Sensory divergence among populations of a southern African endemic horseshoe bat (Chiroptera: Rhinolophidae): a multidisciplinary approach

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This thesis is dedicated to my darling grandmother

Barbara Ellen Odendaal

(28/12/1925 – 26/08/2014)

Mamma, I am so sorry that I did not finish this in time for you to see me graduate. It is, and probably will always be, my biggest regret. I am forever humbled by your utter faith in me, your unwavering support and relentless encouragement and optimism. But most of all, thank you for loving me as only a grandmother could- completely and unconditionally.

~Zellie

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DECLARATION

I, Lizelle Janine Odendaal, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I authorise the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

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ABSTRACT

A fundamental goal of evolutionary biology is to understand how natural selection, random genetic drift and gene flow interact to promote adaptive trait divergence within species. Environmental gradients are ideal opportunities for disentangling the relative effects of selection and gene flow in promoting local adaptation among populations experiencing different selective regimes. In this study a multidisciplinary approach combining the methods of sensory ecology, functional morphology, population genetics and functional genetics was used to explore the relative roles of neutral and adaptive processes in the evolution of sensory divergence in Cape horseshoe bats, *Rhinolophus capensis*. Geographic variation in echolocation resting frequencies (RFs) in this species is characterised by increasing frequency from west (75.7 kHz: xeric habitats) to east (86 kHz: mesic habitats) across their distribution in South Africa. Here the species is found across a wide range of habitats characterised by a gradient of increasing vegetation clutter from xeric habitats in the west, to mesic habitats in the east.

To better understand how selection contributes to the evolution of RF variation in *R. capensis*, the relationships between RF and different ecological and morphological correlates of echolocation frequency were explored. Results revealed some support for the Allometry Hypothesis, which predicts a negative relationship between body size and RF, but body size alone does not account for the observed pattern of RF variation. There was also no support for the Atmospheric Attenuation Hypothesis, which predicts a negative correlation between relative humidity and RF. Instead, RF variation in the Cape horseshoe bat appears to be tightly coupled to differences in vegetation clutter among populations. The detection ranges of large prey and vegetation edge also differed significantly among populations; together these results support the Foraging Habitat Hypothesis and suggest that selection for lower frequencies in more xeric habitats may promote the evolution of RF variation. Geometric morphometric analyses of skulls from different habitats further support this hypothesis where both RF and the size (but not shape) of dorsal nasal chambers covary with differences among habitats. Nasal chamber centroid size explains 70% of the observed variation in RF, supporting

the Morphological Correlates Hypothesis which predicts a tight coupling between RF and cranial features directly involved in echolocation.

Habitat driven selection on RF may act to reduce gene flow among acoustically divergent populations such that sensory variation and neutral population structure covary with habitat discontinuities. Phylogeographic analyses reveal that while genetic drift does contribute to both sensory and genetic differentiation, it is not the dominant driver of divergence in this study system. Although a Bayesian analysis of population structure revealed four spatially defined mitochondrial groups, phylogenetic analysis of mitochondrial sequences did not correlate with RF variation. The distribution of genetic variation among populations is instead characterised by substantial historic gene flow and minimal overall genetic structure, suggesting that adaptive trait divergence has occurred in the face of gene flow. Coalescent analyses reveal a clear pattern of asymmetric gene flow in the recent past with most gene flow occurring among southern and eastern populations at the centre of the species distribution, and very little gene flow towards populations at the edge of the species' range. Classic divergent selection may influence RF at edge populations, while adaptive phenotypic plasticity is a plausible hypothesis explaining RF variation in the face of gene flow among central populations. Gene flow between populations experiencing even marginally different selective environments may favour the evolution of phenotypic plasticity or conversely, plasticity itself may promote gene flow.

To further investigate the relative influence of selection and plasticity on RF variation in this system, functional variation in the hearing gene *Prestin* was explored among acoustically divergent populations and within closely related species in the '*capensis*' clade. Data from 10 exons revealed only two variable amino acid sites; one restricted to *R. simulator*, and the other occurring across acoustically divergent *R. capensis* populations as well as within other species in the clade. *Prestin* is highly conserved within the clade, and sequence variation in the coding regions reported here does not reflect RF differences either within the Cape horseshoe bat or among its close relatives.

This study highlights the complex interactions that characterise adaptive and neutral evolutionary processes that influence patterns of divergence within species; it also

highlights how their relative effects may vary both subtly and substantially across the distribution of species. Only by combining the theoretical and empirical tools from a wide range of disciplines can we achieve a more comprehensive understanding of the nuances of adaptive trait evolution.

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CHAPTER 1

THE EVOLUTION OF SENSORY DIVERGENCE: A REVIEW

Studies investigating the evolution of local adaptation within species distributed across heterogeneous environments can provide important insights into the influence of spatially varying natural selection relative to stochastic processes including random genetic drift and gene flow, in shaping patterns of adaptive trait divergence (Clausen et al. 1940; Mayr 1942; Williams 1966; Schluter 2000; Lenormand 2002; Kawecki & Ebert 2004; Nosil et al. 2005; Garant et al. 2007). Indeed, a fundamental goal of evolutionary biology is to understand how these processes interact to shape patterns of genetic and phenotypic variation within and between species (Dobzhansky & Pavlovsky 1957; Felsenstein 1976; Endler 1977; Slatkin 1987; Nosil & Crespi 2004; Alleaume-Benharira et al. 2006; Nosil et al. 2009; Via 2009; Butlin et al. 2012). The advent of molecular genetic tools and applications has significantly increased our understanding of the interaction between these evolutionary forces, but some issues still remain. For example, the relative influence of the above mentioned processes in the evolution of phenotypic divergence remains unclear. Furthermore, how patterns of genetic variation relate to patterns of phenotypic variation are also poorly understood (Mitchell-Olds et al. 2007). Thus, to understand the relative contributions of neutral and adaptive processes, and the complex interactions between them, in the evolution of adaptive phenotypic variation among populations requires a combination of population genetic tools, functional genetics and the evaluation of the interaction between phenotypic variation and ecology (Mitchell-Olds et al. 2007; Rudh et al. 2007; Sæther et al. 2007; Ohmer et al. 2009). Such population-level analyses are necessary if we are to understand the link between adaptive traits and the underlying ecological factors responsible for them. Furthermore, because ecologically divergent populations of the same species may represent the early stages of speciation, such analyses may also provide insight into speciation processes (Podos & Warren 2007; Via 2009).

The role of landscape features and distance (which separate populations) in the evolution of population differentiation and speciation is well known (Fitzpatrick et al.

2009). Geographic variation in genetic and phenotypic traits among populations may indicate spatially and temporally varying selection pressures to which populations in different environments may adapt (Gómez et al. 2008; Calsbeek et al. 2009) ultimately resulting in local adaptation (Lenormand 2002; Kawecki & Ebert 2004; Schluter & Conte 2009). Geographic variation of traits may however also result from random neutral processes such as genetic drift and founder effects. For example, in the absence of strong selection, populations which are geographically distant from one another are expected to show greater genetic and trait variation than those separated by smaller geographic distances (Wright's Isolation-by-Distance Model: Wright 1943; Slatkin 1993; e.g. Maharadatunkamsi et al. 2000; Chen et al. 2006; Rudh et al. 2007). The extent to which these processes affect population divergence is also dependent on the proportion of the gene pool of the species from which a population has been established (the Founder Effect: Mayr 1963); where a different subset of alleles may characterise populations with different colonisation histories. Divergence among populations is thus also dependent on the degree of isolation and gene flow between them (Rudh et al. 2007). These adaptive and neutral evolutionary processes are not mutually exclusive but interact in a variety of different ways to shape patterns of genetic and phenotypic variation across environments.

The fluctuating migration-selection balance

An on-going debate in studies of adaptive divergence between populations involves the complex interaction between diversifying selection, and homogenising gene flow (Lenormand 2002; Räsänen & Hendry 2008; Edelaar & Bolnick 2012). Traditionally, gene flow is considered to moderate the effect of diversifying selection by reducing the mean fitness of populations due to the introduction of potentially maladaptive alleles into populations that are locally adapted (Räsänen & Hendry 2008; Edelaar & Bolnick 2012). Over time, populations can reach an equilibrium level between diversifying selection and homogenising gene flow, called the migration-selection balance (Haldane 1948; Slatkin 1973; Hendry et al. 2001). Both theoretical and empirical studies (summarised in Räsänen & Hendry 2008) have however shown that the interaction between gene flow and selection is considerably more complex than originally thought

(Crispo 2008). For example, populations experiencing different ecological regimes should experience increasing divergent selection (pathway 1, Figure 1.1) leading to greater adaptive divergence (pathway 2) even in the presence of low levels of gene flow.

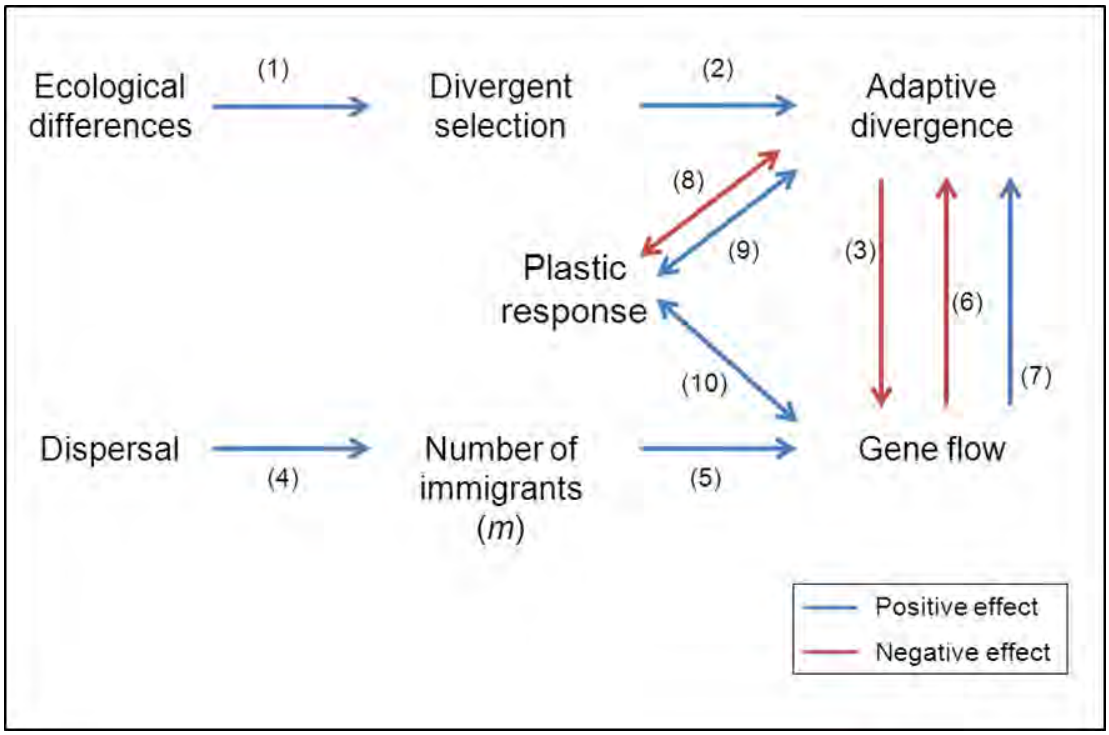

Figure 1.1: A schematic diagram of the potential interactions between divergent selection, adaptive divergence, gene flow and adaptive phenotypic plasticity (modified from Räsänen & Hendry 2008 and Crispo 2008). Blue and red arrows indicate positive and negative influences respectively. The numbers in parentheses relate to pathways discussed in the text.

Adaptive divergence may also reduce gene flow (pathway 3) via the evolution of reproductive isolation (and possibly leading to ecological speciation: Rundle & Nosil 2005; Schluter 2009; Nosil 2012). An increasing number of individuals dispersing between environments (pathway 4) are expected to increase gene flow (pathway 5). An increase in gene flow should limit adaptive divergence by homogenising gene pools that would otherwise diverge in response to selection under different ecological regimes (pathway 6) (Antonovics 1968; Slatkin 1987; Räsänen & Hendry 2008). Pathways 2 and 6 represent the classic migration-selection balance predicted by

theoretical models (Haldane 1948; Slatkin 1973; Hendry et al. 2001) and are supported by a large number of empirical studies which demonstrate a generally negative relationship between levels of connectivity and the degree of adaptive divergence in the traits under study (e.g. Tobias et al. 2010; Wang & Summers 2010; González et al. 2011; Puechmaille et al. 2011; Edelaar et al. 2012).

The relationship is, however, clearly more subtle than this because dispersal and gene flow can also promote adaptive divergence through the spread of advantageous alleles and/or the evolution of non-random dispersal (pathway 7) (Holt et al. 2004; Garant et al. 2005, 2007; Edelaar & Bolnick 2012). Indeed, empirical evidence also supports extensive adaptive divergence in the presence of gene flow (e.g. Niemiller et al. 2008; Milá et al. 2009; Ribeiro et al. 2012; Muñoz et al. 2013) highlighting the role of selection gradients across different environments. If the selection gradient is steep enough to reduce immigrant fitness, the homogenising effects of gene flow on trait variation are likely to be minimised (Nosil 2005). Alternatively, if selection is weak and there are minimal costs to immigrant fitness, even nominal gene flow could constrain divergence (Nosil & Crespi 2004).

Undoubtedly there is a multifaceted and dynamic relationship between selection, fitness and gene flow, but the interaction among these factors, as well as the role of phenotypic plasticity, is seldom considered in studies of adaptive evolutionary divergence (Crispo 2008; Pfennig et al. 2010). Phenotypic plasticity, the ability of a single genotype to produce alternative phenotypes in response to different environmental conditions (West-Eberhard 2003), has traditionally been viewed as an insignificant evolutionary process, largely because environmentally induced phenotypic change does not influence the genes that an individual transfers to its offspring (Pigliucci 2001; Pfennig et al. 2010). Divergent selection therefore cannot act on genetic variants in a population and consequently phenotypic plasticity is seen as constraining rather than promoting adaptive divergence (pathway 8) (Crispo 2008). Recently however, there has been renewed interest in elucidating how plasticity modifies the complex relationships between diversifying selection and gene flow to promote adaptive divergence within and between populations and species (Via et al. 1995; Ghalambor et al. 2007; Crispo & Chapman 2008; Ord et al. 2010; Richter-Boix et

al. 2010; Thibert-Plante & Hendry 2010; Fitzpatrick 2012; Lundsgaard-Hansen et al. 2013). This revival is due in part to the recognition that selection for adaptive phenotypic plasticity can occur if plasticity promotes colonization and survival in different environments (Pigliucci 2001; Price et al. 2003; Yeh & Price 2004), thereby increasing the potential for future adaptive genetic divergence (pathway 9) via selection (Pfennig et al. 2010). High gene flow between selective environments may therefore favour the evolution of increased phenotypic plasticity, or its maintenance, (pathway 10) over adaptive genetic divergence because it could promote adaptation to new conditions within a few generations (Crispo 2008; Jourdan-Pineau et al. 2012). In turn, plasticity may promote gene flow if dispersers are not selected against in their new environments (pathway 10) (Crispo & Chapman 2008). Consequently, adaptation to local conditions could occur faster via plastic responses (pathway 9) because plasticity allows the entire population to adapt simultaneously, whereas natural selection acts on the standing genetic variation among individuals or new mutations that may have evolved (Ghalambor et al. 2007; Crispo 2008).

Geographic variation in acoustic signals

Geographic variation in sensory traits i.e. traits used by organisms to perceive and respond to information about their environment (Sensory Ecology: Ali 1978) can directly impact on individual fitness, e.g. via their role in resource use and species and mate recognition (Dangles et al. 2009), and are often reported to be highly variable across heterogeneous environments (Boughman 2002). An example of such a trait is the acoustic signals used by a wide range of organisms for general communication as well as mate and food acquisition (Wilkins et al. 2013), including fish (Phillips & Johnson 2008), insects (Pinto-Juma et al. 2005), anurans (Lemmon 2009), birds (Podos 2010) and mammals (Campbell et al. 2010). Geographic variation in acoustic signals is common, and roles for phenotypic plasticity (Beckers & Schul 2008), genetic drift, and both sexually and ecologically mediated selection, have been implicated in promoting the evolution of population divergence and speciation across a broad range of taxa (Boughman 2002; Edelaar et al. 2012; Wilkins et al. 2013).

Echolocation, or biosonar, is a form of ultrasonic acoustic signal (normally frequencies > 20 kHz) whereby animals emit calls and analyse the returning echoes to gather information about their surroundings (Griffin 1944, 1953). Echolocation is a complex sensory trait involving the integration of various morphological and neurophysiological adaptations to facilitate the emission of calls and the reception and interpretation of their returning echoes, thereby creating complex auditory scenes of the physical environment (Moss & Surlykke 2010). In mammals, bats (excluding the majority of Old World fruit bats) together with the toothed whales, such as dolphins and porpoises (suborder Odontoceti), have independently evolved ultrasonic hearing coupled with echolocation which they use for communication, orientation and to detect, localise and classify prey (Schnitzler & Kalko 2001; Thomas et al. 2004; Janik 2009).

Echolocation in bats (Chiroptera)

The global success of bats, which account for more than 20% (approximately 1200 species) of extant mammals (Simmons 2005), has largely been attributed to the evolution of sophisticated echolocation coupled with true powered-flight. Most bats produce calls in their larynx and emit these calls either orally (e.g. Family Vespertilionidae) or nasally (e.g. Families Rhinolophidae and Hipposideridae). Bats in the family Pteropodidae however do not use laryngeal echolocation; although cave-dwelling bats in the genus *Rousettus* produce sophisticated brief, broadband tongue-clicks (Holland et al. 2004; Yovel et al. 2011). These clicks are produced in pairs and are used for orientation (Jones & Teeling 2006). All other bats (outside of the Pteropodidae) make use of echolocation calls produced in the larynx. Laryngeal echolocating bats are divided into two broad categories depending on the proportion of time they call relative to the time between calls (the inter-onset interval). The majority of echolocating bats produce calls at a low duty-cycle (ratio of call duration to inter-onset interval is high). These calls are dominated by frequency modulated (FM) signals and the bats separate pulse and echo temporally to avoid self-deafening (Fenton et al. 1995). High duty-cycle bats (ratio of call duration to inter-onset interval is low) viz. those in the families' Rhinolophidae (horseshoe bats) and Hipposideridae (Old World leaf-nosed bats) as well as Parnell's moustached bat, *Pteronotus parnellii*

(Mormoopidae) use calls dominated by a constant frequency (CF) component and they separate pulse and echo in frequency rather than time; they are thus able to transmit and receive echoes simultaneously (Fenton et al. 1995; Jones & Teeling 2006).

Echolocating bats display substantial diversity in call structure (Figure 1.2) with calls typically dominated by either FM or CF components, or a combination of both. Signal structure is generally well correlated with different hunting strategies in diverse foraging habitats (Schnitzler & Kalko 1998) and phylogenetically related species in a particular family tend to have similar call characteristics to one another. However, even distantly related species have evolved convergent call characteristics when experiencing comparable selection pressures due to ecologically similar conditions (Jones & Teeling 2006). For example, specialised high duty-cycle echolocation coupled with Doppler shift compensation evolved independently in the New World Mormoopidae e.g. Parnell's moustached bat, as well as in bats of the Old World families' Rhinolophidae and Hipposideridae (Jones & Teeling 2006).

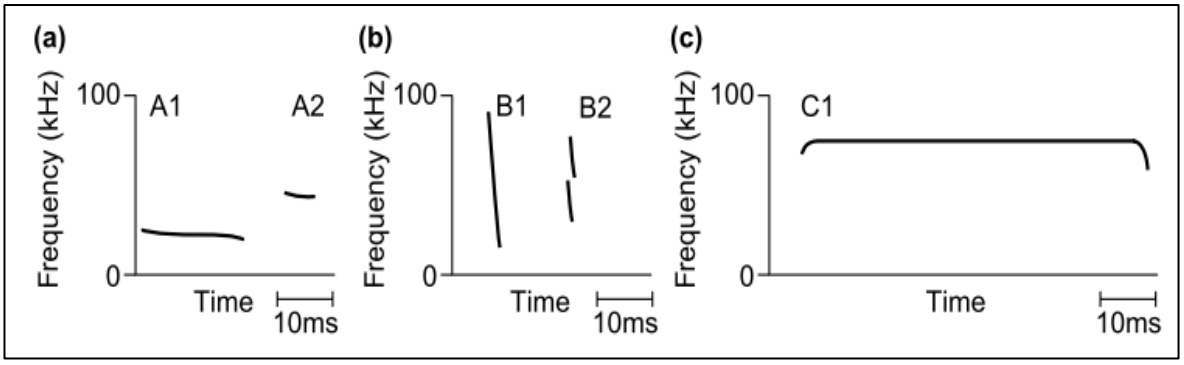

Figure 1.2: Variation in call structure of different echolocating bats. (A1) European free-tailed bat, *Tadarida teniotis* (Molossidae); (A2) pipistrelle bat, *Pipistrellus pipistrellus* (Vespertilionidae); (B1) greater mouse-eared bat, *Myotis myotis* (Vespertilionidae); (B2) fringe-lipped bat, *Trachops cirrhosus* (Phyllostomatidae); (C1) greater horseshoe bat, *Rhinolophus ferrumequinum* (Rhinolophidae) (modified from Schnitzler & Kalko 1998).

Bat echolocation characteristics, in particular frequency, are highly variable both within and between species (Heller & von Helversen 1989; Francis & Habersetzer 1998; Kazial et al. 2001; Hiryu et al. 2006; Armstrong & Coles 2007). Variation may be related

to differences in foraging habitat (Barclay et al. 1999; Guillén et al. 2000) or diet (Houston et al. 2004), possibility promoting geographic variation in echolocation calls (Barclay et al. 1999; Kingston and Rossiter 2004; Gillam & McCracken 2007). For example, differences in foraging habitat structure (typically vegetation cover) may influence the range of frequencies that can function effectively in different habitats, at least for low duty-cycle bats (Foraging Habitat Hypothesis: Jones & Barlow 2004). Low frequency calls allow for long-range detection of targets and are therefore associated with bats foraging in open areas. Higher frequency calls are heavily attenuated (Lawrence & Simmons 1982), but enable greater directionality and provide greater resolution of targets and are thus best suited for short-range detection in cluttered habitats (Neuweiler 1984).

Intrinsic factors such as differences in body size, age, sex or the use of echolocation for communication purposes, may also influence the evolution of geographic variation in echolocation calls. For example, body size scales negatively with call frequency in a number of bat families i.e. Rhinolophidae, Hipposideridae, Emballonuridae, Vespertilionidae, and Molossidae (Jones 1996); and has been found to vary geographically in response to environmental factors such as temperature and rainfall (Burnett 1983; Maharadatunkamsi et al. 2000; Yom-Tov & Geffin 2006). Therefore, variation in body size may cause variation in echolocation calls (Allometry Hypothesis: Stoffberg et al. 2011). While this relationship is also observed in other horseshoe bats, including *Rhinolophus philippinensis* (Kingston & Rossiter 2004), many other species of insectivorous bats deviate from the general allometry between body size and call frequency (Jacobs et al. 2007; Russo et al. 2007). Echolocation may also vary with age and sex; often juveniles produce lower frequency calls than adults (e.g. Moss et al. 1997; Russo et al. 2001; Siemers et al. 2005) and many species show sexual dimorphism in call frequency but not body size (e.g. Neuweiler et al. 1987; Jones et al. 1992, 1994; Russo et al. 2001, 2007; Siemers et al. 2005).

Studies have shown that echolocation can also function in communication (Ma et al. 2006; Siemers et al. 2005; Kazial et al. 2008; reviewed in Jones & Siemers 2011), where calls may convey information about the presence and location of conspecifics, feeding areas and roosts to other “eavesdropping” bats (Fenton & Bell 1981; Barclay 1982;

Jones 2008; Dechmann et al. 2009) or they may convey information about individual identity (Carter et al. 2008), group/colony membership (Hiryu et al. 2006, Voigt-Heucke et al. 2010), sex (Kazial & Masters 2004; Knörnschild et al. 2012; Schuchmann et al. 2012) or species discrimination within multi-species communities (Schuchmann & Siemers 2010a).

