions of the landmark coordinates (Bookstein, 1991), and preserves the geometrics information throughout the analysis (Monteiro *et al.*, 2003). In reviews by Bookstein (1991), Small (1996), Dryden & Mardia (1998) and Rohlf (1999), geometric morphometrics is comprehensively explored and Marcus *et al.* (1996) give more examples of the uses of this method in the biological and medical fields. Using morphometric data for inferring phylogenies, however, has been problematic in the past, and morphometrics has thus been found, for instance, to be a useful postcladistic analytical tool to use for the analysis within clades obtained in phylogenetic analyses (Cardini, 2003).

The general method of converting landmark information into a statistically useable form involves a number of steps. Firstly, homologous landmarks which are biologically meaningful are chosen and they are easily reproducible for each specimen. Scale, position and orientation effects (non-shape variation) are removed from the data (the set of landmark configurations) through superimposition methods, usually the Generalized Procrustes Analysis (GPA) (Adams *et al.*, 2004). This analysis uses the centroid size, defined as the "square root of the summed squared distances from each landmark to the configuration centroid (average landmark)" (Monteiro *et al.*, 2003), to scale the configurations to a unit size (Bookstein, 1986). Rotation of the configurations is then performed to minimize the squared differences between the landmarks (Gower, 1975; Rohlf & Slice, 1990). The mean shape can then be calculated from the rotated, resized superimposed configurations, after which the superimposed configurations are re-superimposed on the mean shape estimate; a process which is repeated until convergence (Adams *et al.*, 2004).

The vertebrate cranium has a number of characteristics highlighted by Kawakami & Yamamura (2008) that make it an ideal indicator of variation in a species. Firstly, since it houses many important features (such as the brain, eyes, hearing apparatus, nose and jaws), the shape of the cranium can be closely correlated to the environment and is thus under strong selective pressure. The vertebrate cranium is made up of many bony segments, held together by sutures, and it is the rigidity of this structure as a whole that enables easy measurement. Lastly, the formation of the shape of the cranium is partly the result of mutations occurring at many loci, and factors affecting these mutations will have an effect on the development of the cranium. Certain parts of the rodent cranium are subject to growth during adulthood, as a result of metabolic and physical stresses (Grüneberg, 1963; Moore & Lavelle, 1974), whilst other parts (e.g. basicranial portion) are not (Caumal & Polly, 2005). Thus, the rodent skull size and shape is driven by both genetic and environmental factors (which may influence gene expression of growth hormones). The sexual dimorphism exhibited by this species may also be driven by both genetic and environmental factors. Examination of the skull shape and size between the genders will provide a clearer picture as to which factors are driving this dimorphism. Arvicolid species which burrow exhibited more angular crania, while above ground species exhibited more elongated skulls (Courant *et al.*, 1997). Since *O. unisulcatus* occasionally digs one or two tunnels underneath the nests, skull shape and size may reflect which one of the two genders does the majority of the digging during development, as well as during adulthood. Environmental factors that affect specific structures/organs through selection may play a major role in speciation processes. The use of

geometric morphometrics within this study will be a useful tool in determining a potential correlation between environmental factors and their effect on the shape of the cranium of *O. unisulcatus.*

Reasoning behind study

Speciation processes involve many steps and most commonly involves population fragmentation (Foster *et al.,* 1998; Turelli *et al.,* 2001). Due to the phylogeographic groupings found in the studies on southern African taxa mentioned above (in the section: "Background on genetic phylogeography"), it has been suggested that climate-driven range expansions and contractions are playing a role in the population genetic structuring of many of these taxa. Since the arid periods in the Plio-Pleistocene favoured arid-adapted species, a genetic signature of population expansion may be present in *O. unisulcatus* during these arid times, with divergences occurring during the more mesic times. More recent agricultural efforts by man may be opening up habitats for species like *O. unisulcatus*, promoting gene flow between populations and expansions of populations.

The observation of differing nesting habits, variation in body colouration, variation in body size, differing dietary intakes, and differing habitats between populations across the species' distribution in South Africa, as well as the historical description of five subspecies within *O. unisulcatus*, suggests that interpopulation genetic and cranial morphological structuring may exist. Integration of morphological and molecular data analyses proved to be useful to identify groupings and to make accurate phylogenetic predictions of spatial structuring of a species (Nice & Shapiro, 1999), for example in *Praomys* (Denys *et al.*, 2003; Lecompte, 2005). It has been suggested that comprehensive integrative studies would provide clearer pictures of African murine biodiversity which may be underestimated for many species (Denys *et al.*, 2003). Thus, in addition to the use of a fast-evolving molecular marker, morphological quantitative characters were integrated within the study.

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CHAPTER 2

MTDNA PHYLOGEOGRAPHIC ANALYSES

Introduction

Phylogeography, briefly outlined in Chapter 1, can be considered as a mixture between population genetics, phylogenetics, as well as biogeography. Since its conception over thirty years ago, phylogeographic studies have provided many useful contributions to knowledge of evolutionary genetic processes (Table 1 in Avise, 2009). Several studies investigating phylogeographic structuring in species with congruent ranges (Bermingham & Moritz, 1998) have been highly informative about historical biogeographic forces. In the South African context phylogeographic patterns have been described for various taxa. The majority of the vertebrate species investigated in southern Africa (see "Background on genetic phylogeography") are rock-dwelling (saxicolous). *Otomys unisulcatus* prefers more open habitats (Roberts, 1951; Vermeulen & Nel, 1988; Du Plessis & Kerley, 1991; Jackson *et al.*, 2004), and so its habitat requirements are different to rock-dwelling species, as well as to those species which require sandy areas for burrowing, such as *P. brantsii*. Whilst Van Dyk (1989) and Van Dyk *et al.* (1991) found no significant genetic differentiation between populations of *O. unisulcatus* based on electrophoretic variation in 20 enzymes and non-enzymatic proteins coded for by 27 loci, as well as karyotypic analyses, the use of these slower evolving markers may not have been able to reflect recent genetic divergences between and within populations. Comparisons between direct DNA sequences have proved to be useful in determining the evolutionary history of a species (for reviews see: Takahata, 1996; Rogers, 1997; Harpending *et al.*, 1998; Cann, 2001). At present, DNA can be extracted from old material, collection and storage of samples is convenient and the data are often more variable than allozymes. Using PCR techniques and direct sequencing, it is easier to determine more fine-scale gene genealogies (Sunnocks, 2000). Organellar DNA, such as mtDNA, is usually uniparentally-inherited, compared to biparentally–inherited nuclear DNA, and the difference in transmission (as well as some other differences in evolution patterns) between organellar and nuclear DNA causes different aspects of population history and biology to be reflected in the respective gene genealogies (Sunnocks, 2000; Avise, 2009). The mtDNA cyt *b* gene has been shown to be an effective marker in intraspecific phylogeographic studies of mammalian taxa (e.g. Mustrangi & Patton, 1997; Harris, Rogers & Sullivan, 2001, Harris & S´a-Sousa, 2002; Kryger *et al.*, 2004; Palma *et al.*, 2005; Smit *et al.*, 2007).

Apart from using more sensitive methods of analyses, populations sampled within the previous *O. unisulcatus* studies included very limited representation from the Little Karoo, as well as along the western coastal regions of South Africa (Van Dyk, 1989; Van Dyk *et al.*, 1990). Through the inclusion of more localities within this study, as well as the utilization of a faster marker, a clearer picture of genetic population differentiation within *O. unisulcatus* may emerge.

Physical attributes of a landscape, such as rivers and mountain ranges, have been shown to act as barriers to gene flow in vertebrates in southern Africa (e.g. Scott *et al.,* 2004). For some southern African species, barriers to gene flow have included plains (e.g. Knersvlakte region: Matthee & Robinson, 1996; Lamb & Bauer, 2000; Matthee & Flemming, 2002; Smit *et al*., 2007), and isolated rocky outcrops have previously been thought to have acted as refuges during unfavourable periods (e.g. Prinsloo & Robinson, 1992; Matthee & Robinson, 1996). Subterranean rodents, such as the Argentinean sand-dune tuco-tuco *Ctenomys aiisiratis* (Mora *et al*., 2006), have been shown to be fragmented at the population level, due to their requirement of sandy environments for their burrows. The Karoo Bush Rat prefers open plains, with dense vegetation cover necessary for shelter and nourishment, and thus it is expected that areas that act as barriers for saxicolous species (such as open plains) may in fact be ideal dispersal routes for *O. unisulcatus*.

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Materials and Methods

Sampling

Sampling of *O. unisulcatus* specimens occurred in two ways. Samples (skins) were first obtained from various museums around South Africa and as far as possible encompassed the entire distribution of *O. unisulcatus* (Appendix A1). Fresh tissue samples, obtained from individuals collected in the field, were sampled and stored in absolute ethanol. Information regarding the sampling localities, such as environmental variables and locations, are detailed in Table 2.1, and Figure 2.1. DNA extraction from all the samples was achieved by making use of the DNeasy Kit (Qiagen). Before DNA extraction of the museum samples, a three-step wash procedure using 100% ethanol, 75% ethanol, and 100% distilled water was utilized to remove surface contamination of foreign DNA.

PCR amplification

The Polymerase Chain Reaction (PCR) was employed to amplify segments of the cyt *b* mtDNA gene from 115 samples obtained from 31 sampled localities (Figure 2.1). Numbers of individuals sampled per locality are detailed in Table 2.1. Primers specific to the Otomyini rodents were designed to amplify a short stretch (approx. 400bp long) of the cyt *b* mitochondrial gene as DNA extracted from museum material is often highly degraded and could be chemically modified (Pääbo, 1989; Pääbo *et al.,* 1989; Austin *et al.*, 1997). The following species specific primers were designed: forward primer OtoF1 (5'-ACAGCATTCTCATCAGTAAC-3') and reverse primer OtoR1 (5'-GCGTCTGAGTTTAGTCCT-3'). This gene region corresponds to the *Mus musculus* cyt *b* gene, stretching from L14325 to H14788.

PCR reaction mixes (25 µl final volume) contained 2.5 µl of 25 mM $MgCl_2$, 2.5 µl 10x reaction buffer, 2.5µl of a 1mM solution of dNTPs, one unit of Taq polymerase (give manufacturer) and 0.5µl each of the 10pmol forward and reverse primers. Since DNA extracted from museum skin samples was often degraded, the volume of the extracted stock DNA was varied in an attempt to standardize the concentration. The PCR technique involved an initial denaturation occurring at 94^{o}C for four minutes, followed by 38 amplification cycles comprising of denaturation at 94^{o}C for 30 sec, annealing at 46^{o}C for 30 sec and extension at 72^{o}C for 45 sec. A final extension hold for eight minutes was performed for each reaction. For the museum samples, an additional annealing hold for two and a half minutes was added at the end, before the final extension.

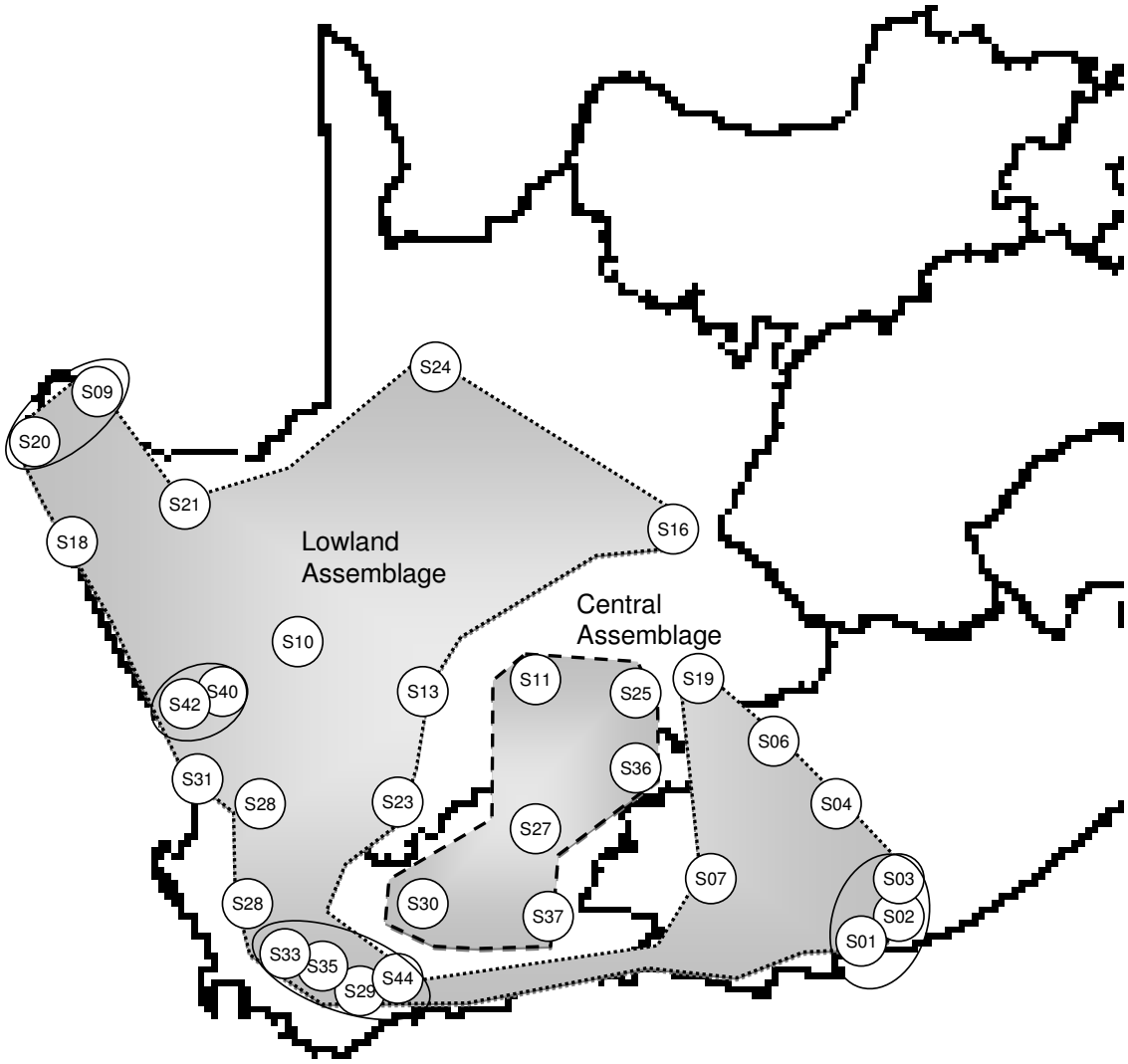

Figure 2.1: Distribution map of sampled localities (circles) and pooled localities (solid lines) of *O. unisulcatus* used in the genetic analyses. Shaded area shows the two assemblages (lowland and central groups) obtained in the Bayesian and Structure analyses. Sampling locality numbers (S01 to S44) correspond to those sampling localities detailed in Table 2.1.

Table 2.1: Specimens used in the present study together with environmental variables of sampling localities in each Province, the pooled population number (see text for details), GPS co-ordinates (in decimal degrees), as well as biome and rainfall seasonality information for sampled localities used in the study, as well as the number of individuals (*n*) sampled at each locality for both the genetic and morphological analyses.

Sampling locality	Location	Province*	Pooled Population number	Biome name	GPS-South	GPS-East	Altitude (m)	Rainfall (mm/ annum)	Rainfall seasonality$	Minimum Temperature (Ave max/ annum; °C)	Maximum Temperature (Ave min/ annum; °C)	Temparature stability (Ave Max – Ave Min)	*n* - genetic analyses	*n* - morphological analyses
S01	Albany	EC	P01	Thicket Bushveld	-33.11	26.45	120	503.1	YR	25.13	10.14	14.99	6	9
S02	Alexandria	EC	P01	Thicket Bushveld	-33.30	25.45	122	594.2	YR	25.13	10.14	14.99	1	3
S03	Bedford	EC	P02	Fynbos	-32.41	26.06	800	595.9	LS	25.13	10.14	14.99	2	4
S04	Cradock	EC	P03	Nama-Karoo	-32.10	25.37	927	304.7	LS	25.13	10.14	14.99	4	5
S05	Fish River Valley	EC	P04	Thicket Bushveld	-33.05	26.42	128	434.3	YR	25.13	10.14	14.99	-	4
S06	Middelburg	EC	P05	Nama-Karoo	-31.36	25.00	1248	274.7	LS	25.13	10.14	14.99	2	4
S07	Steytlerville	EC	P06	Nama-Karoo	-33.14	24.22	570	240.3	LS	25.13	10.14	14.99	3	5
S08	Tarkastad	EC	P07	Fynbos	-31.01	26.16	610	440.3	LS	25.13	10.14	14.99	-	3
S09	Alexander Bay	NC	P14	Succulent-Karoo	-28.29	17.04	356	39.6	WN	22.62	12.08	10.54	1	2
S10	Calvinia	NC	P15	Succulent-Karoo	-31.26	19.49	1049	212.9	WN	24.5	8.49	16.01	10	11
S11	Carnarvon	NC	P08	Nama-Karoo	-30.21	21.49	1000	219.4	VL	23.87	8.27	15.60	2	5
S12	De Aar	NC	P09	Nama-Karoo	-30.45	23.54	1200	290.0	VL	24.74	9.29	15.45	-	1
S13	Fraserburg	NC	P28	Nama-Karoo	-31.92	21.51	1006	203.7	LS	24.74	9.29	15.45	6	-
S14	Garies	NC	P16	Succulent-Karoo	-30.32	18.28	600	148.0	WN	18.79	11.23	7.56	-	2
S15	Hanover	NC	P10	Nama-Karoo	-31.07	24.45	1103	306.8	VL	24.74	9.29	15.45	-	3
S16	Hopetown	NC	P27	Nama-Karoo	-29.62	24.08	760	325.1	VL	24.74	9.29	15.45	1	2
S17	Kamiesberg	NC	P29	Succulent-Karoo	-30.23	18.11	1200	258.7	WN	18.79	11.23	7.56	-	1
S18	Port_Nolloth	NC	P19	Succulent-Karoo	-29.16	16.52	10	66.5	WN	19.08	10.89	8.19	4	8
S19	Richmond	NC	P09	Nama-Karoo	-31.02	23.46	1359	333.5	LS	24.74	9.29	15.45	2	3
S20	Richtersveld	NC	P14	Succulent-Karoo	-28.21	17.06	380	48.1	WN	22.62	12.08	10.54	6	2
S21	Springbok	NC	P17	Succulent-Karoo	-29.41	18.01	950	181.1	WN	24.11	12.07	12.04	14	2
S22	Steinkopf	NC	P18	Succulent-Karoo	-29.12	17.49	914	135.6	WN	24.11	12.07	12.04	-	4
S23	Sutherland	NC	P30	Succulent-Karoo	-32.34	20.40	1550	261.4	WN	20.36	3.32	17.04	1	2
S24	Upington	NC	P25	Succulent-Karoo	-28.45	21.23	2548	167.3	WN	28.79	12.64	16.15	1	-
S25	Victoria West	NC	P10	Nama-Karoo	-31.27	23.09	1219	266.7	LS	24.74	9.29	15.45	3	11
S26	Williston	NC	P26	Nama-Karoo	-31.34	20.92	1006	171.8	LS	24.74	9.29	15.45	-	3
S27	Beaufort West	WC	P11	Nama-Karoo	-32.14	21.37	1040	371.1	LS	24.99	10.72	14.27	6	10
S28	Clanwilliam	WC	P31	Transition Zone	-32.04	19.05	152	203.7	WN	28.67	12.72	15.95	2	2
S29	Darling	WC	P20	Transition Zone	-33.24	18.19	140	438.9	WN	24.98	11.01	13.97	1	1
S30	Laingsburg	WC	P12	Fynbos	-33.20	20.86	650	128.6	WN	25.54	11.24	14.30	1	4
S31	Lamberts Bay	WC	P22	Succulent-Karoo	-32.07	18.27	100	142.4	WN	22.35	11.25	10.53	11	7
S32	Langebaan	WC	P24	Transition Zone	-32.97	18.16	14	260.1	WN	23.53	11.25	12.28	-	1
S33	Malmesbury	WC	P20	Transition Zone	-33.24	18.17	140	467.1	WN	24.57	10.55	14.02	1	1
S34	Matjiesfontein	WC	P12	Fynbos	-33.13	20.34	655	443.4	YR	25.54	11.24	14.30	-	2
S35	Montagu	WC	P20	Transition Zone	-33.46	20.05	365	289.3	WN	24.98	11.01	13.97	1	4
S36	Murraysburg	WC	P13	Nama-Karoo	-32.18	23.28	884	266.1	LS	24.74	9.29	15.45	2	2
S37	Oudtshoorn	WC	P12	Nama-Karoo	-33.58	22.20	332	238.6	WN	23.31	9.16	14.15	5	-
S38	Piquetburg	WC	P21	Transition Zone	-32.36	18.18	12	320.1	WN	25.90	11.51	14.39	3	5
S39	Roberston	WC	P20	Transition Zone	-33.55	19.51	200	267.5	WN	24.98	11.01	13.97	-	2
S40	Van Rhynsdorp	WC	P23	Succulent-Karoo	-31.35	18.59	66	200.0	WN	26.23	11.21	15.02	8	1
S41	Vredenburg	WC	P24	Transition Zone	-32.91	18.50	105	309.5	WN	23.53	11.25	12.28	-	2
S42	Vredendal	WC	P23	Succulent-Karoo	-31.42	18.12	90	152.0	WN	26.23	11.21	15.02	1	2
S43	Willowmore	WC	P06	Transition Zone	-33.28	23.50	600	257.2	WN	23.31	9.16	14.15	-	1
S44	Worcester	WC	P20	Transition Zone	-33.38	19.39	360	244.5	WN	25.27	11.95	13.32	1	1

** Key to Provinces:* *EC = Eastern Cape; NC = Northern Cape; WC = Western Cape*

$ Key to Rainfall Seasonality: *WN = Winter rainfall; YR = Year round; LS = Late summer; VL = Very late summer*

DNA sequencing and alignment

The gene-specific PCR products were purified with a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Biosciences) and were then subjected to cycle-sequencing by using BigDye terminator chemistry (Applied Biosystems), following the recommendations of the manufacturer. Centri-sep 96 multi-well filter plates (Princeton Separations) were used to clean the samples, which were then analysed using an ABI Prism 3100 16-capillary genetic analyser (Applied Biosystems). The reverse strand was sequenced in all cases and where the electropherograms produced ambiguous results, the forward strand was also sequenced. Once the sequences were obtained, they were compared with sequences stored in GenBank by using BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were viewed in BioEdit Sequence Alignment Editor v. 7.0.5.2 and aligned manually (by eye).

Phylogenetic and phylogeographic analyses

To ensure that population level studies in both the genetic and the morphological studies would not be biased through small sample sizes, geographically contiguous localities were pooled, based upon their geographic proximity, and their similarity in environmental variables and other factors (including altitude). Essentially, those populations which possessed a small sample size (below two individuals), were pooled with the locality closest to it (>20km), ensuring the pooled localities were in the same biome. Individuals from Upington (S24) and Hopetown (S15) were not pooled with another population, as no locality was sampled within 20km of these populations.

To obtain broad scale phylogenetic patterns, Bayesian analyses were performed on *O. unisulcatus* sequences using the default settings in the program MrBayes v.3.1.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). *Parotomys brantsii* was specified to root the tree and one *P. littledalei* individual was also included as outgroup. Two simultaneous runs of one cold and three heated Metropolis-coupled MCMC chains were run for two million generations beginning from a random tree. Trees were sampled every 100 generations. The GTR+ Γ model (GTR model with gamma-distributed rate variation across sites (Γ)) was utilized. The model was selected by executing the program MrModeltest v.2.2 (Nylander, 2004) in the program PAUP* v.4.0b10 (Swofford, 2001), using the AIC criteria. The analyses were repeated three times to ensure that the posterior probabilities (PP) surface was explored adequately. The number of cycles to discard as burn-in approximated 10% of the total samples produced (i.e. 2 000 of 20 000 samples), empirically determined from plots of the fluctuating value of the log-likelihood of the cold chain in the program Microsoft Excel.

Potential geographic groupings were identified *a priori* by using the program Structure v.2.2 (Pritchard *et al.*, 2000a), by coding single nucleotide polymorphisms with a value of two (or three if more than two nucleotides were present at the locus), and the rest of the loci were coded as one. One million cycles per run, with $\lambda = 1$, using an admixture model and a burn-in period of 250 000 cycles, was performed, and the number of groups (*K*) was varied. However, the program Structure may not be useful on its own to identify historical entities (Zink & Barrowclough, 2008), even though it is useful for detecting population

management units (e.g. Latch *et al.*, 2006). Thus, a Spatial Analysis of Molecular Variance (SAMOVA) in the program SAMOVA v.1.0 (Dupanloup *et al.*, 2002) was also used to distinguish historically isolated geographic groups of populations. The *K*–value (number of groups) was varied to determine the maximum value of Φ_{CT} (measure of genetic variation among groups).

A three-dimensional (3D) surface plot of the interpolation analyses, performed using the program AIS v.1.0 (Miller, 2005), was used to visualise the patterns of genetic diversity. Peaks in the graph represent localities which possess large genetic distances between themselves and other localities.

Mean sequence divergence for the entire dataset and within groups was calculated in MEGA v.4 (Tamura *et al.*, 2007), using the uncorrected *p*-distance matrix. The *p*-distance is the proportion of loci of compared sequences which differ from one another (Tamura *et al.*, 2007). The program Arlequin v.3.01 (Excoffier *et al.*, 2005) was used to calculate the nucleotide diversity, and heterozygosity values, as well as to perform an analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992), and pairwise fixation comparisons among populations. The haplotype diversity (h) was calculated using the formula $h = (1 - \Sigma xi^{2})\ n / (n - 1)$, where xi is the haplotype frequency and n is the sample size (Nei & Tajima, 1981) in Microsoft Excel. A statistical parsimony network of the maternal haplotypes was constructed in TCS v.1.21 (Clement *et al.*, 2000), as finer-scale spatial and temporal population structure can be better estimated using haplotype networks, compared to other tree-based methods (Bermingham & Moritz, 1998; Goldstein *et al.*, 2000; Posada & Crandall, 2001).

Demographic changes

To determine whether any demographic changes have occurred, various statistical tests have been devised in the past. The mismatch distribution (Roger & Harpending, 1992), the raggedness statistic *rg* (Harpending *et al.*, 1993), as well as the Tajima's *D* statistic (Tajima, 1989) have been used in the past, however Fu's F_S test of neutrality (Fu, 1997) has proved to be the most powerful statistical test in detecting population growth for a variety of cases (Ramos-Onsins & Rozas, 2002). Fu's F_S (Fu, 1997) test of selective neutrality, performed in Arlequin v.3.01 (Excoffier *et al.*, 2005), was used to determine the potential departure from neutrality; interpreted as demographic changes in population numbers. A significantly negative value of Fu's F_S would denote a recent increase in population size, because rare alleles are seen to be more abundant, whilst a null value would indicate that the different populations have remained similar in size and thus indicate stability (Mahoney, 2004). A positive value for Fu's F_S would indicate that an elimination of rare alleles had recently occurred, such as would be the case following a population bottleneck or a selective sweep of the gene of interest or genes linked to the gene of interest (Mahoney, 2004).

The program Multidivtime (MDIV; Nielsen & Wakeley, 2001) was used to identify values for theta ($\theta = 2N_{ef}\mu$), migration rate ($M=2N_{ef}m$), time since divergence ($T = t/N_{ef}$) and time to most recent common ancestor ($TMRCA = t\mu$), where N_{ef} is the female-effective population size, μ is the mutation rate per gene and t is the generation time. The program was run for 2 million generations, under a finite sites model, with

10 migrants per generation and 10 units of time of population divergence used as the upper bounds (see also Nielsen, 2002). Two chains were run for each analysis in order to ensure accuracy in estimation of parameters. The calculation of the time since divergence (*t*) in millions of years before present (Mya) involved using the following formula: $t = T * \theta/(2u) * g$ (Brown *et al.,* 2007) where *T* and θ are generated by MDIV, and *g* is the generation time (0.3 years; Pillay, 2001). The value of u was obtained by the following formula: $2*\mu*k$. The mutation rate (μ) used was 8.25×10^{-09} substitutions per site per generation, or 3.3×10^{-08} substitutions per site per year, for rodent cyt *b* region (Veyrunes *et al.*, 2005; Abdel Rahman Ahmed *et al.*, 2008) and *k* was the sequence length (400bp).

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Results

Geographic genetic variation

The nucleotide composition corresponds with values found for the cyt *b* gene in other mammals (e.g. *Rhabdomys pumilio:* Rambau *et al.*, 2003): Guanine = 15%; Cytosine = 24%, Adenine = 29% and Thymine = 32%. Even though the sequences were relatively short (approx 400 bp), 40 haplotypes were identified from the 115 specimens collected from the 23 pooled localities (referred to hereon as populations; Appendix A2).

The Bayesian analysis produced an unresolved mtDNA phylogenetic tree, the internal clades of which were not significantly supported by the posterior probabilities (Figure 2.2A). One clade corresponded to populations from the middle part of the range, called a central assemblage (Beaufort West (S27), Victoria West (S25), Murraysburg (S36), Oudtshoorn (S37) and Laingsburg (S30)). This clustering was supported by the program Structure ($K = 2$; Figure 2.2B) even when when K was set to a value greater than two (only $K = 3$ is shown: Figure 2.2C). This indicates that the central assemblage is different enough to be assigned to a separate group. The other clade detected by the Bayesian analysis was not supported by the posterior probabilities, and did not consistently group into separate groups in the Structure analyses when more than two groups were specified. It is thus proposed that only two groups can be identified. The first group contained populations along the coastal plains (Succulent Karoo, Fynbos and Thicket Bushveld biomes), as well as inland to Bushmanland and Namaqualand (Nama Karoo biome). This group will be referred to as the coastal assemblage from now on. The populations of the second group are situated inland in the Little Karoo region (Nama Karoo Biome), and will be referred to as the central assemblage from hereon (Figure 2.2).

In contrast to the findings above, SAMOVA divided the haplotypes into five groups (Φ_{CT} value maximised at 0.640 ($P < 0.001$)). The first group contained populations from the western and eastern parts of the species' range (coastal assemblage specified above in Bayesian analyses), whilst the remaining four groups contained populations from the central assemblage (Little Karoo region). Individuals from Beaufort West (S27), Victoria West (S25) and Carnarvon (S11) grouped into separate groups (groups *a*, *b* and *c*). Individuals from Murraysburg (S36), Oudtshoorn (S37) and Laingsburg (S30) grouped into a single group (group *d*), whilst the remaining specimens grouped together (group *e*). The four groups (groups *a* – *d*) are probably indicative of additional small scale structure within the central group but given the small sample sizes for the populations in these groups (and some groups consist of a single sampling point), only the two main groups outlined above will be further considered.

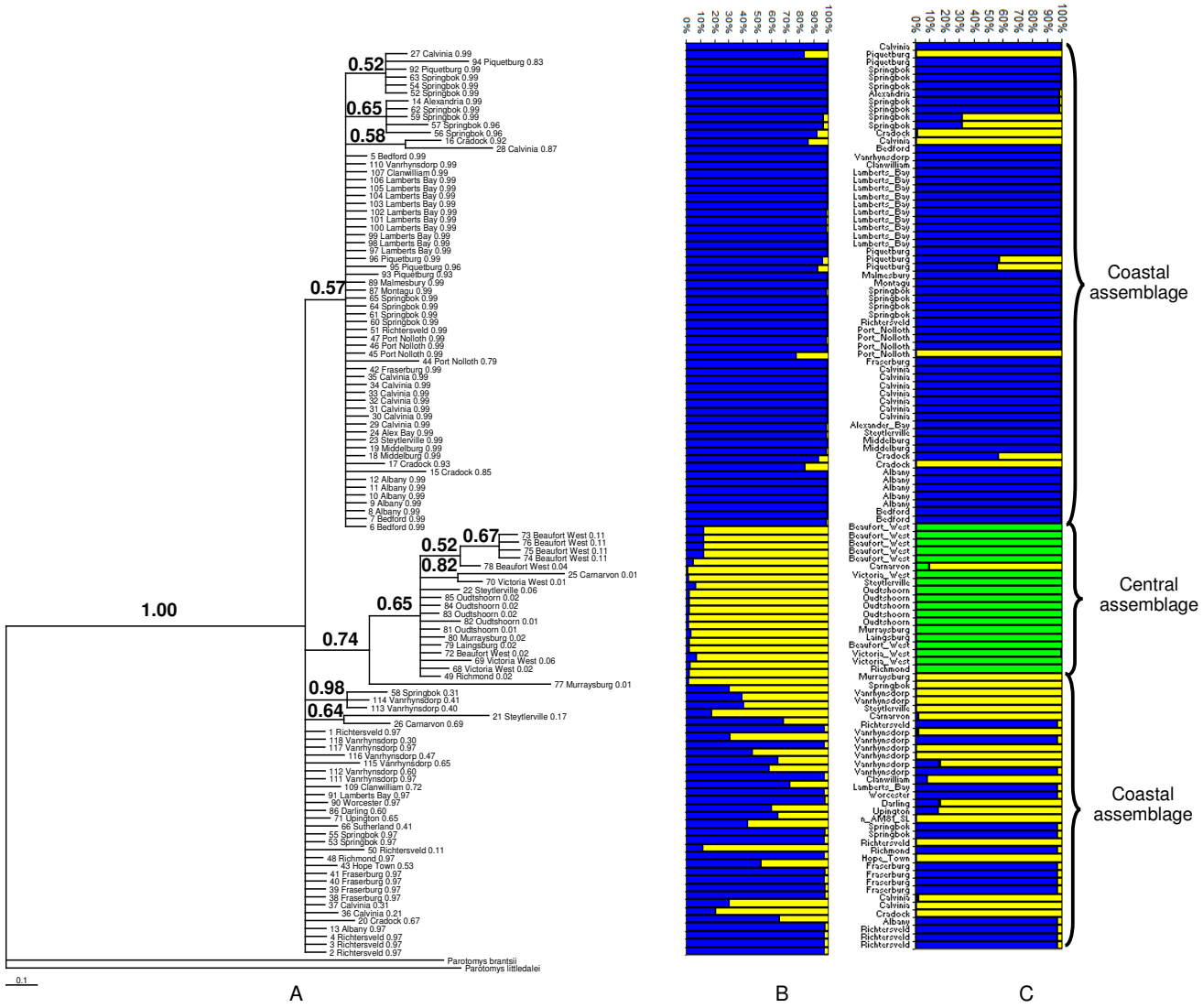

Figure 2.2: Results of a Bayesian analysis depicting the phylogenetic structuring of *O. unisulcatus* (A). Values in bold above branches are Bayesian posterior probability values. Labels for the specimens in the tree indicate the specimen number, its locality, and the probability of the specimen belonging to the coastal assemblage, as opposed to the central assemblage. Results of the sequential cluster analysis of the program Structure when *K*=2 (B) and *K*=3 (C) showing the clustering of individuals into either the coastal or the central assemblage. Lengths of the bars indicate the percentage probability of an individual belonging to one assemblage or the other.

The spatial distribution of genetic distances between populations can be visualised in Figure 2.3, which is a three-dimensional plot of geographic coordinates (X- and Y-axes) and genetic distances between populations (Z-axis). From these analyses it seems that the central assemblage is characterized by individuals that have greater residual genetic distances between populations than those in either the western or the eastern parts of the range (coastal assemblage). This can be explained as indicative of an older more stable population. It is also this same region where SAMOVA indicated potential substructure (see above).

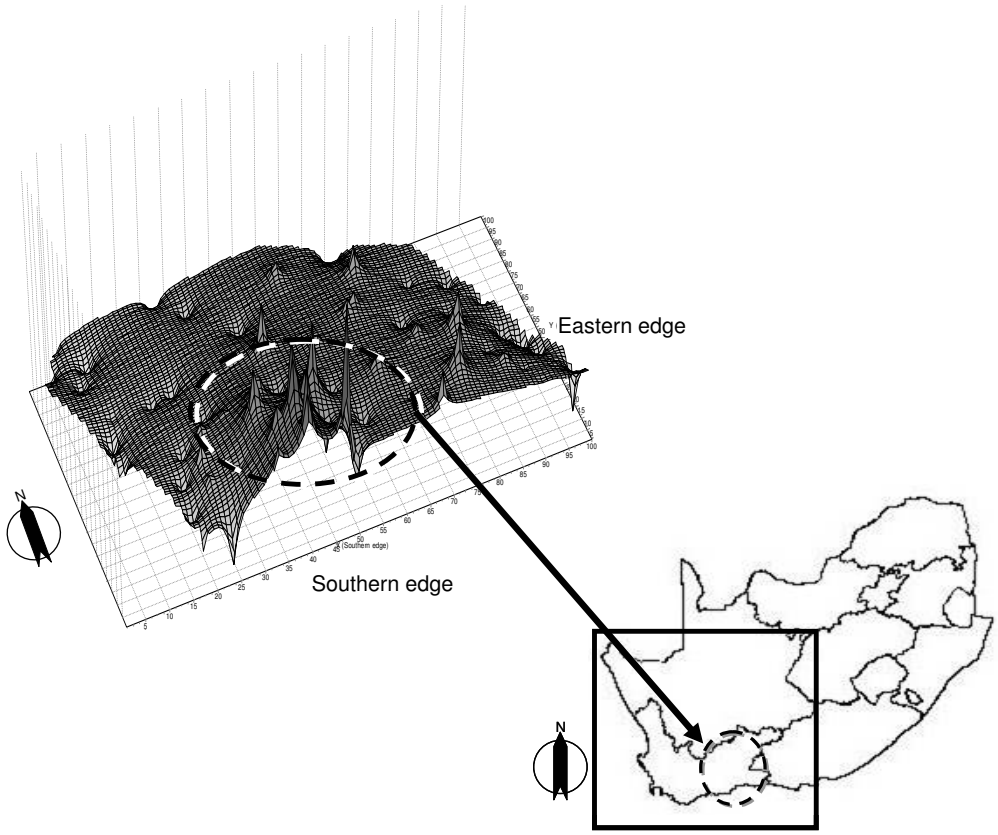

Figure 2.3: Three-dimensional surface plot of the geographical coordinates (*X*- and *Y*-axis) and residual genetic distances (*Z*-axis), displaying areas within the species' distribution which show large genetic distances (peaks) between localities.

When the two assemblages were specified in AMOVA, the among-groups variance component was identified as a major source of genetic variation (60.78%). The within-populations variance component (30.70%) was low relative to the among-groups component, but large relative to the among-populations/within-groups variance component (8.52%). Fixation indexes found were as follows: Φ_{SC} = 0.217, Φ_{ST} = 0.693 and Φ_{CT} = 0.608 and the tests comparing the variance components and fixation indices (1023 permutations) were all highly significant ($P < 0.001$), thus indicating a strong differentiation between the two groups, as well as among populations within the assemblages. Pairwise Φ_{ST} values between populations consisting of more than four individuals, fell between 0.0 and 0.97, with the most number of significant values occurring in the Beaufort West and Oudtshoorn/Laingsburg populations (Table 2.2; above diagonal). The mean Φ_{ST} value between the two assemblages was 0.79±0.11, the highest values of which was found between Oudtshoorn (S12) and Lamberts Bay (S22; 0.97, $P < 0.001$), and Fraserburg (S28), and Albany (S01; 0.94, $P < 0.001$). The mean Φ_{ST} value between the populations of the coastal assemblage was 0.15±0.16, and between populations of the central assemblage was 0.69±0.00.

Table 2.2: Sequence divergences, sequence diversities and pairwise Φ_{ST} values for populations which contain four or more individuals (*n*). Sequence divergences (below diagonal; standard deviations in brackets), intrapopulation sequence diversity (diagonal elements shaded in black; standard deviations in brackets) and pairwise Φ_{ST} values (above diagonal) shown. Significant values shown in bold-italic font. Populations classified into the central assemblage (groups *a* and *b* obtained in the Structure and Bayesian analyses) are shaded light grey.

Location	*n*		1	2	3	4	5	6	7	8	9	10	11
Albany (P01)	7	**1**	0.19% (0.17%)	0.18	0.00	0.00	0.07	0.00	***0.11***	***0.38***	***0.42***	***0.82***	***0.94***
Richtersveld (P14)	7	**2**	0.42% (0.54%)	0.53% (0.63%)	0.04	***0.13***	0.00	***0.32***	***0.22***	0.06	0.00	***0.65***	***0.82***
Calvinia (P15)	12	**3**	0.45% (0.51%)	0.64% (0.61%)	0.70% (0.65%)	0.01	0.00	0.06	0.05	***0.17***	0.10	***0.62***	***0.75***
Springbok (P17)	14	**4**	0.34% (0.30%)	0.60% (0.56%)	0.61% (0.53%)	0.52% (0.39%)	0.00	0.06	0.05	***0.28***	***0.16***	***0.69***	***0.82***
Western Cape Populations (P20)	4	**5**	0.25% (0.24%)	0.39% (0.48%)	0.49% (0.48%)	0.43% (0.32%)	0.32% (0.21%)	0.28	0.10	0.03	0.00	***0.72***	***0.90***
Lamberts Bay (P22)	11	**6**	0.11% (0.15%)	0.38% (0.53%)	0.40% (0.51%)	0.31% (0.29%)	0.21% (0.22%)	0.05% (0.11%)	***0.22***	***0.49***	***0.66***	***0.88***	***0.97***
Piquetburg (P21)	5	**7**	0.48% (0.40%)	0.78% (0.66%)	0.74% (0.62%)	0.64% (0.47%)	0.59% (0.45%)	0.41% (0.38%)	0.72% (0.43%)	***0.36***	***0.32***	***0.70***	***0.84***
Vanrhynsdorp (P23)	9	**8**	0.59% (0.33%)	0.57% (0.43%)	0.76% (0.48%)	0.80% (0.35%)	0.47% (0.29%)	0.56% (0.31%)	0.97% (0.49%)	0.55% (0.27%)	0.13	***0.64***	***0.78***
Fraserburg (P28)	5	**9**	0.24% (0.17%)	0.30% (0.49%)	0.50% (0.43%)	0.42% (0.28%)	0.21% (0.15%)	0.21% (0.12%)	0.61% (0.40%)	0.41% (0.26%)	0.11% (0.14%)	***0.78***	***0.94***
Beaufort West (P11)	6	**10**	1.29% (0.35%)	1.25% (0.47%)	1.47% (0.47%)	1.45% (0.39%)	1.77% (0.26%)	1.95% (0.13%)	2.36% (0.39%)	1.70% (0.31%	1.07% (0.33%)	0.33% (0.35%)	***0.69***
Oudtshoorn & Laingsburg (P12)	6	**11**	1.97% (0.19%)	1.82% (0.17%)	1.95% (0.53%)	2.11% (0.27%)	1.17% (0.31%)	1.26% (0.32%)	1.67% (0.49%)	1.27% (0.29%)	1.75% (0.15%)	0.69% (0.33%)	0.09% (0.14%)

The mean sequence diversity for the dataset as a whole was 0.95±0.83%, whilst intragroup diversity was lower at 0.71±0.72% and 0.90±0.75% for the coastal and central assemblages, respectively (Table 2.3). The mean sequence divergence between the two geographic assemblages was 1.72±0.70%. When the dataset was divided into biomes (Table 2.4), it was found that within the Succulent Karoo biome the population with highest sequence diversity between members was Calvinia (S10: 0.78±0.74%), whilst in the Nama Karoo biome it was between members of the Richmond population (S19: 1.90±0.00%). In the Fynbos biome, the highest sequence diversity was found between members of the Steytlerville population (S07: 0.93±0.81%), and the overall highest sequence diversity was found between members of the Richmond population (S19: 1.90±0.00%). The lowest sequence diversity within each biome and for the dataset overall was 0.00%.