Echolocation and genes

Recent advances in molecular technology and the accompanying abundance of comparative DNA sequence data have led to the discovery of a wide range of candidate genes directly involved in pathways that influence sensory perception in various organisms (e.g. olfactory genes: Johansson & Banks 2011, visual genes: Zhao et al. 2009, auditory genes: Davies et al. 2012). Investigating mutations in these genes from individuals with divergent phenotypes may suggest selection for particular phenotypes in different environments (Stapley et al. 2010). Echolocation is a highly complex trait because it involves the integration of many morphological and neurophysiological adaptations (Teeling et al. 2012) and thus it is likely that several genes contribute to the trait. Using a candidate gene approach, recent studies have revealed interesting relationships between echolocation divergence and functional genetic variation between echolocating lineages. For example, *FOXP2,* dubbed the 'vocalisation gene' (Lai et al. 2001; Liegeois et al. 2003) is associated with vocalisation and sensory-motor integration in mammals (Fisher & Marcus 2006). Li et al. (2007) found that *FOXP2* is highly conserved in mammals but very variable in echolocating bats and thus proposed that differences in echolocation call characteristics among species may be related to variability in this gene.

Another candidate gene involved in echolocation is the 'hearing gene' *Prestin* that encodes the membrane motor protein which drives the mechanical amplification of sound in the outer hairs cells of the mammalian cochlear (Zheng et al. 2000). Thus, *Prestin* is directly responsible for the acoustic sensitivity of the cochlea (Dallos et al. 2006). Although, *Prestin* is highly conserved in mammals (Franchini & Elgoyhen 2006), studies have found significant convergent amino acid substitutions in all bats which use laryngeal echolocation (Li et al. 2008). Likewise, evidence of strong positive

selection was found in the lineages leading to horseshoe (Rhinolophidae) and Old World leaf-nosed (Hipposideridae) bats (Li et al. 2008). Additionally, echolocating dolphins and whales share many amino acid mutations in *Prestin* with echolocating bats and surprisingly, the majority of these substitutions are shared with horseshoe bats which use sophisticated CF echolocation coupled with Doppler shift compensation (Li et al. 2010; Liu et al. 2010). At the time, the studies by Liu et al. (2010) and Li et al. (2010) represented one of the best examples of convergent molecular evolution, and suggested that the amino acid mutations present in *Prestin* may be driven by natural selection and directly related to the evolution of high frequency hearing in these echolocating lineages (Rossiter et al. 2011). Remarkably, subsequent studies have shown that this phenomenon is not restricted to *Prestin*. As many as five other genes involved in hearing show signatures of parallel convergent evolution between echolocating bats and whales (Liu et al. 2011; Davies et al. 2012; Shen et al. 2012), suggesting that many hearing genes underlie the evolution of echolocation in these lineages.

High duty-cycle echolocation in the Rhinolophidae

High duty-cycle echolocating bats (horseshoe bats, Old World leaf-nosed bats and *Pteronotus parnellii*) produce calls of long duration and which have a prominent constant frequency (CF) component followed by a brief frequency modulated (FM) component (Neuweiler 1989). This has evolved as a unique auditory system which functions in both resource acquisition and intraspecific communication (Kanwal et al. 1994; Kingston et al. 2001; Jones & Teeling 2006). Horseshoe bats emit echolocation calls through their nostrils, and they place most of the energy into the second harmonic of their calls (Sales & Pye 1974). During flight horseshoe bats compensate for flight-induced Doppler shifts by lowering the frequency of their emitted pulse. This ensures that the returning echo falls within the narrow frequency range of their acoustic fovea - a region of the cochlea with sharply tuned neurons sensitive to a unique frequency called the reference frequency (Schuller & Pollak 1979). Horseshoe bats are able to couple the frequency of the echoes from their emitted calls to their reference frequency independent of the size of Doppler shifts with extreme precision

(Schuller et al. 1974). The reference frequency is always 150 – 200 Hz higher than the frequency these bats emit when stationary, often referred to as the 'resting frequency' (RF) (Schnitzler & Denzinger 2011). Horseshoe bats typically forage for insect prey in narrow-space cluttered environments (Schnitzler & Kalko 2001) either on the wing (aerial hawking), or from a perch (flycatcher style) (Schnitzler et al. 1985; Neuweiler et al. 1987; Jones & Rayner 1989). In both cases, the long CF component of their echolocation calls generates echoes from flying insects that are characterised by amplitude and frequency modulations called acoustic glints (Neuweiler 1989). This long CF call emitted at a high duty-cycle, together with Doppler shift compensation and the auditory fovea allows horseshoe bats to detect fluttering insects in cluttered space (reviewed in Schnitzler & Denzinger 2011). The characteristic echolocation frequency of horseshoe bats is largely a genetically determined trait (Rübsamen 1987). However, the final RF of juvenile rhinolophids is also partly influenced by that of its mother (Matsumura 1979; Jones & Ransome 1993) and the fine-tuning of echolocation frequency may have a learnt component as a result of mother-to-offspring transmission (Jones & Ransome 1993; Boughman & Moss 2003). Cultural learning has been established for the genus *Rhinolophus* (Jones & Ransome 1993; Boughman & Moss 2003; Ma et al. 2006) and horseshoe bats incorporate the stable CF component of their echolocation calls into calls used for intraspecific communication (Matsumura 1979, 1981; Andrews & Andrews 2003; Ma et al. 2006; Liu et al. 2013).

The sophisticated echolocation system of horseshoe bats makes them ideal model organisms for the study of geographic variation in sensory traits. For example, because the RF can be recorded from handheld individuals, recording methods for different populations can be standardized; measurement error due to variations in flight speed and the direction of the bat relative to the microphone can also be minimised (Armstrong & Coles 2007).

Sensory diversification in horseshoe bats

Several studies have investigated sensory divergence in the echolocation frequencies of rhinolophid and hipposiderid bats (e.g. Kingston et al. 2001; Jacobs et al. 2007; Russo et al. 2007; Yoshino et al. 2008; Chen et al. 2009; Jiang et al. 2010). Population

divergence in echolocation has predominantly been attributed to differences in diet (Kingston & Rossiter 2004) and environmental humidity (Atmospheric Attenuation Hypothesis: Guillén et al. 2000; Jiang et al. 2010; Armstrong & Kerry 2011). Furthermore, because divergence in echolocation calls requires changes in the morphology associated with the production, emission and reception of echolocation calls, divergence has also been correlated with several morphological features directly involved in echolocation production (Morphological Correlates Hypothesis: Armstrong & Coles 2007; Odendaal & Jacobs 2011) and reception (Francis & Habersetzer 1998), as well as to adaptive changes in body size that reflect prevailing environmental conditions (Taylor et al. 2012). Among sympatric species, call divergence may also have evolved under regimes of social selection where character displacement has maintained private bandwidths for intraspecific communication (Acoustic Communication Hypothesis: Duellman & Pyles 1983; Heller & von Helversen 1989; Jacobs et al. 2007; Russo et al. 2007). In this scenario, within a particular community, the echolocation frequencies used by any one species may be a consequence of surrounding frequencies used by others (Heller & von Helversen 1989; Russo et al. 2007).

Although echolocation is clearly an adaptive trait, acoustic divergence may nonetheless be subject to neutral evolutionary processes like gene flow and genetic drift. More recently, neutral evolutionary processes have become a focus of research centred on understanding the factors responsible for shaping current population genetic structure in bats (Flanders et al. 2009, 2011; Xu et al. 2010; Dool et al. 2013; Lin et al. 2014) and their potential role in patterns of echolocation variation within species (Yoshino et al. 2008; Chen et al. 2009; Puechmaille et al. 2011; Stoffberg et al. 2012; Clare et al. 2013). A number of studies report significant population genetic structure and limited gene flow between acoustically divergent populations (Thabah et al. 2006; Ramasindrazana et al. 2011), often revealing the presence of cryptic lineages (Chattopadhyay et al. 2012; Clare et al. 2013). However, few studies have specifically evaluated sensory divergence within a phylogeographic and population genetic framework in the Rhinolophidae (Yoshino et al. 2008; Chen et al. 2009; Sun et al. 2013). Those that have reveal a number of intriguing relationships between

echolocation frequency variation and for example, female philopatry (Yoshino et al. 2008), geographic isolation (Sun et al. 2013) and Pleistocene climatic cycling events (Stoffberg et al. 2012). Despite great progress in our understanding of sensory divergence in horseshoe bats, the relative influence of neutral and adaptive processes remain largely unknown, mainly due to studies demonstrating the importance of a single evolutionary process. To gain a comprehensive understanding of the evolution of sensory divergence therefore requires an evaluation of the interaction between echolocation variation and ecology within the context of both neutral and functional genetic variation. In this study, such a holistic approach was used to understand sensory diversification within a southern African endemic species, the Cape horseshoe bat*, Rhinolophus capensis* Lichtenstein 1823.

Study system: the Cape horseshoe bat, *Rhinolophus capensis*

Rhinolophus capensis is a medium-small (forearm: 48.84 – 51.45 mm: Odendaal & Jacobs 2011) horseshoe bat endemic to the extreme southwest of southern Africa (Monadjem et al. 2010) (Figure 1.3). The south-west Cape region of southern Africa is characterized by exceptionally high floral endemism and diversity and is comprised of several major biomes including Desert, Succulent Karoo, Fynbos, Forest and areas of transition (ecotones: Smith 1997) between Fynbos and neighbouring biomes (Albany thicket, Nama-Karoo and Grassland) (Mucina & Rutherford 2006). Because of its ecological success across a wide range of habitats, which are likely to exert a gradient of selection pressures, variation in the echolocation system of *R. capensis* presents an ideal system for the investigation of sensory diversification.

The relatively restricted distribution of this species to the low-lying coastal plain of southern Africa, at least compared to that of other African horseshoe bat species co-occurring in the region (e.g. Figure 1.3), also facilitates thorough sampling across its geographic range, minimising the common problem of under-sampling trait variation within a species. *Rhinolophus capensis* co-occurs with a larger congeneric species, *Rhinolophus clivosus*, over much of its range (Figure 1.3). At the northwest limit of its range, it also co-occurs with *Rhinolophus darlingi damarensis* (Monadjem et al. 2010).

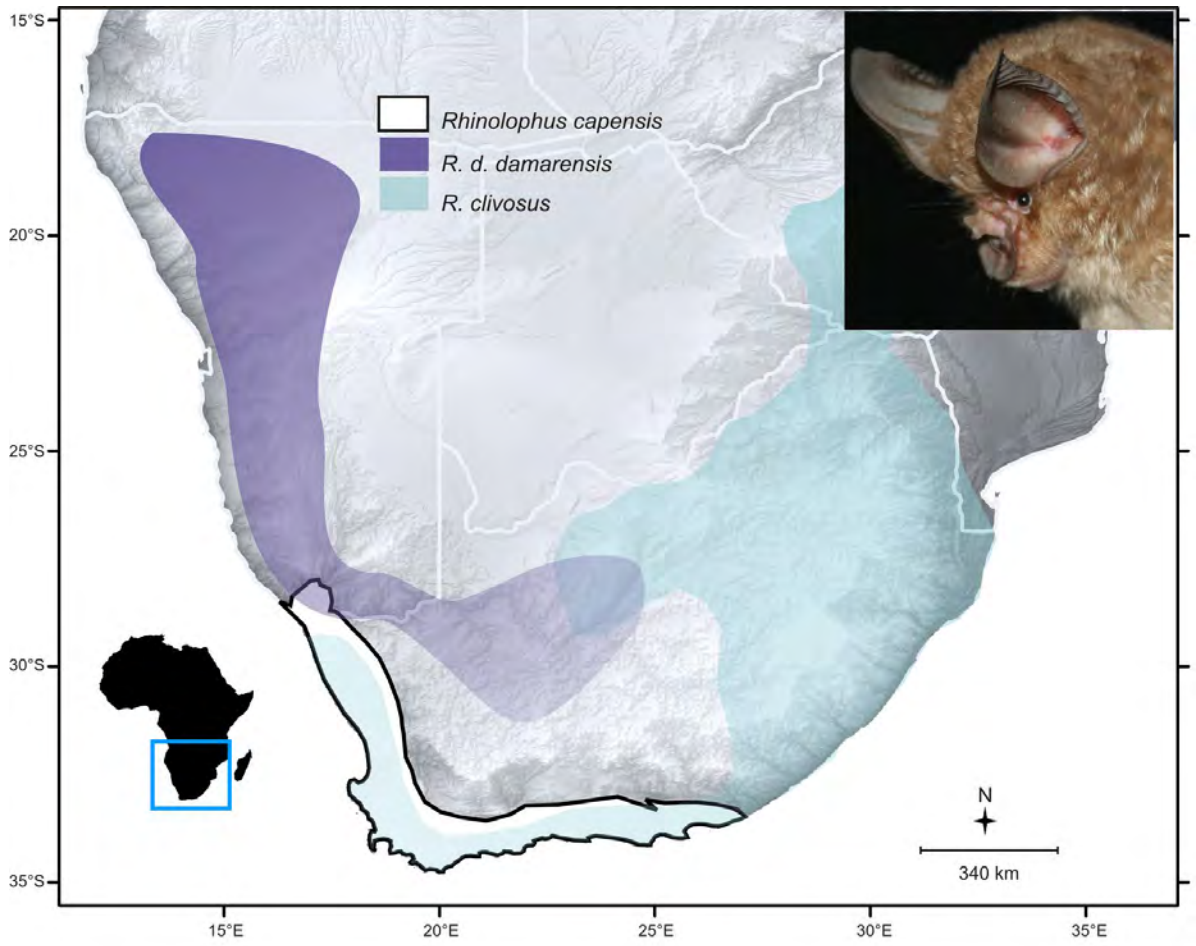

Figure 1.3: The geographic distribution of the Cape horseshoe bat, *Rhinolophus capensis* (inset) together with the distribution of co-occurring horseshoe bat species, *R. d. damarensis* and *R. clivosus*.

Like all horseshoe bats, *R. capensis* forages in or near cluttered habitats (Csorba et al. 2003; Jacobs et al. 2007). These bats have short, broad wings with intermediate wingloading (6.6 – 8.6 Nm^2) and low aspect ratio (5.5 – 6) (Odendaal & Jacobs 2011) and their diet consists primarily of Coleoptera and Lepidoptera (Jacobs et al. 2007). Previous research revealed significant differences in the RFs of *R. capensis* from three populations situated in three different biomes; bats from De Hoop (Fynbos Biome) echolocated at 84 kHz, whereas Steenkampskraal (Succulent Karoo Biome) and Table Farm (ecotone containing elements of Albany Thicket, Nama-Karoo, Grassland and Fynbos Biomes) bats echolocated at 80 kHz and 86 kHz respectively (Odendaal & Jacobs 2011). Call variation among these populations were unrelated to differences in

foraging habitat, rainfall or geographic variation in body size. Instead, there was a close association between RF and cranial features directly related to echolocation (dorsal nasal chambers) independent of differences in body sizes, suggesting a role for natural selection in the evolution of acoustic divergence in this species (Odendaal & Jacobs 2011). However, the full extent of acoustic variation within this species was unknown and previous research ignored any role for neutral processes in the evolution of echolocation variation in this system.

Research aims

In this study a comprehensive and interdisciplinary approach combining ecology, functional morphology, phylogeographic structure and functional genetic sequence data is used to investigate the relative influences of neutral and adaptive processes in the evolution of sensory divergence in echolocating bats. Using *R. capensis* as a model, this study tests a number of prevailing hypotheses about the roles of morphology, gene flow, habitat structure and functional gene variation in the evolution and maintenance of echolocation variation in bats and in so doing seeks to answer a number of current questions in the field of adaptive trait evolution:

1) In species distributed across heterogeneous landscapes, how do spatially varying divergent selection and random genetic drift interact to shape patterns of adaptive trait divergence?

2) Is adaptive trait divergence reflected in patterns of neutral population structure as would be expected if strong divergent selection constrains gene flow among populations adapted to local environmental conditions?

3) Are patterns of adaptive trait divergence reflected in protein-coding sequence differences in functional genes related to the trait?

Thesis outline

Chapter 2 describes the degree of geographic variation in resting frequency (RF) among populations of *R. capensis* and tests the relationship between variation in RF and both ecological and morphological correlates of echolocation. To identify the

selection pressures which may drive sensory diversification in this system, ecological models together with a geometric morphometric analysis of skull size and shape variation were used to explore the following hypotheses to explain variation in bat echolocation: (i) the Foraging Habitat Hypothesis, which predicts a close association between the degree of vegetation clutter and echolocation frequency to facilitate effective prey detection in habitats characterised by different degrees of clutter, (ii) the Atmospheric Attenuation Hypothesis, which predicts a negative correlation between relative humidity and echolocation frequency due to the rapid attenuation of high frequency sounds in humid environments, (iii) the Allometry Hypothesis, which predicts a negative correlation between body size and echolocation frequency and (iv) the Morphological Correlates Hypothesis which predicts a tight coupling between RF and cranial features directly involved in echolocation production and emission (i.e. dorsal nasal chambers: Hartley & Suthers 1988).

Chapter 3 then evaluates RF variation within a neutral evolutionary genetic framework to (i) determine whether observed patterns of RF variation across the distribution *of R. capensis* are influenced by random genetic drift, (ii) explore the evolutionary relationships among maternally inherited mitochondrial DNA lineages of *R. capensis* and test whether these maternal lineages are geographically structured, (iii) explore the spatial distribution of neutral genetic variation using a Bayesian approach, and test whether the spatial distribution of RF variation uncovered in the previous chapter is reflected in the population genetic structure of the species, and (iv) use coalescent methods to estimate historic levels of gene flow between populations to determine whether divergence in RF is associated with reduced gene flow among populations of *R. capensis*.

To determine whether sensory divergence and neutral genetic variation are influenced in any way by functional gene variation, Chapter 4 explores the evolution of the 'hearing gene' *Prestin* among species closely related to *R. capensis* (the *R. capensis* clade: *R. capensis*, *R. denti*, *R. simulator* and *R. swinnyi*: Stoffberg et al. 2010), and among populations of *R. capensis* with divergent RFs. Classic tests for signatures of selection were conducted to determine whether *Prestin* has evolved under positive or purifying selection within the *R. capensis* clade.

The thesis concludes with Chapter 5, the synthesis. Here the results of the previous chapters are integrated and discussed, enabling a comprehensive response to the research questions outlined above. The implications of this research for our understanding of the relative roles of neutral and adaptive processes in the evolution of sensory divergence in bats and other vertebrates are discussed. This chapter also highlights the benefits of using the Cape horseshoe bat as a model system to provide novel insights into the broader fields of sensory ecology and adaptive trait evolution, and provides direction for future research.

CHAPTER 2

GEOGRAPHIC VARIATION IN RESTING FREQUENCY OF THE CAPE HORSESHOE BAT: ECOLOGICAL AND MORPHOLOGICAL CORRELATES

INTRODUCTION

Species distributed across heterogeneous environments are likely to experience temporally and spatially varying selection pressures and therefore different phenotypes may be favoured to better exploit local environmental conditions (Clausen et al. 1940; Mayr 1942; Willams 1966; Schluter 2000; West-Eberhard 2003; Nosil et al. 2005; Ghalambor et al. 2007; Badyaev et al. 2008; Seehausen et al. 2008; Hereford 2009; Leinonen et al. 2010; Tobias et al. 2010; Edelaar et al. 2012; Cole 2013). As a result, geographic variation in phenotypic traits is relatively common, particularly when those traits are important for fitness (Thorpe 1987). Because the phenotype is the expression of the interaction between genes themselves together with gene flow, genetic drift and natural selection, phenotypic differences may represent a window into the evolutionary processes that can lead to population divergence and speciation.

Few phenotypes are as diverse as the signals animals use for communication (Endler 1992; Bradbury & Vehrencamp 1998; Ptacek 2000; Campbell et al. 2010). A phylogenetically broad range of animals use acoustic signals to mediate mate choice, resource defence, and species discrimination (Bradbury & Vehrencamp 1998; Slabbekoorn & Smith 2002; Wilkins et al. 2013), and as a consequence, the information transferred between signallers and receivers may be important for reproduction and survival (Endler 1992; Endler & Basolo 1998; Ey & Fischer 2009). Divergence in acoustic signals may therefore play a crucial role in the evolution of population divergence and could ultimately lead to speciation if divergence is closely coupled with assortative mating (Cole 2013; Wilkins et al. 2013).

Much of our current knowledge of the causes and consequences of acoustic signal variation within, and between, species has come from studies investigating geographic

variation in bird song and the calls of insects and anurans (Podos & Warren 2007; Ey & Fischer 2009), where research has predominantly focused on investigations of acoustic divergence within the Sensory Drive Framework (Wilkins et al. 2013). Sensory drive was first described by Endler (1992) as an explanation for the link between habitat choice and the coevolution of communication signals, sensory systems and behaviour as a function of the physics of signal production and transmission, and the neurobiology of perception. Different environmental conditions will favour different sensory characteristics, leading to divergence in communication signals that can function effectively in different habitats (Boughman 2002; Wilkins et al. 2013). Although much of the evidence for sensory drive is derived from visual communication systems (e.g. Cummings 2007; Seehausen et al. 2008; Morrongiello et al. 2010; Ng et al. 2012), its role in the evolution of acoustic divergence has received increased attention, where studies discuss patterns of acoustic divergence as a function of e.g. habitat structure (Henry & Lucas 2010; Tobias et al. 2010), community composition (Grant & Grant 2010), local ambient noise profiles (Kirschel et al. 2009) and sender/receiver morphology (Huber & Podos 2006). Nestled within the Sensory Drive Framework, the Acoustic Adaptation Hypothesis (Morton 1975) states that acoustic signals are shaped by habitat-driven selection to optimise sound propagation in different habitats, and support both for (Perla & Slobodchikoff 2002;Tobias et al. 2010; Ziegler et al. 2011) and against (Jain & Balakrishnan 2012; Vargas-Salinas & Amézquita 2014) this hypothesis have been reported.

Acoustic divergence can also evolve as a by-product of selection on other ecology-related traits such as body size (Gingras et al. 2013) or morphological traits directly involved in signal production (Huber & Podos 2006). Wilkins et al. (2013) argue that the Sensory Drive Framework is not itself an explanation for the underlying mechanisms which shape patterns of acoustic divergence within and between populations, but rather a means to delimit the amount of standing acoustic variation available within particular habitats on which ecological selection, sexual selection and random genetic drift can act. Thus both stochastic processes (Irwin et al. 2008; Campbell et al. 2010) and habitat-driven selection on acoustic signals associated with e.g. mate choice and individual fitness can influence variation (Tobias et al. 2010;

Kirschel et al. 2011). Although great progress has been made in understanding the causes and consequences of acoustic divergence (reviewed in Ey & Fischer 2008 and Wilkins et al. 2013), little is known about how neutral and adaptive processes interact to produce a given pattern of divergence among populations or phylogenetic lineages, especially with respect to vocalisations emitted by mammals, including bats.

The dual function of echolocation

Unlike bird song or the advertisement calls emitted by insects and anurans which are generally indicators of mate quality and purely used for communication (Podos & Warren 2007; Wells & Schwartz 2007), bat echolocation calls have a dual function in foraging and communication (Fenton 1985; reviewed in Jones & Siemers 2011). Horseshoe bats (genus *Rhinolophus*) in the family Rhinolophidae have perhaps evolved the most sophisticated form of echolocation, with calls characterised by a long constant frequency (CF) component preceded and followed by a brief frequency modulated (FM) component (Neuweiler 1989). Horseshoe bats are high duty-cycle echolocators and compensate for Doppler shifts induced by their own flight speed by lowering the frequency of their emitted pulse. This ensures that the returning echo falls within the narrow frequency range of their acoustic fovea – a region of the cochlea with sharply tuned neurons sensitive to the reference frequency (Schuller & Pollak 1979). This is similar to the frequency individual bats emit when stationary, called the 'resting frequency' (RF) (Schnitzler & Denzinger 2011). Research focussed on understanding the remarkable echolocation system of horseshoe bats has yielded a comprehensive understanding of the morphological and neurophysiological basis of echolocation production, emission and perception (Schuller et al. 1974; Schuller & Pollak 1979; Vater et al. 1985; Rübsamen 1987; Rübsamen & Schäfer 1990; Pedersen 1996; Metzner et al. 2002; Smotherman & Metzner 2005; Odendaal & Jacobs 2011; Santana & Lofgren 2013; Liu et al. 2013) , as well as the functional role of echolocation in prey detection (Link et al. 1986; von der Emde & Schnitzler 1990; Koselj et al. 2011) and, more recently, communication in these bats (e.g. Matsumura 1981; Heller & von Helversen 1989; Jones & Ransome 1993; Siemers et al. 2005; Ma et al. 2006; Schuchmann & Siemers 2010a,b; Puechmaille et al. 2014). Thus, horseshoe bats are

ideal models to investigate the complex interactions between geography, ecology, functional morphology and sensory divergence.