Heterozygosity values for the entire dataset, as well as for the coastal and central assemblages separately were 0.047±0.05, 0.038±0.05, 0.093±0.07, respectively (Table 2.3). Heterozygosity was lowest in the Thicket Bushveld biome, slightly higher in the Succulent Karoo biome, and the highest in the Nama Karoo and Fynbos biomes (Table 2.4). In the Fynbos biome the population with the highest heterozygosity was Oudtshoorn (S12). In the Nama Karoo biome, it was Fraserburg (S28), and in the Succulent Karoo biome it was Calvinia (S10). The population with the highest heterozygosity overall was Fraserburg.

Table 2.3: Molecular indices for groups 1 and 2, as well as the dataset as a whole. Values in bold indicate significant values (95% level of significance). Key to headings: Number of individuals (n), haplotype diversity (*h*), heterozygosity (*H*) and its standard deviation (s.d.), mean sequence divergence (Seq. Div.) and its standard deviation (s.d.), number of polymorphic sites (S), mean number of pairwise differences (*d*) and its standard deviation, ratio of segregating sites to pairwise differences (*S/d*), nucleotide diversity (π), Fu's F_S-value (F_S).

Groups	n	*h*	*H* (s.d.)	Seq. Div. (s.d.)	*S*	*d* (s.d.)	*S/d*	π	F_S
1	98	0.839	0.038 (0.05)	0.71% (0.72%)	43	4.32 (1.91)	9.95	2.37	**-26.98**
2	17	0.796	0.093 (0.07)	0.90% (0.75%)	13	6.00 (4.48)	2.17	2.72	**-21.04**
All	115		0.047 (0.05)	0.95% (0.83%)	49	5.91 (3.17)	8.29	3.36	**-26.19**

Table 2.4: Sequence diversities (Seq. div.) and heterozygosity values (*H*) for biomes, as well as their standard deviations (s.d.). Sequence diversities and heterozygosity values, and standard deviations for populations within the biomes with the highest values.

Biome	Seq. Div. (s.d.)	Populations with highest seq.div. within biome	Locality number	Seq. Div. (s.d.)
Thicket Bushveld	0.3% (0.3%)	Albany	S01	0.40% (0.37%)
Fynbos	0.8% (0.8%)	Steytlerville	S07	0.93% (0.81%)
Nama Karoo	1.4% (0.9%)	Richmond	S19	1.90% (0.00%)
Succulent Karoo	0.7% (0.7%)	Calvinia	S10	0.78% (0.74%)
	H **(s.d.)**	**Populations with highest heterozygosity within biome**	**Population number**	***H*** **(s.d.)**
Thicket Bushveld	0.001 (0.001)	Albany	S01	0.001 (0.001)
Fynbos	0.093 (0.14)	Oudtshoorn	S37	0.33 (0.00)
Nama Karoo	0.050 (0.12)	Fraserburg	S13	0.40 (0.00)
Succulent Karoo	0.006 (0.001)	Calvinia	S10	0.007 (0.05)

The parsimony network obtained in the program TCS (Figure 2.4) showed some correlation with geography in that most individuals belonging to the central assemblage cluster together. The two most common haplotypes (labelled *H1* and *H2*) differed from one another by one mutational step. Haplotype *H1* was shared among 44 individuals from 17 populations, the largest representation from Lamberts Bay (S31: 10 individuals), Calvinia (S10: 7 individuals) and the Albany region (S01: 5 individuals). Haplotype *H2* was shared amongst 16 individuals from 8 populations, the largest representation from the Richtersveld (S20: 4 individuals), Fraserburg (S13: 4 individuals), Springbok (S21: 2 individuals) and Vanrhynsdorp (S40: 2 individuals). Populations in the eastern and western regions of the species range possessed these two common haplotypes (except Calvinia), whilst the middle region (the central assemblage) did not share these common haplotypes (Figure 2.5). The central assemblage, indicated in Figure 2.4, was separated from the *H2* haplotype by three mutation steps, indicating that while haplotypes were not shared between this region and the rest of populations, the difference between the haplotypes was relatively small. The distribution of the populations which did not possess the two most common haplotypes corresponds loosely with the subspecies boundaries of *O. unisulcatus grantii* and *O. u. unisulcatus* (Figure 1.1). Those populations which do possess the two most common haplotypes occur in areas which contain the subspecies boundaries of *O. u. broomi, O. u. bergensis* and *O. u. albiensis.*

Demographic changes

Values for Fu's F_S test for neutrality were negative and significantly different from zero for both groups, as well as for the dataset as a whole (Table 2.3) indicating expansion. When molecular clock analyses were performed between groups 1 and 2, it was found that the central and coastal assemblages diverged approximately 1.48±0.57 Mya.

Subspecies validation

When populations were grouped according to previously described subspecies boundaries (a) including a separate group for those that were not placed in any subspecies group, and (b) excluding populations not falling within subspecies boundaries, the AMOVA produced the following results:

(a) Fixation indices obtained were $F_{SC} = 0.28$ ($P < 0.001$); $F_{ST} = 0.47$ ($P < 0.001$); $F_{CT} = 0.26$ ($P < 0.01$). The within-populations variance component (53.09%) was identified as a major source of genetic variation; whilst the among-groups (26.32%) and among-populations/within-groups (20.59%) variance components contributed approximately the same percentage to the total genetic variation.

(b) Fixation indices obtained were $F_{SC}=0.42$ ($P < 0.001$); $F_{ST}=0.59$ ($P < 0.001$); $F_{CT}=0.30$ ($P = 0.05$). The within-populations variance component (40.44%) was identified as the largest source of genetic variation; whilst the among-groups (30.11%) and among-populations/within-groups (29.45%) variance components contributed approximately the same percentage to the total genetic variation.

Pairwise comparisons of Φ_{ST}-values supported the distinctiveness of *O. u. grantii* from all other previously described subspecies (except *O. u. unisulcatus*) with significant values of between 0.27 and 0.39. *O. u. unisulcatus* is also significantly distinct from *O. u. broomi* and those specimens from the western parts of the range not previously as a subspecies, with values of 0.68 and 0.67 respectively.

Table 2.5: Pairwise comparisons of Φ_{ST}-values between previously described subspecies.

	O. u. albiensis	*O. u. unisulcatus*	*O. u. bergensis*	*O. u. grantii*	*O. u. broomi*	None (east)	None (west)
O. u. albiensis	-						
O. u. unisulcatus	0.92	-					
O. u. bergensis	0.00	0.91	-				
O. u. grantii	**0.35**	0.00	**0.39**	-			
O. u. broomi	0.01	**0.68**	0.04	**0.34**	-		
None (east)	0.00	0.46	0.03	**0.27**	0.04	-	
None (west)	0.00	**0.67**	0.00	**0.34**	0.01	0.02	-

✧

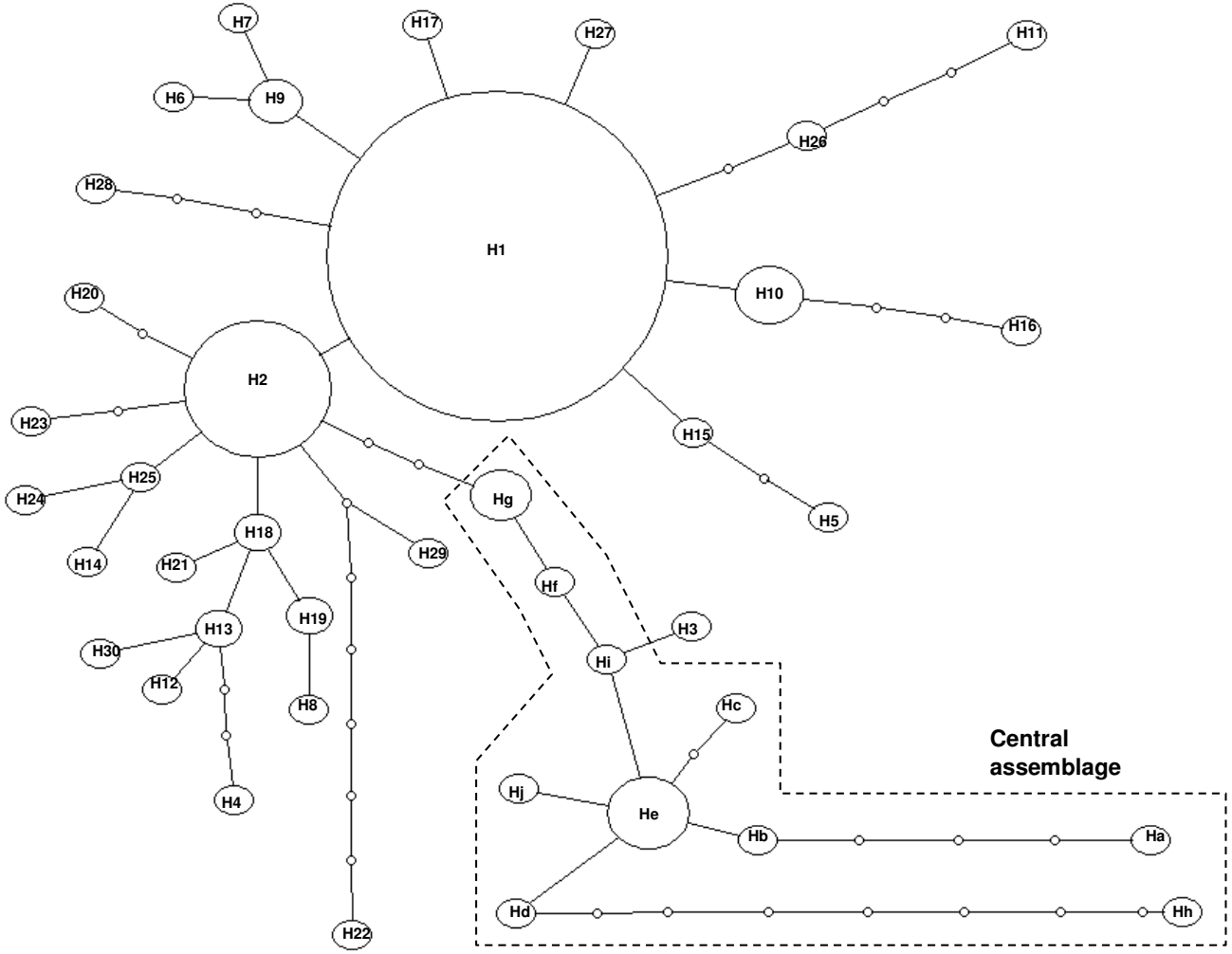

Figure 2.4: Network of maternal haplotypes produced in TCS. Haplotypes (scaled to reflect the number of specimens possessing a particular haplotype) are separated by single mutational steps, and small empty circles indicate missing haplotypes.

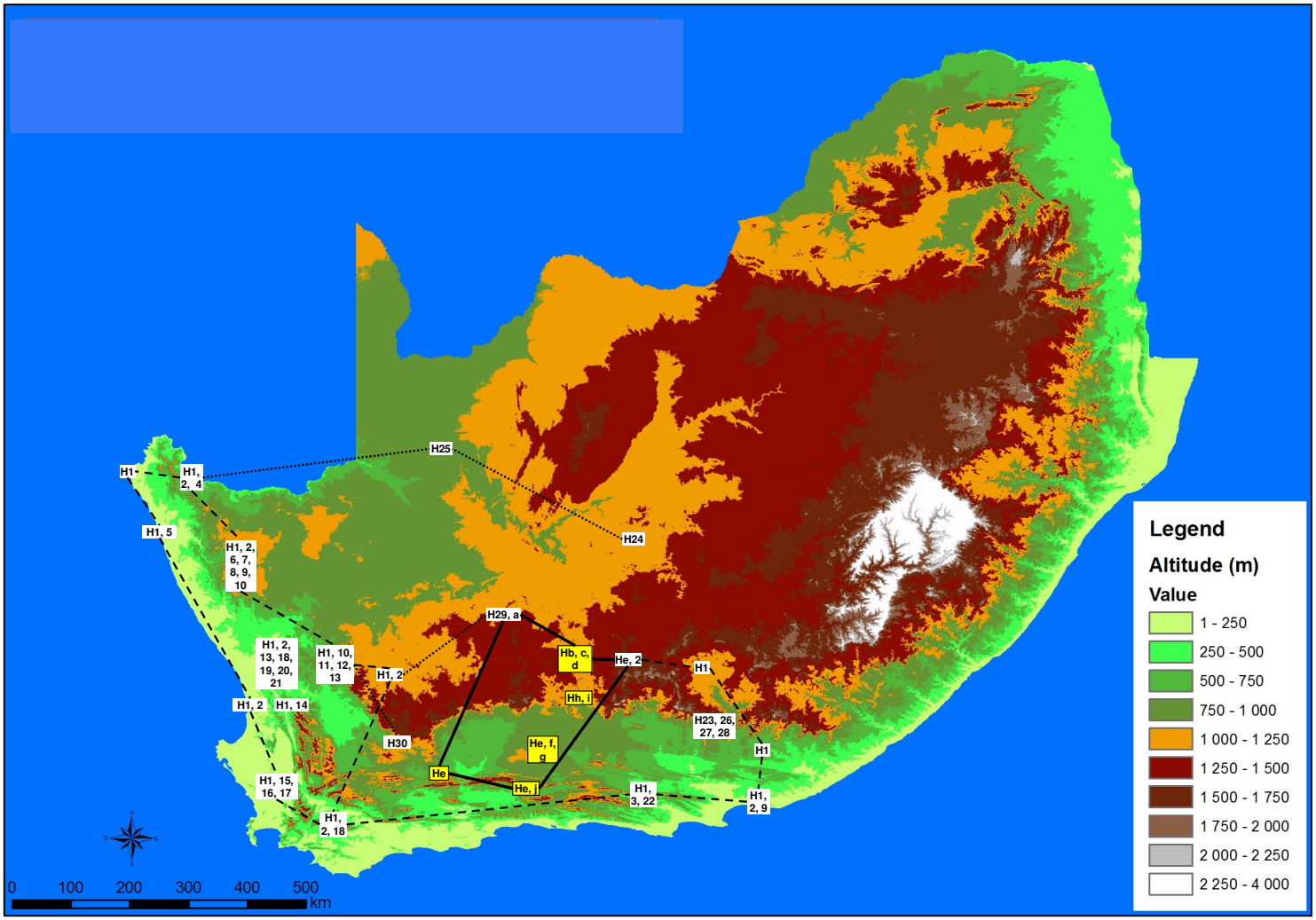

Figure 2.5: Haplotype distributions overlaid on an elevation map of South Africa. Key to haplotypes provided in Appendix A1. Locations in yellow indicate those locations in the central assemblage. Dashed lines connect localities which possess Haplotype *H1* (most common haplotype), solid line joins those localities which do not possess either Haplotype *H1* or *H2*. Dotted lines join those populations which do not possess the most common haplotypes, but which were not grouped into the central assemblage. (Map created by Hadley Remas, CSIR Satellite Applications Centre).

Discussion

Genetic variation

It appears that there exist two genetic assemblages within *O. unisulcatus*. The first assemblage (coastal) encompasses both the eastern and western populations within the species' range, whilst the second assemblage (central) occurs in the Little Karoo region of the range. Average sequence divergence and population differentiation parameters support the genetic distinctiveness of these geographic assemblages. However, the mean overall sequence divergences, as well as mean intragroup sequence diversities were low, when compared to intraspecific sequence diversities exhibited by other rodents (between 1.4% and 13.7% in the Bathyergidae; Faulkes *et al.*, 2004). Heterozygosity for the species as a whole (0.047) approximated that found for mammals (0.039; Avise & Aquadro, 1982). The central assemblage had a higher heterozygosity than this mean value, indicating that it is perhaps an older lineage/provided a refugia for the species during times of unfavourable conditions. The fact that the SAMOVA also further subdivided this assemblage into an additional four groups also shows how different the populations of this central assemblage are from one another.

Populations within the coastal assemblage share a number of haplotypes, and it is these populations which possess the common haplotypes (*H*1 and *H*2) spread throughout the range. Such connectedness at the genetic level indicates a recent shared ancestry for the populations in the coastal assemblage. It also implies a fairly high level of historic geneflow. In contrast, there are no shared haplotypes between the coastal and central assemblages, signifying that there is restricted geneflow between them. This is supported by the AMOVA, in which most of the variance was shown to exist between assemblages rather than within them.

An historical population expansion event would be indicated by a high haplotype diversity accompanied by a low nucleotide diversity (π), a significantly negative F_S-value, as well as a high ratio of number of segregating sites to average number of pairwise difference (*d*) (*S*/*d*) (Russell *et al.*, 2005). The coastal assemblage may have thus undergone a recent population expansion event as it fulfils many of these criteria. Even though the haplotype diversity is high, the heterozygosity and sequence divergences among haplotypes are low. For the central group, the higher peaks within the landscape interpolation plot (Figure 2.3), larger pairwise differences, as well as the higher heterozygosity, Φ_{ST}-values between populations, nucleotide diversity (π), and intragroup sequence divergence values indicate a more ancient divergence for this group. The significantly negative Fu's F_S-value for this group, however, indicates that this group nonetheless still retains a signature of population expansion, albeit older.

Congruent patterns in other southern African species

The shallow genetic structure obtained in the Karoo Bush Rat is in contrast to most previous studies (e.g. invertebrates: Daniels *et al.*, 2001; Gouws *et al.*, 2003; reptiles: Branch *et al.*, 1995, 1996; Lamb & Bauer, 2000; Matthee & Flemming, 2002; Daniels *et al.*, 2004; Tolley *et al.*, 2004, 2006; Swart *et al.*, 2009;

Makokha, 2006; birds: Bowie *et al.*, 2005; mammals: Prinsloo & Robinson, 1992; Matthee & Robinson, 1996; Rambau *et al.*, 2003; Kryger *et al.*, 2004; Smit *et al.*, 2007) and the mtDNA data provide new insights into the recent evolutionary past of this species. Geneflow between populations did not appear to be impeded by previously identified barriers (the Knersvlakte plains - Matthee & Robinson 1996; Matthee & Flemming, 2002; Smit *et al.*, 2007; the Kalahari sandflows - Deacon & Lancaster, 1988; Haacke, 1989). Whilst the Orange River appears to be a factor in limiting the species distribution northwards (forms the edge of the described species' range), other rivers throughout the species' range have not influenced the genetic population structuring of *O. unisulcatus*.

Avise *et al.* (1987) suggested that species that are connected through gene flow (as a result of the absence of barriers) would exhibit shallow genetic structure. Shallow phygeographic structuring of *O. unisulcatus* has also been mirrored in other predominantly plains dwelling southern African mammal species: the yellow mongoose *Cynictus penicillata* (Jansen van Vuuren & Robinson, 1997), the springhare *Pedetes capensis* (Matthee & Robinson, 1997), and the Tete veld rat *Aethomys ineptus* (Russo *et al.,* 2006). All these species are predominantly grassland species and are considered to be habitat generalists.

A similar pattern of genetic differentiation to that found in this study, in the south-central part of South Africa, has also been observed for two other mammals, and one reptile, with different life-histories: the rock hyrax, *Procavia capensis* (Prinsloo & Robinson, 1992), and the southern African scrub hare, *Lepus saxatilis* (Kryger *et al.*, 2004), as well as the southern rock agama, *Agama atra* (Swart *et al.*, 2009). The rock hyrax and the rock agama are rock-dwelling species', whilst scrub hares prefer scrub or savanna woodland habitat with grass cover. Within the three species, a genetic break was found between the Great Karoo, and the coastal plain of the Little Karoo. It was suggested that the *P. capensis* clade occurring in the Great Karoo was the ancestral population, and recent dispersal occurred from this region into the rest of South Africa, distributing a common haplotype throughout geographically distant populations. No barrier was suggested to have existed between the Western Cape clade and the Central clade in the southern African scrub hares, *L. saxatilis* (Kryger *et al.*, 2004), however since scrub hares may also prefer more open habitat (scrub or savanna woodland), elevation may be limiting gene flow within the species. Climatic shifts were thought to be the driving force of the genetic differentiation between clades within *A. atra*, and no readily apparent barriers were suggested to occur between the Cape clades (Swart *et al.,* 2009). Clearly, this central area (Little Karoo) appears to have a factor which is limiting gene flow between itself and the rest of South Africa. In *Elephantulus edwardii*, individuals from the central parts of the Karoo (Beaufort West and Williston) were genetically (12.2% uncorrected cyt *b* sequence divergence) and morphologically (tuft length on tail; ventral and dorsal pelage and flank colour; colour and shape of eye ring) different enough from *E. edwardii sensu stricto* to warrant the suggestion that these specimens be recognised as a separate species.