Hypotheses for variation in echolocation frequencies

Geographic variation in echolocation calls has been reported for both low duty-cycle (e.g. Barclay et al. 1999; Law et al. 2002; Gillam & McCracken 2007) and high duty-cycle (e.g. Armstrong & Coles 2007; Yoshino et al. 2008; Chen et al. 2009; Stoffberg et al. 2012; Sun et al. 2013) bat species. While each echolocation call is composed of different call parameters which all play a role in how bats detect, localise and classify objects (e.g. call duration, pulse interval, bandwidth and call intensity: reviewed in Jones & Holderied 2007 and Yovel et al. 2011), studies on geographic variation in echolocation usually focus on frequency differences among populations. This is mainly because echolocation frequencies are often species-specific and, due to the physics of sound propagation, call frequency and the associated wavelength of the emitted pulse is directly related to how outgoing pulses, and their returning echoes, are transmitted through the environment. In bats, the Foraging Habitat Hypothesis (Jones & Barlow 2004), a variation of the Acoustic Adaptation Hypothesis, describes the association between foraging habitat structure and the range of echolocation frequencies low duty-cycle bats can use to forage and avoid obstacles effectively in habitats characterised by different degrees of vegetation density, i.e. “clutter”. Lower frequency calls with longer wavelengths allow for long-range detection of targets and are therefore associated with bats foraging in open spaces such as above tree canopies or over open landscapes (Norberg & Rayner 1987; Neuweiler 1990; Barclay & Brigham 1991; Jung et al. 2014). Higher frequency calls are more susceptible to atmospheric attenuation and does not travel as far as low frequency calls (Lawrence & Simmons 1982). However, higher frequencies have greater directionality, providing greater resolution of targets, and are therefore best suited for short-range detection in obstacle-rich habitats (highly cluttered) such as forests and dense vegetation (Neuweiler 1984; Norberg & Rayner 1987; Schnitzler & Kalko 2001; Jones & Barlow 2004). The hypothesis predicts that a gradient of increasing vegetation clutter can drive the evolution of increasing echolocation frequencies for effective prey detection

in different habitats and many species across bat families indeed increase their echolocation frequencies with increasing clutter (e.g. *Myotis lucifugus* (Vespertilionidae): Wund 2006; *Macrophyllum macrophyllum* (Phyllostomidae): Brinkløv et al. 2010; reviewed in Schnitzler & Kalko 2001). But the Foraging Habitat hypothesis can also be applied to high duty-cycle echolocators; this is because the frequency of the CF portion may still be constrained by the transmission properties of the physical habitat these bats use. For example, within the same nature reserve in the Jilin Province of China, Greater horseshoe bats (*Rhinolophus ferrumequinum*) use a variety of habitats with differing degrees of vegetation density and emit significantly lower echolocation frequencies in relatively open habitats compared to cluttered habitats (Xu et al. 2008).

Acoustic divergence in horseshoe bats may also have evolved in response to resource competition, promoting the partitioning of prey resources by their size classes (the Prey Detection Hypothesis: Jacobs et al. 2007). Echoes from insect prey are strongest when the wavelength of the emitted call is equal to or shorter than the wing length of the target insect (Houston et al. 2004). As a result echo strength is greatly diminished when the wavelength exceeds target circumference, because much of the call's energy is lost to the environment (Pye 1983). Because lower frequencies reflect weakly from smaller targets (Jones 1997), bats using high frequencies should be able to detect a wider range of insect prey sizes than bats using lower frequencies (Jacobs et al. 2007). Because of this, resource partitioning via fine scale discrimination of prey size could have important implications for the evolution of sensory divergence (Jones 1997). For example, disruptive selection on frequencies used by three co-occurring size morphs of *Rhinolophus philippinensis*, a cryptic species trio, enable individuals to forage more effectively on different prey size classes in their shared environment; thereby promoting assortative mating and speciation in these lineages (Kingston & Rossiter 2004). Nevertheless, in cases where frequency and the corresponding wavelength differences are too small to result in substantial differences in target echo strengths, acoustic divergence may instead evolve to maintain private frequency bandwidths to facilitate intraspecific communication (Acoustic Communication Hypothesis: Duellman & Pyles 1983; Kingston et al. 2001; Thabah et al. 2006; Jacobs et al. 2007; Russo et al.

2007; Schuchmann & Siemers 2010a). This hypothesis proposes that within a particular community, the echolocation frequency of horseshoe bats may also be influenced by the frequencies of other closely related species (Kingston & Rossiter 2004; Russo et al. 2007).

Related to the both the Prey Detection and Foraging Habitat Hypotheses, the Atmospheric Attenuation Hypothesis, first proposed by Guillén et al. (2000), argues that differences in relative humidity between habitats can influence the call frequencies used by CF bats because high frequencies are heavily attenuated in very humid conditions (Griffin 1971; Lawrence & Simmons 1982; Hartley 1989). The frequencies used by CF bats may therefore represent a trade-off between higher frequencies to improve prey resolution, and lower frequencies to increase prey detection range (Armstrong & Kerry 2011), and some studies report close associations between measures of humidity and call frequency in CF bats (Guillén et al. 2000; Jiang et al. 2010).

Alternative hypotheses

Because variation in the sizes and shapes of organisms can impose different constraints on the sounds they are able to produce, acoustic divergence may also be caused by morphological variation (Ryan & Brenowitz 1985; Irwin et al. 2008). The positive relationship between body size and the size of vocal organs results in an inverse correlation between call frequency and body size: larger animals generally produce lower frequency calls (Pye 1979). This relationship is found in a broad range of taxa including echolocating bats (Jones 1996). Where body size has evolved in response to environmental variation, echolocation frequencies are also likely to demonstrate the same relationship. For example, Taylor et al. (2012) proposed that adaptive shifts in body size in ancestral populations of species belonging to the *Rhinolophus hildebrandtii* complex was driven by paleoenvironmental changes during the Neogene; these have led to concomitant changes in echolocation frequencies, promoting allopatric speciation in this species complex.

Echolocation frequency scales negatively with body size in five families of bats including the Rhinolophidae (Allometry Hypothesis: Jones 1997; Stoffberg et al. 2011). While this relationship is maintained at the intraspecific level in some horseshoe bat species (e.g. Kingston & Rossiter 2004), other species deviate from the allometric scaling between body size and frequency (e.g. *Rhinolophus clivosus*: Jacobs et al. 2007). Call frequency may also vary with age and sex where juveniles emit lower frequencies than adults (Siemers et al. 2005) and some species display sexual dimorphism in call frequency but not body size (e.g. Jones et al. 1992, 1994; Siemers et al. 2005; Russo et al. 2007). Thus differences in body size do not always explain differences in echolocation frequency in horseshoe bats (Siemers et al. 2005). Instead, selection may act on echolocation independently of body size, and if so, we would expect a stronger correlation between echolocation frequency and the morphological features directly related to echolocation (cf. Robinson 1996; Armstrong & Coles 2007; Odendaal & Jacobs 2011) and its perception (Francis & Habersetzer 1998; Davies et al. 2013). Horseshoe and leaf-nosed bats emit calls through their nasal cavity; this mode of echolocation is often considered a key innovation because it required a substantial redesign of the chiropteran rostrum and skull base (Pedersen & Müller 2013; Santana & Lofgren 2013). Their skulls are characterised by grossly enlarged nasal cavities and paranasal sinuses which act as band-pass filters, supressing the fundamental harmonic and concentrating most of the energy into the second harmonic of the emitted pulse (reviewed in Pedersen & Müller 2013). Studies show that geographic variation in echolocation frequency is often better explained by differences in the size and dimensions of the nasal chambers in both horseshoe (Odendaal & Jacobs 2011) and leaf-nosed (Armstrong & Coles 2007) bats than by differences in body size (Morphological Correlates Hypothesis: Armstrong & Coles 2007; Odendaal & Jacobs 2011). The use of geometric morphometric methods in the analysis of dorsal nasal chamber shape variation across the Rhinolophidae reveals another possible explanation; that broad scale patterns of cranial shape variation may also reflect adaptations to environmental differences (Santana & Lofgren 2013).

It is clear that numerous morphological (body size, skull morphology) and ecological (foraging habitat characteristics) factors influence the evolution of sensory divergence

in horseshoe bats. These factors are not mutually exclusive and because they are also likely to vary across heterogeneous landscapes, multiple datasets are required to determine how their complex interactions shape patterns of sensory divergence within species. *Rhinolophus capensis* is distributed across a wide range of habitats ranging from xeric in the west to mesic in the east and resting frequency (RF) is therefore likely to also vary among populations across its distribution. Here, multiple datasets on morphological and ecological correlates of echolocation are used to explore questions relating to a range of different hypotheses which can explain patterns of variation in RF within and between populations of *R. capensis*:

i) Is divergence in RF correlated to differences in body size between populations as predicted by the Allometry Hypothesis?

ii) Do habitat discontinuities (biomes) between populations shape the observed pattern of RF variation in *R. capensis*, and if so, is this reflected in significant differences in detection ranges of prey and background targets between habitats characterised by different degrees of vegetation clutter (Foraging Habitat Hypothesis) and/or relative humidity (Atmospheric Attenuation Hypothesis)?

iii) Do patterns of variation in the dorsal nasal chambers, the only part of the vocal tract evident in skulls, covary with habitat discontinuities? If so, is there a stronger correlation between RF and the size and shape of the nasal chambers than between RF and body size as predicted by the Morphological Correlates Hypothesis?

MATERIALS AND METHODS

Sampling sites

Rhinolophus capensis individuals were sampled from 11 populations across the full distribution of the species, spanning several major biomes. These include Desert at the extreme northern edge of its distribution, through Succulent Karoo, Fynbos and Forest and finally to areas of transition (ecotones: Smith 1997) between Fynbos and neighbouring biomes (Albany Thicket, Nama-Karoo and Grassland Biomes) at the eastern limit of its range (Figure 2.1; Table 2.1). These biomes are characterised by two distinct geographical rainfall gradients; (i) a latitudinal gradient of increasing aridity as

one moves north towards the Namib Desert, and (ii) a longitudinal rainfall seasonality shift from a predominantly winter to an aseasonal rainfall regime from west to east, and another shift to a summer rainfall regime at the extreme eastern edge of the species' distribution (Cowling et al. 1997; Proches & Cowling 2006; Linder et al. 2010; Figure 2.1). These rainfall gradients across the region translate into a clear habitat gradient from relatively open and sparse habitats in the north and west characterised by low rainfall levels (Desert and Succulent Karoo Biomes), to wetter and more cluttered and dense habitats in the east (Fynbos, Albany Thicket and Forest Biomes).

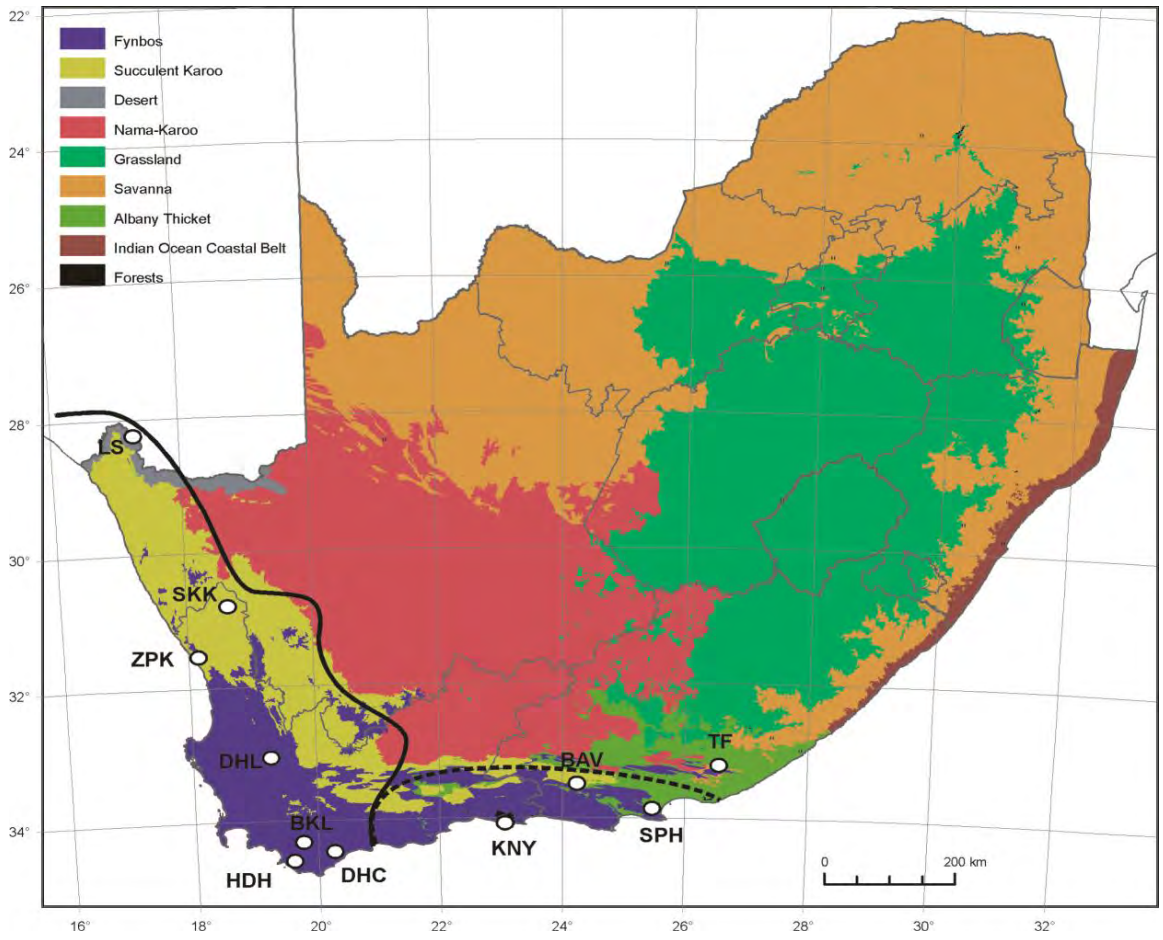

Figure 2.1: A map of the biomes of South Africa (Mucina and Rutherford 2006) together with the geographic locations of the 11 populations of *Rhinolophus capensis* sampled in this study. Lines indicate the approximate positions of the different rainfall zones of South Africa. Solid line indicates the winter rainfall zone; dashed line indicates all-year rainfall zone. The key to population acronyms are detailed in Table 2.1.

Lekkersing (LS; Figure 2.1) represents the northern edge of the species' distribution, situated in the Richtersveld region in the southern limit of the unique coastal Desert Biome of the Namib Desert (Jürgens 1991; Jürgens et al. 1997; Figure 2.1). This biome is extremely arid (< 100 mm rain per annum) and characterised by dwarf, open, sparsely distributed succulent shrubland (Jürgens et al. 1997).

Steenkampskraal (SKK) and Zoutpansklipheuwel (ZPK) are situated in the Succulent Karoo Biome and Succulent Karoo/Fynbos ecotone respectively (Figure 2.1). The area is arid with an average rainfall of less than 150 mm per annum (Cowling et al. 1999). The major vegetation type is Lowland Succulent Karoo characterised by sparse, dwarf (ca. 30 cm in height) succulents together with elements of Strandveld Succulent Karoo and Sandplain Fynbos (Cowling et al. 1999).

De Hel (DHL), Boskloof (BKL), Heidehof (HDH) and De Hoop (DHC) are all situated within the Fynbos Biome (Figure 2.1), an evergreen, sclerophyllous, fire-prone shrubland (Born et al. 2007) with an average vegetation height not exceeding 3m (Okitsu 2010). Knysna (KNY) is situated in the largest continuous block of forest situated on the southern Cape coast which covers an area of less than 600 km^2. The region receives the most rainfall (1000-1500 mm per annum) of all the sampling sites and has an average canopy height of 25m (Midgley et al. 1997).

Among all the sampling locations, Baviaanskloof (BAV) displays the greatest spatial heterogeneity of vegetation structure because elements of all eight southern African biomes occur within a relatively small area of the Baviaanskloof Valley (Proches & Cowling 2006). The region is characterised by steep rugged mountains surrounding a relatively flat, open floodplain with high seasonal and inter-annual variation in rainfall (Jansen 2008). The main vegetation types are Fynbos, Grassland, Albany Thicket (dense, evergreen, spiny shrubland dominated by succulents) and Nama-Karoo (dwarf, deciduous shrubland) but Desert, Forest, Succulent Karoo and Savanna elements occur marginally in the region (Proches & Cowling 2006). Table Farm (TF) and Sleepy Hollow (SPH) also occur in transitional areas between several biomes, including Albany Thicket, Nama-Karoo, Grassland and Fynbos (Lubke et al. 1986; Dold 2003).

Table 2.1: Locality data and sample size information for echolocation recordings and skulls of *Rhinolophus capensis* individuals used in this study.

Population	Population acronym	Biome category	GPS (decimal degrees)	*n* Males	*n* Females	*n* Skulls	
						Males	Females
Lekkersing	LS	Desert	-28.42, 16.88	10	9	2	6
Steenkampskraal	SKK	Succulent Karoo	-30.98, 18.63	11	13	5	4
*Zoutpansklipheuwel	ZPK	Succulent Karoo/Fynbos	-31.63, 18.21	25	-	2	-
De Hel	DHL	Fynbos	-33.08, 19.08	10	1	1	1
*Boskloof	BKL	Fynbos	-34.39, 19.68	15	-	-	-
Heidehof	HDH	Fynbos	-34.62. 19.50	6	11	1	1
De Hoop	DHC	Fynbos	-34.42, 20.36	35	23	5	2
Knysna	KNY	Forest	-33.88, 23.00	5	2	-	-
Baviaanskloof	BAV	Multiple Biomes	-33.63, 24.24	17	10	-	-
Sleepy Hollow	SPH	Multiple Biomes	-33.96, 25.28	1	1	1	1
Table Farm	TF	Multiple Biomes	-33.283, 26.42	18	18	5	5

*Populations where only males were found in the colony

Field methods

The capture, handling and voucher collection methods used in this study comply with the guidelines recommended by the American Society of Mammalogists (Gannon et al. 2007) and were approved by the University of Cape Town's Science Faculty Animal Ethics Committee (approval number: 2008/V18/LO). Bats were captured from their roosts during the day with hand nets, or as they emerged from roosts at dusk using mist nets and/or harp traps. Mist nets were checked regularly throughout trapping periods to ensure that bats were not injured by being in the net for too long.

The age (adult or juvenile) and sex of each bat was recorded. Juveniles were distinguished from adults by the presence of cartilaginous epiphyseal plates in their finger bones (Anthony 1988) and excluded from subsequent analyses. Body mass (to the nearest 0.01 g) using a portable electronic balance, and forearm length (to the nearest 0.01 mm) using dial callipers, were measured. Seasonal and diurnal variation in body mass was controlled for by excluding pregnant females, sampling only in the southern hemisphere spring and summer seasons and measuring bats only after their gut was emptied by keeping them overnight in soft cotton bags.

Echolocation recordings and analyses

Echolocation calls were recorded from hand-held bats positioned 10 cm in front of an Avisoft Ultrasound Gate 416 (Avisoft Bioacoustics, Berlin, Germany) microphone connected to a notebook computer with Avisoft SasLab Pro software (sampling rate 500 kHz). Resting peak frequency (RF, where the constant-frequency (CF) component is stable and inter-pulse variation is low: Armstrong & Coles 2007) was recorded from hand-held individuals because it eliminates differences in peak frequency that may be due to Doppler shift compensation during flight. RF is a reliable indicator of the reference frequency because the difference between the reference and resting frequency is stable in horseshoe bats (within 150 – 200 Hz: Schnitzler & Denzinger 2011). Horseshoe bats may also initially emit lower frequency calls before reaching their final RF (Siemers et al. 2005), and therefore only calls recorded ten seconds after the bat started to echolocate were used in analyses. Recordings were slowed down by

ten and analysed using BatSound Pro software (Pettersson Elektronik AB, Uppsala, Sweden) with a sampling rate of 500 kHz.

The peak frequency (frequency of maximum intensity, kHz) of the dominant second harmonic of the CF component was measured from the fast Fourier transformation power spectrum (FFT = 1024, frequency resolution 684 Hz) with a Hanning window. To identify the typical echolocation parameters for each bat the mean call duration, RF, lowest frequency of the FM component, bandwidth and inter-pulse interval was calculated from 5 – 10 high quality calls (amplitude of the signal at least three times higher than that of the background noise as displayed on the oscillogram). The RF from an original call sequence that was most similar to the calculated mean parameters was chosen for all subsequent analyses. Thus the 'true' RF instead of a constructed statistical value was used in all analyses (e.g. Odendaal & Jacobs 2011).

Skull morphology

Skulls were extracted from 42 voucher specimens stored in 99% ethanol which were collected after their echolocation calls were recorded. Due to ethical considerations it was not possible to collect a large number of vouchers from each population sampled in this study. Instead, vouchers were collected to i) represent the range of RFs used across the distribution of *R. capensis* and ii) represent at least four major biome classifications.

The extracted skulls were scanned at the Micro-Focus X-ray Tomography Facility (MIXRAD) at the South African Nuclear Energy Corporation (Necsa) in Pretoria, South Africa (Hoffman & De Beer 2012). Each skull was scanned using a 0.25 mm aluminium filter at 100 kV and 100 mA while being rotated 360° to produce 1000 X-ray radiographs. The 2D radiographs were transformed to produce raw 3D volume files which were directly imported into AVIZO Fire software (version 8, Visualization Sciences Group, France). Here, the volume rendering tool was used to reconstruct high quality 3D models of the skulls onto which various landmarks were placed. Eighteen homologous 3D landmarks were digitised (Figure 2.2) to encompass the braincase (blue), rostrum (orange) and the nasal chambers (green) – a region of the skull directly involved in echolocation emission and the only part of the vocal tract recognized in the

skull (Hartley & Suthers 1988; Armstrong & Coles 2007). All landmarks were well-defined characters/positions which were clearly and consistently identified on each specimen (Zelditch et al. 2012). Only landmarks positioned on the right half of the skull were digitised to avoid any effects of bilateral asymmetry (Zelditch et al. 2012). All landmarks were digitized by one person (the author), and described in Appendix 1.

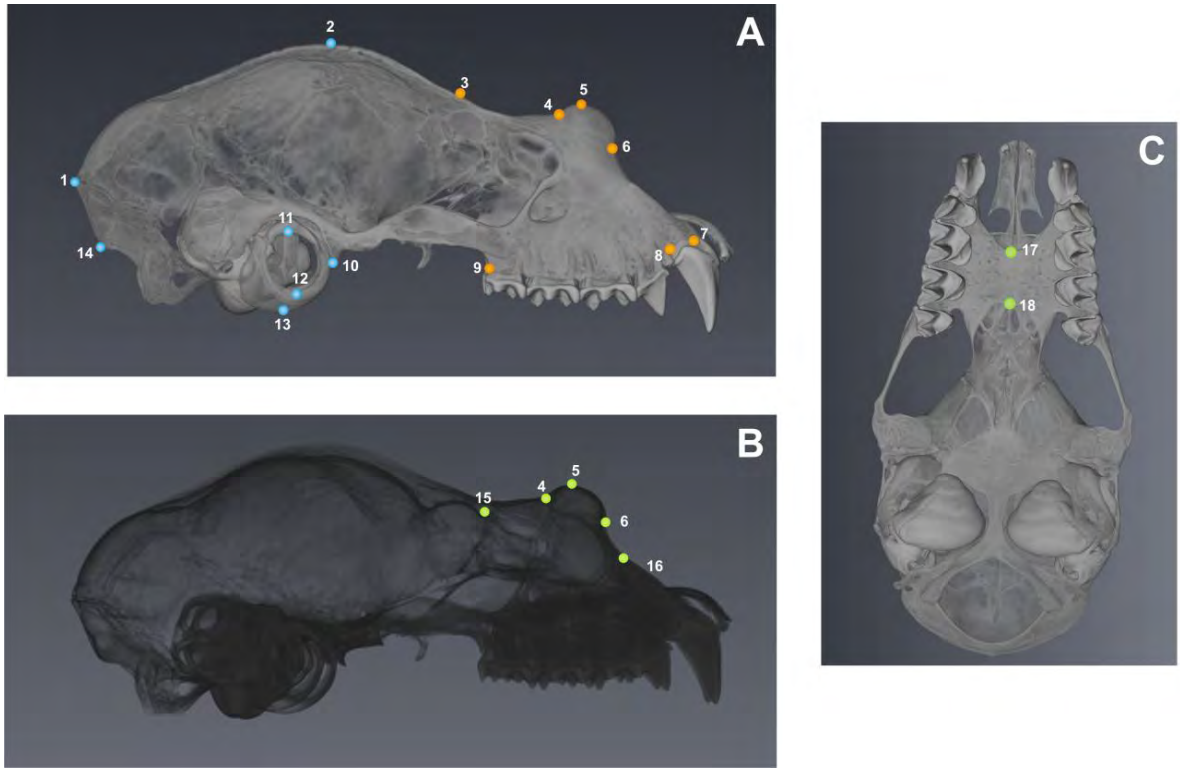

Figure 2.2: Homologous landmarks of the braincase (blue) and rostrum (orange) (A); and the nasal chambers (green) (B and C) of *Rhinolophus capensis* crania. The nasal chamber and rostrum configurations share landmarks 4-6.

Statistical analyses

Several intrinsic (e.g. body size, sex and skull morphological correlates) and extrinsic (e.g. habitat characteristics) factors influence patterns of geographic variation in acoustic signals. To separate the effects of these factors on RF divergence in *R. capensis* required a series of statistical tests. To ensure the data met the assumptions

of subsequent analyses, a Kolmogorov-Smirnov test for normality, Levene's test for homogeneity of variances, and the regression of means against standard deviations were conducted for all grouping levels using STATISTICA (version 10, StatSoft Inc., USA). Where analyses required additional assumptions to be met, these are described in the text.

(i) Exploring the influence of body size, sex and foraging habitat on RF variation

To quantify variation in RF and body size within and between populations the mean ± standard deviation (SD) was calculated for males and females in each population. An equal number of males and females were used to calculate the mean body mass and RF of each population. The coefficient of variation (CV = SD/mean x 100) in RF for each population was also calculated.

A general linear model (GLM) with RF as the dependent variable and population, sex and their interaction as categorical predictors was conducted to determine whether RF differed between locations and sexes in STATISTICA. Next SPSS (version 21, IBM, USA) was used to conduct linear mixed models (LMMs) performed with type III sums of squares to determine the ecological factors which best predict RF in *R. capensis*. Mixed models are robust to unbalanced designs and thus data from single-sex populations could be included (McCulloch & Searle 2001). LMMs also estimate the effects of both fixed and random factors to models of data that are normally distributed (McCulloch & Searle 2001). The GLM results revealed significant differences between populations and sexes, but no significant interaction between them (see results). Therefore, population and sex were included as random factors to control for spatial clustering of samples and intrinsic sexual differences in RF (McCulloch & Searle 2001). Fixed factors included biome category and body mass. Even though the presence or absence of congeneric species may also influence RF variation across different populations of horseshoe bats, it was not possible to explicitly test the Acoustic Communication Hypothesis for several reasons. First, the range of *Rhinolophus darlingi damarensis* (mean RF of 85.1 kHz in the region of overlap: Jacobs et al. 2013), only overlaps with that of *R. capensis* at LS (Figure 1.3; Chapter 1) and therefore its presence cannot be replicated across populations. Second, the presence of *R. d. damarensis* and the Desert

Biome category are collinear and therefore cannot be included in the same model (Freckleton 2011). Lastly, with the exception of the LS site, *R. capensis* co-occurs with a larger congeneric species, *Rhinolophus clivosus*, over much of its range. Five geographical lineages of *R. clivosus* corresponding to previously described sub-species based on genetic, acoustic and morphological differences have been identified, and of these, *R. capensis* overlaps with two lineages which echolocate between 92.2 and 92.5 kHz (Stoffberg et al. 2012; Figure 1.3). At these frequencies the RF of *R. clivosus* is unlikely to influence the RF of *R. capensis* and the presence of *R. clivosus* was subsequently excluded from the analyses.

A model selection approach based on Akaike's information criterion (AIC) (Akaike 1973) was used to determine which model, out of a range of candidate models, best explained RF divergence in *R. capensis* (Burnham & Anderson 2002). The model with the lowest AIC value was considered the most parsimonious and the difference in AIC scores (Δ_i) were calculated to determine the likelihood that a given model was the best model relative to other candidate models (Burnham & Anderson 2002). A Δ_i value of zero indicates the best fit model; values up to two indicate models with significant empirical support; values between four and seven indicate less support and models with values > 10 have essentially no support (Burnham & Anderson 2002). Akaike weights (w_i) were also used to calculate the probability that a given model is the best among a candidate set of models. Thus the best model has the lowest Δ_i and highest w_i (Burnham & Anderson 2002). Once the best-fit model was identified, the effect of each factor level on RF variation in *R. capensis* was estimated using the restricted maximum likelihood method. Heteroskedasticity was checked by performing a scatterplot of predicted residuals against observed residuals in SPSS. To determine the importance of each variable included in the best model, the summed Akaike weight (w_+) for all models containing that particular variable was calculated. The variable with the largest w_+ is likely to be the most important variable in the model (Burnham & Anderson 2004).

Based on the model selection results, the influence of habitat structure (typically vegetation cover and density) and body size on RF variation was further explored. The Normalized Difference Vegetation Index (NDVI) was used as a measure of vegetation

cover. NDVI is a suitable measure because it provides an estimate of above ground primary productivity (Turner et al. 2003) and it has been shown to be associated with a wide range of vegetation properties including photosynthetic activity (Myneni et al. 1995), vegetation cover (Carlson & Ripley 1997; Purevdorj et al. 1998) and vegetation biomass (Borowik et al. 2013). Because of this, NDVI is commonly used to link vegetation dynamics to various aspects of animal ecology (reviewed in Pettorelli et al. 2005, 2011). NDVI is a measure of the density of chlorophyll contained in vegetation and it is calculated as (NIR – RED) / (NIR + RED), where NIR is the near-infrared light, and RED is the visible-red light, reflected by vegetation and captured by the satellite. The values of NDVI range from -1 to 1, where negative values correspond to an absence of vegetation. Green and/or dense vegetation has high RED absorption together with high NIR, leading to high, positive NDVI values. In contrast, sparse vegetation absorbs substantially more NIR, leading to lower NDVI values (Myneni et al. 1995). Bare soils, snow and cloud have NDVI values close to zero (Carlson et al. 1997; Neigh et al. 2008). The Expedited Moderate Resolution Imaging Spectroradiometer (eMODIS) Vegetation Indices dataset from NASA was used and it provides the maximum value for NDVI images for composites over a 10-day period at a resolution of 250 m from the year 2000 to present. Average NDVI values from a 20 km radius around each sampling site were extracted and compiled in ArcGIS 9.3.1 (ESRI®). Because echolocation frequency scales negatively with body size in horseshoe bats (Jones 1997), variation in body size could cause concomitant changes in RF. To test whether RF variation is associated with differences in NDVI while controlling for the potential influence of body size, SPSS was used to conduct a hierarchal multiple regression analysis (HMRA). The first step of a HMRA is to add the independent variable that you wish to control for (in this case, body size). The second step examines the relationship between an independent (NDVI) and dependent (RF) variable while controlling for the effect of the first independent variable. To control for the effect of sex, this analysis was limited to males (n = 153), and the correlations between RF, body mass and NDVI as well as the change in the correlation coefficients between the model including body size and the model including NDVI but controlling for body size, were evaluated.

Differences in humidity between populations of *R. capensis* may also influence RF variation because higher frequency calls are heavily attenuated in humid conditions (Lawrence & Simmons 1982; Hartley 1989). Regression analysis was used to determine whether RF scaled negatively with relative humidity as predicted by the Atmospheric Attenuation Hypothesis (Guillén et al. 2000; Armstrong & Kerry 2011). Relative humidity (%) data for each sampling site were obtained from the literature or from the nearest weather stations and provided by the South African Weather Service.

Like most other rhinolophids, *R. capensis* forage in or near highly cluttered habitats (Csorba et al. 2003; Jacobs et al. 2007). While the long CF portion of their echolocation calls allows for the detection of fluttering prey against structurally complex backgrounds such as dense vegetation (Neuweiler 1989), differences in vegetation cover between sampling sites could still influence the sound transmission properties of the echolocation call frequency used in each habitat. To better understand the effects of vegetation cover on variation in echolocation frequencies the mean detection distances for prey and vertical background targets (e.g. leafy vegetation edge) for each population was calculated according to the method developed in Stilz & Schnitzler (2012). This method depends on the dynamic range and frequency of the sonar system, local atmospheric conditions (including temperature and relative humidity) and target type. The dynamic range was calculated as the difference between peak intensity (dB SPL) at 1m (79.1 dB SPL for *R. capensis* at De Hoop: Jacobs & Parsons, unpublished data) and the auditory threshold of the bat (assumed to be 0 dB SPL for horseshoe bats: Kingston & Rossiter 2004). Because peak intensity data were not available for each population, it was assumed that different populations have similar dynamic ranges. The different prey size categories tested in Stilz & Schnitzler (2012) were derived from Houston et al. (2004) and included small, medium and large categories- all within the size range of prey consumed by *R. capensis*, at least at De Hoop (2mm – 19 mm: Jacobs et al. 2007). Mean minimum temperature (°C) and relative humidity (%) data for each population were obtained from the nearest weather stations and provided by the South African Weather Service.

(ii) Exploring the relationship between skull morphology and resting frequency: a geometric morphometric approach

Geometric morphometrics were used to explore whether RF variation across populations of *R. capensis* is correlated with skull size and shape differences among individuals. To confidently detect the subtle differences in skull size and shape within a species requires extensive sampling to obtain a large enough sample size to ensure robust statistical inference (Cardini & Elton 2007), but due to ethical considerations this was not feasible. Instead, the voucher specimens collected during this study characterised the gradient of increasing RF from west to east across the distribution of *R. capensis* (see results), and specimens from different populations were pooled to represent the four major biome classifications used in the previous LMM analyses, viz. Desert, Succulent Karoo, Fynbos, and Multiple Biomes. Under this sampling regime it was possible to assess patterns of sexual dimorphism in skull size across biome categories, but impossible to explicitly test whether patterns of shape differences between sexes are the same across regions due to the multivariate nature of shape variables and the unbalanced sample size. The analytical approach used was thus limited to exploring general patterns of skull shape variation within *R. capensis*. Data for males and females were pooled in the analyses of shape variation to incorporate all of the observed variation within each biome category. This approach is a compromise between obtaining a dataset in which one can directly match form (skull shape and size) to function (RF) while exploring patterns of morphological variation within a species.

Three different sets of landmark configurations representing i) the nasal chambers (a region of the skull directly involved in echolocation and the only part of the vocal tract recognized in the skull: Armstrong & Coles 2007), ii) the rostrum (excluding the unique nasal chamber landmarks) and iii) the entire skull (including the braincase and rostrum, but excluding the subset of landmarks unique to the nasal chambers) were analysed. This approach was used to evaluate whether patterns of overall skull variation differed from patterns observed in the region of the skull directly related to echolocation, particularly, the nasal chambers as well as allowing a direct comparison between skull features and RF between individuals.

For each dataset the raw landmark coordinates from each landmark configuration were standardized in MorphoJ (version 1.05f: Klingenberg 2011) using a full Procrustes superimposition (Rohlf & Slice 1990; Klingenberg 2011) to mathematically extract size and shape information. Procrustes superimposition removes the effects of position, scaling (size differences) and orientation to generate Procrustes shape coordinates which contain information about the shape of the configuration (Rohlf & Slice 1990; Zelditch et al. 2012). Information about the overall size of the specimen is preserved in the centroid size (CS) (Bookstein 1991; Dryden & Mardia 1998) which is calculated as the square root of the summed squared deviations of landmark configurations from their centroid (Mitteroecker & Gunz 2009). Following superimposition, no outliers (specimens with landmark configurations that strongly deviate from the average shape) were detected and all data were analysed.

The CS of each landmark configuration was compared between sexes and biomes using a 2-way analysis of variance (ANOVA), and differences in CS were visualised using box plots in STATISTICA. The relationships between RF variation and CS of the parts of the skull directly involved in echolocation (nasal chambers embedded in the rostrum) was assessed in a 2-step hierarchal multiple regression model conducted in SPSS. RF was the dependent variable and the CSs of the rostrum and nasal chamber were the covariates entered into the model respectively. Thus the percentage of variance in RF explained by the variables entered in each step after accounting for the influence of variables already entered in the model could be determined.

The nearly linear relationship between size and shape (allometry) may cause substantial size-related allometric effects on total shape variation (Mitteroecker & Bookstein 2007; Klingenberg 2013). The association between size and shape of each landmark configuration was evaluated through multivariate linear regression of the Procrustes shape coordinates onto natural log-transformed CS, and the significance was tested using permutation tests with 10 000 iterations (Mitteroecker & Gunz 2009) in MorphoJ. These tests yielded significant positive correlations between size and shape (see results) for each configuration. Subsequently, the shape variables were size-corrected by computing the shape residuals (e.g. Klingenberg 2009; Kulemeyer et

al. 2009; Mlenković et al. 2010; Evin et al. 2011; Alvarado-Serrano et al. 2013; Santana & Lofgren 2013) which were used in the analyses described below.

A principal component analysis (PCA) calculated from the covariance matrix of the residual shape coordinates was performed in MorphoJ and scatterplots of the main axes of variation across individuals were examined for each configuration. Differences in shapes were measured as Procrustes distances between mean shapes and were visualized using wireframe diagrams (Viscosi & Cardini 2011; Klingenberg 2013). To explore the association between RF divergence and shape, two independent two-block Partial Least Squares analyses (PLS) were performed in MorphoJ. PLS explores the patterns of covariance between two sets of variables by finding linear combinations of variables within blocks that account for as much covariance between sets as possible. The covariation between log RF and the residual shape coordinates of both the rostrum and nasal chamber was calculated, and its statistical significance was evaluated through a randomisation test with 10 000 permutations. The RV coefficient was assessed to determine the overall strength of the covariation between RF and shape of the different landmark configurations. This metric is a multivariate statistic analogue to the squared correlation and ranges from 0 (no covariation) to 1 (complete covariation) (Klingenberg 2011). The RV coefficient is therefore a measure of the strength of association between two datasets.

RESULTS

The influences of body size, sex and foraging ecology on RF variation

The RF of 242 individuals distributed across multiple biomes was measured and a clinal increase in mean RF across the distribution of the species ranging from 75.7 kHz (LS) in the west, to 86.5 kHz (BAV) in the east (Figure 2.3; Table 2.2) was observed. Resting frequencies differed significantly among populations (GLM: $F_{18,486} = 120.5$, $P < 0.001$; Tukey HSD tests: P's < 0.005) with the exception of KNY which used similar frequencies to HDH and DHC (Tukey HSD tests: P's > 0.05; Table 2.2). Sex significantly influenced echolocation variation within populations with females emitting higher frequencies than males (GLM: $F_{3,172} = 33.5$, $P < 0.001$; Table 2.2). However, there was no significant

interaction between sex and population (GLM, $F_{18,486}$ = 1.1, P > 0.05), suggesting that the degree of sexual dimorphism in RF was similar across populations. The CV of call frequencies for each population was low and at most 1% (Table 2.2).

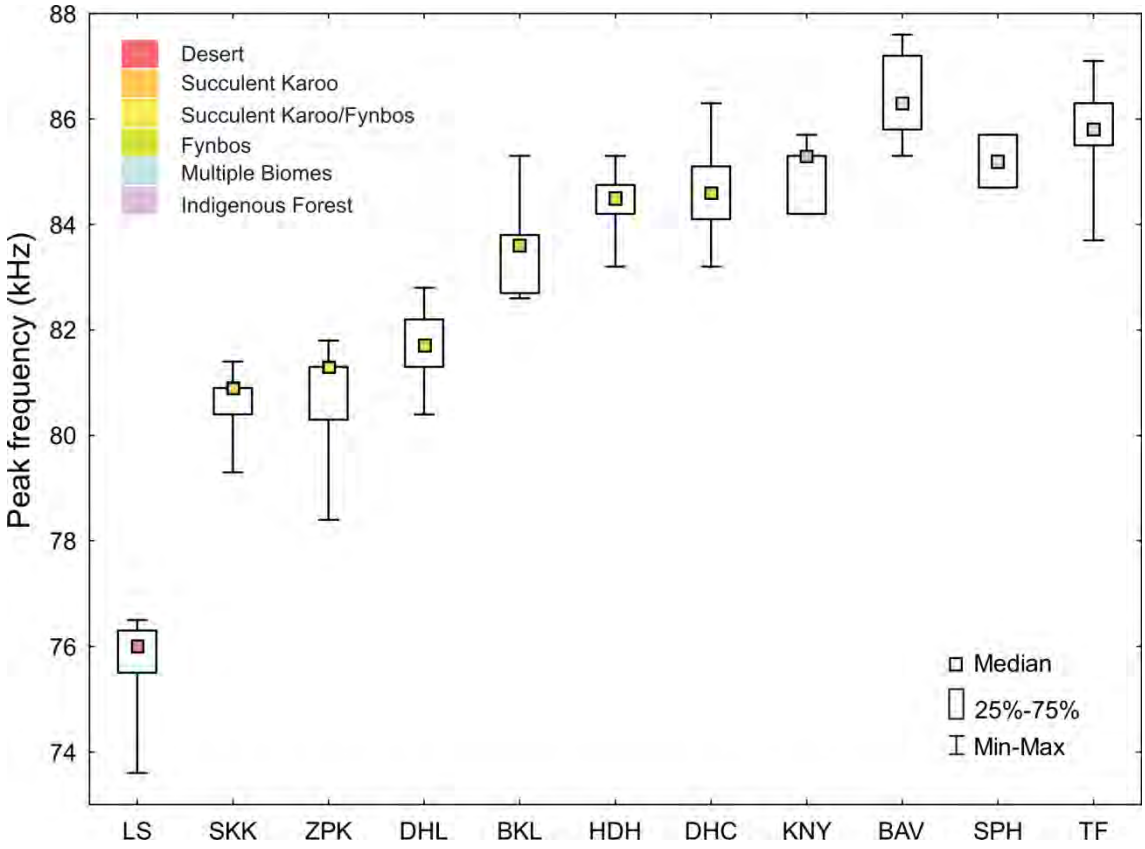

Figure 2.3: Box and whisker plots of median resting frequencies across sampled populations of *Rhinolophus capensis*. Colours represent biome classifications and a key to acronyms is given in Table 2.1. Sampling localities are represented from western to eastern localities.

Table 2.2: Mean (± SD) body mass and resting frequencies for populations and sexes are shown. No females were captured at ZPK and BKL. An equal number of males and females were used to calculate the mean population mass and RF, except at DHL, where the single female captured was included when calculating the population mean. Multiple biomes occur at BAV, TF and SPH (combinations of Albany Thicket, Nama-Karoo, Fynbos and Savanna).

Population	Biome category	Population mass (g) (*n*) (Mean ± SD)	Male mass (g) (*n*) (Mean ± SD)	Female mass (g) (*n*) (Mean ± SD)	Population RF (kHz) (*n*) (Mean ± SD)	CV (%)	Male RF (kHz) (*n*) (Mean ± SD)	Female RF (kHz) (*n*) (Mean ± SD)
LS	Desert	(18) 13.2 ± 1.1	(10) 12.6 ± 0.9	(9) 13.8 ± 0.9	(18) 75.7 ± 0.8	1.06	(10) 75.5 ± 1	(9) 75.9 ± 0.5
SKK	Succulent Karoo	(22) 13.1 ± 0.9	(11) 12.6 ± 0.7	(13) 13.5 ± 0.9	(22) 80.6 ± 0.5	0.62	(11) 80.5 ± 0.6	(13) 80.8 ± 0.3
ZPK	Succulent Karoo/Fynbos	(25) 12.3 ± 0.6	Male only colony		(25) 80.8 ± 0.8	1.08		
DHL	Fynbos	(10) 12 ± 0.45	(10) 12.0 ± 0.4	12.12	(10) 81.5 ± 0.6	0.83	(10) 81.5 ± 0.6	(1) 82.8
BKL	Fynbos	(15) 11.7 ± 0.5	Male only colony		(15) 83.4 ± 0.7	0.90		
HDH	Fynbos	(12) 11.31 ± 0.5	(6) 11.2 ± 0.5	(11) 11.6 ± 0.4	(12) 84.5 ± 0.6	0.67	(6) 84.1 ± 0.5	(11) 84.8 ± 0.6
DHC	Fynbos	(46) 10.4 ± 1.1	(35) 9.6 ± 1	(23) 10.8 ± 1.1	(46) 84.6 ± 0.7	0.90	(35) 84.1 ± 0.65	(23) 85.1 ± 0.6
KNY	Forest	(4) 11.6 ± 1.5	(5) 10.9 ± 0.6	(2) 12.3 ± 1.8	(4) 84.7 ± 0.7	0.7	(5) 84.8 ± 0.6	(2) 85.2 ± 0.7
BAV	Multiple Biomes	(20) 10.8 ± 1.4	(17) 10.2 ± 0.8	(10) 11.5 ± 1.5	(20) 86.5 ± 0.7	0.90	(17) 86 ± 0.9	(10) 87 ± 0.5
SPH	Multiple Biomes	(2) 11.5 ± 0	(1) 11.51	(1) 11.52	(2) 85.2 ± 0.7	0.82	(1) 84.7	(1) 85.7
TF	Multiple Biomes	(36) 13.9 ± 0.8	(19) 13.6 ± 0.7	(18) 14.2 ± 0.9	(36) 85.8 ± 0.75	0.90	(19) 85.4 ± 0.65	(18) 86 ± 0.6

The most parsimonious model explaining variation in RF included the factors biome category ($F_{5, 12}$ = 40.3, P < 0.005) and body mass ($F_{1, 232}$ = 10.4, P < 0.001) of which the former was the most important variable in the model (w_+ biome = 0.99, w_+ body mass = 0.95; Table 2.3). Generally, bats from the Desert Biome use significantly lower frequencies than Succulent Karoo bats (point of reference automatically selected by SPSS). Also, bats inhabiting Forests or regions comprised of multiple biomes use significantly higher frequencies (Table 2.4).

Table 2.3: Model selection results for three candidate models explaining variation in resting frequencies. The most parsimonious model is highlighted in **bold.**

Model	AIC	Δ_i	Weights (w_i)
Biome	540.86	6.14	0.044
Body mass	588.46	53.74	2.04E-12
Biome + body mass	**534.72**	**0**	**0.95**

Table 2.4: Restricted maximum likelihood estimates and confidence intervals for the best-fit linear mixed effects model. The best-fit linear mixed effects model describes resting frequency variation as a function of biome and body mass in *R. capensis*.

Parameter	Estimate (SE)	*df*	t-value	P- value	95% CI
Intercept	80.85 (0.6)	11.8	125.9	<0.001	79.4, 82.3
Biome					
Desert	-4.9 (0.9)	11.7	-5.5	<0.001	-6.9, -2.9
Forest	4.1 (0.9)	13.3	4.4	<0.001	2.1, 6.1
Fynbos	2.8 (0.7)	12.1	3.8	<0.005	1.2, 4.4
Multiple Biomes	5.4 (0.8)	11.5	6.9	<0.001	3.7, 7.1
Succulent Karoo/Fynbos	.09 (1)	11.2	0.08	0.9	-2.3, 2.5
Succulent Karoo[+]	0	0			
Body mass *	-0.17 (0.05)	231.9	-3.2	<0.001	-0.2, -.06

[+]Reference parameter; *Centred variable

In the first stage of the HMRA, body size significantly influenced RF (R^2 = 0.121, $F_{1, 150}$ = 20.6, P < 0.0001) but it only explained 12 % of the variation in RF (Figure 2.4). Bats from LS use relatively lower RFs given their body size when compared to other sampled populations. The inclusion of NDVI in the second stage of the regression model significantly increased the proportion of variance explained in the model (Δ R^2 = 0.68, $F_{1, 149}$ = 528.7.9, P < 0.0001), with NDVI accounting for 80 % of the variation in RF (R^2 = 0.80, $F_{2, 149}$ = 310.9, P < 0.0001) after controlling for the effect of body size (Figure 2.5). The results reported (not shown) where were very similar to an analysis excluding LS samples.