What caused the shallow genetic structure between the two *O. unisulcatus* clades is not clear. It is possible, however, that it could be attributed to altitudinal gradients probably provided by the Great Escarpment leading onto the African Plateau (also see Matthee & Robinson 1996). For a plains-dwelling species, elevated areas, such as mountain ranges, may be playing a role in creating genetic breaks in the species. A few areas of increased elevation separate the two groups found in this study. Firstly, separating the Little Karoo in the south and the Great Karoo in the south-central regions of South Africa is the Grootswartberge. Between the Grootswartberge and the Nuweveldberge (forming the natural border of the Western Cape and Northern Cape provinces), is an area of lower elevation (Weimarck, 1941; Oliver *et al.*, 1983; Linder, 2001), where the central assemblage is located. The Nuweveldberge forms part of the Great Escarpment and north of this is the Nama Karoo, and the beginning of the African Plateau (McCarthy & Rubidge, 2005). The Hottentots Holland Mountains in the south-western Cape separate the plains into coastal plains (West Coast and Agulhas plains; Weimarck, 1941; Oliver *et al.*, 1983; Linder, 2001). These areas of higher elevation may have resulted in forming barriers, and allowing isolation between populations. As has been mentioned before, the populations forming the two assemblages appear to not share haplotypes, even though they are only separated by a few mutation steps. This indicates that the two assemblages have been previously, and may still be, isolated from one another. Populations on the eastern and the western parts of the CFR (Cape Floristic Region) do share haplotypes, and it is possible that geneflow may be occurring along the coastal plain to the south of the Grootswartberge

Demographic history

Two periods of upliftment of the interior portion of southern Africa are thought to have occurred. The most recent upliftment is though to have occurred at approximately 5 Mya, resulting in an upliftment on the western side of southern Africa of about 100m (McCarthy & Rubidge, 2005). As a consequence of the upliftment, the east-to-west rainfall gradient became more pronounced as the western regions became more arid (McCarthy & Rubidge, 2005). The increase in grassland led to a radiation of the grazers, and a drying phase began during the Late Miocene (Cerling *et al.*, 1997), during which C_4 plants progressively replaced C_3 plants (Cerling *et al.*, 1997; Bond, 2008). During the late Pliocene and early Pleistocene Periods (approximately 4 – 1.5 Mya; Lavocat, 1978; Carroll, 1988), the climate of the African continent oscillated between cold, dry periods and warm, humid phases (Grubb, 1978; deMenocal, 1995). The habitat changed with the climate, with savanna habitats extending and humid forests contracting during dry phases, and *vice versa* occurring during humid phases (Coe & Skinner, 1993). The evolutionary history of southern African species is reflected in the geographic structuring of the species' genetic variation, and it has been found that the habitat contractions and expansions during the Pliocene and Pleistocene Periods have influenced diversification within southern African species (Ewer & Cooke, 1964; Grant & Leslie, 1993; Kryger *et al.*, 2004; deMenocal, 2004). For many species dependant on more mesic conditions, arid periods would have resulted in population fragmentation. For highly mobile, but habitat specific species (such as montane birds; Bowie *et al.*, 2004; Bowie *et al.*, 2006), these aridification periods influenced the spatial structure of populations, causing vicariant breaks. Gene flow between extant populations is occurring as a result of the

most recent, present-day mesic inter-glacial period (Bowie *et al.*, 2006). However, for an arid-adapted species such as *O. unisulcatus,* periods of aridification would favour range expansion, as well as secondary contacts between possible previous refugia.

Aridification cycles on the African continent coincide with Pliocene glacial periods in the Northern Hemisphere (Dupont & Leroy, 1995). Three Plio-Pleistocene peaks of aridification in Africa were estimated to have occurred at approximately 2.8, 1.7 and 1.0 Mya (deMenocal, 2004), interspersed by humid periods (deMenocal, 1995; 2004). Divergence times, then, indicate that populations in the central assemblage diverged from those of the coastal assemblage during the mesic period between the two most recent cycles of aridification during the Plio-Pleistocene Periods at 1.48 Mya. During the following arid cycle, dispersal and contact between the previously isolated populations may have occurred, causing the dispersal of the common haplotypes as well as some of the rare haplotypes. As a result of the present-day mesic inter-glacial period, beginning within the last million years, the populations may once again be isolated, allowing for rare alleles to be produced. These climatic fluctuations may be the cause of the shallow genetic structuring within this species, and perhaps why the two assemblages, may be accumulating rare alleles, whilst still showing indications of a recent expansion and low sequence divergences.

Subspecies validation

A study conducted by Van Dyk (1989) investigating the intraspecific genetic variation between populations of *O. unisulcatus*, found that there were no significant genetic differences between populations and therefore discounted any subspecies delimitations. The distribution of the populations that do not contain either of the two most common haplotypes (*H1* and *H2*), corresponds well to the previously described subspecies boundaries for *O. unisulcatus unisulcatus* and to the southern regions of the subspecies boundary of *O. u. grantii* (Meester *et al.*, 1986). Indeed, the latter two subspecies were found to be significantly distinct from all other subspecies. Populations which fell within the boundaries of the other three described subspecies (*O. u. broomi, O. u. bergensis,* and *O. u. albiensis*) all possessed haplotypes *H1* and *H2*, as well as some unique haplotypes separated from the latter by only a few steps.

Populations in Upington and Hopetown fell outside the previously described distribution for the species as a whole, and these two locations possessed haplotypes unique unto themselves. This finding may be biased, however, by the limited sampling (only one individual from each population was sampled). Further work, utilising perhaps a combination of mtDNA and nuclear markers evolving at an appropriately fast rate, is needed to test the validity of the previously described subspecies. Meester and colleagues (1986) claimed that too many subspecies have been historically recognised. From this study, it can be tentatively suggested that only one genetic subspecies should be recognised. Those individuals belonging to populations clustering into the central assemblage should be grouped into a separate subspecies *O. unisulcatus unisulcatus*.

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CHAPTER 3

CRANIAL VARIATION IN *OTOMYS UNISULCATUS*

Introduction

General background

The application of geometric morphometrics in investigations of clinal variation in shape and size has proved to be valuable for investigating morphological differences between individuals (e.g. Viguier, 2002; Frost *et al.*, 2003; Monteiro *et al.*, 2003; Marroig *et al.*, 2004; Santos *et al.*, 2004; Cardini *et al.*, 2007). When compared to linear distance measures, geometric morphometric analyses have been seen to produce more accurate estimations of spatial patterns produced by morphogenesis, due to the spatial relationships associated with the landmark configurations (Monteiro *et al.*, 2003). This is due to the fact that the differences between morphological characters are measured in terms of size and shape. Shape variables, in particular, have been shown to be informative for ecological and evolutionary processes influencing and shaping the morphological characters in question (Atchley & Hall, 1991; Atchley *et al.*, 1992; Malhortra & Thorpe, 1997).

Bergmann postulated, in 1847, that endothermic vertebrates from cooler climates would tend to be larger than congeners from warmer environments; a rule which was revised by Rensch in 1938 (Mayr, 1942, 1956, 1963; Meiri & Dayan, 2003). It was suggested that, in an animal of a larger size, the increased volume relative to its surface area would result in a higher heat production, as well as reduced heat loss – attributes which would be advantageous in a cooler environment (Meiri & Dayan, 2003). The usefulness of this rule as a predictor of size variation across temperature gradients has, however, been questioned by a number of authors (e.g. Scholander, 1955, 1956; Irving, 1957; McNab, 1971; Geist, 1987, Meiri & Dayan, 2003). In general, approximately a third of mammal orders comply with the rule, however the order Rodentia was one of those which did not (Meiri & Dayan, 2003). It has been found that environmental factors, such as rainfall (e.g. Popp, 1983; Dunbar, 1990; Barrett & Henzi, 1997; Ashton *et al.*, 2000; Millien *et al.*, 2006) and altitude (Anderson, 1982) play a role in the development of vertebrates.

Southern African rodent species found to exhibit variations in size across their distributions include species of *Acomys* (Dippenaar & Rautenbach, 1986), *Otomys* (Taylor, Meester & Kearney, 1993), *Saccostomus* (Ellison *et al.*, 1992) and *Aethomys* (Chimimba, Dippenaar & Robinson, 1998; Chimimba, 2000a, b). Size was shown to be positively correlated with longitude in the Tete veld rat *Aethomys ineptus* (Chimimba, 2001). It would appear that the intraspecific variation in size of southern African mammals is frequently existent and it is probably influenced by a number of factors. Determining which factors are influential in an evolutionary context will aid in future conservation efforts.

Cardini and colleagues (2007) used rainfall as a proxy for habitat productivity, a factor which has increasingly been seen to be an important predictor of mammalian body size (Dunbar, 1990; Ashton *et al.*, 2000; Lehman *et al.*, 2005; Millien *et al.*, 2006). The results of the study indicated that rainfall was the main predictor of clinal variation in shape and size of craniums of vervet monkeys, rather than temperature. Latitudinal gradients also seem to play a role in size variation (Cardini *et al.*, 2007). It is thus advantageous to conduct analyses using a combined dataset of a number of environmental factors, to include some possible influences that may be having an effect on the size and shape of the Karoo Bush Rat skull.

Armitage (1999) claimed that in order for an organism to survive in harsher environments, body size would have to increase in order to accumulate more resources, and this can be seen in the clinal increase in body size from the eastern populations to the more arid north-western populations of *O. unisulcatus* (Roberts, 1951; Meester *et al.,* 1986). This increase in body size is most likely to have caused an increase in cranium size, as well as a possible change in shape. Cranium structure may be under selection in the Karoo Bush Rat due to the differing dietary intakes amongst the populations; with succulent vegetation forming the dominant dietary component in the more north-western populations (Succulent-Karoo biome), whilst leaves from dwarf deciduous shrubs and grasses (Rutherford & Westfal, 1986), may form a larger part of their diet in the more eastern Nama-Karoo biome (Du Plessis & Kerley, 1991). The cranium shapes may also differ due to selective forces acting on the sensory organs and the brain housed within it. Those parts of the skull which do not exhibit growth during adulthood may be influenced more by factors affecting the genetic components of the species, than by environmental factors influencing the species during its life.

A recent study performed on domestic rats (*Rattus rattus*; Rae *et al.*, 2006), which were either reared in warm or cold environments, showed a significant difference in shapes of the craniofacial structures. The overall size and shape of the external craniofacial structures differed, causing an orientation shift in the nasal and premaxillary portions of the cranium, as well as an accompanying shift in the zygomaxillary suture, and the medial movement of the paraoccipital process. These differences resulted in a change in the location of the masticatory muscle attachments, thereby causing changes in the relative orientation of the orbit and zygomatic arch. It was concluded by the authors that the changes in sinus volume due to cold stress exhibited in *Rattus*, as well as in other mammalian taxa, may be a common occurrence within mammals in response to cold. This study shows that some craniofacial elements are phenotypically plastic, and that development of certain of the anterior portions of the rodent crania are influenced by environmental factors.

Taylor *et al.* (2004a) performed a study using geometric morphometric analyses to investigate cranial evolution in the southern African members of the Otomyini tribe. It was found that the dorsal cranial shapes of *O. unisulcatus* and *O. sloggetti* were seen to be morphologically intermediate between the *Parotomys* species and the other *Otomys* species studied. The ventral cranial and mandible shapes analyses, however, showed that only *O. sloggetti* was distinct from the other *Otomys* species and that *Parotomys* and *Otomys*

were distinct groups. It was also shown that habitat, climate, phylogeny and burrowing behaviour could explain the variation in cranial size and shape.

The present study attempted to investigate the patterns of covariation in cranial shape among populations of *O. unisulcatus* in relation to climatic and habitat variation, using geometric morphometrics, and, in particular, the two-block partial least-squares (2B-PLS) analysis (Sampson *et al.*, 1989; Streissguth *et al.*, 1993; McIntosh *et al.*, 1996; Rohlf & Corti, 2000). This analysis is theoretically similar to Canonical Variates Analysis, which also models the relationship between two blocks of data to describe patterns of covariation which exist between two sets of variables (Simoglou, 1999; Rohlf & Corti, 2000). The morphological character of shape is considered to be multi-dimensional, and thus can contribute toward the determination of the ecological factors and evolutionary processes underlying observed inter- and intraspecific diversity (Thorpe, 1976; Atchley & Hall, 1991; Atchley *et al.*, 1992; Raff, 1996; Malhortra & Thorpe, 1997). This method treats the variables symmetrically, rather than using the independent variables to predict the dependant variables, like in regression analyses (Rohlf & Corti, 2000). The utilization of the 2B-PLS analysis within this study is then a useful approach to investigating and visualising covariances between skull shape and environmental variables for *O. unisulcatus*.

The historical subspecies descriptions indicate that overall body size has changed in response to harsher environments (Meester *et al.*, 1986) and Armitage (1999) suggested that body size and shape should increase in response to harsher environments. The patterns of covariation between cranium shape and environmental variables are expected to show a clinal increase in size from the eastern to the more arid, north-western populations. Previous ecomorphological studies have demonstrated the applications of this technique, including Corti *et al.* (1996), Klingenberg & Ekau (1996), Rohlf & Corti (2000), Adams & Rohlf (2000), Ruber & Adams (2001) and Monteiro *et al*. (2003).

This section of the study aims to determine the morphological relationships between the populations of *O. unisulcatus* with regards to the changes in shapes and sizes of their craniums. I also investigated whether the possible resultant morphological relationships relate to the genetic groupings and the previously described subspecies boundaries (which were determined according to external morphological characters). This study also aims to explain patterns of variation by determining the environmental factors most affecting the shape and size of the *O. unisulcatus* cranium.

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Materials and Methods

Environmental variables

Environmental variables (Table 2.1) used in the analyses included: rainfall (mm/annum), rainfall seasonality, mean maximum temperature (^{o}C), mean minimum temperature (^{o}C), temperature stability (difference between maximum and minimum temperature; ^{o}C), altitude (m), and vegetative biomes (Mucina & Rutherford, 2006). Data such as mean rainfall, mean maximum and minimum temperature were obtained from the South African Weather Service. Temperature stability was determined by calculating the difference between the average maximum and minimum annual temperatures for each sampling locality, and this value was used as an indication of the constancy of the environment. The five categorical vegetation types used were obtained from Mucina & Rutherford (2006), and were delineated according to the biomes which occur throughout the species range. The vegetation types were divided into 'dummy' variables (linear orthogonal contrasts; Sokal & Rohlf, 1995) where each vegetation type assumes a value of 2 (Thicket Bushveld biome), 1 (Fynbos biome), 0 (Transition zone between biomes), -1 (Nama Karoo biome) or -2 (Succulent Karoo biome) and thus are easily combined with continuous variables in linear models (e.g. Monteiro *et al.*, 2003). The same procedure was employed to information pertaining to rainfall seasonality. Altitude variables were obtained from the locality information linked to the museum specimens.

Landmark determination

Images of the craniums of *O. unisulcatus* were obtained from those skulls housed in the Transvaal Museum, Durban Museum, South African Museum and Amathole Museum collections (Appendix A1). The ventral and dorsal views of the craniums were photographed using a Panasonic DMC-LC40. Age classes were determined using premolar wear (see Taylor *et al.,* 1993), and only adult specimens (age classes 4 and 5) were used. Specimens that were whole, and were minimally damaged were selected and photographed. The skulls were placed on a horizontal plane, and the camera was placed above the cranium, and a spirit level was used to ensure that the lens and the specimen were parallel. The camera height was adjusted until a focal distance of 0.3m from the camera to the specimen was achieved, which ensured a standard constant focal distance for each specimen. To ensure that distortion did not occur (Mullin & Taylor, 2002), skulls were placed in the middle of the focal area. For the dorsal and ventral analyses, 148 and 117 *O. unisulcatus* individuals respectively were photographed. The photographs of *O. unisulcatus* craniums were then viewed, and homologous landmarks were chosen (Figure 3.1) and digitised using the program tpsDig (Rohlf 1998b - 1998d, 1999). Landmarks on the specimens were analysed by utilizing geometric morphometric analyses (Bookstein, 1991; Rohlf & Marcus, 1993; Dryden & Mardia, 1998; Rohlf, 1998a) by using programs from the TPS series developed by Rohlf (1998b - 1998d, 1999) and the program R v.2.7.0 (R Development Core Team, 2008).

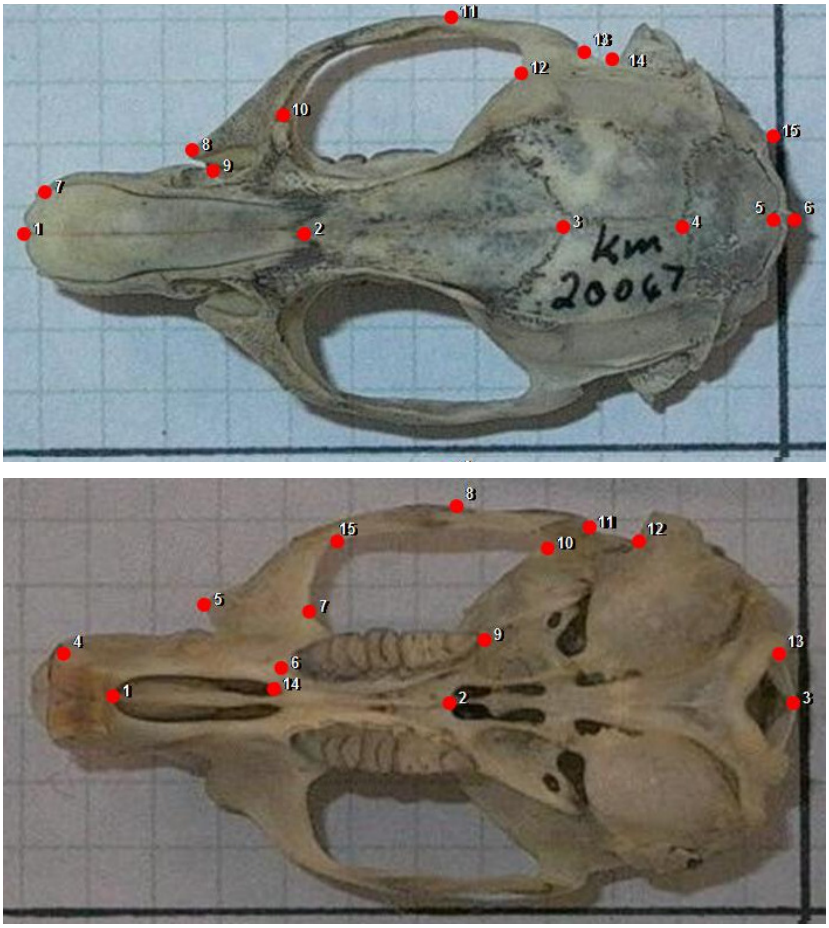

Figure 3.1: Homologous landmarks chosen for dorsal (top) and ventral (bottom) views of *O. unisulcatus* craniums.

To ensure that photographing techniques were consistently performed, skulls of three adult males from Montagu were photographed on three separate days. The photographs produced were copied five times over to ensure correct landmark placement, and landmarks were digitised for the 45 photographs. The three resultant distinct, non-overlapping groups produced from a Principal Components Analysis (PCA) (not shown here) verified that individual variation was not strongly influenced by the methods used to photograph the skulls and to digitize the landmarks.

Once the landmark coordinates had been digitised, the program R v.2.7.0 (R Development Core Team, 2008) was utilised for all analyses, unless otherwise specified. A Generalized Procrustes Analysis consensus (GPA; equivalent to the generalized least squares method, GLS (Rohlf & Slice, 1990) was performed. The analysis involves finding the coordinates for centroids (centre of gravity) for each landmark, rescaling of images to a standard unit centroid size (CS), and then rotation of resultant configurations so that the Procrustes chord distances among corresponding landmarks are at a minimum. The average or consensus configuration is then computed and Procrustes chord distances, defined as the sum of squared distances between the corresponding landmarks of the consensus configuration and that of the specimen, were determined. Then superimposed coordinates are projected into the tangent space using an orthogonal projection (Dryden & Mardia, 1998). The percentage of variation contributed by allometric

growth was calculated for each view, for each sex. The effect that allometric growth may have on the shape analysis of the skull was removed using multivariate regression and a linear model (Claude, 2008). The residual variation would then correspond to shape variation.

Once the Procrustes chord distances were obtained, the configurations were projected into an Euclidean space tangent to the shape space, the error of which was minimized by the use of the sample mean shape as the point of tangency. The Procrustes distances in the shape space were compared with the Euclidean distances in the tangent space, in order to test the closeness of the tangent space to the curved shape (Rohlf, 1998d).

Genetic and environmental effect on Centroid Size

The significance of the variation in centroid size among genetic assemblages was tested using an ANOVA (Chambers & Hastie, 1992). Centroid Size (CS) was plotted against environmental variables, with the aim of determining which environmental factors play a role in CS variation. Linear regression analyses of the centroid sizes against the separate environmental variables were performed. To identify potential outliers, which would influence the regression slope, Cook's Distance (D*i*) and the leverage values were also investigated. R^2-values (fraction of variance explained by the model) and F-values were calculated. An ANOVA (Chambers & Hastie, 1992) was performed between each environmental variable and the centroid size. The correlation coefficient and the significance of the relationship were calculated using Pearson's product-moment correlation (Pearson, 1895) between each environmental variable and the centroid size.

Genetic effect on cranium shape

The mahalanobis distances were computed between populations and a hierarchical clustering algorithm (average method) was performed using the obtained distances. The significance of the variation in skull shape of males and females among genetic assemblages was tested using a MANOVA (R Development Core Team, 2008). Principal Components Analyses (PCAs) were performed on the mean shape variables of the populations for the split-sex datasets (both views), to investigate the variation between the two genetic assemblages found in the previous chapter. The means of the populations were used to avoid over-weighting some populations as sample sizes were different. The same datasets were employed in Linear Discriminant Analyses (LDAs), to investigate whether the two genetic assemblages could be placed into two discriminant clusters.

Environmental effect on cranium shape: Two-Block Partial Least-Squares analysis

The 2B-PLS analysis is used to explore patterns of covariation between two sets of variables by constructing pairs of variables that are linear combinations of the variables within each of the two sets, and this accounts for as much as possible of the covariation between the two original sets (Rohlf & Corti, 2000).