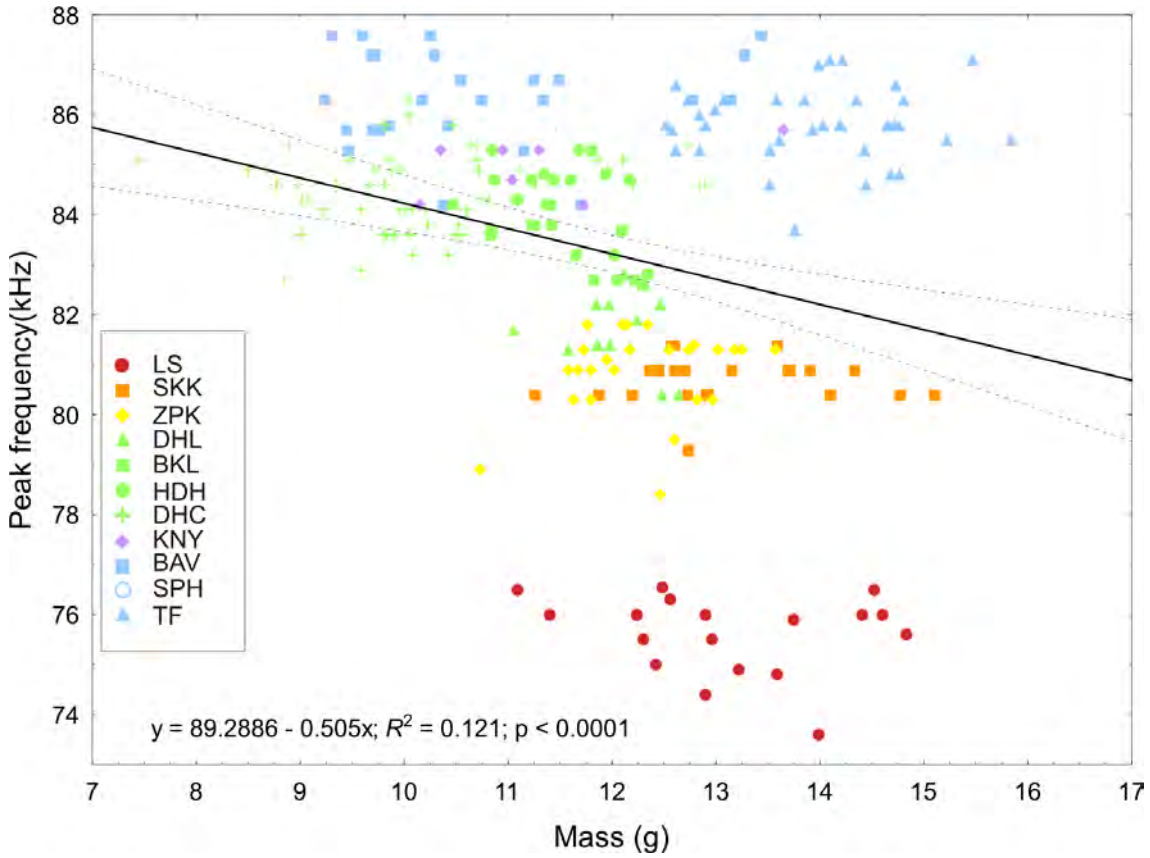

Figure 2.4: The regression of mass (g) on resting frequency (kHz) for male *R. capensis* (n = 153) from 11 populations. Colours represent the biome category of each population and shapes represent different populations. The key to population acronyms are given in Table 2.1.

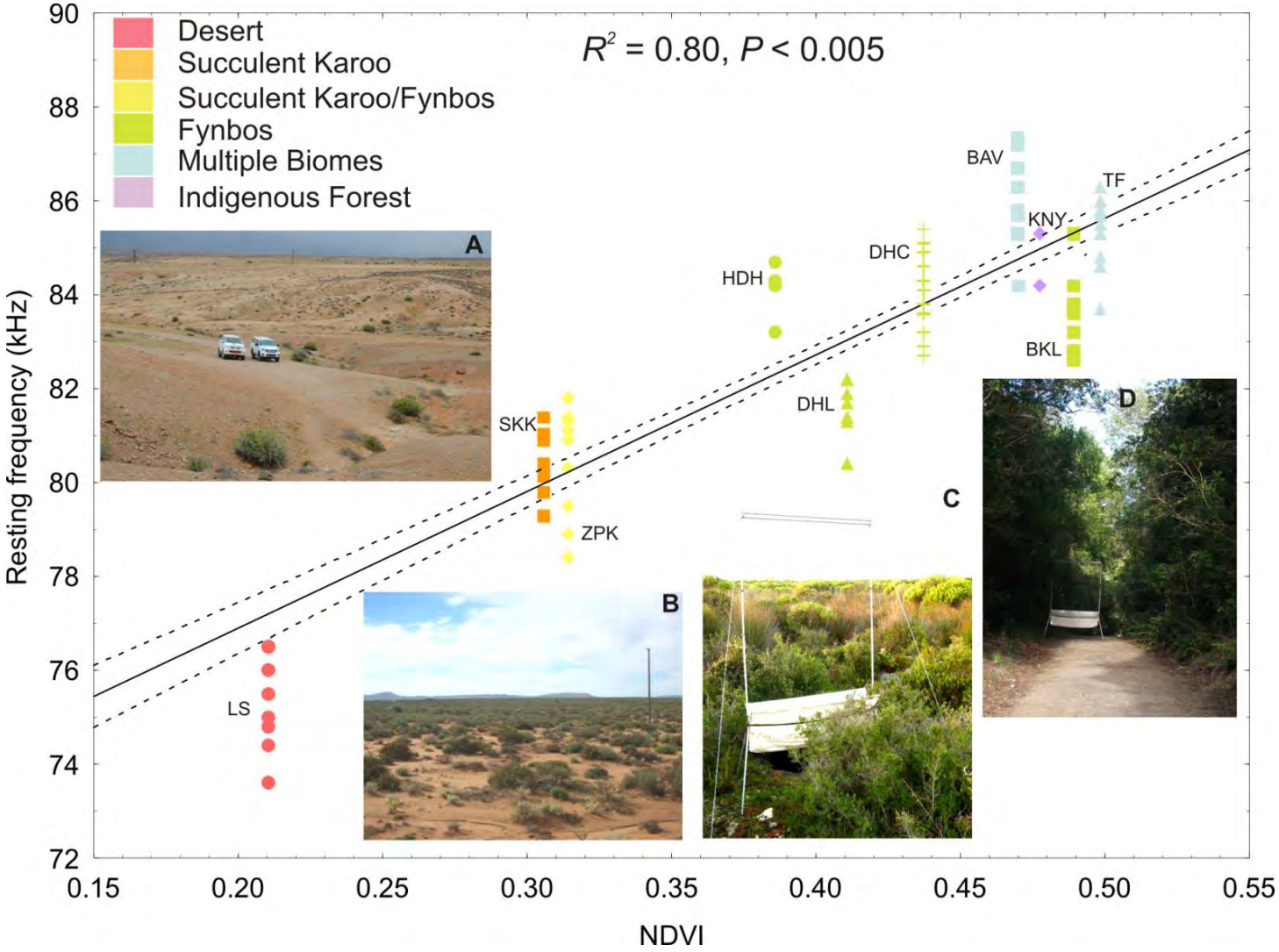

Figure 2.5: The regression of RF (kHz) and NDVI (as a proxy for habitat clutter) across populations of *R. capensis* (males only, *n* = 153). Habitat photographs show the vegetation cover and structure for selected populations, where A: Lekkersing (Desert), B: Steenkampskraal (Succulent Karoo), C: De Hoop (Fynbos), D: Knysna (Forest). A key to acronyms used is given in Table 2.1.

While there was no relationship between relative humidity and RF (R^2 = 0.04, P > 0.5) there were significant differences in the detection distances for large prey and vegetation edge (GLM: $F_{16, 306}$ = 275, P < 0.01; Tukey HSD tests: P's < 0.05), but not for small or medium sized prey (Tukey HSD tests: P's > 0.05) between populations (Table 2.5). There was, however, no difference in estimated detection ranges associated with vegetation or large prey between ZPK, SKK and DHL and between TF and BKL (Tukey HSD tests: P's > 0.05; Table 2.5). Bats inhabiting more open habitats used lower frequencies and had longer detection distances than bats in more cluttered habitats, with LS bats (Desert Biome) having the longest detection distances of both large insects and background vegetation (Table 2.5).

Table 2.5: Climatic variables for each population and the mean estimated detection distances for prey and background vertical targets (leafy vegetation edge). Detection ranges were calculated using the method of Stilz & Schnitzler (2012) (http://www.biosonarlab.unituebingen.de/rangecalculator/index.html). Climatic data were obtained from the nearest weather stations and provided by the South African Weather Service.

Population	Minimum temperature (°C)	Relative humidity (%)	Estimated detection distance (m)			
			Small prey	Medium prey	Large prey	Vegetation edge
LS	12.05	73.31	1.5	2.7	3.7	8.1
SKK	11.24	82.27	1.5	2.6	3.6	7.6
ZPK	11.24	82.27	1.5	2.6	3.6	7.5
DHL	11.36	78.30	1.5	2.6	3.6	7.6
BKL	10.81	85.33	1.4	2.6	3.5	7.3
HDH	13.66	81.95	1.4	2.4	3.3	6.6
DHC	13.49	83.90	1.4	2.4	3.3	6.6
KNY	13.31	83.14	1.4	2.4	3.3	6.6
BAV	12.18	81.19	1.4	2.5	3.4	6.9
SPH	13.11	79.74	1.4	2.5	3.4	6.8
TF	11.20	78.17	1.4	2.6	3.5	7.3

The influence of skull morphology on RF variation

Overall skull, rostrum and nasal chamber sizes were significantly different between sexes (ANOVA: $F_{3,32}$ = 3.27, P < 0.05) and biomes (ANOVA: $F_{9,78}$ = 10.41, P <0.0001), however there was no significant interaction between them (ANOVA: $F_{9,78}$ = 0.75, P > 0.05). Females had significantly larger skulls than males in all three landmark configurations (Tukey HSD test: P < 0.005; Figure 2.6).

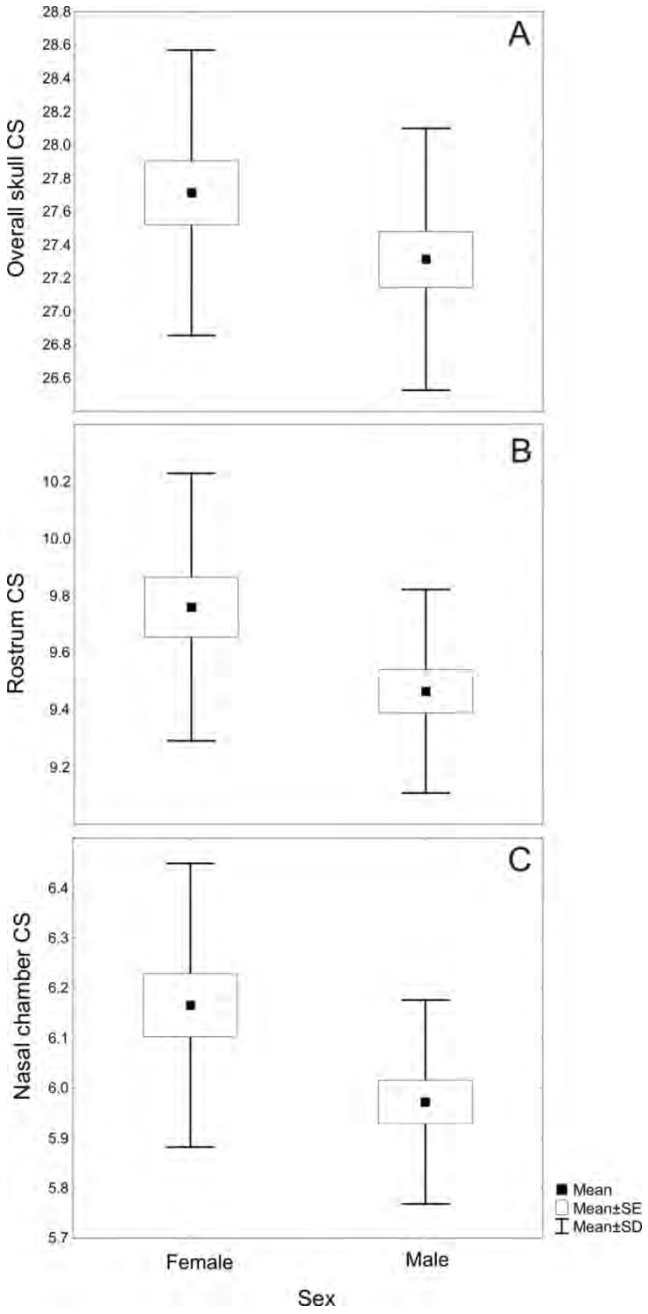

Figure 2.6: Plot of overall skull (A), rostrum (B) and nasal chamber (C) centroid size means, standard deviations, and standard errors between sexes for all bats sampled.

Bats from the Desert Biome had significantly larger CSs than bats inhabiting other biomes (Tukey HSD tests: *P*'s < 0.005), although there was essentially no difference in CSs between bats inhabiting Fynbos and Multiple Biomes (Tukey HSD test: *P* > 0.05; Figure 2.7).

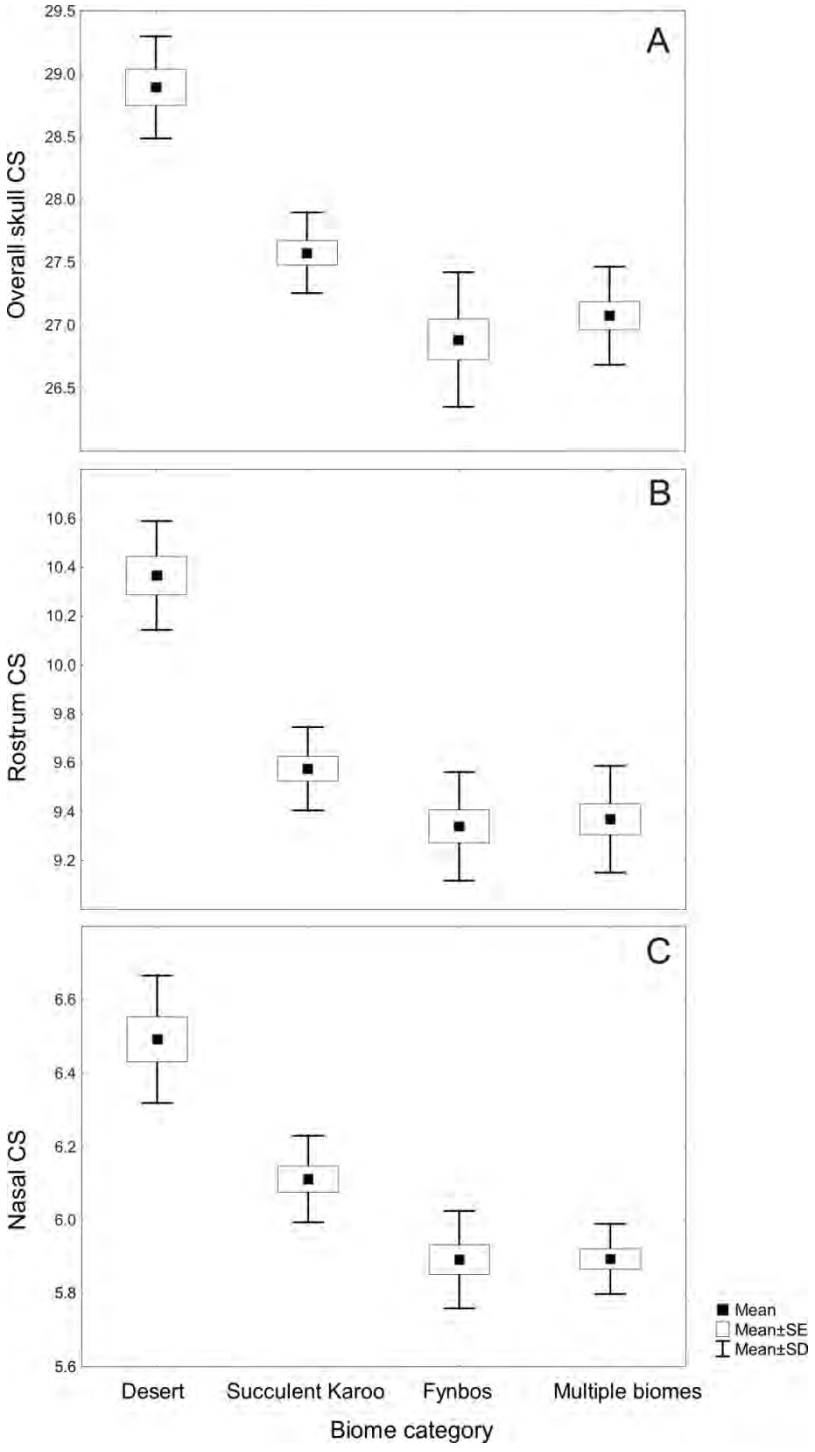

Figure 2.7: Plot of overall skull (A), rostrum (B) and nasal chamber (C) centroid size means, standard deviations, and standard errors between biome categories.

The first step of the HMRA analysis revealed a significant negative correlation between rostrum CS and RF (R^2 = 0.66, $F_{1, 40}$ = 78.8, P < 0.0001) with rostrum CS accounting for 66% of the variation in RF. The inclusion of nasal chamber CS in the second stage of the regression model (while controlling for the effect of rostrum CS) significantly increased the proportion of variance explained in the model, but only by 4 % (ΔR^2 = 0.04, $F_{1,39}$ = 5.23, P < 0.05), with nasal chamber CS accounting for 70% of the variation in RF (R^2 = 0.70, $F_{2,39}$ = 46.2, P < 0.0001; Figure 2.8). Bats inhabiting the more cluttered habitats of Fynbos and ecotones have smaller nasal chambers, and corresponding higher frequencies than Succulent Karoo and Desert bats (Figure 2.8).

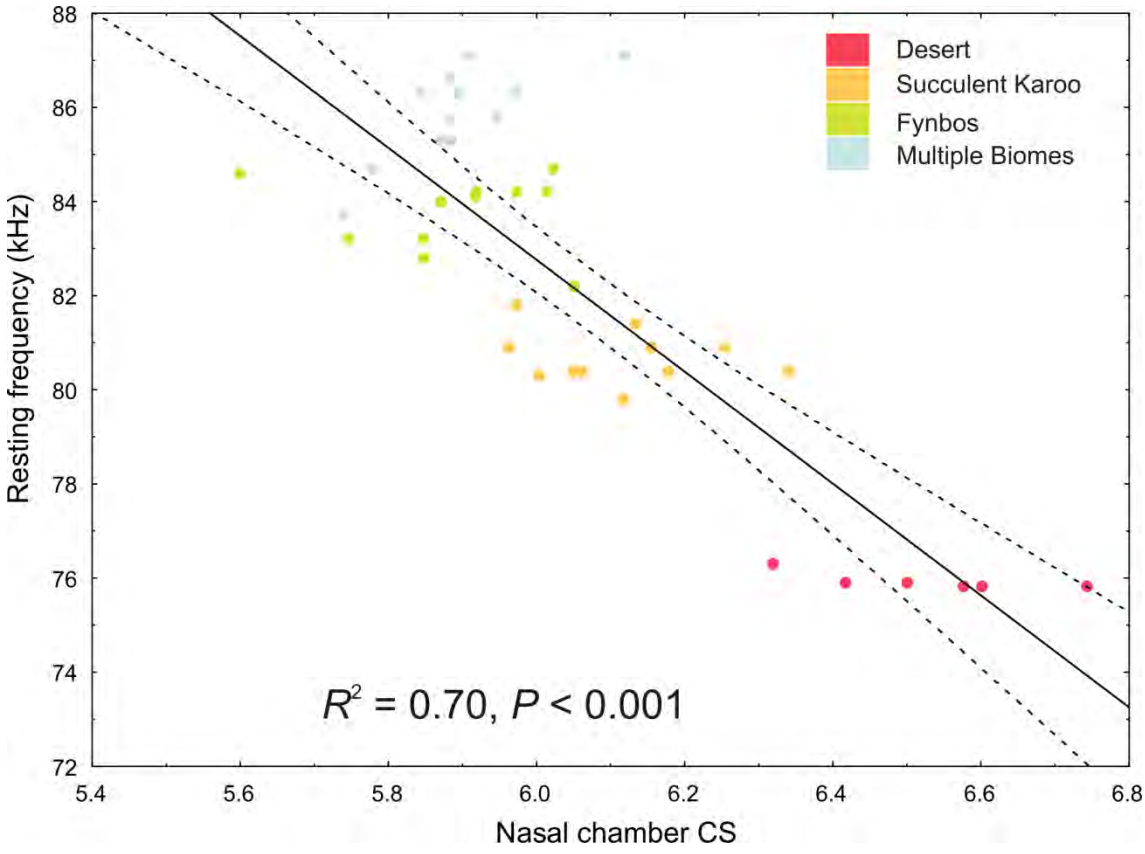

Figure 2.8: The regression of resting frequency (kHz) against nasal chamber centroid size across individuals of *R. capensis*. Each specimen is colour-coded according to the biome category from which it was collected.

Permutation tests revealed that the regressions of shape against log CSs were significant (P's < 0.005) and allometry accounted for 7.43 %, 14.37% and 12.78% for the variation in overall skull, rostrum and nasal chamber shape respectively. The PCA

analyses based on the non-allometric shape components revealed that the first three principal component (PC) axes explained 56.4 %, 60.8% and 57.9% of the total variation in of the overall skull, rostrum and nasal chamber of *R. capensis* respectively, and shape changes along the first two PC axes were visualised using wireframes (Figure 2.9). Generally, the PCA analyses revealed that shape variation in *R. capensis* was not structured according to biomes in either landmark configuration since none of the PC axes clearly separated bats according to the biome they inhabited (Figure 2.9 A – C). However, while rostrum shape variation between individuals inhabiting Succulent Karoo, Fynbos and Multiple Biomes covered the entire spectrum of shape variation along PC1 and PC2, bats inhabiting the Desert Biome were all characterised by having shorter rostra (Figure 2.9 B).

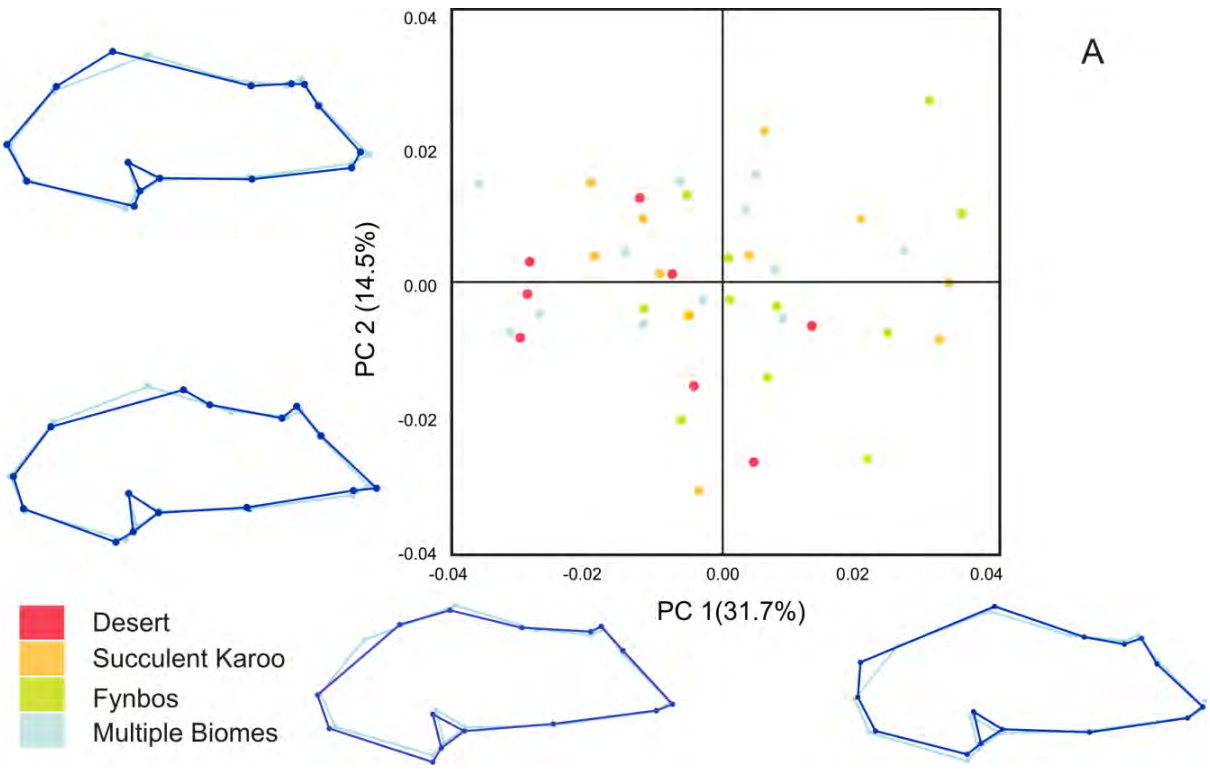

Figure 2.9 A: Scatterplot of residual shape coordinates across *R.capensis* individuals coded by biome for overall skull shape. Wireframe graphs (dark blue outlines) show a change in 0.1 units of Procrustes distance in both the negative and positive directions of the PC with respect to the mean shape (light blue outline).