This method is useful in analysing the covariation with shape by using shape variables as one of the sets of variables, and environmental variables as the other set (e.g. Monteiro *et al.*, 2003).

Specimens were divided into their pooled sampling localities and a mean configuration for each locality was calculated using the program R v.2.7.0 (R Development Core Team, 2008). The program tpsPLS v.1.18 (Rohlf, 2003) was used for further 2B-PLS analyses. These mean configurations (consensus configurations) were superimposed, yielding a grand mean shape, which was used as a tangent configuration (Rohlf, 1996) in order to calculate partial warp and uniform component scores (Bookstein, 1991; Rohlf, 1996), assuming the exponent alpha=0 (Rohlf, 1993). Partial warps are shape variables used to describe localized shape changes, and when combined with uniform components, they span a tangent shape space that can be utilised to depict shape variation (Monteiro *et al.*, 2003). The 2B-PLS analysis computes linear combinations, constructed to maximize the covariation between the shape variables and the environmental variables, by using a singular-value decomposition of the matrix of covariance between the two sets of variables (Monteiro, 2003). The total covariation between the two sets of variables is computed with the formula: (squared sing. value of first latent variable) / (sum of squared sing. value of first latent variable and squared sing. value of second latent variable) (Rohlf & Corti, 2000).

The one limitation of the 2B-PLS method is that there is no direct test of significance, however permutation tests seem to suffice (Hoskuldsson, 1988; Rohlf & Corti, 2000). Statistical tests for significant associations between the 2 sets of variables were conducted using permutation tests, which were carried out repeating the analyses with 999 independent random permutations of specimen ordering in the two datasets. Deformed grids were used to visualize the shape changes associated with the linear combinations obtained by 2B-PLS (otherwise known as PLS shape vectors). The linear combination of the environmental variables (Table 2.1) were used to represent the environmental gradient, and were obtained using linear correlation between the original environmental variables and the linear combination scores of environmental variables (Monteiro, 2003). Clustering of the populations was achieved by utilising a hierarchical clustering algorithm in the program R v.2.7.0 (R Development Core Team, 2008), using the complete method.

Subspecies validation

The specimens in the split-sex datasets were divided according to the approximate subspecies delineations described previously (Roberts, 1951). The specimens which did not occur within any subspecies geographic boundaries were divided into three groups: (1) Specimens occurring in the Western Cape Province, (2) specimens occurring in the Eastern Cape Province and (3) specimens occurring in the western regions of the Nama Karoo biome. MANOVAs were performed in the program R v.2.7.0 (R Development Core Team, 2008) between the subspecies delineations and the shape variables, whilst ANOVAs were performed between the centroid sizes and the subspecies delineations. Boxplots were constructed in the same program to visualise the variation in the centroid sizes between the previously described subspecies (Roberts, 1951). The mean shapes of the members of each subspecies was calculated in R v.2.7.0 (R Development Core

Team, 2008), and plotted to visualise which landmark contributed the most variation between the subspecies.

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Results

Procrustes and Euclidean distances were highly correlated for all datasets (correlation coefficients all above 0.99). The ANOVA revealed that there were significant differences in the group means between the genders' centroid sizes in the ventral view ($F = 16.12$, $P < 0.001$), whereas the differences in the dorsal view approached significance ($F = 3.34$, $P = 0.07$). The mean centroid size of males was larger, with a greater variance around the mean, than females in both views. There were significant differences in the MANOVA (Hotelling-Lawley test) for the shape variables between the sexes (ventral view: $F = 2.76$, $P < 0.001$; dorsal view: $F = 1.87$, $P < 0.001$). Males had more bulbous zygomatic arches, whilst females had flattened, elongated zygomatic arches in both views. As in the mtDNA chapter, pooled sampling localities are referred to as populations in the text.

When variation explained by size and allometric growth effects were removed from the shape variables using a multivariate regression utilizing a linear model in which the intercept was calculated by the linear model, the amount of variation that was explained by size was between 12% and 14%.

Genetic and environmental influences on CS

The ANOVAs investigating the significance of variance between CS values and genetic assemblages produced no significant relationships for any of the datasets. Correlations between centroid size and environmental variables revealed only one significant correlative association: between the centroid sizes of the ventral view of the females and rainfall (Table 3.1). Scatterplots of the environmental variables (rainfall seasonality, minimum temperature, biomes, altitude, and maximum temperature) did not produce any discernible patterns of variation, and thus further analyses were not conducted on these variables. Scatterplots of centroid size against temperature stability did reveal that those specimens in locations with low values of the respective environmental variables did possess larger centroid sizes, however there were no significant correlations between the variables.

The CS values of the ventral view of the female individuals had a greater variation at rainfall values between 180 mm and 350 mm. Sampling localities which possess values that fall within this range include population P05 (Middelburg), P06 (Steytlerville), P08 (Carnarvon), P10 (Victoria West), P11 (Beaufort West), P13 (Murraysburg), P15 (Calvinia) and P20 (Western Cape pooled sample). It appears that at lower rainfall levels the CS values do not drop lower than 350 for the ventral view. This indicates that at lower levels of rainfall, the centroid size will tend to be larger, and less varied than at higher levels. Sampling localities which possess values that fall into the lower ranges include population P14 (Richtersveld), P17 (Springbok), P19 (Port Nolloth), P22 (Lamberts Bay), and P23 (Vredendal). These sampling localities are all situated in the Succulent Karoo biome.

The R^2-value (fraction of variance explained by the model) was the highest in the female ventral view dataset, indicating that a larger amount of variance between the centroid sizes and rainfall could be explained by the model in this dataset, than in any of the others. There was a significantly positive correlation between the centroid sizes and the rainfall variables (t-value = 2.38, P = 0.02), as well as a significant relationship between the variances of the two variables (F = 5.667, P = 0.02). There were three outliers from Albany (P01), Bedford (P02), and Port Nolloth (P19) (Figure 3.2). All three had Cook's Distances larger than 1.0, however only the individual from Port Nolloth had a very large leverage value (as well as a large Cook's Distance; Figure 3.2B). These individuals could have influenced the regression line and the correlations, so they were sequentially excluded from the analyses. It was found that excluding the Albany (number 12) and the Port Nolloth (number 40) individuals produced the most significant correlations (t-value = 3.58, $P < 0.001$) and regression analyses (F = 9.18, $P < 0.01$) (Table 3.1). The amount of variance that could be explained by the linear model (R^2-value) also increased to 16% from 6% when the outliers were excluded. For the remaining datasets, even when the outliers were removed, there were no significant correlations and regressions between the centroid sizes and the environmental variables. In the regression plot of the female ventral view dataset (Figure 3.2C), the distribution is skewed around the mean, indicating that the variance spreads increasingly around the regression line.

Table 3.1: Correlations between centroid size and environmental variables (only rainfall and temperature stability shown) for the split-sex datasets (with and without outliers). ANOVA results (with degrees of freedom (d.f.), F-values, and significance levels (P-value)), correlation coefficients (Corr. coeff.) and R^2-values shown. Significant values shown in bold font. Analyses with and without the identified outliers are shown.

		ANOVA on ventral view datasets				ANOVA on dorsal view datasets			
		F-value (1 df)	***P*-value**	**Corr. coeff.**	**R^2-value**	**F-value (1 df)**	***P*-value**	**Corr. coeff.**	**R^2-value**
Rainfall	**Females**	**5.667**	**0.020**	0.274	0.062	2.864	0.094	0.183	0.022
	Females (without outliers)	**9.178**	**0.004**	0.347	0.159	1.1514	0.286	0.118	0.014
	Males	0.085	0.772	-0.042	0.019	0.755	0.388	0.110	0.004
	Males (without outliers)	0.0534	0.818	0.033	0.001	3.3171	0.074	0.229	0.052
Temperature stability	**Females**	0.152	0.698	-0.046	0.012	0.034	0.855	-0.020	0.012
	Females (without outliers)	0.013	0.9086	0.014	0.0002	0.099	0.753	-0.0348	0.001
	Males	1.150	0.289	-0.151	0.003	3.114	0.083	-0.219	0.032
	Males (without outliers)	0.979	0.328	-0.141	0.020	0.533	0.468	-0.0938	0.009

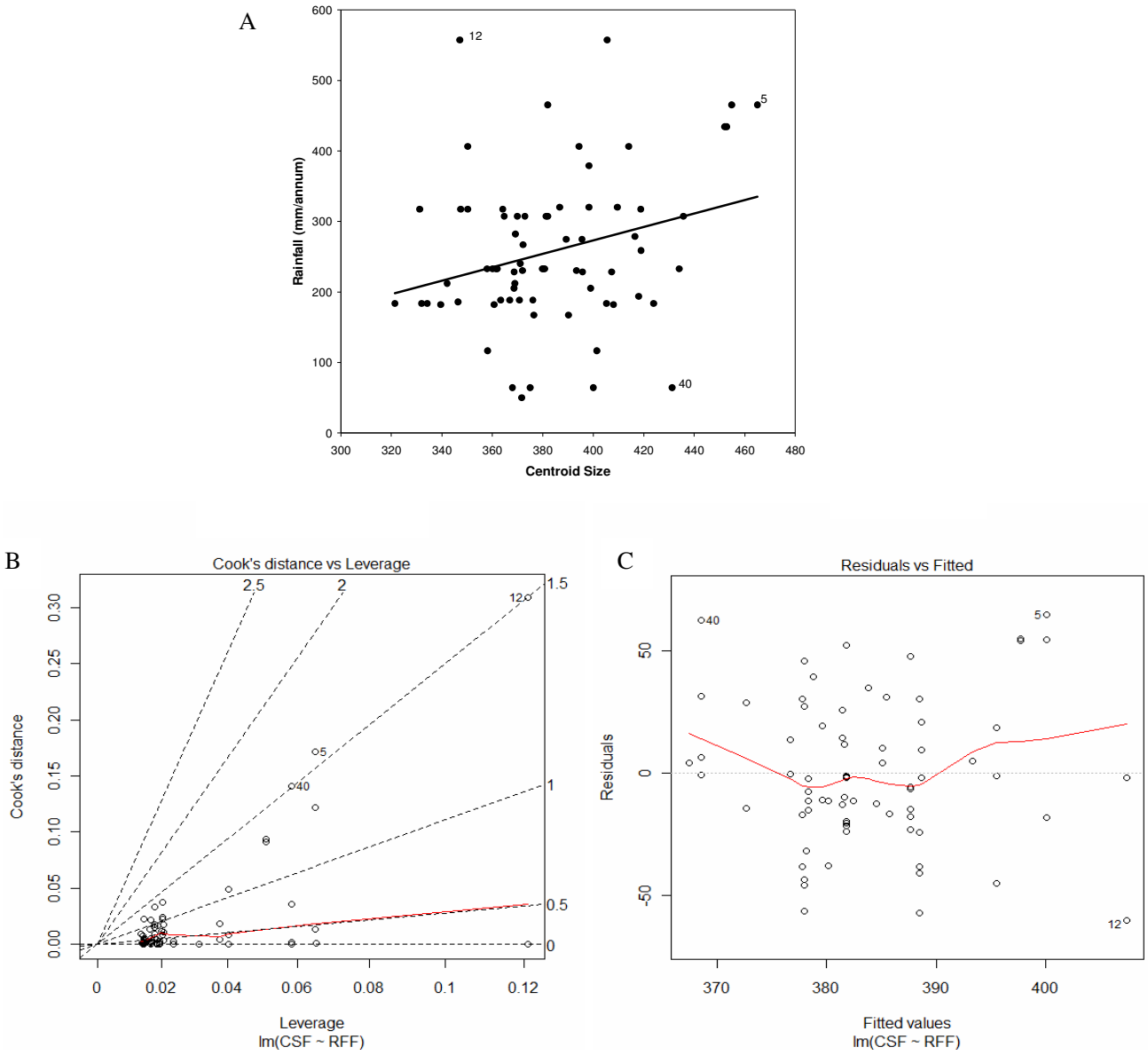

Figure 3.2: Correlation scatterplot (A), Cook's Distance (D*i*) (B) and linear regression plot (C) comparing centroid size (CS) against rainfall variables for the ventral views of female *O. unisulcatus* crania. The Cook's Distance (D*i*) was plotted against the leverage values to identify outliers which may have caused the regression to be biased. Those individuals identified as the outliers originate from: [5] Bedford (P02); [12] Albany (P01) and [40] Port Nolloth (P19).

Genetic effect on cranial shape

There were no significant F-values in MANOVAs in which the differences in variances of shape variables between genetic assemblages were examined. Cluster dendrograms of the mahalanobis distances between populations (Figure 3.3) indicate that the shapes of the crania are not solely influenced by genetic influences.

PCAs and LDAs were conducted on mean shape variables for populations, for the split-sex datasets (both views) (Figure 3.4). In the PCAs, the first pair of PC axes contributed the most variation (>45%) for all datasets. Those populations which were grouped into the central assemblage in the genetic analyses consistently did not fall into distinct clusters for both views. In the LDAs performed on the same datasets also verified that those populations which clustered into the two distinct genetic assemblages did not fall into discriminant groups, when the shape variables were analysed.

The two assemblages found in the genetic analyses are not recovered in these morphometric analyses, and this means that environmental influences may be a greater influence on skull shape than common ancestry.

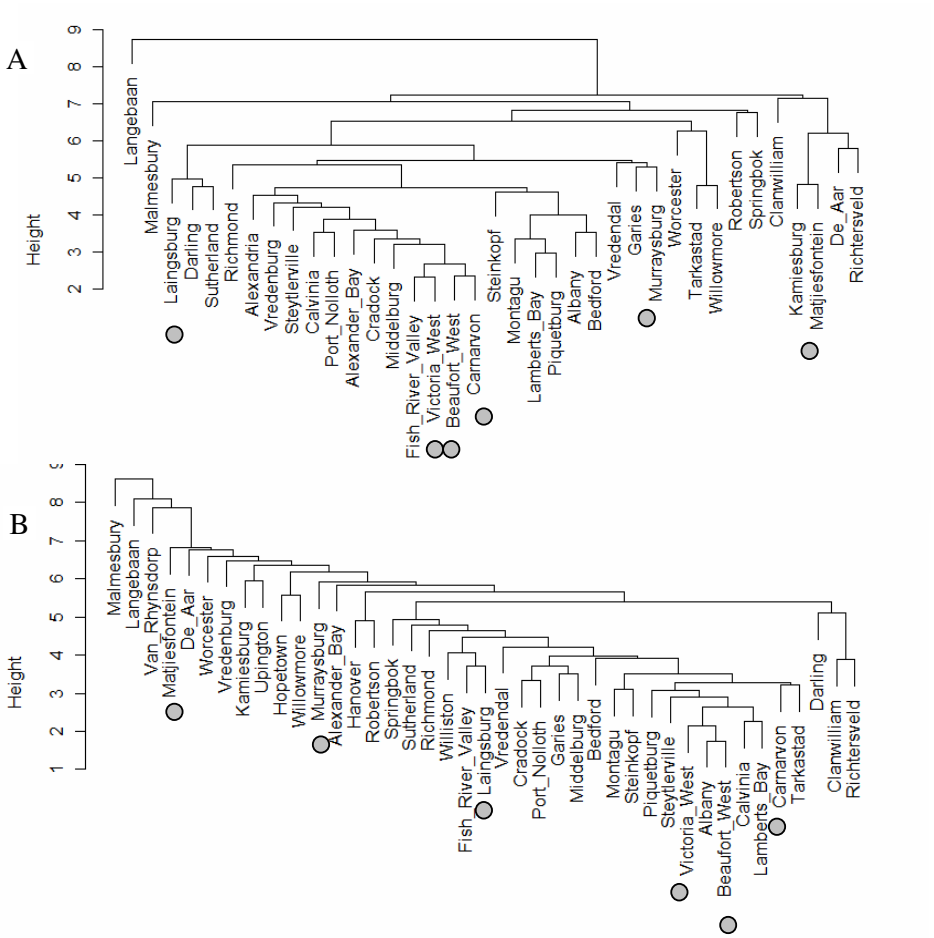

Figure 3.3: Hierarchical clustering dendrogram of the mahalanobis distances of the shape variables for the ventral view (A) and the dorsal view (B) of *O. unisulcatus* crania. Grey dots indicate those populations which were classified as the central assemblage.

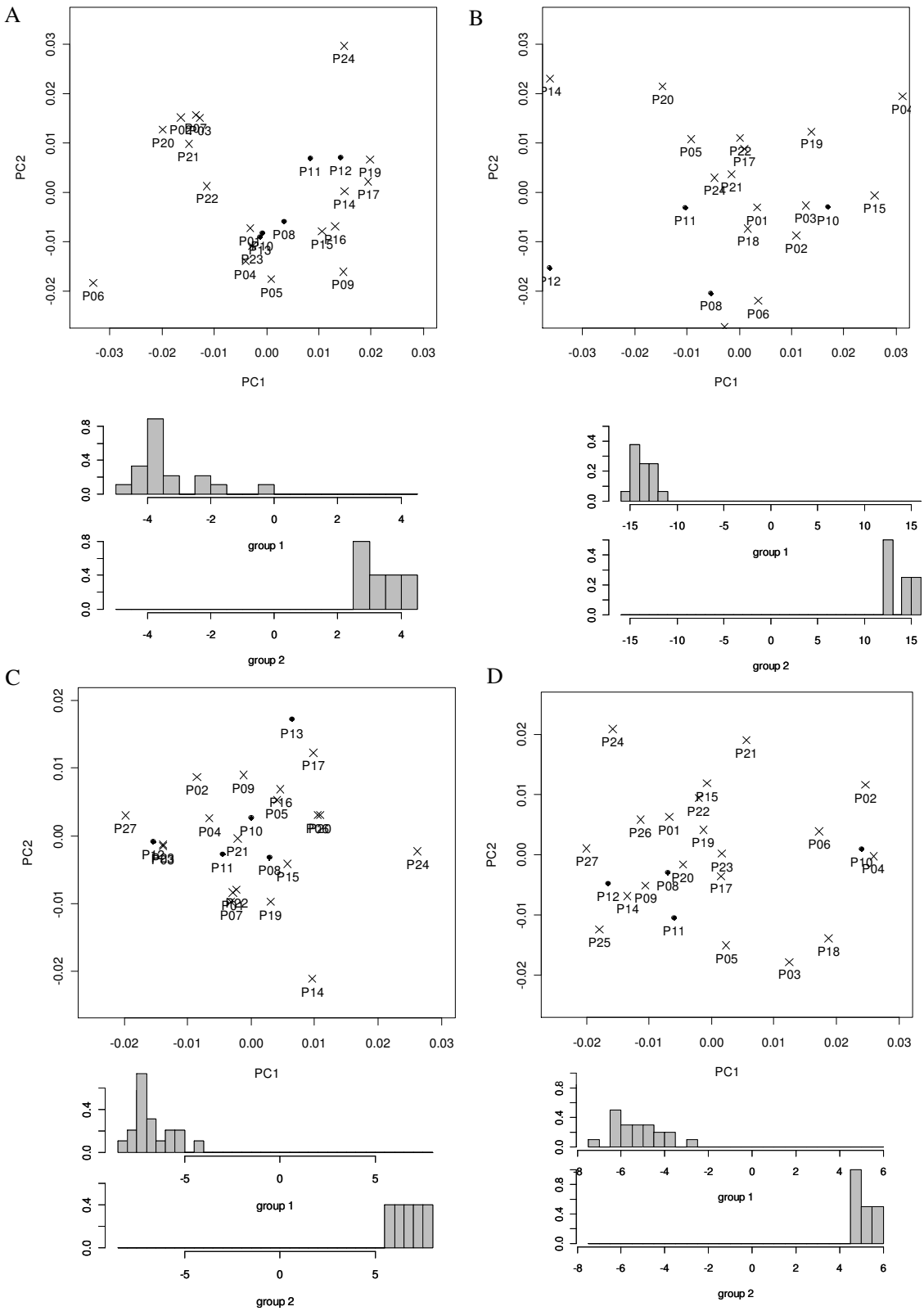

Figure 3.4: Principal Components Analyses (PCAs) and Linear Discriminant Analyses (LDAs) of mean shape variables for female (A, C) and male (B, D) datasets for the ventral (A, B) and dorsal (C, D) views. PCA analyses (only the first two PC axes examined) show the populations which were placed in the coastal (✕) and the central (●) assemblages in the genetic analyses. The first two PC axes consistently contributed the largest amount of variation when the compared to the other axes (above 45%). In the LDA barplots (beneath PCA figures), the top barplots represent the coastal assemblage, and the bottom barplots, the central assemblage.

Two Block Partial Least Squares Analysis

In the 2B-PLS analysis (Table 3.2, Figure 3.5), the first dimension described between approximately half and two thirds of the covariance between the two blocks (shape variables and environmental variables) for the split-sex datasets (66% and 71% for ventral view; 66% and 47% for dorsal view for females and males respectively). The only significant permutation for covariation values for the first dimension was found for the male dataset for the ventral view. The first dimension for all datasets also possessed the highest singular values; the remaining dimensions all possessed progressively smaller singular values than that of the first. The second dimension only contributed between 15% and 31% of the covariation, and the only permutation that approached significance for this dimension was found in the female dataset for the ventral view. It was at the third dimension that 96% of the total covariance between skull shape and environmental variables was explained, however no significant covariance was found for any of the datasets in this dimension. Since the fourth dimension in all datasets contributed little to the cumulative covariance (only between 2% and 8%), it was assumed that shape changes contributed by this dimension would not be significant and thus also not being biologically meaningful. The remaining dimensions (fifth to seventh) contributed very little to the cumulative covariation (0% to 2%) and thus these dimensions were not included in the analyses.