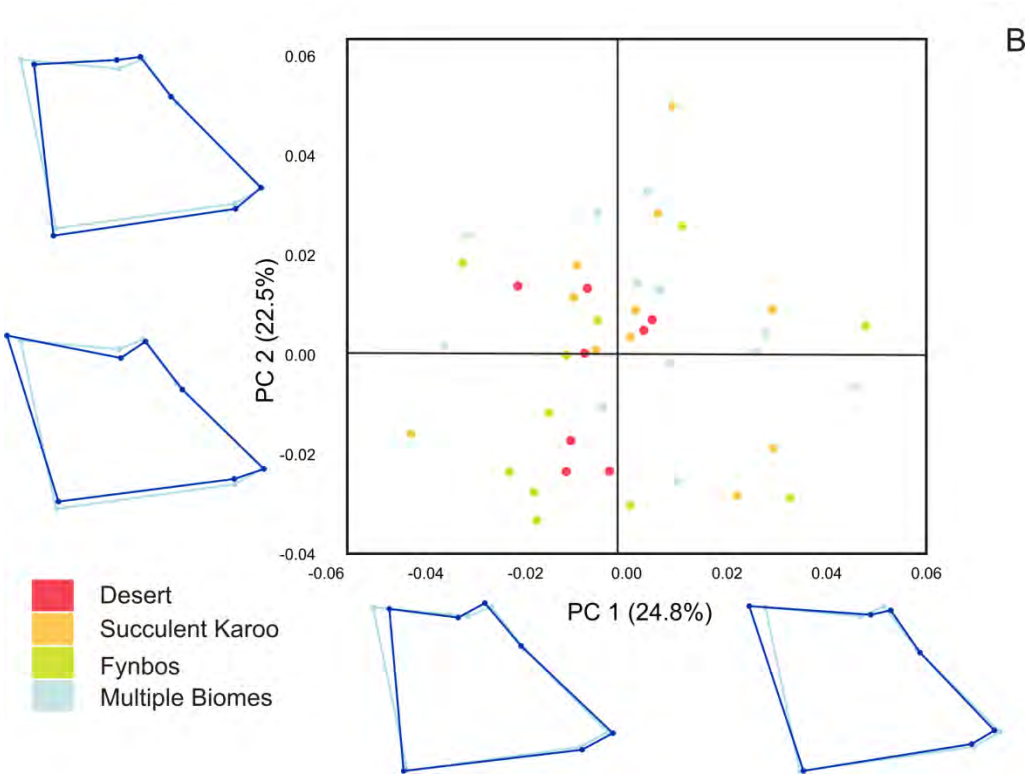

Figure 2.9 B: Scatterplot of residual shape coordinates across *R.capensis* individuals coded by biome for rostrum shape.

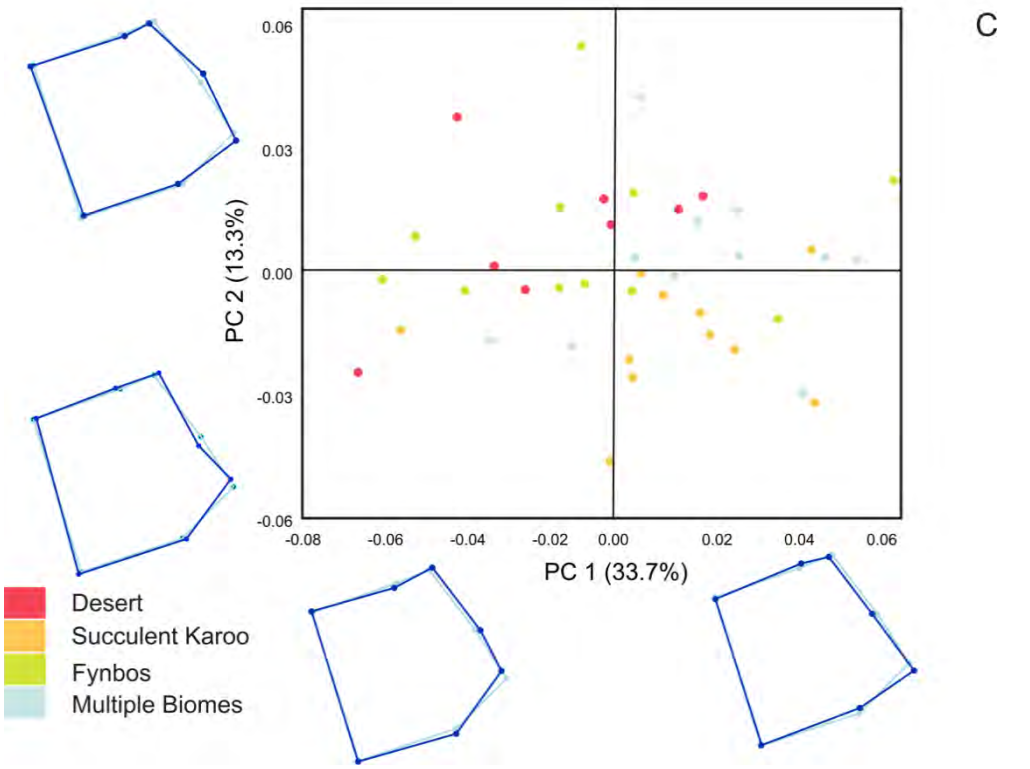

Figure 2.9 C: Scatterplot of residual shape coordinates across *R.capensis* individuals coded by biome for nasal chamber shape.

The PLS analyses indicate that the subtle differences in skull shape among individuals do not covary with RF variation in *R. capensis* for either landmark configuration (Table 2.6). In contrast, when repeating the PLS analyses on non-sized corrected shape coordinates, shape variation in all landmark configurations significantly covaried with RF across individuals with the covariation between nasal chamber shape and RF having the highest RV coefficient (Table 2.6).

Table 2.6: Results of PLS analyses showing the RV coefficients and statistical significance of the covariation between Log RF and the rostrum and nasal chamber shape variation across *R. capensis* individuals.

	Configuration	**RV coefficient**	***P* value**
Size-corrected shape residuals	Rostrum	0.04	0.69
	Nasal chamber	0.10	0.09
Non size-corrected shape coordinates	Rostrum	0.24	0.0002
	Nasal chamber	0.30	0.0001

DISCUSSION

The spatial distribution of variation in RFs of *Rhinolophus capensis* is characterised by a strong gradient of increasing frequency from west to east across the range of the species (Figure 2.3). Although body size plays a minor role in explaining RF variation, biome is identified as the best predictor of RF in this species. In support of this, a significant relationship was found between RF and increasing vegetation clutter from west to east across the distribution of *R. capensis*, and this is reflected in significant differences in the prey and vegetation detection ranges between populations characterised by different degrees of vegetation clutter. Geometric morphometric analyses revealed broad scale differences in skull, rostrum and dorsal nasal chamber size between biomes. However, no such differences were detected in shape,

suggesting that the size of skull features directly related to echolocation may be more important than shape in explaining variation in RF in this species. Results also indicate that RF and the size of dorsal nasal chambers covary with differences between foraging habitats. This gives credence to the hypothesis that the evolution of geographic variation in RF may be shaped by differences in foraging habitat structure, where echolocation frequency covaries with the degree of habitat clutter.

The association between body size and resting frequency

The frequency of acoustic signals scales negatively with body size in several organisms (e.g. Genevois & Bretagnolle 1994; Castellano et al. 2002) including bats (Jones 1997); larger bat species produce echolocation calls of lower frequencies than smaller species. This relationship is also recovered in *R. capensis* with larger bats generally using lower RFs. However, body size only explained a minor proportion of the variation in RF because concomitant changes in body size and RF were not evident across all populations. For example, although BAV bats were not the smallest, they used the highest echolocation frequencies. In contrast, individuals in populations at both ends of the distribution (LS in the west and TF in the east) were similar in size but echolocated at the lowest and second highest frequencies respectively (Table 2.2; Figure 2.4). This may explain why previous research on echolocation variation in the species did not support a relationship between body size and RF, instead suggesting the decoupling of echolocation divergence from the evolution of body size (Odendaal & Jacobs 2011). Undersampling trait variation in species with broad geographic ranges highlights the weakness of inferring evolutionary processes from data sets which inadequately sample the true distribution of a trait.

The results presented here suggest a far more complex scenario than simply the decoupling of two traits in *R. capensis*; rather, the allometric relationship between body size and RF breaks down in populations situated towards the edge of the species' range. This is perhaps not unexpected given that range edges and ecotones provide novel environments to which species can become locally adapted, leading to significant phenotypic divergence of edge populations from those at the centre of the distribution of a species (Smith et al. 1997). It is at the edge of species' distribution

where dynamic selection regimes act most likely to shape variation in unexpected ways (Sexton et al. 2009).

The influence of environmental heterogeneity

Because echolocation in bats is a short range detection system used primarily for prey detection and orientation, local environmental conditions may delimit the acoustic parameter space (termed the 'acoustic window': Wilkins et al. 2013) available in a given habitat. For example, the rate at which sound energy is absorbed in the atmosphere is directly related to air humidity and the frequency of the sound, where higher frequencies are more attenuated in humid environments than lower frequencies (Lawrence & Simmons 1982; Harley 1989; Guillén et al. 2000). Here, the Atmospheric Attenuation Hypothesis, which proposes that divergence in RF is the result of selection against higher frequencies in humid environments (Armstrong & Kerry 2011), does not appear to contribute to geographic variation in RF; despite the clear humidity gradient from west to east. Instead, bats in the Desert Biome use significantly lower frequencies than those occupying Forest and ecotones between multiple biomes (Table 2.4; Figure 2.5) such that the clinal increase in RF across populations may be in response to increasing vegetation cover and density from west to east (Figure 2.5). At LS in the west the vegetation is very sparse, consisting of low shrubs (< 1m in height), whereas the vegetation in the east ranges from dense Fynbos to Forest (Figure 2.5). Indeed, NDVI correlates positively with RF, providing support for habitat clutter as an important explanatory variable of variation in RF.

The mean estimated detection distance for large prey and background vegetation edge was also significantly different between populations; bats occupying more open habitats have lower RFs and longer detection distances than those in more cluttered habitats, allowing them to detect larger prey or background targets at greater distances. While this result was statistically significant, LS bats only had a 10 cm and 50 cm greater detection distance than their nearest neighbours for large prey and vegetation edge, respectively (Table 2.5). Although all horseshoe bats supposedly fly close to vegetation and may therefore experience even relatively open habitats as cluttered, the 50 cm greater detection distances of the vegetation edge may be

advantageous during orientation and commuting flight in the sparse vegetation of LS where the distance between clumps of vegetation are greater than in the Fynbos or the Forest (Figure 2.5). Prey density is also likely to be lower at LS and selection may therefore favour the evolution of lower RFs to allow for the detection of larger prey at greater distances.

There is sufficient empirical evidence to suggest that even rhinolophids, constrained by their echolocation to hunt in narrow space (Schnitzler & Kalko 2001), display some degree of flexibility in the foraging habitat they exploit (Goiti et al. 2003, 2008; Xu et al. 2008; Salsamendi et al. 2012) or the foraging style they adopt (ground gleaning: Siemers & Ivanova 2004; aerial hawking vs. perch hunting: Jones & Rayner 1989; Lee et al. 2012) as a result of resource partitioning (Goiti et al. 2008; Russo et al. 2005), habitat structure (Xu et al. 2008) and seasonal changes in prey resources (Goiti et al. 2008). At least one species appears to vary its echolocation frequency in response to different degrees of clutter. Greater horseshoe bats (*R. ferrumequinum*) foraging in a variety of habitats with differing degrees of clutter were found to use significantly lower echolocation frequencies in relatively open habitats compared to cluttered habitats (Xu et al. 2008). It is likely that *R. capensis* may use both aerial hawking and perch hunting styles to different degrees in the different habitats, perhaps also altering its call frequency to deal with different degrees of clutter.

Although differences in habitat structure provide a compelling explanation for different RFs in *R. capensis*, an interesting anomaly has also emerged; *R. d. damarensis* (forearm length 49.5 ± 1.7 mm; n = 20) is sympatric with *R. capensis* in the extremely arid area of LS but uses frequencies (mean ± SD = 85.4 ± 1.4 kHz in region of overlap; range = 82 – 89 kHz: Jacobs et al. 2013) as high as those used by *R. capensis* in highly cluttered habitats, as for example, at TF. It seems reasonable to assume that in sympatry selection would favour similar call frequencies, and therefore detection distances in these two species, such as occurs in the arid region of LS. However, it is also possible that *R. d. damarensis* exploits a different foraging niche to *R. capensis* at LS. Alternatively, this species may use higher intensity calls to achieve more or less the same detection distances as *R. capensis* (Surlykke & Kalko 2008). Of course it is also possible that the presence of *R. d. damarensis* at LS has selected for the low

frequencies observed in *R. capensis* at this site. The frequencies of bats from the LS population are much lower than in the other arid populations (Figure 2.5) despite similarities in body size (Table 2.2). On the basis of its body size, *R. capensis* at LS should echolocate at approximately 82 kHz instead of the observed mean RF of 75.7 kHz (Figure 2.4). Bats at LS may have shifted their frequency below 82 kHz to avoid acoustic overlap with *R. d. damarensis* for the maintenance of a private frequency bandwidth for effective intraspecific communication. However, the closest known roosts of these two species are 40 km apart and it is not known if their foraging areas overlap, i.e. if they are syntopic, as required by the Acoustic Communication Hypothesis. Furthermore, high duty-cycle bats in species rich communities are often characterised by small differences in echolocation frequency among them (although the entire range of frequencies within the community may be large; e.g. Heller & von Helversen 1989) and even within the same community horseshoe bats are able to discriminate between conspecifics emitting echolocation calls with overlapping frequencies (e.g. European horseshoe bats, *R. euryale*, *R. mehelyi* and *R. hipposideros*: Schuchmann & Siemers 2010a). Given that *R. capensis* and *R. d. damarensis* are the only two horseshoe bat species which co-occur around LS, the 10 kHz difference in frequency between them appears extreme, especially since even a 5 kHz shift would result in sufficient sonar partitioning for the maintenance of private frequency bands (Russo et al. 2007; Schuchmann & Siemers 2010a). It is therefore more likely that lower call frequencies affords LS bats with a greater detection distance in the stark habitat which characterises LS because if LS bats were to echolocate at the frequency dictated by their body size, they would have the same detection distance as SKK bats (7.6 m for vegetation edge).

Covariation between skull morphology and resting frequency divergence

Although cranial size and body mass are both considered good descriptors of body size, they may have evolved under different selective pressures and therefore are likely to respond differently to intrinsic and extrinsic factors (Tomassini et al. 2014). For example, in bats body size is closely associated with the aerodynamics of flight, roosting ecology, reproductive behaviour and physiology (Barclay & Brigham 1991; Fleming 1991; Swartz et al. 2003; Avila-Flores & Medellín 2004) and various factors can

therefore influence its evolution. Conversely, the cranium of bats functions as an efficient transmitter and receiver of echolocation calls (Pedersen 1993), as well as being co-opted for trophic specialisations (Freeman 1981). Female *R. capensis* are characterised by larger skull dimensions than males (Figure 2.6), and this may be due to the allometric scaling between body size and cranial size rather than RF differences between sexes. At the population level however, large scale environmental differences between biomes may drive the evolution of skull size variation in *R. capensis*. Although body mass accounted for a significant (but small) proportion of the observed variation in RF, it is not surprising that a much stronger correlation was found between RF variation and the size of dorsal nasal chambers across individuals of *R. capensis*; bats with larger dorsal nasal chambers emit significantly lower RFs (Figure 2.8). The spatial distribution of variation in the CS of all three landmark configurations (overall skull, rostrum and dorsal nasal chamber) reflects the pattern of increasing RF from west to east across biomes (Figure 2.7). However, the significantly larger skull dimensions of bats inhabiting arid regions is unlikely due to allometric scaling with body mass because Desert and Succulent Karoo bats were similar in body mass to bats from ecotone populations (Table 2.2). This, together with the close association between RF and dorsal nasal chamber size in particular, further support the hypothesis that habitat-related differences between biomes may drive the evolution of RF variation in *R. capensis*. In contrast, the subtle differences in the size-corrected shape of the three landmark configurations were not related to broad scale differences between habitats (Figure 2.9). Also, differences in rostrum and dorsal nasal chamber shape did not covary with RF variation, and therefore skull shape variation may not be functionally relevant to the evolution of variation in echolocation within *R. capensis*. This may be because size is often considered more evolutionary labile than shape (Stanley 1979, 1985) because changes in size can occur over short time scales (over a few generations) as a result of spatially and temporally varying selection pressures (Cardini et al. 2013; Tomassini et al. 2014). Studies have reported differences in the rates of evolution between size and shape for various organisms (Marroig & Cheverud 2005, 2010; Hunt 2007; Pie & Tschá 2013) including bats (Dzeverin 2008) and conclude that shape is often constrained by strong stabilising selection whereas size is driven by directional selection and thus evolutionary change is more likely to occur along a size-

axis (Marroig & Cheverud 2005, 2010). In bats, empirical evidence supports that cranial size can respond to selection over very short time frames; in response to strong directional selection cranial size in *Pipistrellus kuhlii* has significantly increased over a period of 133 years, allowing bats to exploit new and profitable prey sources in urban environments (Tomassini et al. 2014). Thus, the close association between cranial morphology, bite force and feeding ecology in bats (Freeman 1981, 1984; Aguirre et al. 2002; Dumont et al. 2009; Nogueira et al. 2009; Santana et al. 2010; Santana et al. 2012) may also shape variation in skull size between populations of *R. capensis*. For example, the much larger skull size of arid bats (but most notably LS bats) may be the result of consuming harder-bodied prey than their counterparts inhabiting Fynbos and Multiple Biomes. Arid adapted insects are usually characterised by harder cuticles to minimise desiccation. As an adaptation to consuming harder prey items larger skulls and shorter rostrums might be expected to characterise bats inhabiting arid regions, producing a relatively higher bite force (Aguirre et al. 2002; Dumont et al. 2009). Since the differences in wavelength (and therefore minimum detectable prey size) between populations situated in Multiple Biomes (87 kHz) and Desert (75 kHz) are small (0.6 mm), arid bats are not necessarily constrained to consuming only large prey. Indeed, the maximum detection distances calculated for small and medium sized prey were similar across populations. Also, given that arid regions are often characterised by patchy prey distributions, it is unlikely that in arid regions *R. capensis* would selectively choose only larger insect prey. Swartz et al. (2003) argued that bite force in bats is influenced by absolute skull size rather than by specific traits of the skull because larger skulls have larger mastoid muscles which increases the cross sectional area of muscles, resulting in a higher bite force. An in-depth experimental approach is required to determine whether selection has acted on skull and rostrum size to allow arid bats to consume harder bodied prey with greater efficiency, thereby influencing the dimensions of the dorsal nasal chambers, and ultimately resulting in the much lower RFs of arid bats. This is likely given that the skull of horseshoe bats does not deviate from the mammalian norm of two integrated and evolutionary stable cranial modules (crania and rostrum), despite the novel function of the dorsal nasal chambers in nasal echolocation (Santana & Lofgren 2013).

Ecologically adaptive traits can also promote divergence if divergence has a pleiotropic effect on reproductive isolation via assortative mating; so called 'magic traits' (Wilkins et al. 2013). In *R. philippinensis* assortative mating has evolved between size morphs as a by-product of selection for different frequencies used to exploit different prey sizes (Kingston & Rossiter 2004). The significant and consistent sexual dimorphism in the RFs observed in *R. capensis* (female's echolocate at higher frequencies than males: Table 2.2), may indicate that RF may serve a role in sex-specific communication in the species. Recent experimental evidence reveals that horseshoe bats (*R. euryale* and *R. mehelyi*: Schuchmann et al. 2012) are not only able to recognise the sex of conspecifics based on their echolocation calls, but also that echolocation may play an important role in female mate choice (Puechmaille et al. 2014). Thus, it is possible that LS bats may not be able to effectively recognise other *R. capensis* as potential mates, which could reduce gene flow between LS and other populations.

Conclusions

While RF scales negatively with body size in *R. capensis*, differences in habitat complexity across the distribution of the species appears to be the dominant driver of sensory divergence in this system. The clinal increase in RF from west to east reflects the distinct habitat gradient of increasing vegetation clutter which characterises the distribution of this species. Furthermore, RF and the size of skull features directly related to echolocation covaried with differences between habitats and suggest that selection for lower echolocation frequencies in less cluttered habitats and/or selection for consuming hard-bodied prey may drive the evolution of RF divergence in this species. If so, adaptive divergence in RF may result in reduced gene flow between acoustically divergent populations.

CHAPTER 3

EXPLORING SENSORY TRAIT VARIATION IN THE CAPE HORSESHOE BAT WITHIN A PHYLOGEOGRAPHIC FRAMEWORK

INTRODUCTION

Intraspecific genetic variation is shaped by historic and contemporary demographic processes such as dispersal, sex-biased behaviour, population size changes and mating systems (e.g. Hailer et al. 2007; Barrientos et al. 2009; Watanabe et al. 2010; Masello et al. 2011; Rossiter et al. 2012; Lin et al. 2014), as well as by the interplay between evolutionary processes such as gene flow, genetic drift and selection (Boughman 2002; Michell-Olds et al. 2007; Via 2009). Deciphering how these processes shape the spatial and temporal distribution of genetic variation within and among closely related species is a fundamental goal of the field of phylogeography (Avise et al. 1987; Avise 2000, 2009; Beheregaray 2008; Knowles 2009).

The discipline was first developed by Avise and colleagues (1987) as a means to integrate the methods of phylogenetics and population genetics, and in recent years significant advances in population genetic and statistical methodologies (reviewed in: Knowles 2004, 2009; Templeton 2004; Kidd & Richie 2006; Edwards 2009; Nielsen & Beaumont 2009; François & Durand 2010; Garrick et al. 2010; Chan et al. 2011) has led to the rapid expansion of the field, subsequently enabling robust hypothesis testing in ecology and evolution. For example, current phylogeographic studies employ a wide range of molecular, ecological, climatological and geospatial tools to make meaningful inferences about (i) the underlying historical processes responsible for the origin, distribution and maintenance of biodiversity (Clare 2011; González et al. 2011; Willows-Munro & Matthee 2011), (ii) the evolution of reproductive isolation (Racey et al. 2007; Kirschel et al. 2011), and (iii) the factors shaping broad-scale biogeographic patterns of co-distributed species (Burney & Brumfield 2009; McGovern et al. 2010; Tobias et al. 2010; Gonzalez et al. 2014).

Using aspects of population genetic theory and phylogeography has provided important insights into not only the relative roles of genetic drift and selection in the

evolution of population differentiation (e.g. Antoniazza et al. 2010; Tobias et al. 2010; González et al. 2011; Ng & Glor 2011; Puechmaille et al. 2011), but also into how divergence might proceed in the face of gene flow (Knowles 2009). Although a number of recent empirical studies indicate that adaptive divergence even with gene flow is more common than initially thought (e.g. Niemiller et al. 2008; Drovetski et al. 2009; Milá et al. 2009; Ballentine & Greenburg 2010; Richter-Biox et al. 2010, Ribeiro et al. 2012; Frédérich et al. 2012; Galligan et al. 2012; Morgans et al. 2014), it remains the subject of much debate (Hey 2006). The main challenge is that commonly employed neutral genes used to infer population structure do not encode the traits under selection. As a result, while divergent selection may act against maladaptive alleles, immigrant neutral alleles can become established in the population (Hey 2006; Nosil et al. 2009). Still, selection can also cause genome-wide variation in allele frequencies if it promotes reproductive isolation (Nosil et al. 2009; Freeland et al. 2010). Under this scenario it is reasonable to predict adaptive and neutral loci would covary among populations and this can be explored by assessing trait divergence (as a proxy for adaptive genetic divergence) in comparison to neutral genetic variation measured directly from neutral genetic markers (Galligan et al. 2012). If the trait evolves neutrally, a strong positive correlation would suggest a role for random genetic drift and a pattern of isolation-by-distance is expected. Discordance between adaptive trait divergence and neutral genetic divergence may be evidence for divergent selection in the presence of gene flow (Galligan et al. 2012). Furthermore, a strong positive correlation independent of geographic distance may represent ecological divergence and a pattern of isolation-by-adaptation may be expected (Nosil 2008; Nosil et al. 2009; Edelaar et al. 2012).