The only statistically significant correlation (as well as the largest) was found in the male dorsal view dataset, even though this dataset possessed the lowest percentage of covariation between the first two latent variables. After examining the loadings, this could be as a result of the minimum temperature variable (largest positive loading). This significant relationship has not been confounded by a correlation with size, as this relationship was not significant (R = 0.50; P = 0.81). The larger positive loadings in the first dimension in all datasets are related to temperature variables, even though only the male dorsal view dataset shows a significant correlation to environmental variables. It appears that factors that affect the delimitation of biomes are affecting the shape variables in the female ventral view dataset, as the largest positive loading was related to the biome boundaries.

Table 3.2: Covariation between the environmental variables and the partial warps produced by a 2B-PLS analysis (first two latent variables shown). Random permutations (999 iterations) were performed. Significance of permutation results (P) are shown in brackets and significant results are shown in bold. The symbol λ_i^2 presents the ith squared singular values, $\sum\lambda_i^2$ denotes the cumulative sum of squared values, whilst r_i refers to the cross-set correlation values between the first two latent variables.

	Dimensions							
	Female specimens				**Male specimens**			
	Ventral View		**Dorsal View**		**Ventral View**		**Dorsal View**	
	1	**2**	**1**	**2**	**1**	**2**	**1**	**2**
1-Rainfall	0.44	0.43	0.23	0.06	0.30	0.65	0.25	0.31
2-Biome	0.52	0.29	0.01	0.46	0.10	0.53	0.28	0.57
3-Altitude	0.02	-0.47	0.38	-0.46	0.47	-0.42	-0.61	0.02
4-Rainfall Seasonality	-0.28	0.13	-0.35	0.47	-0.37	0.15	0.30	-0.25
5-Maximum Temperature	0.47	-0.07	0.57	0.46	0.18	0.25	0.14	0.48
6-Minimum Temperature	0.19	0.62	-0.23	0.30	-0.45	0.16	0.51	-0.08
7-Temperature Stability	0.46	-0.33	0.56	0.23	0.56	0.06	-0.33	0.53
λ_i^2	0.66 (0.12)	0.23 (0.60)	0.62 (0.23)	0.16 (0.94)	**0.71 (0.04)**	0.15 (0.96)	0.47 (0.68)	0.31 (0.21)
$\sum\lambda_i^2$	0.66 (0.12)	0.90 (0.06)	0.62 (0.23)	0.78 (0.76)	**0.71 (0.04)**	0.86 (0.26)	0.47 (0.68)	0.78 (0.58)
r_i	0.69 (0.11)		0.71 (0.19)		0.77 (0.08)		**0.82 (0.02)**	
% of covariance between variables	89.17%		93.76%		95.73%		69.68%	

In the two-block partial least squares (2B-PLS) ordinations (Figures 3.6 to 3.7), four major clusters were found for all datasets, with a few exceptions. The first major cluster included specimens from the Eastern Cape and from the Fynbos region in the Western Cape (Albany (P01), Fish River Valley (P04), Montagu (P20), Robertson (P20), Worcester (P20) Darling (P20), Piquetburg (P21), Langebaan (P24) and Vredenburg (P24)). This cluster corresponds to the southern part (south coast) of the mtDNA coastal assemblage. It tended to be positively correlated with all environmental variables, except that of altitude (variable 3). The second cluster included specimens from localities along the west coast of South Africa, and these localities all fall within the Succulent Karoo biome (Richterveld (P14),Garies (P16), Port Nolloth (P19), and Lamberts Bay (P22)). This cluster corresponded to the western coastal region of the genetic coastal assemblage. This cluster tended to be negatively correlated with all environmental variables, except that of rainfall seasonality (variable 4) and minimum temperature (variable 6). It was this cluster that had the greatest variation in skull shape between populations. The third major cluster found consisted of specimens from localities located within the more inland Nama Karoo biome (Middelburg (P05), Carnarvon (P08), Richmond (P09), Victoria West (P10), Beaufort West (P11), Murraysburg (P13), Calvinia (P15), and Hope Town (P27)). These populations occur in the higher altitude region on the African Plateau. This cluster was shown to be positively correlated with altitude (variable 3), maximum temperature (variable 5) and temperature stability (variable 7). Those populations which occur along the escarpment (Bedford (P02), Cradock (P03), Steytlerville (P06), Tarkastad (P07), Laingsburg (P12), Springbok (P17), Van Rhynsdorp (P23), and Sutherland (P30)), were in various clusters depending on the dataset used. Populations from Laingsburg, Springbok and Van Rhynsdorp (occurring along the western region of the escarpment in the Succulent Karoo biome) could either be placed in the second or the fourth cluster. Even though they are placed in the fourth group in some datasets, these populations cluster closely (in terms of shape), and thus their cranium shapes closely resemble, the second cluster. Populations P07 (Tarkastad) and P30 (Sutherland) could be placed either in the third or fourth cluster. Two populations occurring along the south-eastern region of the escarpment (P02-Bedford and P06-Steytlerville) could be either placed in the fourth or a fifth cluster.

In the ventral view, the first cluster showed a cranium shape in which the maxillary bones tended to be broader, causing the zygomatic arches to, in turn, be relatively broader elongating anteriorly. The second cluster possessed narrower zygomatic arches that are elongated posteriorly, and the palatine bones are longer (producing a shorter anterior palatal foramen (a.p.f.)). The third cluster possessed narrower maxillary bones and broader zygomatic arches that elongated outwards. The fourth cluster possessed broader zygomatic arches that are elongated posteriorly, and possess narrower maxillary bones.

For the dorsal view, the first cluster tended to have broader zygomatic arches which elongated anteriorly, as well as a narrower braincase. The second cluster possessed relatively narrower zygomatic arches, longer nasal section, broader braincase and seemingly larger tympanic bullae (landmarks 13 and 14 were closer together). The third cluster's zygomatic arches were relatively broader elongating outwards, as well as

seemingly larger tympanic bullae (landmarks 13 and 14 were closer together). The fourth cluster appeared to approximate the mean shape in both females and males. The fifth cluster (female dataset) seems to be intermediate between the first and third cluster.

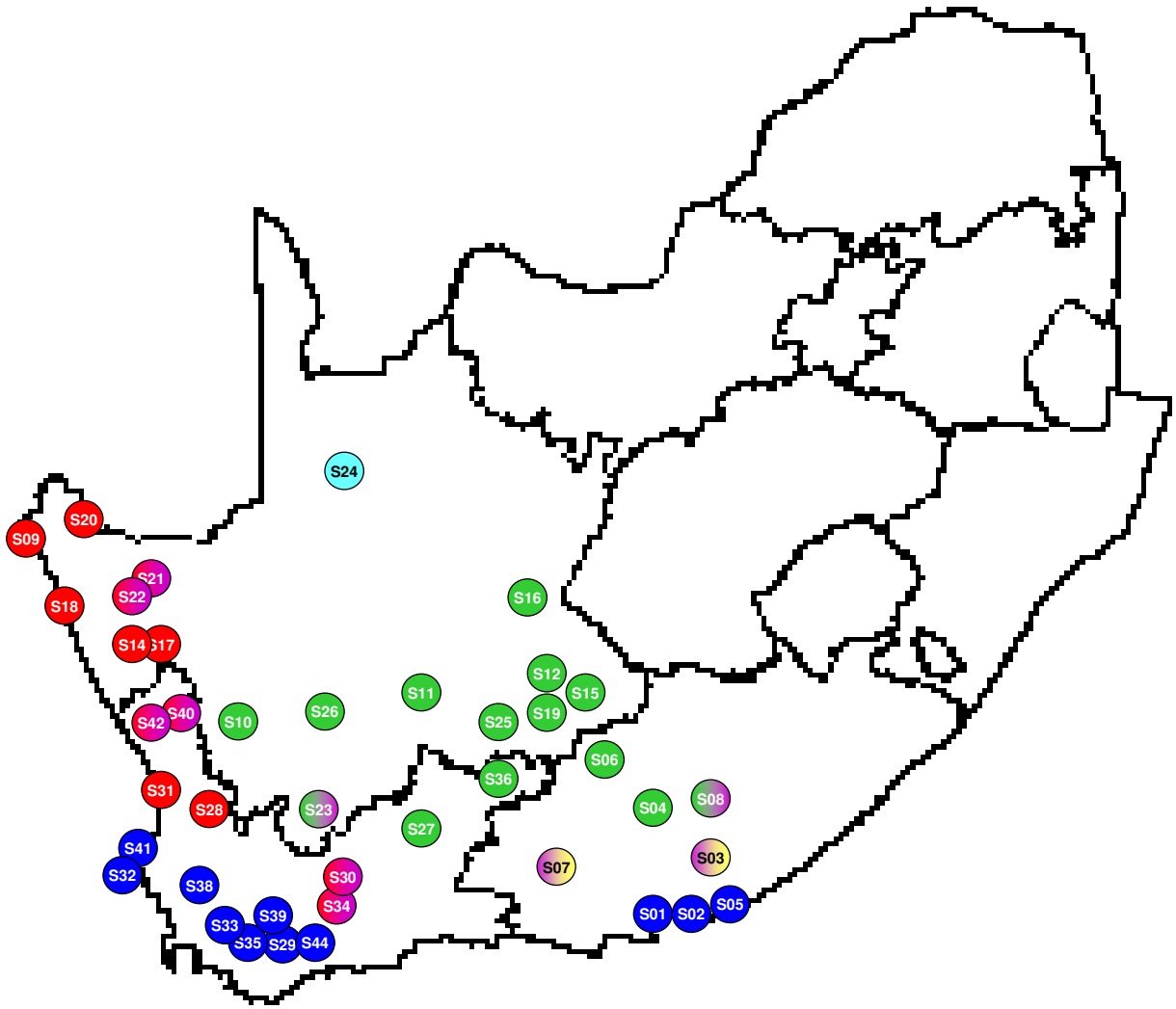

Figure 3.5: Distribution map of sampled localities (circles) of *O. unisulcatus* used in the morphological analyses. Colours relate to the main clusters obtained in the Two-Block Partial Least Squares analysis. The text shown within the localities refer to Table 2.1. Key to colours: Cluster 1 = blue, cluster 2 = red, cluster 3= green, cluster 4 = purple, cluster 5 = yellow, cluster 6 = light blue. Localities with two colours indicate that the hierarchical clustering placed them into different clusters, when the datasets were analysed.

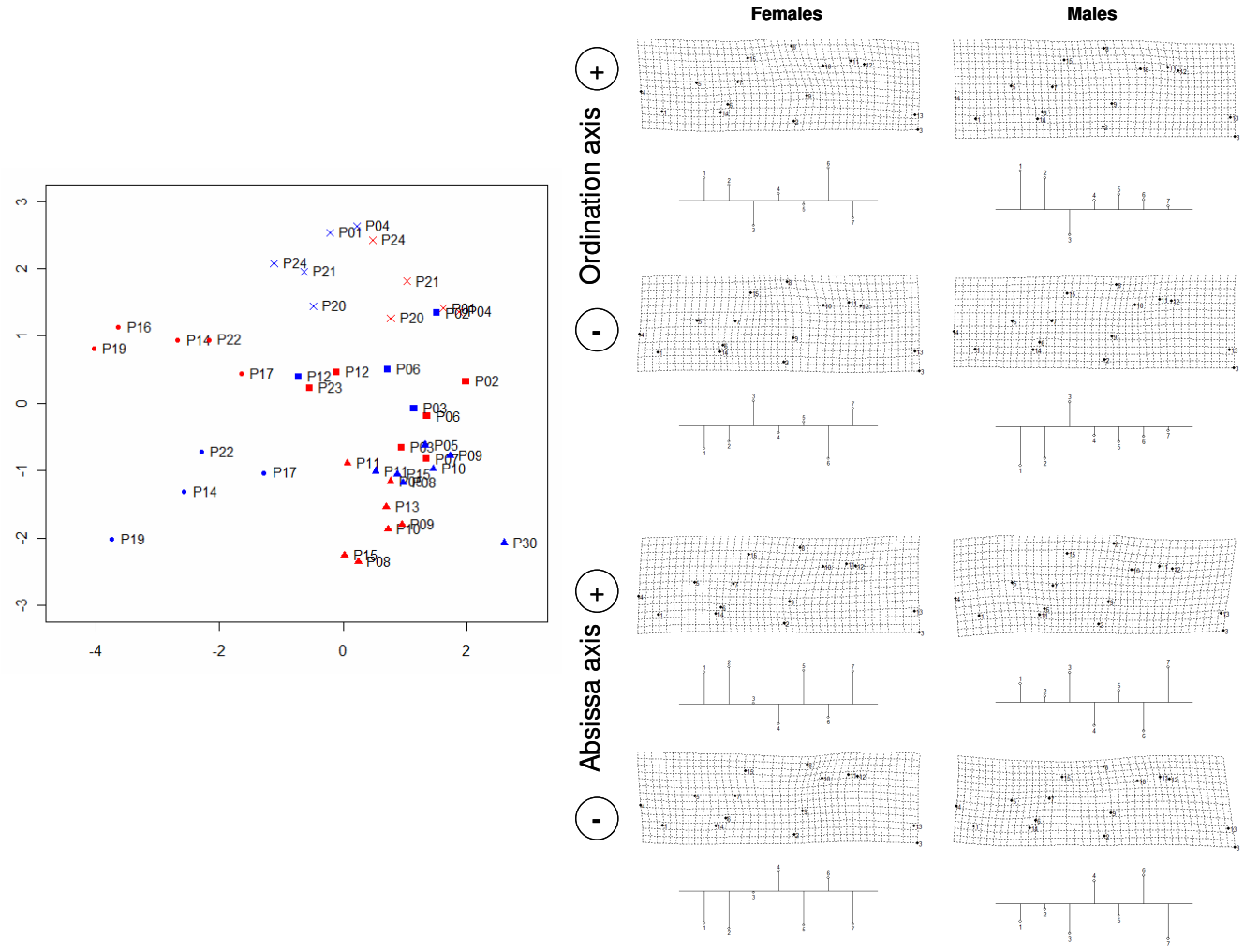

Figure 3.6: Ordinations of the results of a 2B-PLS regression analysis on the ventral view mean shape variables of *O. unisulcatus* and associated environmental variables for female (red points) and male (blue points) specimens, for the first pair of latent variables. The points are labelled according to the pooled sampling localities in Table 2.1. Deformed grids (used to show shape change), and correlation plots are shown for the respective axes (Ordination axis = X-axis; Abscissa axis = Y-axis). Key to labels in the correlation plots: 1 = Rainfall (mm/annum), 2 = Biome boundaries, Altitude (m), 4 = Rainfall seasonality, 5= Maximum temperature (^{o}C), 6 = Minimum temperature (^{o}C), 7 = Temperature Stability. Key to clusters obtained through hierarchical clustering: cluster 1 = ✕, cluster 2 = ●, cluster 3 = ▲, cluster 4 = ■.

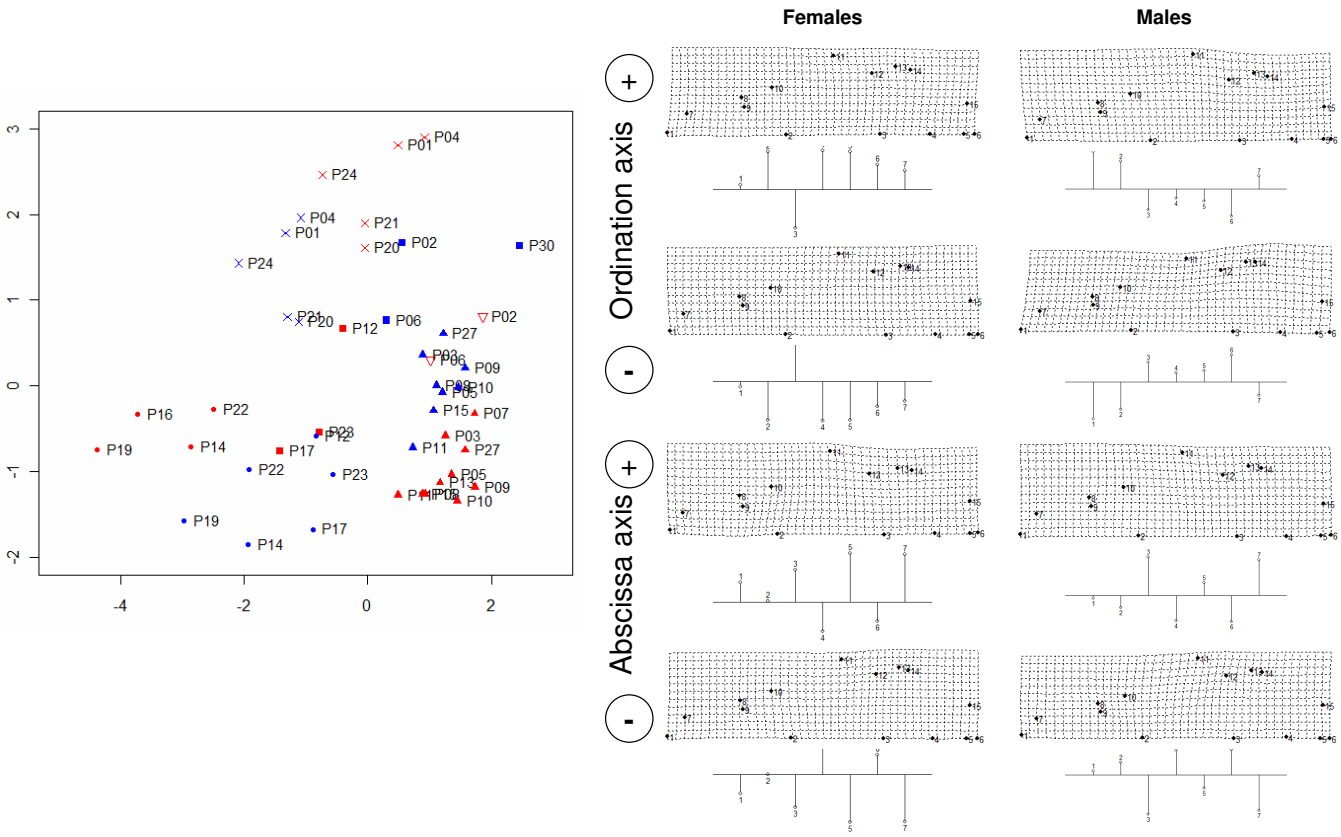

Figure 3.7: Ordinations of the results of a 2B-PLS regression analysis on the dorsal view mean shape variables of *O. unisulcatus* and associated environmental variables for female (red points) and male (blue) specimens for the first pair of latent variables. The points are labelled according to the pooled sampling localities in Table 2.1. Deformed grids (used to show shape change), and correlation plots are shown for the respective axes (Ordination axis = X-axis; Abscissa axis = Y-axis). Key to labels in the correlation plots: 1 = Rainfall (mm/annum), 2 = Biome boundaries, Altitude (m), 4 = Rainfall seasonality, 5= Maximum temperature (^{o}C), 6 = Minimum temperature (^{o}C), 7 = Temperature Stability. Key to clusters obtained through hierarchical clustering: cluster 1 = ✕, cluster 2 = ●, cluster 3 = ▲, cluster 4 = ■, cluster 5 = ▽.

Subspecies validation

Since there were significant differences between the shapes of male and female specimens, the subspecies analyses were divided into the two different genders. On the whole, males tended to have larger ranges of centroid sizes than females, even though an ANOVA performed produced a non-significant result in the dorsal view datasets when the relationship between the sexes and the centroid sizes were investigated. The only significant relationship between the centroid sizes and the subspecies delineations occurred in the female ventral view dataset, however that of the male dorsal view dataset approached significance (Figure 3.8). Those specimens of *O. u. unisulcatus* consistently had smaller mean centroid sizes relative to the other subspecies, and the geographic boundary for this subspecies occurs in the area in which the central assemblage was found in the mtDNA study (Chapter 2). The variances in the *O. u. albiensis* and *O. u. grantii* were consistently large in all datasets analysed. Females of the *O. u. bergensis*, as well as *O. u. broomi*, exhibited smaller centroid sizes when compared to the males, for both views.

The relationships between the shape variables and the subspecies delineations were significant in all datasets in the MANOVAs, with F-values ranging between 1.27 and 1.69. It appears that the landmark number 8 in the ventral view and the landmark number 11 in the dorsal view (both the outer edge of the zygomatic arch) are showing the most variation between the subspecies. In the ventral view datasets, *O. u. broomi, O. u. albiensis* and the Western Cape specimens tend to have zygomatic arches which are broader anteriorly (landmark 8 is more anterior relative to the other specimens). These specimens fall into the first and the second cluster found in the 2B-PLS analysis. The Eastern Cape and the *O. u. unisulcatus* specimens tended to have a more posteriorly flattened zygomatic arch. These specimens fall into the fourth cluster found in the 2B-PLS analysis. Specimens from the Nama Karoo biome and *O. u. bergensis* specimens exhibit posteriorly flattened zygomatic arches in the female datasets, and anteriorly rounded zygomatic arches in the male datasets. These specimens occur in the third and the second 2B-PLS clusters, respectively. The *O. u. grantii* specimens have zygomatic arches which are intermediate between the anteriorly rounded and the posteriorly flattened arches, and these specimens fall into the third 2B-PLS cluster.

Specifically in the male dorsal view dataset, landmarks 12, 13 and 14 are also showing a large amount of variation relative to the other landmarks. It is also in this dataset that *O. u. unisulcatus* specimens exhibit a wider braincase (landmarks 13, 14, 15, 6), and a shorter, narrower nasal section (landmarks 1, 7 and 9). The male *O. u. unisulcatus* specimens also exhibited a wider braincase in the ventral view dataset (landmarks 11 and 12).