Relationship between population structure and acoustic divergence

Such holistic approaches as demonstrated above have been effective in investigating the causes and consequences of divergence in sensory traits (Wang & Summers 2010; Muñoz et al. 2013), particularly in the acoustic signals animals use for communication, resource use and mate choice (Wilkins et al. 2013). Geographic variation in acoustic signals and their associated sensory systems often plays a role in sexual selection and

may promote population divergence and speciation (Boughman 2002; Wilkins et al. 2013). Acoustic divergence may also be influenced by cultural factors such as the mode and degree of cultural transmission of acoustic signals, which further confound the relationship between gene flow, divergent selection and genetic drift (Slabbekoorn & Smith 2002; Yoshino et al. 2008). In birds, studies have found little correlation between acoustic and genetic differentiation (e.g. Wright & Wilkinson 2001; Soha et al. 2004; Saranathan et al. 2007; Leader et al. 2008), highlighting the influence of horizontal transmission of signals after dispersal via immigrant males learning local song variants from conspecifics (Slabbekoorn & Smith 2002). In contrast, acoustic signals in some bats (Jones & Ransome 1993; Esser & Schubert 1998) and marine mammals (Rendell & Whitehead 2002; Yurk et al. 2002) are transmitted vertically from mother to offspring (but see Deecke et al. 2000). Acoustic similarity therefore indicates common ancestry in matrilineal lineages, such that divergence in the trait should reflect genetic structure (Chen et al. 2009).

While birdsong and vocalisations emitted by anurans and insects are purely used for communication (Gerhardt & Huber 2002; Podos & Warren 2007), bat echolocation calls function in both foraging and communication (Fenton 1985). Their gregarious and nocturnal lifestyle often makes bats difficult to study by direct observation (Burland & Worthington Wilmer 2001). An increasing number of researchers are therefore employing molecular genetics and phylogeographic tools when studying bat populations, yielding extraordinary insights into various aspects of their biology; these include the genetic consequences of: migration and dispersal (Dechmann et al. 2007; Bryja et al. 2009; Chen et al. 2010; Rodrigues et al. 2010; Burns & Broders 2014; reviewed in Moussy et al. 2012), habitat fragmentation (Campbell et al. 2009; Meyer et al. 2009; Struebig et al. 2011), the roles of social systems (Rossiter et al. 2012), colonisation histories (Rebelo et al. 2012; Dool et al. 2013; Lin et al. 2014), foraging ecology (Clare et al. 2014; Sedlock et al. 2014) and the evolution of cryptic species diversity (Taylor et al. 2009, 2012; Chattopadhyay et al. 2012; Murray et al. 2012; Clare et al. 2013). These approaches have rarely been applied to reveal the historical processes that likely shape contemporary patterns of echolocation divergence, even though geographic variation in echolocation call characteristics (most notably in

echolocation frequency) appears to be relatively common (e.g. Barclay et al. 1999; Armstrong & Coles 2007; Gillam & McCracken 2007; Yoshino et al. 2008; Chen et al. 2009; Puechmaille et al. 2011). Indeed, the potential contribution of neutral processes is rarely considered in most studies of acoustic variation even though genetic drift is the fundamental alternative explanation to adaptation (Campbell et al. 2010).

Population structure and echolocation divergence in horseshoe bats

Neutral evolutionary processes have recently become a focus of research centred on understanding the factors that shape patterns of acoustic population structure in high duty-cycle bats (e.g. Old World leaf-nosed bats: Kingston et al. 2001; Thabah et al. 2006, New World Parnell's moustached bat: Clare et al. 2013, Old World horseshoe bats: Yoshino et al. 2008; Chen et al. 2009; Stoffberg et al. 2012; Sun et al. 2013). Although these studies report strong associations between acoustic and genetic population structure, they vary in how demographic and evolutionary processes interact to produce a given pattern of acoustic divergence. For example, in horseshoe bats the species-specific echolocation frequency is genetically determined (Rübsamen 1987), but the final RF of young horseshoe bats is also partly influenced by the frequency of its mother (Matsumura 1979). The final frequency may therefore have a learned component as a result of mother-to-offspring transmission during postnatal development (Jones & Ransome 1993). Furthermore, investigations of dispersal behaviour in horseshoe bats suggest that dispersal is generally characterised by female philopatry and male-biased dispersal, resulting in strong genetic structure in maternally inherited markers but weaker structure in bi-parentally inherited markers (Chen et al. 2008; Yoshino et al. 2008; Flanders et al. 2009; Mao et al. 2010). The maternal transmission hypothesis is proposed to explain regional divergence in peak frequency in *Rhinolophus cornutus pumilus* in Taiwan (Yoshino et al. 2008). Because of the limited dispersal of females, echolocation differences may have resulted from random genetic drift and maintained by mother-to-offspring transmission of peak frequency because of the limited dispersal of females i.e. female philopatry (Yoshino et al. 2008). Similarly, echolocation differences and neutral genetic divergence covary with geographic distance in the Formosan lesser horseshoe bat, *Rhinolophus*

monoceros, suggesting that population divergence may have arisen by maternal transmission followed by genetic drift or selection (Chen et al. 2009). In contrast Sun et al. (2013) argue that some degree of cultural drift and ecological selection best explain patterns of RF divergence in Greater horseshoe bats, *Rhinolophus ferrumequinum*, in China. Correlated differences in echolocation frequency, morphology and population genetic structure characterise regional lineages of *Rhinolophus clivosus* in southern Africa, which may have evolved in response to climate-driven vegetation changes (Stoffberg et al. 2012).

Exploring RF divergence within a neutral evolutionary framework in the Cape horseshoe bat

In this chapter echolocation divergence in *R. capensis* is explored within a neutral evolutionary framework. The main aim is to quantify the extent to which population genetic structure and gene flow contribute to the evolution of echolocation variation in this species. RF variation in *R. capensis* is characterised by an increase in frequency from xeric to mesic habitats, and sensory variation appears to be shaped by both ecological (habitat structure) and morphological factors (body size and skull morphology) (Chapter 2), suggesting that selection for lower frequencies in xeric habitats may contribute to RF variation in this species. The covariation between biomes and the size of skull features directly related to echolocation further suggest that selection may have acted directly on RF in *R. capensis* (Chapter 2). If so, adaptive divergence in RF may result in restricted gene flow between populations such that acoustic differences are reflected in genetically structured populations. Using data from the mitochondrial D-loop, the hypothesis that selection on RF has resulted in reduced gene flow is tested by quantifying the degree of historical gene flow among acoustically divergent populations. The distribution of mtDNA haplotypes and RF variation is explored within a spatially explicit phylogeographic framework to also test a prediction of the maternal transmission hypothesis; i.e. due to the vertical transmission of echolocation frequencies from mother-to-offspring in horseshoe bats (Yoshino et al. 2008), there should be a positive correlation between maternal population structure and RF variation. Lastly, to determine whether genetic drift also

influences RF variation, the correlations between neutral population structure, RF divergence and geographic distance are also explored.

MATERIALS AND METHODS

Field collection of tissue samples

Tissue samples for DNA extraction and sequencing were collected from 203 individuals captured from 11 populations after their echolocation calls were recorded. Biopsy punches (3mm) were taken from the wing or tail membrane (Worthington & Barratt 1996). Membranes were illuminated to ensure that no blood vessels were ruptured during sampling and tissues were stored in molecular grade (99%) ethanol at room temperature in the field, and at 4°C until extraction.

Mitochondrial DNA extraction and amplification by PCR

Total genomic DNA was extracted using standard protocols of the Qiagen DNeasy Blood and Tissue Kit. A 519 base pair (bp) region of the hypervariable mitochondrial control region (D-loop) was amplified using polymerase chain reaction (PCR) with the primers N777 (5'TACACTGGTCTTGTAAAACC) and E (3' CCTGAAGTAGGAACCAGATG) from Hoelzel et al. (1991) and Wilkinson & Chapman (1991) respectively. PCR conditions consisted of an initial cycle of 94°C for 5 minutes, followed by 35 cycles of 94°C, 50 – 55°C and 72°C each for 30 seconds and a final step of 72°C for 7 minutes. All PCR reactions included a negative control consisting of all reagents except DNA to check for contamination. PCR products including the negative control and a positive control (PCR product of a previous successful amplification) were separated by electrophoresis in a 1% agrose gel with ethidium bromide and gel purified using a Wizard SV Gel and PCR Clean-up System (Promega). Samples were sequenced in both directions using BigDye 3.1 chemistry on an ABI 3730 XL DNA Analyser (Applied Biosystems) at the Central Analytical Facility at Stellenbosch University, South Africa. Chromatograms were edited and aligned using BioEdit version 7.1.3.0 (Hall 1999).

Data analysis

Geographic variation in phenotypic traits may be a consequence of neutral evolutionary processes, particularly when dispersal distances result in a pattern of predominantly nearest-neighbour gene exchange (Petren et al. 2005; Meyer et al. 2009). To better understand the role of random genetic drift in the evolution of RF divergence in *R. capensis* a number of statistical approaches were used to (i) understand the evolutionary relationships among maternal lineages in a spatial context, (ii) determine the degree to which genetic variation is spatially structured and (iii) quantify levels of historic gene flow among populations.

(i) Estimates of genetic diversity

The number of haplotypes, haplotype diversity (*Hd*), nucleotide diversity (π) (Nei 1987) and number of polymorphic sites were calculated for each population using DnaSP version 5.10.01 (Librado & Rozas 2009). Haplotype frequencies were mapped across sampling localities to visualise the spatial distribution of haplotypes.

(ii) Evolutionary relationships among maternal lineages of *R. capensis*

A phylogenetic network approach (Huson & Bryant 2006; Huson & Scornavacca 2010) was used to explore the evolutionary relationships among haplotypes and determine whether observed relationships reflect either the geographic sampling of populations or specific biome discontinuities across the species' range. Networks allow reticulations among branches, instead of imposing a strictly bifurcating tree-like structure on the evolutionary history of lineages (Cassens et al. 2003). Conflicting evolutionary patterns cannot be visualised using the phylogenetic tree approach because only the predominant pattern is displayed on the tree, i.e., there is only one path connecting any two taxa. Population-level processes like gene flow, inbreeding, recombination and lineage sorting, can however result in reticulate genealogies (Morrison 2005). Consequently networks are more informative at the population-level because they display multiple pathways connecting lineages (Morrison 2005). A Neighbour-Net network was constructed in SplitsTree version 4.12.6 (Huson & Bryant 2006) using uncorrected '*p*' distances. Neighbour-Net is a distance based method and

has its foundations in the Neighbour-Joining algorithm. It produces a splits graph which is more robust than splits-decomposition methods because it shows the complexity of the inter-relationships among populations (Bryant & Moulton 2004; Morrison 2005).

(iii) Spatial genetic structure

To assess whether significant genetic differentiation occurs among populations and biomes an Analysis of Molecular Variance (AMOVA) was performed in GenAlEx version 6.5 (Peakall & Smouse 2012). Significance was estimated at the 0.05% level with 1000 random permutations. AMOVA allows the hierarchal partitioning of genetic variation within populations, among populations and among regions (in this study, biomes) by calculating Φ_{ST}, a measure of population genetic differentiation analogous to the fixation index F_{ST} (Wright 1965; Excoffier et al. 1992). Pairwise population comparisons of Φ_{ST} are presented with probability values based on 99 permutations. The fixation index ranges from 0 (no genetic differentiation) to 1, although F_{ST} is rarely higher than 0.5, even in highly structured populations (Wright 1965).

To further explore whether maternal lineages were geographically structured the spatially explicit Bayesian clustering mixture model for DNA sequence data in BAPS version 6.0 (Cheng et al. 2013) was used to determine the most probable number of genetic clusters among *R. capensis* populations. Bayesian clustering models which incorporate spatial information like GPS coordinates of sampling localities, have been shown to perform better than non-spatial clustering models in several studies (e.g. François & Durand 2010; McKay et al. 2010). Spatial clustering for groups was performed with the proposed number of clusters (*K*) ranging from 4 –11. The analysis was repeated ten times for each maximum *K* and the log marginal likelihood values for each genetic partition was checked to correctly determine the number of genetic clusters given the data. Final spatial clusters were visualized using a Voronoi tessellation and represented by different colours.

To visualise patterns of spatial genetic structure across the entire sampling region, a 3-D surface plot of genetic diversity was generated in the program Alleles in Space (AIS, Miller 2005). The first step in the AIS analysis is the creation of a connectivity network

between sampling sites based on Delaunay triangulation of the geographic coordinates of sampling sites. The genetic distances between connections in the network are then placed at the geographic midpoints of each segment (Watson 1992; Brouns et al. 2003). These distances are interpolated to create a 3-D surface plot where the x- and y-axes correspond to geographic coordinates and the z-axis represents genetic distance at a particular point on the landscape. To ensure that large genetic distances were not erroneously identified due to possible geographic isolation between sampling sites, the residual genetic distances instead of raw genetic distances was used (Manni et al. 2004; Miller et al. 2006). Peaks represent areas of high pairwise genetic distances among nearest neighbours and may indicate genetic breaks or possible barriers to gene flow. Alternatively, troughs represent areas of low genetic distance among nearest neighbours and therefore may indicate regions with significant gene flow (Miller 2005; Miller et al. 2006).

(iv) Estimating patterns of historic gene flow

To obtain estimates of historic gene flow among populations a maximum likelihood method based on the coalescent as implemented in Migrate-N version 3.3.2 (Beerli 2009) was used. Migrate-N uses an equilibrium model that estimates migration rates averaged across the coalescent history using a Markov chain Monte Carlo (MCMC) sampling scheme. The program simultaneously estimates Θ, the effective population size scaled by mutation rate where $\Theta = N_e\mu$, together with pairwise migration rates summarised as $M = m/\mu$, where m is the effective immigration rate per generation between populations. While Migrate-N does allow for unequal sample sizes and asymmetric gene glow, it also assumes that populations are in migration–mutation equilibrium (Beerli 1998). Although MCMC coalescent methods are sensitive to the presence of "ghost" (i.e. unsampled) populations (Slatkin 2005), simulation studies have shown that the migration rates generated by Migrate-N are robust; whether or not ghost populations are included in analyses, the migration rates between sampled populations are very similar (Bittner & King 2003; Beerli 2004; Slatkin 2005). Although ghost populations might exist, this is unlikely since the geographic range of *R. capensis* has been extensively surveyed over many years. Furthermore, migration estimates generated in Migrate-N are also robust to small population sizes; in a simulation study,

migration estimates were similar for 3 populations consisting of 10, 50 and 100 individuals (Beerli 2004).

Banding data from European horseshoe bats reveal generally small home ranges where maximum dispersal distances rarely exceed 100 km over the course of an individual's life time (Hutterer et al. 2005). If similar, dispersal in *R. capensis* likely occurs over shorter distances. Therefore Θ and *M* between populations was estimated using a custom designed migration matrix model where migration was only allowed between neighbouring populations. Populations separated by large geographic distances were not directly connected except in cases where unique haplotypes were shared. Initial Θ and *M* values were obtained from F_{ST} calculations and the following search parameters were used: 10 short chains with 500 000 gene trees sampled and 5000 trees recorded; 3 long chains with 50 million sampled trees of which 50 000 were recorded. The first 10 000 trees were discarded as burn-in and a static heating scheme with six temperatures and a swapping interval of 1 was used; results were averaged over five replicate runs.

(v) Correlation between RF divergence, geographic distance and genetic distance

To explore the relationships between genetic structure, RF divergence and geographic distance, Mantel (Mantel 1967) and partial Mantel (Smouse et al. 1986) tests were used to investigate whether genetic and RF divergence is associated with geographic distance. Matrix correlations were calculated in GenAlex v6.5 (Peakall & Smouse 2012) and XLStat (v2013, Addinsoft) with 1000 random permutations. The three matrices were geographic distance (straight line distance in kilometres calculated from geographic coordinates using the program Geographic Distance Matrix Generator version 1.2.1 (Ersts, Internet)); genetic distance using Slatkin's linearized Φ_{ST} (Slatkin 1993) and call frequency differences (kHz) among populations. Log-transformed geographic distance was regressed against genetic distance and peak frequency difference. The partial Mantel test determined whether there was a correlation between RF difference and genetic distance while controlling for the effect of geographic distance.

RESULTS

Genetic diversity and haplotype distribution

A total of 39 unique mtDNA haplotypes were identified from 203 individuals (Table 3.1; Figure 3.1; Appendix 2). Most populations shared haplotypes with their nearest neighbours and a few haplotypes were shared between more distant populations (e.g. between ZPK and BAV approximately 600 km apart; Figure 3.1). Three genetically isolated populations (i.e. LS, TF, and KNY) consisting largely of unique mitochondrial lineages were identified. Populations displaying the greatest variation in the different genetic diversity indices were BKL (situated in the Fynbos Biome), TF and BAV (both in Multiple Biomes) (Table 3.1). Haplotype diversity ranged from 0.57 (SPH) to 0.88 (BKL) and the highest number of unique haplotypes (n=12) was found at Baviaanskloof (BAV).

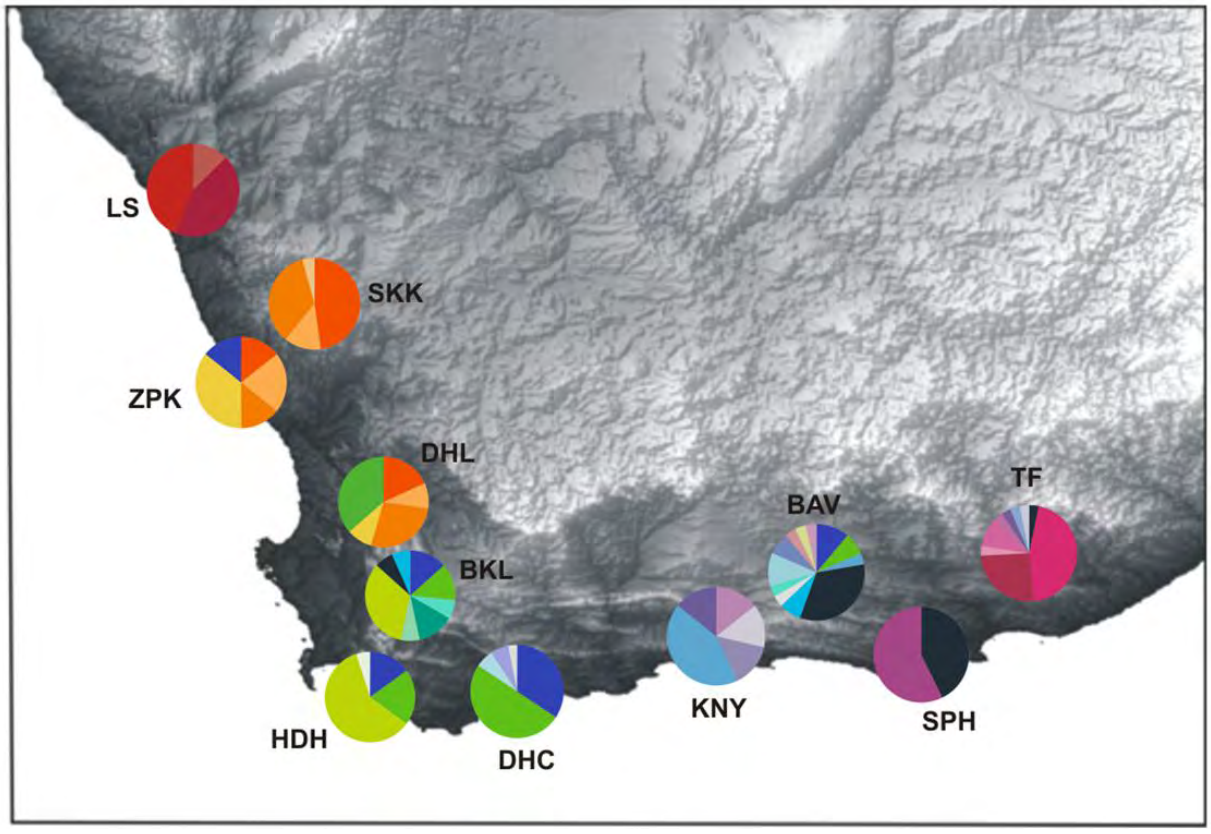

Figure 3.1: The distribution of 39 unique haplotypes (each a unique colour) across the 11 populations of *Rhinolophus capensis* sampled in this study. A key to acronyms is provided in Table 3.1

Table 3.1: Genetic variability in 11 populations of *Rhinolophus capensis* based on 519 bp of the mitochondrial control region. Haplotype diversity (*Hd*), number of haplotypes, nucleotide diversity (π) and number are shown.

Population	*n*	*Hd*	Number of haplotypes	π
Lekkersing (LS)	16	0.64	3	0.001
Steenkampskraal (SKK)	23	0.66	4	0.004
Zoutpansklipheuwel (ZPK)	14	0.82	5	0.005
De Hel (DHL)	11	0.82	5	0.005
Boskloof (BKL)	15	0.88	8	0.007
Heidehof (HDH)	20	0.61	4	0.006
De Hoop (DHC)	32	0.64	5	0.003
Knysna (KNY)	7	0.86	5	0.004
Baviaanskloof (BAV)	27	0.87	12	0.007
Sleepy hollow (SPH)	7	0.57	2	0.006
Table Farm (TF)	31	0.74	9	0.010
All populations	203	0.73 ± (SE) 0.03	39	0.005 ± (SE) 0.007

Spatial population genetic structure and evolution

Intraspecific gene genealogy

Complex network relationships characterise the evolutionary history of *R. capensis* populations sampled in this study. The Neighbour-Net network revealed numerous reticulations between haplotypes, suggesting several alternative evolutionary pathways among them (Figure 3.2) (Bryant & Moulton 2004).While the network recovered a number of clear clades these were not generally structured by biome or geographic proximity. Only haplotypes from the Desert Biome and populations

occurring in regions where multiple biomes are connected formed discrete genetic clusters (Figure 3.2).

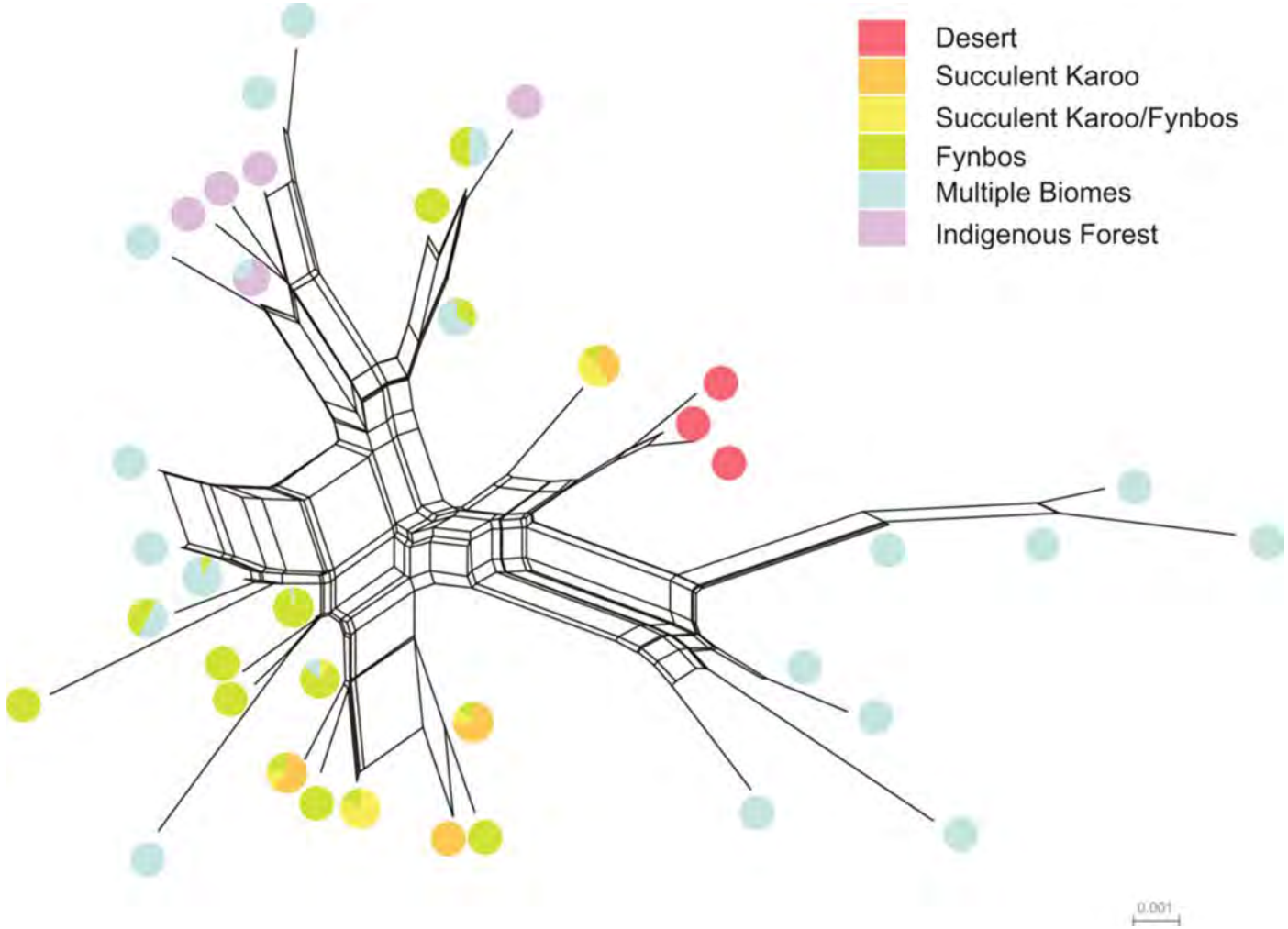

Figure 3.2: Neighbour-Net network based on *p*-corrected distances of the 39 unique haplotypes isolated in this study. Each circle represents a unique haplotype coloured according to the biome/s in which it occurs. Pie graphs indicate where haplotypes are shared across various biome categories.