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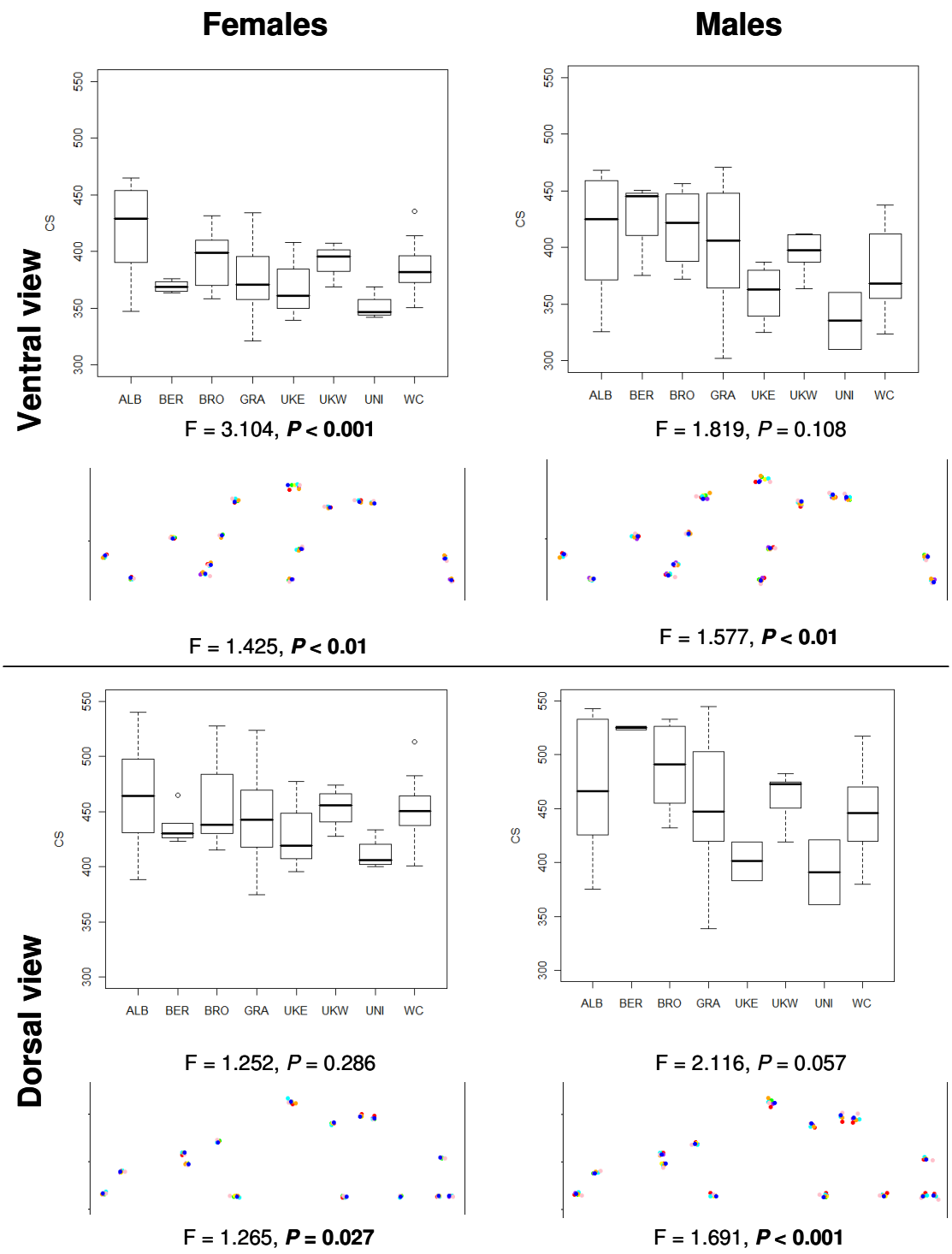

Figure 3.8: Visualisations of the variation in the centroid sizes and of landmark variation between the subspecies in the ventral views (above) and the dorsal views (below) for the split-sex datasets. Boxplots (median and the inter-quartile range) show the variances in the centroid sizes between subspecies. F-values and significance values (*P*-values, significant values shown in bold) obtained from the respective ANOVAs and MANOVAs are shown below the figures. Mean shapes of the various subspecies are shown below the boxplots and indicate which landmarks vary according to the subspecies delimitations. Key to subspecies (abbreviations/colours): *O. unisulcatus albiensis* = ALB/green, *O. u. bergensis* = BER/purple, *O. u. broomi* = BRO/red, *O. u. grantii* = GRA/yellow, *O. u. unisulcatus* = UNI/pink. Key to specimens which do not fall into any species delineation: Western Cape specimens = WC/blue, Nama Karoo specimens = UKW/orange, Eastern Cape specimens = UKE/light blue.

✧

Discussion

Populations of *O. unisulcatus* have been found to be genetically indistinct (Van Dyk, 1990), or divided into two assemblages based on mtDNA analyses (this study). It has been shown that morphological traits can evolve at a quicker rate than mtDNA markers can coalesce, due to their polygenic nature and the fact that they are often under greater selection (Zink & Barrowclough, 2008). The size and shape of *O. unisulcatus* skulls show responses to environmental factors, with minimum temperature significantly influencing the clusters of shape variables in the male dorsal view dataset, and rainfall significantly influencing the size variables in the female ventral view dataset.

Genetic influences on size and shape

It has been suggested that size clines may be used as indicators of admixture and gene flow, as a result of the genetic factors influencing size clines (Storz, 2002), however due to the plastic and highly adaptive nature of size (Cardini *et al.*, 2007) this conclusion should be treated with caution. Population processes, such as population bottlenecks have also been shown to affect skull shape in vertebrates (Cardini, 2003). Thus, genetic and environmental factors play a role in the processes determining cranium shape and size.

Otomys unisulcatus cranial size and shape appeared to not cluster according to the assemblages found in the phylogeographic genetic analyses. The genetic makeup of the species may be affecting other cranial structures not assessed in this study (e.g. soft anatomy, internal nasal cavities, molar shape, and mandible shape), however the shape and size of the ventral and dorsal portions of the cranium appear to not be reflecting the same phylogeographic structure obtained from the analysis of the cytochrome *b* gene.

Environmental effects on size variation

Clinal size variation has been observed to occur in mammals (e.g. Smithers, 1971; Smith, 1979; Robinson, 1981; Searle, 1984; Quin, Smith & Norton, 1996; Chimimba, 2001; Monteiro *et al.*, 2003). Variation in cranium shape and size can be produced by a combination of genetic and environmental (nongenetic) factors (Cardini *et al.*, 2007). Some environmental factors that have been shown to affect body size include predation rate (Isbell, 1994; Hill & Dunbar, 1998), extreme summer temperatures (Yom-Tov, 1993; Smith & Charnov, 2001), and longitude (Chimimba, 2001). Size clines may therefore be affected by many factors (Sokal & Rinkel, 1963; Gould & Johnston, 1972; Chimimba, 2001).

Centroid sizes in this study have been used as a proxy for body size. Longitude did not seem to be factor in the variation in centroid size, which is in contrast with results found for *Aethomys* (Chimimba, 2001), a southern African rock-dwelling rodent. In the latter study, specimens found in hotter low-rainfall regions tended to have larger centroid sizes and showed little variation, relative to the other regions. *Otomys unisulcatus* does not conform to Bergmann's rule, as specimens from the hotter areas did not possess smaller sizes than those from the cooler environments. This result supports the conclusion of Meiri and

Dayan (2003) that rodents in general do not follow Bergmann's rule. Instead, it appears that rainfall (and not temperature) is a factor influencing centroid size, and by proxy body size. The only significant relationship found between centroid sizes and environmental factors was the correlation between the centroid sizes in the ventral view of the females and rainfall. Since the females require more nutrients when producing offspring, the significant correlation between rainfall and centroid size may be indicating that the decreased habitat productivity in low rainfall areas may be influencing the body size of females (while not significantly influencing males).

The suggestion by Armitage (1999) that body size increases with harsher environments may hold true for those specimens from the Succulent Karoo, as this biome exhibits a harsher environment for a small mammal than other areas of South Africa. If this species is truly adhering to Armitage's suggestion, then the larger centroid sizes of those specimens from the Thicket Bushveld biome (Eastern Cape Province) are an interesting finding. There is a larger range of sizes exhibited by *O. unisulcatus* in this biome, compared to the other biomes, so there may be factors (not investigated in this study) which may be influencing size within this region.

Environmental effects on shape variation

The bones associated with the basicranial aspect of the cranium have been seen to change little during adulthood (Caumal & Polly, 2005). Therefore, environmental variance may be reflected in changes in the shapes of the zygomatic arches, palate, and other bones associated with masticatory muscles, as well as those bones which house the sensory organs (such as the eyes and the hearing apparatus; Rae *et al.*, 2006). The ventral side of the cranium contains many more bones which are related to sensory and masticatory organs and therefore those bones may be under greater selective pressures than any features on the dorsal side of the cranium. The selection against variation (stabilizing selection) in the dorsal portions of the cranium may in fact be the reason why these bones do not vary as much as the ventral portions. Since this model of evolution was not tested in this study, the latter is pure speculation.

Variations in mammalian skull-shape have been shown to be related to diet (e.g. Milne & O'Higgins, 2002; Goheen *et al.*, 2003; Lieberman *et al.*, 2004; Mavropoulos *et al.*, 2004; Semprebon *et al.*, 2004; Viguier, 2004; Wright, 2005; Taylor, 2006). A strong correlation between skull shape and burrowing behaviour has been found in arvicolid rodents (Courant *et al.* 1997). Those species which burrow tended to exhibit a more angular shape, whilst those that remained above ground had more elongated skulls (Courant *et al.*, 1997). Due to the highly plastic nature of skull shape, this interspecific pattern may be evident at species level. Looking specifically at the Otomyini tribe members, it would seem that an evolutionary trend of progressive elongation of the nasal bones, and of the brain-case is occurring within the tribe, with *Parotomys* and *O. unisulcatus* basal to the remaining members (Taylor *et al.*, 2004a). Only three of the Otomyini members construct permanent burrow systems (*Parotomys brantsii*, *P. littledalei* and *O.*

sloggetti), with the remaining members constructing above-ground nests. So it is expected, and also found, that *O. unisulcatus* has a more elongated skull, relative to the burrowing species (Taylor *et al.* 2004a).

The male shape variable datasets produced the only significant permutations, as well as producing better defined correlations and covariation coefficients. It appeared that the most variation in shape when performing a regression analysis against environmental variables was in the zygomatic arches. The broader maxillary bones and zygomatic arches observed in the first cluster may be the result of a change in dietary composition. This cluster occurs in the Cape Floristic Region where fynbos (predominantly heathy, evergreen shrubs; Verboom *et al*., 2008) makes up the majority of the vegetation. Thus the need for stronger masticatory muscles (such as the masseter originating on the zygomatic arch; Walker & Liem, 1994) may have led to the widening in the maxillary and zygomatic arch. Succulents and annuals form the majority of the diet in the second cluster and the eastern parts of the fourth cluster, so a broader maxillary bone is not necessary as the diet is not as abrasive as in the first cluster. Interestingly, the second cluster (Succulent Karoo biome) has narrower zygomatic arches, which is expected if strong masticatory muscles are not essential, whilst the fourth cluster (populations occurring along the escarpment) possesses broader zygomatic arches in the ventral view. In the dorsal view, the fourth cluster approximates the mean shape, thus this cluster exhibits zygomatic arches which are intermediate (neither broad nor narrow relatively).

The third cluster occurs in the Nama Karoo, which is a grassy, dwarf shrubland (Mucina & Rutherford, 2006), so the broad zygomatic arch exhibited by the cluster may reflect the group's need for stronger masticatory muscles. However, since the maxillary bone is narrower in this cluster, the broader zygomatic arch may indicate that this cluster may burrow more than those individuals in the other clusters. The eastern parts of the Nama Karoo are dominated by grasses (Mucina & Rutherford, 2006), so there may not be an adequate number of shrubs required for their stick nests present, thereby necessitating the digging of burrows under bushes to escape predators.

Skull shape, and not size, followed an environmental gradient in *Thrichomys apereoides* (Brazilian punaré rat; Monteiro *et al.*, 2003). It appears that the clusters found within this study do not follow latitudinal or longitudinal gradients, but rather they correspond to biome boundaries. They are also not showing a significant relationship to the genetic assemblages, and therefore this species may be morphologically flexible according to its environment.

Cardini *et al.* (2007) found that rainfall seasonality was an influential factor in the size of the vervet monkey *Cercopithecus aethiops*, however mean body size in Malagasy sifakas *Propithecus verreauxi* was seen to be negatively correlated with rainfall seasonality and positively correlated with rainfall (Lehman *et al.*, 2005). Even though the 2B-PLS analysis showed that the clusters correlated positively or negatively with the separate environmental variables, only one dataset (male ventral view) showed a significant

correlation with the environmental variable dataset, possible due to the minimum temperature variable (largest positive loading in this dataset).

The larger tympanic bullae found in the second and third cluster may relate to the fact that they occur in the more arid regions of the species' range. It has been suggested that the reason for the presence of an enlarged tympanic bullae is to improve hearing, enabling the animal to better avoid predators in an open habitat (Taylor *et al.*, 2004a). The bullae of *O. unisulcatus* in general are not as large as those of *Parotomys*, and it is thought that the enlarged bulla is an ancestral trait that has been lost in the *Otomys* genus (Taylor *et al.*, 2004a). The fact that *O. unisulcatus* specimens in the arid-occurring regions of South Africa still retain the smaller bullae (relative to *Parotomys*) indicates a more mesic origin for the species (Pocock, 1976; Taylor *et al.*, 1989). Since *O. unisulcatus* inhabits extensive stick-lodges, predator-avoidance is provided by the protection of the nest, and enhanced hearing is not as necessary in this species, as it is in *Parotomys brantsii*, for predator avoidance (Sheets, 1989; Taylor *et al.*, 2004a). However, since those individuals that inhabit the more arid regions (Succulent Karoo and Nama Karoo) exhibit larger bullae relative to the rest of the populations may be as a result of lower density of cover in these areas, thereby necessitating enhanced hearing capabilities for predator avoidance. In fossorial species', enlarged tympanic bullae are thought to increase the individual's sensitivity to low frequencies (1-3 KHz range) (Webster, 1962; Vernon *et al.*, 1971; Webster & Strother, 1972; Webster & Webster, 1972, 1980), which is an advantage underground as higher frequencies tend to get absorbed by the walls of tunnels (Heth *et al.*, 1986).

Subspecies validation

The centroid sizes of the females in the ventral view differed significantly between subspecies. The same dataset was found to be significantly positively correlated with rainfall, indicating that the amount of annual rainfall (and by proxy habitat productivity) is significantly influencing the size of female Karoo Bush Rats. The cranial shapes exhibited by the various subspecies correspond well with the clusters found in the 2B-PLS analysis. This indicates that the environmental variables which are influencing the clustering of the shape variables in the 2B-PLS analysis, may be influencing the cranial shapes exhibited by the various subspecies.

On the whole, it appears that the cranial shapes differed significantly between subspecies. The zygomatic arch was once again the most variable portion of the cranium, however the variation within this structure did not show the same pattern as found in the 2B-PLS analysis. This could be as a result of the fact that the analysis was comparing the mean shapes of the subspecies relative to one another, whilst the 2B-PLS analysis was comparing the mean shapes of specific populations with environmental variables.

The *O. u. unisulcatus* specimens (which fall into the central genetic assemblage) tended to have cranial shapes which differed from the other specimens. Posteriorly flattened zygomatic arches were found in the ventral datasets, whilst the dorsal view datasets exhibited wider braincases, and shorter, narrower nasal

sections for this subspecies. Thus, the geographic clustering of the cranial shape appears to adhere to the previously described subspecies boundaries, which were primarily based on morphological characters. Since the subspecies boundaries were not recovered in the genetic analysis for this species, further genetic work needs to be done to investigate the validity of the subspecies boundaries, utilizing more genes (possibly the combination of nuclear and mitochondrial genes).

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CHAPTER 4

DISCUSSION OF PHYLOGEOGRAPHY AND GEOMETRIC MORPHOMETRIC RESULTS

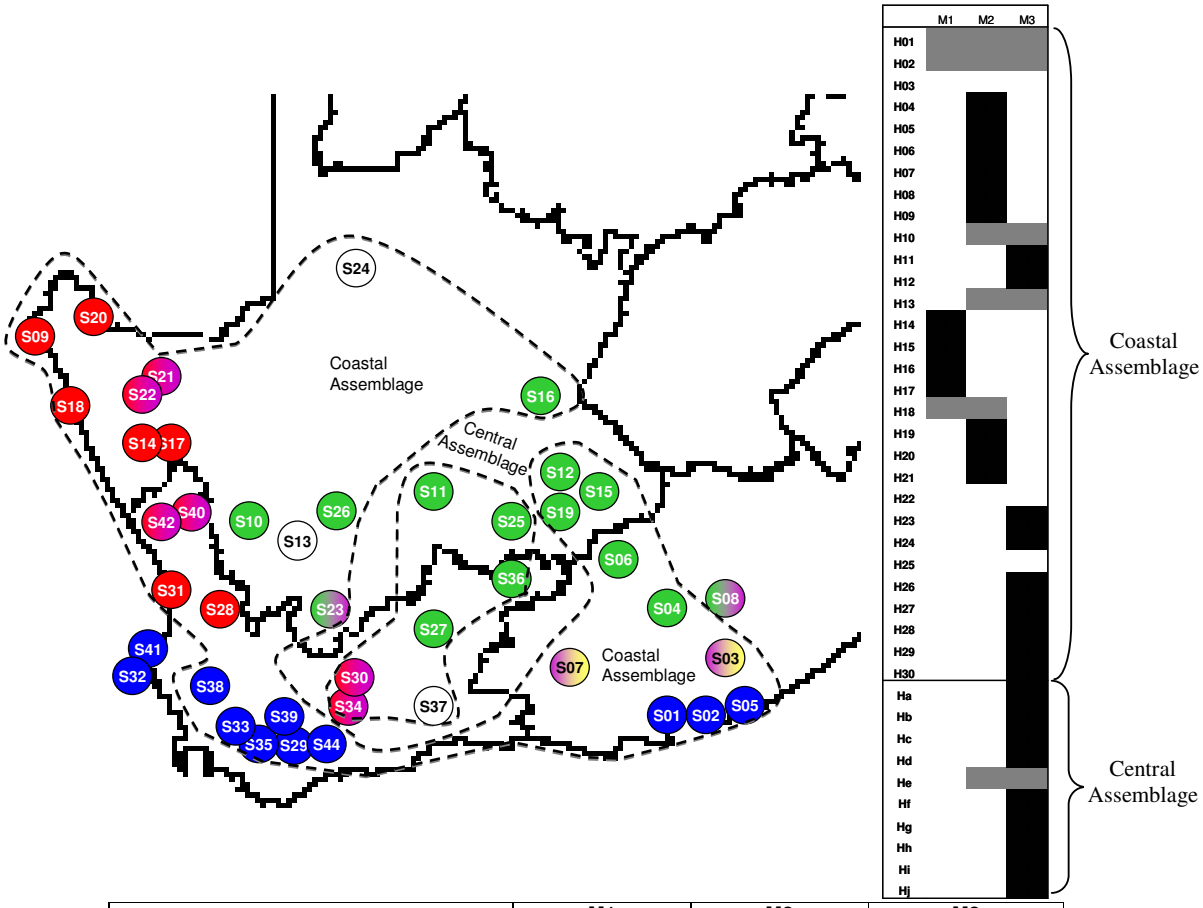
The use of both genetic and morphological analyses to investigate potential phylogeographic breaks in *O. unisulcatus* proved to be effective, enabling various evolutionary processes associated with this species to come to light. The results of the genetic and the morphological analyses both point to the fact that populations of *O. unisulcatus* are structured, with the morphological geographic groupings showing more subdivision than the genetic phylogeographic assemblages. The morphological clusters do not follow latitudinal or longitudinal gradients, but rather adhere to biome boundaries. Elevation appears to be playing a role in the division of populations in this plains-dwelling species, as can be observed in both genetic and morphological analyses. Interestingly, in both analyses, populations from the Fynbos biome and the Thicket Bushveld biome are clustered together, with the above-mentioned central assemblage intermediate (Figure 4.1). This association in both sets of analyses suggests that gene flow probably exists along the southern coastal plain.

When the genetic haplotype composition of each morphological cluster was examined (Figure 4.1), it was found that in the first three morphological clusters the percentage of haplotypes unique to the respective clusters were larger than the percentage of haplotypes shared between the clusters. The fourth and fifth morphological clusters were not included in the examination as these populations exhibited the same haplotypes as morphological clusters two and three. Even though the MANOVAs did not show that there were any significant relationships between the cranial shape clusters and the genetic assemblages, there appears to be a large percentage of haplotypes that are unique to each morphological cluster, with a few shared.

The fourth and fifth morphological clusters were not included in the examination as these populations exhibited the same haplotypes as morphological clusters two and three. The unique haplotypes within the first morphological cluster (M1) occurred in Clanwilliam (P31: H14) and Piquetburg (P21: H15, H16, H17) in the Western Cape Province. The unique haplotypes within the second morphological cluster (M2) were found in populations in the Succulent Karoo biome: Richtersveld (P14: H04), Port Nolloth (P19: H05), Springbok (P17: H06, H07, H08, H09), and Vanrhynsdorp (P23: H19, H20, H21). Haplotypes unique to the third morphological cluster were found in Calvinia (P15: H11, H12), Hope Town (P27: H24), Cradock (P03: H23, H26, H27, H28), Carnarvon (P08: H29) and Sutherland (P30: H30), populations which occur in either the Nama Karoo biome (P15, P27, P08) or the Thicket Bushveld biome (P03). Also unique to M3 were all of the central genetic assemblage haplotypes (Ha to Hj), except He which is shared with M2.

The haplotypes which were shared between the clusters were as follows: H01, H02, H10, H13, H18 and He. Haplotypes H01 and H02 were shared between all three clusters (though they were found at different

localities). Haplotype H18 was shared between M1 and M2, and this haplotype occurred in Darling (P20: M1) and Vanrhynsdorp (P23: M2). Haplotypes H10 and H13 were shared between morphological clusters two (M2) and three (M3). Haplotype H10 occurred at Springbok (P17: M2) and Calvinia (P15: M3), whilst H13 occurred at Vanrhynsdorp (P23: M2) and Calvinia (P15: M3). Haplotype He was found in Laingsburg (P12: M2), Richmond (P09: M3) and Beaufort West (P11: M3). M1 and M2 had a very high percentage of haplotypes which belonged to the coastal genetic assemblage (100 % and 93% respectively), while almost half of the haplotypes which occurred in M3 belonged to the central genetic assemblage. The ratio of haplotypes to populations was lowest in M3, indicating that fewer haplotypes occurred in each population relative to the other two morphological clusters. For all three clusters though the number of haplotypes per populations was at between 2.0 and 2.5.



	M1	M2	M3
No of haplotypes	7	15	23
No of populations	3	6	11
Ratio haplotypes/populations	2.33	2.50	2.09
% unique haplotypes	57,14%	66.67%	78.26%
% shared haplotypes	42.86%	33.33%	21.74%
% haplotypes from coastal assemblage	100%	93.33%	56.52%
% haplotypes from central assemblage	0%	6.67%	43.48%

Figure 4.1: Distribution map of clusters found in the phylogeographic (Genetic assemblages, dashed lines) and morphological (circles) analyses. Key to colours: Morphological cluster (Morph. cluster) 1 = blue, Morph. cluster 2 = red, Morph. cluster 3 = green, Morph. cluster 4 = purple, Morph. cluster 5 = yellow, locations only used in genetic analyses = white. Inset table to the right indicates which morphological clusters (M1, M2, M3) possess which genetic haplotypes (H01 to H30, Ha to Hj). Black blocks indicate haplotypes unique to the cluster, while grey blocks indicate shared haplotypes between the clusters (coastal genetic assemblage is separated from the central assemblage by a line). Summary statistics for each morphological cluster shown below figure.

Collectively, rainfall (and not temperature, latitude or longitude) appeared to be influencing the skull size of the females of *O. unisulcatus*. High elevation areas (such as mountain ranges) were proposed to be the main dividing factor in population structuring. Morphological clusters adhered to biome boundaries, as well as showing that there is mixing of individuals with various skull shapes along the western and south-eastern escarpment. The genetic phylogeographic structuring of this species indicates that the central assemblage has been isolated from the rest of the populations by mountain ranges leading up to the escarpment (Nuweveldberge) and the Grootswartberge separating the Little Karoo from the coastal plains in the south. Divergence of the two genetic assemblages probably occurred in one of the more mesic periods of the Pleistocene, and dispersal of the two most common haplotypes throughout the coastal parts of the range occurred during the following arid cycle. Between that time and the present-day, conditions have become progressively more mesic, thereby causing the populations to become more fragmented and allowing for rare alleles to be accumulated in certain areas.

It is striking how closely the morphological groupings match those of the subspecies boundaries. The second morphological group falls into the *O. unisulcatus broomi, O. u. unisulcatus* and *O. u. bergensis* boundaries, whilst the first morphological group contains the species from *O. u. albiensis*, as well as populations previously undefined in terms of subspecies from the Fynbos biome. The third morphological group contains all of the populations defined to be part of the *O. u. grantii* subspecies, as well as those specimens in the Eastern Cape Province and the western part of the Nama Karoo that were previously undefined in terms of subspecies. This group also contained populations found to be part of the central assemblage (except *O. u. unisulcatus* specimens, which clustered into the morphological cluster 2). At this stage, there may be weak support for the classification of subspecies according to the genetic results, and further analyses are required to verify the existence of a possible subspecies in the central part of the range. Since the subspecies were originally identified according to morphological characteristics, it was expected, and found, that the geometric morphometric analyses would recover in broad terms the same subspecies boundaries.

One possible reason for the greater structuring found in the morphological analyses is the polygenic nature of morphological traits, which may evolve at a quicker pace than neutral mtDNA markers can coalesce to achieve reciprocal monophyly (Zink & Barrowclough, 2008). Morphological traits are also more plastic, and changes in skull shape during development may be influenced to a large extent by environmental factors. This species therefore appears to be more morphologically adaptable, changing its size and skull shape according to the environment in which it is reared.

This species is currently classified as being of “Least Concern” (Friedmann & Daly, 2004). However, to a large extent the habitat of *O. unisulcatus* are being used increasingly for livestock farming, and the overgrazing of livestock (particularly browsers) may be causing the availability of suitable plants for nesting purposes to be decreasing. In the future, this may cause the habitat to become patchier, thereby causing fragmentation of populations. The encroachment of human settlements on the Karoo Bush Rat’s

natural habitat may also be a factor in the future which may influence the connectivity of populations, as well as reduce the availability of suitable habitat for the species. It is vital that the Succulent Karoo biome, being the biome with the least amount of natural reserves (Rebelo, 1997; Lombard *et al.*, 1999), have more protected areas designated within it. It is an area that contains many unique haplotypes of saxicolous species (e.g. Matthee & Robinson, 1996; Lamb & Bauer, 2000; Matthee & Flemming, 2002; Miller-Butterworth *et al.,* 2003; Smit *et al*., 2007), as well being an important area for the arid-adapted species (e.g. *Parotomys* and *O. unisulcatus*) of southern Africa.

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Appendix A

Appendix A1: Sampling localities, information regarding age class, gender, collection numbers, and whether specimens were used in the genetic or morphological or both analyses.

Location Name	Museum / Collectors Numbers*	Personal Numbers	Used in Dorsal analyses	Used in Ventral analyses	Sequenced	Age Class category$	Gender**
Albany	AM20009	AM93	-	-	Y	3	M
Albany	AM33796	AM211	-	-	Y	3	F
Albany	TM22778	TM05	Y	-	-	4	F
Albany	TM6720	TM57	Y	-	Y	4	M
Albany	TM6726	TM25	Y	-	-	5	M
Albany	AM33804	AM210	Y	Y	-	4	F
Albany	TM6717	TM54	Y	Y	-	4	F
Albany	AM33802	AM206	Y	Y	Y	4	M
Albany	AM33803	AM207	Y	Y	Y	4	M
Albany	AM33808	AM208	Y	Y	Y	4	M
Albany	TM22781	TM20	Y	Y	-	4	M
Alexander Bay	TM22729	TM64	Y	Y	-	4	F
Alexander Bay	AM28775	AM115	Y	Y	Y	5	M
Alexandria	AM19911	AM91	Y	Y	Y	4	F
Alexandria	AM20047	AM98	Y	Y	-	5	M
Alexandria	AM20343	AM201	Y	Y	-	5	M
Beaufort West	AM29682	AM17	-	-	Y	4	F
Beaufort West	AM18192	AM200	-	-	Y	1	M
Beaufort West	AM33819	AM204	Y	-	-	4	M
Beaufort West	AM29683	AM121	Y	Y	Y	4	F
Beaufort West	TM29622	TM87	Y	Y	-	4	F
Beaufort West	TM29625	TM90	Y	Y	-	4	F
Beaufort West	AM18182	AM198	Y	Y	Y	4	M
Beaufort West	AM18193	AM203	Y	Y	-	4	M
Beaufort West	TM29610	TM86	Y	Y	-	4	M
Beaufort West	TM29623	TM88	Y	Y	-	4	M
Beaufort West	AM18183	AM199	Y	Y	Y	5	F
Beaufort West	TM29588	TM37	Y	Y	Y	5	F
Bedford	AM9020	AM04	Y	Y	-	4	F
Bedford	AM9063	AM197	Y	Y	Y	4	F
Bedford	AM8835	AM182	Y	Y	-	5	F
Bedford	AM9043	AM143	Y	Y	Y	5	M
Calvinia	AM12984	AM190	-	-	Y	4	M
Calvinia	AM12969	AM188	-	-	Y	3	M
Calvinia	TM39371	TM39	-	-	Y	5	M
Calvinia	AM12944	AM76	-	-	Y	U	U
Calvinia	AM12941	AM75	-	-	Y	3	F
Calvinia	AM12963	AM77	Y	-	Y	4	F
Calvinia	AM12964	AM78	Y	-	Y	4	F
Calvinia	AM12945	AM145	Y	Y	Y	5	F
Calvinia	AM12966	AM186	Y	Y	Y	5	F
Calvinia	TM39353	TM16	Y	-	Y	5	F
Calvinia	TM5084	TM53	Y	Y	-	5	F
Calvinia	AM12968	AM187	Y	Y	-	5	M
Calvinia	AM12975	AM192	Y	Y	-	5	M
Calvinia	AM12983	AM189	Y	-	-	5	M
Calvinia	TM39371	TM09	Y	Y	-	5	M
Calvinia	TM39374	TM40	Y	Y	Y	5	M
Carnarvon	AM18190	AM88	-	-	Y	4	F
Carnarvon	AM18184	AM14	-	-	Y	2	M
Carnarvon	AM18190	AM88	Y	Y	-	4	F
Carnarvon	TM27359	TM80	Y	Y	-	4	F
Carnarvon	TM27360	TM81	Y	Y	-	4	M
Carnarvon	TM27376	TM82	Y	Y	-	4	M
Carnarvon	TM27352	TM77	Y	Y	-	5	F

Location Name	Museum / Collectors Numbers*	Personal Numbers	Used in Dorsal analyses	Used in Ventral analyses	Sequenced	Age Class category$	Gender**
Clanwilliam	AM8804	AM184	-	-	Y	3	F
Clanwilliam	TM2241	TM46	Y	Y	-	4	M
Clanwilliam	AM8844	AM43	Y	Y	Y	5	M
Cradock	AM8947	AM44	-	-	Y	2	F
Cradock	AM8950	AM47	-	-	Y	3	M
Cradock	AM8948	AM45	Y	-	Y	4	F
Cradock	AM8953	AM50	Y	Y	Y	4	F
Cradock	TM22780	TM70	Y	Y	-	4	F
Cradock	AM8951	AM48	Y	-	-	4	M
Cradock	AM8949	AM46	Y	Y	-	5	M
Darling	AM30343	AM08	Y	Y	Y	4	F
De Aar	AM23195	AM147	Y	Y	-	4	F
Fish River Valley	DM2995	DM07	Y	Y	-	4	F
Fish River Valley	DM2996	DM08	Y	Y	-	4	M
Fish River Valley	DM2997	DM09	Y	Y	-	4	M
Fish River Valley	DM2971	DM12	Y	Y	-	5	F
Fraserburg	AM29688	AM124	-	-	Y	3	F
Fraserburg	AM29687	AM123	-	-	Y	2	F
Fraserburg	AM29688	AM124	-	-	Y	3	F
Fraserburg	AM29690	AM148	-	-	Y	2	M
Fraserburg	AM29685	AM149	-	-	Y	3	U
Fraserburg	AM29686	AM122	-	-	Y	3	F
Garies	AM8707	AM217	Y	Y	-	5	F
Garies	AM8712	AM27	Y	Y	-	5	F
Hanover	SAM7361	SAM12	Y	-	-	4	U
Hanover	SAM7491	SAM13	Y	-	-	4	U
Hanover	TM74	TM97	Y	-	-	5	F
Hopetown	AM11081	AM60	-	-	Y	U	F
Hopetown	TM22753	TM68	Y	-	-	4	F
Hopetown	TM22799	TM74	Y	-	-	5	M
Laingsburg	AM26494	AM15	-	-	Y	2	M
Laingsburg	TM9065	TM63	Y	Y	-	4	F
Laingsburg	TM9060	TM59	Y	-	-	5	F
Laingsburg	TM9061	TM60	Y	Y	-	5	F
Laingsburg	TM9062	TM61	Y	Y	-	5	M
Lamberts Bay	AM8836	AM183	-	-	Y	4	F
Lamberts Bay	AM8835	AM182	-	-	Y	5	F
Lamberts Bay	AM8832	AM179	-	-	Y	5	F
Lamberts Bay	AM8841	AM178	-	-	Y	3	M
Lamberts Bay	AM8838	AM175	-	-	Y	3	M
Lamberts Bay	AM8837	AM174	-	-	Y	3	M
Lamberts Bay	AM8833	AM180	-	-	Y	4	F
Lamberts Bay	AM8837	AM146	-	-	Y	3	M
Lamberts Bay	AM8834	AM181	Y	Y	Y	4	F
Lamberts Bay	AM8839	AM176	Y	Y	Y	4	F
Lamberts Bay	AM8840	AM177	Y	Y	Y	4	F
Lamberts Bay	TM2242	TM23	Y	-	-	4	F
Lamberts Bay	NM318	DM24	Y	Y	-	4	M
Lamberts Bay	AM8815	AM41	Y	Y	-	5	F
Lamberts Bay	NM317	DM25	Y	Y	-	5	M
Langebaan	TM41477	TM19	Y	Y	-	4	F
Malmesbury	AM30345	AM128	Y	Y	Y	4	M
Matjiesfontein	TM9058	TM30	Y	Y	-	5	F
Matjiesfontein	TM9057	TM29	Y	Y	-	5	M
Middelburg	AM11381	AM68	Y	-	Y	5	M
Middelburg	AM11380	AM67	Y	Y	-	4	M
Middelburg	AM11375	AM62	Y	Y	Y	5	F
Middelburg	AM11382	AM69	Y	Y	-	5	F
Montagu	AM33822	AM172	Y	-	-	5	M
Montagu	AM31084	AM132	Y	Y	Y	5	F

Location Name	Museum / Collectors Numbers*	Personal Numbers	Used in Dorsal analyses	Used in Ventral analyses	Sequenced	Age Class category$	Gender**
Montagu	AM31087	AM134	Y	Y	-	5	F
Montagu	AM31085	AM133	Y	Y	-	5	M
Murraysburg	AM23370	AM20	Y	Y	Y	4	F
Murraysburg	AM23371	AM102	Y	Y	Y	4	F
Oudtshoorn	OHOU01	OU18	-	-	Y	U	U
Oudtshoorn	OHOU02	OU19	-	-	Y	U	U
Oudtshoorn	OHOU03	OU20	-	-	Y	U	U
Oudtshoorn	OHOIR02	OU29	-	-	Y	U	U
Oudtshoorn	OHOIR04	OU30	-	-	Y	U	U
Piquetburg	AM8807	AM36	Y	Y	Y	4	F
Piquetburg	AM29348	AM168	Y	Y	-	4	M
Piquetburg	AM29349	AM120	Y	Y	Y	4	M
Piquetburg	AM8716	AM31	Y	Y	-	5	F
Piquetburg	AM8806	AM35	Y	Y	Y	5	F
Port Nolloth	AM8703	AM214	-	-	Y	3	M
Port Nolloth	AM8708	AM212	-	-	Y	5	F
Port Nolloth	AM8694	AM21	Y	Y	Y	4	M
Port Nolloth	DM263	DM19	Y	Y	-	4	F
Port Nolloth	AM8690	AM12	Y	Y	-	4	M
Port Nolloth	DM2988	DM21	Y	Y	-	4	M
Port Nolloth	DM2989	DM22	Y	Y	-	4	M
Port Nolloth	AM8702	AM213	Y	-	-	5	F
Port Nolloth	AM8706	AM216	Y	Y	Y	5	F
Port Nolloth	TM7921	TM27	-	Y	-	5	F
Richmond	AM11391	AM72	Y	Y	Y	5	F
Richmond	TM4991	TM52	Y	-	Y	5	F
Richmond	TM4990	TM51	Y	Y	-	5	M
Richtersveld	RVPL10	PL10	-	-	Y	U	U
Richtersveld	RVPL13	PL13	-	-	Y	U	U
Richtersveld	RVPL01	PL01	-	-	Y	U	U
Richtersveld	RVPL15	PL15	-	-	Y	U	U
Richtersveld	AM11392	AM73	-	-	Y	3	F
Richtersveld	AM11389	AM70	-	-	Y	3	F
Richtersveld	TM43864	TM103	Y	Y	-	4	F
Richtersveld	AM28768	AM16	Y	Y	-	5	F
Roberston	TM22750	TM03	Y	-	-	4	F
Roberston	AM26499	AM165	Y	Y	-	4	M
Springbok	GPSP02	OU04	-	-	Y	U	U
Springbok	GPSP03	OU05	-	-	Y	U	U
Springbok	GPSP04	OU06	-	-	Y	U	U
Springbok	GPSP05	OU07	-	-	Y	U	U
Springbok	GPSP06	OU08	-	-	Y	U	U
Springbok	GPSP07	OU09	-	-	Y	U	U
Springbok	GPSP08	OU10	-	-	Y	U	U
Springbok	GPSP09	OU11	-	-	Y	U	U
Springbok	GPSP10	OU12	-	-	Y	U	U
Springbok	GPSP11	OU13	-	-	Y	U	U
Springbok	GPSP12	OU14	-	-	Y	U	U
Springbok	GPSP13	OU15	-	-	Y	U	U
Springbok	GPSP14	OU16	-	-	Y	U	U
Springbok	GPSP15	OU17	-	-	Y	U	U
Springbok	DM3054	DM04	Y	Y	-	4	M
Springbok	DM3053	DM03	Y	-	-	4	U
Steinkopf	SAM19114C	SAM03	Y	Y	-	4	M
Steinkopf	DM2998	DM10	Y	Y	-	5	F
Steinkopf	SAM19114A	SAM01	Y	Y	-	5	F
Steinkopf	TM22604	TM71	Y	-	-	5	F
Steytlerville	AM24121	AM106	-	-	Y	4	F
Steytlerville	AM24123	AM108	-	-	Y	3	M
Steytlerville	AM24120	AM105	Y	-	-	4	F

Location Name	Museum / Collectors Numbers*	Personal Numbers	Used in Dorsal analyses	Used in Ventral analyses	Sequenced	Age Class category$	Gender**
Steytlerville	AM24118	AM103	Y	-	-	4	F
Steytlerville	AM24122	AM107	Y	-	-	4	F
Steytlerville	AM24124	AM109	Y	Y	-	4	F
Steytlerville	AM24117	AM03	Y	Y	Y	5	M
Sutherland	AM14727	AM79	Y	Y	-	4	M
Sutherland	AM14729	AM81	Y	Y	Y	5	M
Tarkastad	TM22749	TM04	Y	Y	-	4	F
Tarkastad	TM22761	TM11	Y	Y	-	4	F
Tarkastad	TM22770	TM95	Y	Y	-	4	F
Upington	TM35384	TM91	-	-	Y	4	M
Van Rhynsdorp	TM2234	TM02	Y	-	-	5	M
Van Rhynsdorp	VROU01	OU21	-	-	Y	U	U
Van Rhynsdorp	VROU02	OU22	-	-	Y	U	U
Van Rhynsdorp	VROU03	OU24	-	-	Y	U	U
Van Rhynsdorp	VROU04	OU25	-	-	Y	U	U
Van Rhynsdorp	VROU05	OU26	-	-	Y	U	U
Van Rhynsdorp	VROU01	OU27	-	-	Y	U	U
Van Rhynsdorp	VROU01	OU28	-	-	Y	U	U
Van Rhynsdorp	AM2236	TM22	-	-	Y	U	U
Victoria West	N/A	OU02	-	-	Y	U	U
Victoria West	AM23196	AM99	Y	Y	-	4	F
Victoria West	AM23197	AM100	Y	Y	-	4	F
Victoria West	AM23199	AM151	Y	Y	Y	4	F
Victoria West	AM23372	AM152	Y	Y	Y	4	F
Victoria West	AM23374	AM163	Y	Y	-	4	F
Victoria West	TM22797	TM73	Y	-	-	4	F
Victoria West	DM2984	DM15	Y	Y	-	4	M
Victoria West	DM2985	DM16	Y	Y	-	4	M
Victoria West	AM23373	AM162	Y	Y	-	5	F
Victoria West	AM9032	AM58	Y	Y	-	5	F
Victoria West	TM22794	TM72	Y	Y	-	4	F
Vredenburg	TM22795	TM110	Y	Y	-	4	M
Vredenburg	TM22741	TM105	Y	-	-	U	F
Vredenburg	TM22760	TM109	-	Y	-	4	F
Vredendal	AM33842	AM153	Y	-	-	5	M
Vredendal	AM29352	AM155	Y	Y	-	4	F
Vredendal	AM29353	AM154	Y	Y	Y	4	F
Williston	TM7926	TM98	Y	-	-	4	M
Williston	TM27323	TM08	Y	Y	-	5	M
Williston	TM7927	TM99	Y	Y	-	5	M
Willowmore	DM2962	DM01	Y	Y	-	4	M
Worcester	AM31091	AM137	Y	Y	-	5	F
Worcester	AM31092	AM138	-	-	Y	U	M
Parotomys brantsii	GPSP03PB	PB03	-	-	Y	U	U
P. littledalei	RVPL02	PL02	-	-	Y	U	U

* ***Key to Museum numbers***: *AM = Amathole Museum; TM = Transvaal Museum; DM = Durban Museum; NM = Natal Museum; SAM = South African Museum*

** ***Key to gender codes***: *M = Male; F = Female; U = Unknown gender*

$ ***Key to age class categories codes***: *1,2,3 = Juveniles; 4,5 = Adults; U = Unknown age*