Spatial distribution of genetic variation

The investigation of hierarchal population genetic structure revealed significant partitioning of genetic variation at all three levels but most variation occurred within populations (Φ_{ST} = 0.54) rather than between populations (Φ_{ST} = 0.33) or biomes (Φ_{ST} = 0.13) (*P*'s < 0.005). However, among population genetic differentiation was variable, and pairwise population comparisons of Φ_{ST} values were significantly different from zero (*P*'s < 0.05) except for between DHL and SKK, and between BKL and HDH (Table 3.2).

Table 3.2: Pairwise values Φ_{ST} among populations of *R.capensis* (below diagonal) and probability values based on 99 permutations (above diagonal).

	LS	ZPK	SKK	DHL	BKL	HDH	DHC	KNY	BAV	SPH	TF
LS		0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010
ZPK	0.660		0.040	0.050	0.010	0.010	0.010	0.010	0.010	0.010	0.010
SKK	0.640	0.081		0.330	0.010	0.010	0.010	0.010	0.010	0.010	0.010
DHL	0.723	0.089	0.000		0.030	0.010	0.020	0.010	0.010	0.010	0.010
BKL	0.620	0.335	0.227	0.173		0.180	0.010	0.010	0.020	0.010	0.010
HDH	0.638	0.442	0.377	0.359	0.038		0.010	0.010	0.010	0.010	0.010
DHC	0.775	0.381	0.233	0.106	0.213	0.437		0.010	0.010	0.010	0.010
KNY	0.829	0.627	0.594	0.603	0.343	0.337	0.685		0.010	0.010	0.010
BAV	0.592	0.344	0.237	0.169	0.080	0.271	0.154	0.418		0.020	0.010
SPH	0.786	0.552	0.438	0.414	0.314	0.466	0.470	0.636	0.239		0.010
TF	0.511	0.499	0.457	0.455	0.424	0.461	0.550	0.517	0.440	0.336	

Bayesian clustering identified four spatially explicit genetic clusters across populations (Figure 3.3). Only individuals situated at opposite ends of the species distribution (populations LS and TF), were assigned to unique clusters while the nine populations between them were assigned to two spatially structured clusters (Figure 3.3).

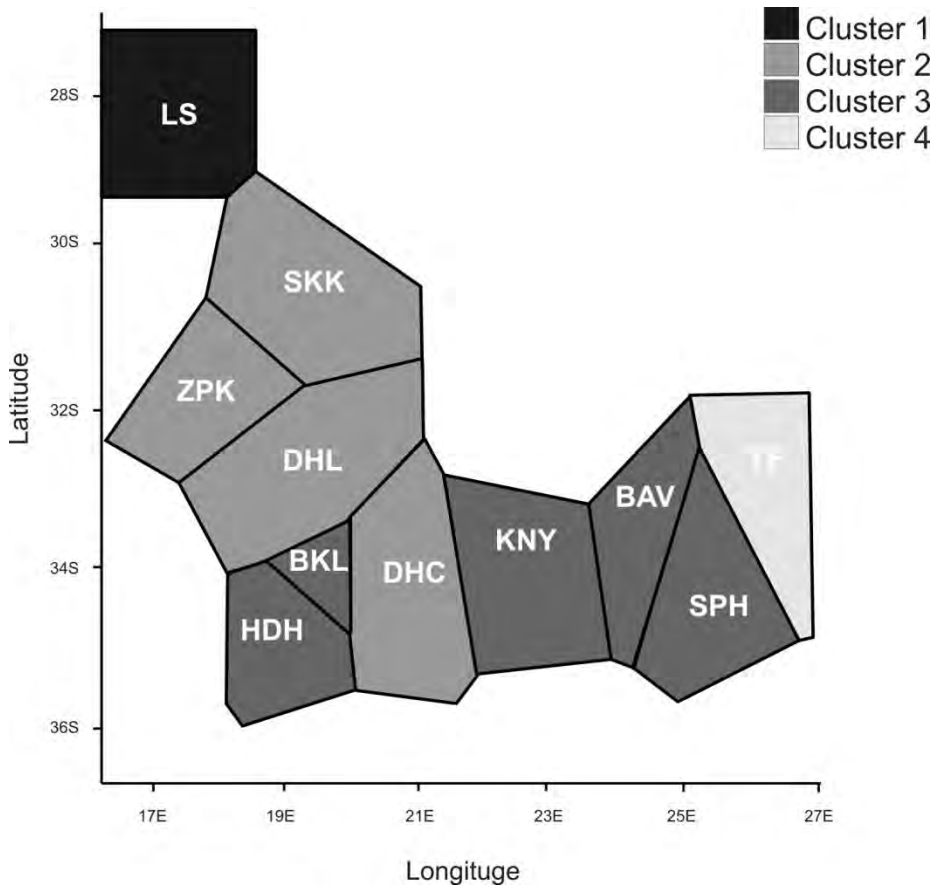

Figure 3.3: Voronoi tessellation of the Bayesian clustering population analysis as implemented in BAPS. Four genetic clusters were identified and populations assigned to the same clusters, share the same colours. A key to acronyms is provided Table 3.1.

The landscape interpolation plot showed areas of high genetic differentiation (as indicated by peaks) occur in the east (where populations are situated in mixed biomes), in the region around LS (Desert) and between the coastal populations of DHC (Fynbos) and KNY (Forest). Areas of low genetic variation (as indicated by troughs) occur among populations situated in the Fynbos Biome (Figure 3.4). Generally few peaks were evident across the landscape, suggesting that few genetic barriers occur across the distribution of *R. capensis*. The AIS and BAPs analyses reveal that the spatial distribution of genetic variation within *R. capensis* is characterised by an overall pattern of low population genetic structure.

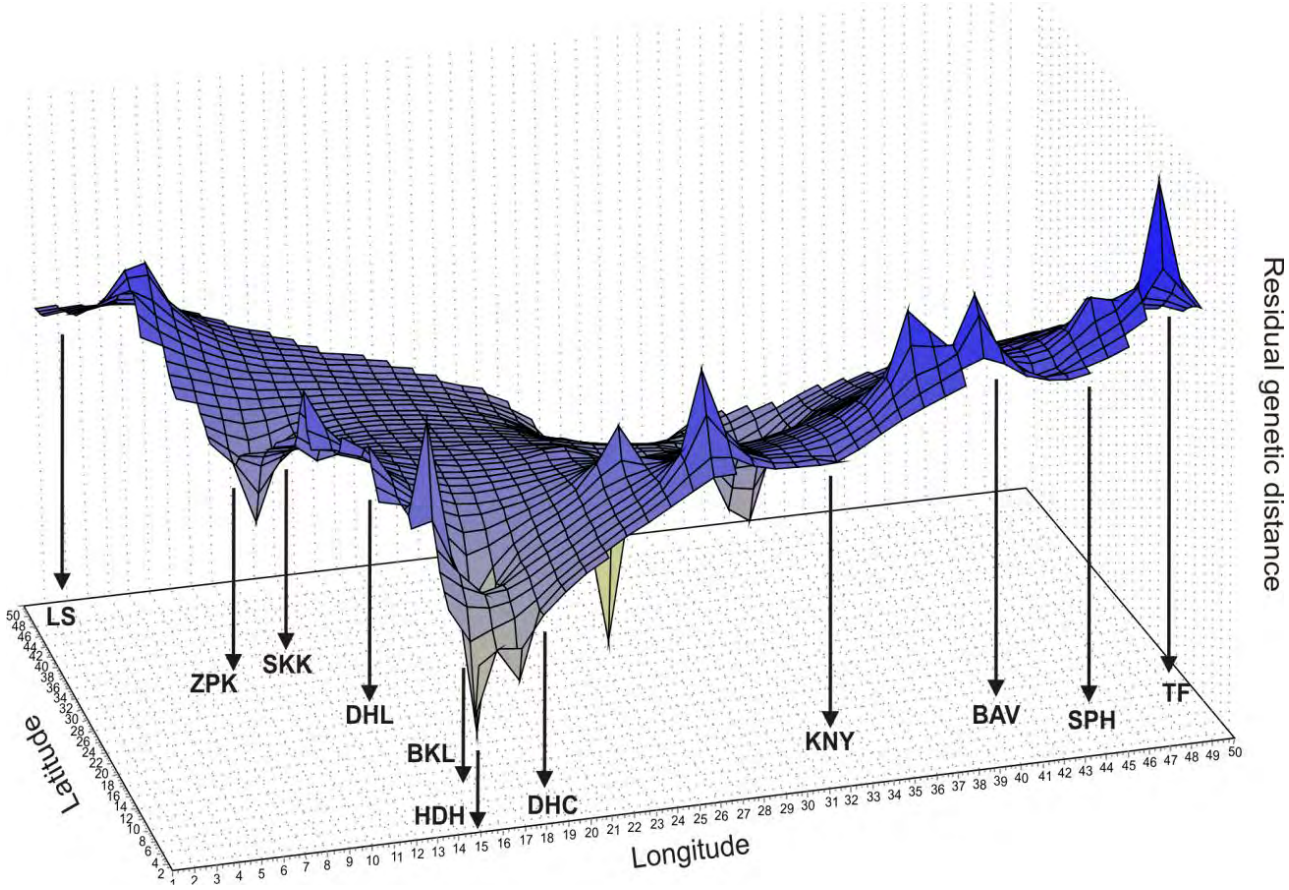

Figure 3.4: Landscape interpolation surface plot using a distance weighting parameter of 1. Latitude and longitude coordinates are divided into a 50 x 50 grid and surface plot heights (z-axis) represent residual genetic distances. A key to acronyms is provided Table 3.1.

Patterns of historical gene flow

Estimates of historical migration rates (*M*) revealed generally asymmetrical patterns of gene flow between populations situated in different biomes (Figure 3.5). The main source populations were ZPK, BKL and BAV and gene flow generally occurred between neighbouring populations. There was also evidence for long distance gene flow from BAV to BKL (430 km straight line distance) and from DHC to BAV (370 km) but this occurred at relatively low levels (Figure 3.5). Together, these results support a pattern of relatively high regional connectivity and therefore moderate fine-scale population structure in *R. capensis*.

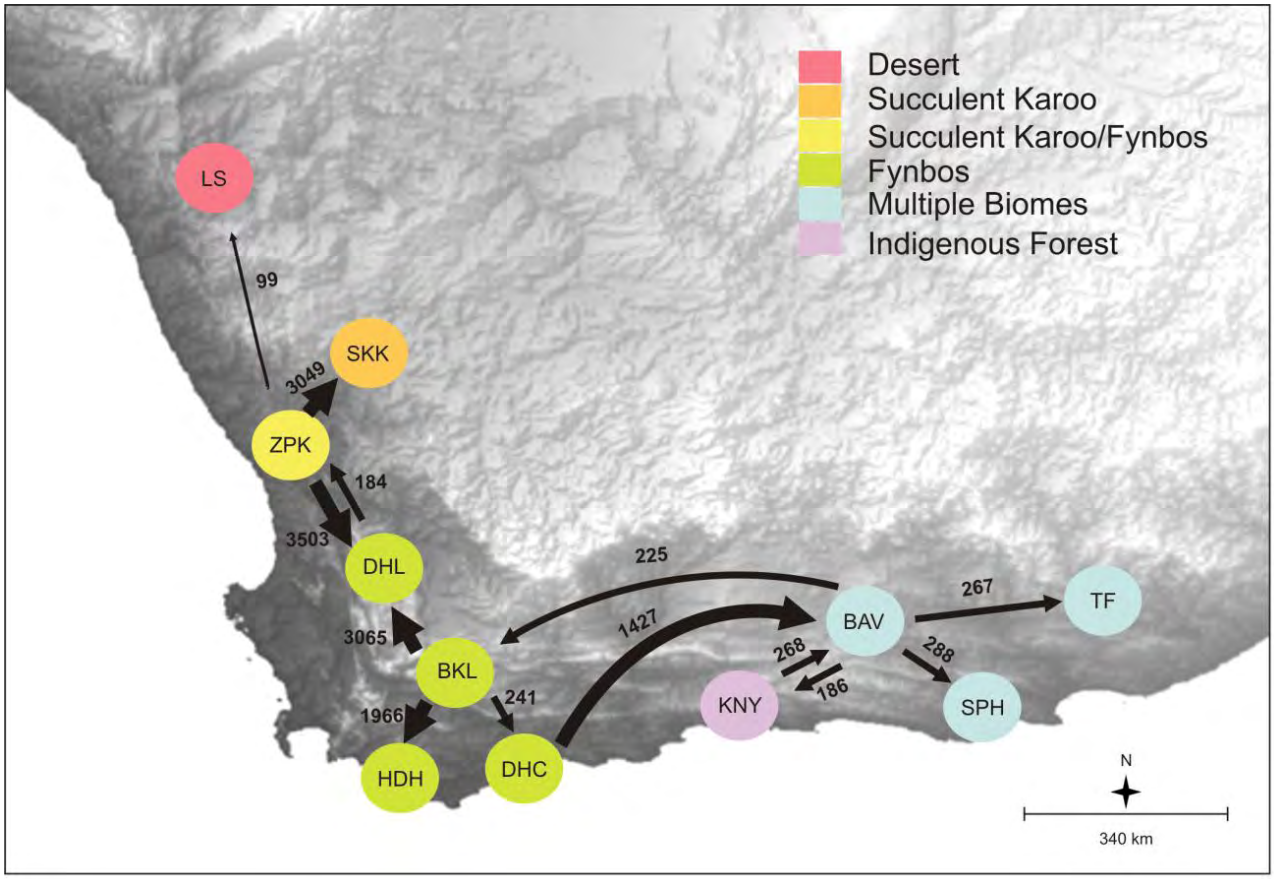

Figure 3.5: Estimated migration patterns among populations of *R. capensis*. The thickness of the arrows indicates the relative migration rates; values indicate *M*, the number of immigrants per generation scaled by mutation rate. The 95% confidence intervals are provided in Appendix 3.

Correlations between RF variation, geographic distance and genetic distance

Pairwise differences in both RF and genetic distance were characterised by significant positive relationships with geographic distance (Figure 3.6). Thus RF and genetic distance co-varied with geographic distance and followed an IBD pattern. However, geographic distance only explained a small proportion of the variation in RF and genetic distance (44% and 30%, respectively (Figure 3.6A and B). There was a significant correlation between RF difference and genetic distance among populations (Figure 3.6C) and this remained when the effect of geographic distance was controlled for (Partial Mantel Test: $R^2 = 0.075$, $P < 0.001$). Despite the significance of the correlation, genetic distance only explained a very small amount of the variation in echolocation frequencies (7.5%).

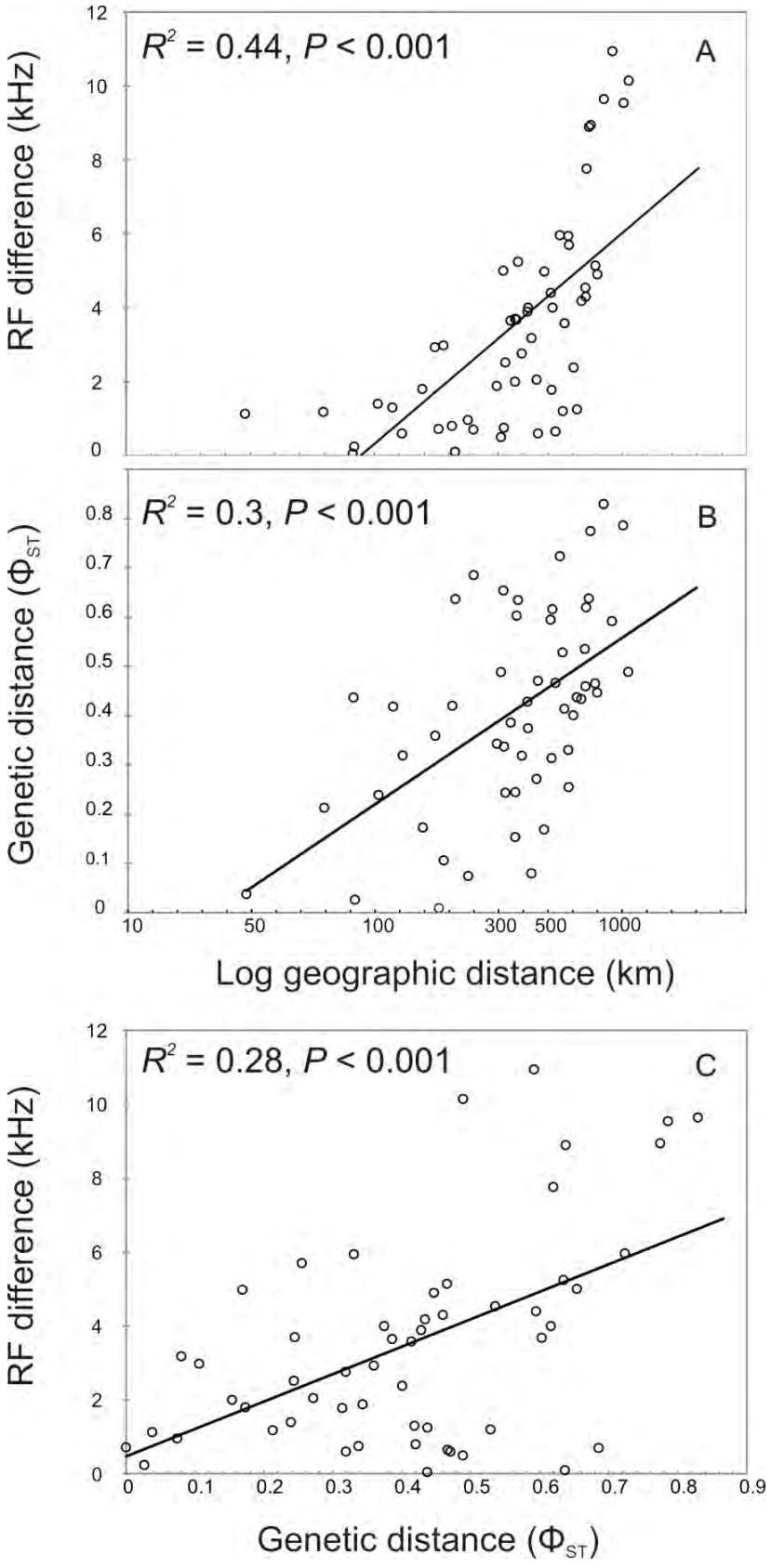

Figure 3.6: Pairwise geographic distance versus RF difference (A) and genetic distance (B) and genetic distance versus RF difference (C).

DISCUSSION

Across heterogeneous environments, the interaction between variable selection pressures and gene flow determine the rate and extent to which populations become locally adapted (Räsänen & Hendry 2008). To tease apart the complex interplay between natural selection and gene flow requires an investigation of the association between adaptive traits and local environmental conditions, and the quantification of gene flow between populations experiencing different selection regimes (Räsänen & Hendry 2008; Cheviron & Brumfield 2009). Surprisingly, very little is known about how patterns of gene flow vary across species' distributions, or how it relates to patterns of adaptive trait divergence across heterogeneous landscapes (Sexton et al. 2014). Results from this study suggests that selection for lower frequencies (and associated skull morphological correlates) in more xeric habitats may contribute to the evolution of RF divergence in *R. capensis*. Populations characterised by low levels of gene flow might then be expected to show greater levels genetic differentiation than populations with high levels of gene flow. Results presented here indicate that the spatial distribution of neutral genetic variation among populations of *R. capensis* is instead characterised by considerable historical gene flow and moderate overall genetic structure, revealing an unexpected pattern of significant sensory variation in the face of homogenising gene flow.

Moderate structure characterises the spatial distribution of mtDNA variation in *R. capensis*

Studies investigating the evolutionary forces shaping phenotypic and genetic divergence between populations often describe trait divergence in the context of substantial population structure and limited gene flow (Yoshino et al. 2008; Wang & Summers 2010; González et al. 2011; Puechmaille et al. 2011). Within a southern African context, emerging literature for the region reveal paleoenvironmental change from the Miocene through to the Pleistocene profoundly impacted the evolution of population divergence and speciation in a wide range of taxa. Several studies report a strong link between divergent genetic lineages of different taxa and the biomes or ecogeographical regions of southern Africa (summarised in Table 3.3)

Table 3.3: A brief summary of selected studies to illustrate how paleoenvironmental changes (e.g. the establishment of the current biomes, climatic zones and/or ecogeographical regions) in southern Africa have shaped patterns of population genetic divergence and/or speciation in different species.

Species or group	Taxon	Time period	Summary	References
Forest shrew *Myosorex varius*	mammal	Pleistocene	Using mtDNA and nuclear intron sequence data, this study found that changes in the rainfall regime and habitat discontinuities between biomes strongly influenced the rapid radiation of geographically structured lineages of *M. varius* during the Pleistocene.	Willows-Munro & Matthee 2011.
Karoo bush rat *Myotomys unisulcatus*	mammal	Miocene-Pliocene boundary	A Bayesian analysis of population structure based on mtDNA sequences in *M. unisulcatus* sampled across its range revealed two distinct genetic clusters. The Great Escarpment together with vegetation differences between biomes likely acted as a significant barrier to gene flow between the two lineages.	Edwards et al. 2011.
Namaqua rock mouse *Micaelamys namaquensis*	mammal	Pliocene-Pleistocene	MtDNA sequence data revealed the presence of eight genetically distinct maternal lineages in *M. namaquensis*, suggesting that this taxon possibly represents a cryptic species complex. Lineages are strongly associated with different biomes, and lineage diversification is associated with the establishment of the current biomes during the Pleistocene.	Russo et al. 2010.
Four-striped mouse *Rhabdomys pumilio* and *R. dilectus*	mammal	Miocene-Pliocene boundary	The diversification of four genetically-distinct monophyletic mitochondrial clades comprised of *R. dilectus* and three evolutionary distinct lineages of *R. pumilio* is correlated with changes in vegetation distributions due to climatic oscillations and the establishment of the Great Escarpment during the Miocene-Pliocene boundary.	du Toit et al. 2012
Chacma baboon *Papio ursinus*	mammal	Pliocene-Pleistocene	Exploring the evolutionary relationships between Chacma baboon morphotypes revealed that Chacma arose in the early Pleistocene, and diversified into two distinct genetic clades. The timing and pattern of diversification suggests that diversification in Chacma was likely driven by the increase in aridity over much of Africa due to climatic fluctuations during the Pleistocene.	Sithaldeen et al. 2009.

Table 3.2 continued.

Hildebrandt's horseshoe bat *Rhinolophus hildebrandtii c*omplex	mammal	Miocene-Pliocene	Four new species of the *R. hildebrandtii* complex in eastern and southern Africa were discovered. Lineages from East Africa diverged from southern African lineages during the Pliocene, whereas the latter diversified further during the Pleistocene. Tectonic uplift of the East African Rift Valley together with climatic cycling during these periods may have driven diversification in this species complex	Taylor et al. 2012.
Geoffroy's horseshoe bat *Rhinolophus clivosus*	mammal	Pleistocene	MtDNA sequences revealed the presence of five geographically structured and genetically and ecology distinct lineages of *R. clivosus* in South Africa. Diversification was associated with climatic cycling and subsequent vegetation changes during the Pleistocene.	Stoffberg et al. 2012.
Dwarf chameleons *Bradypodion*: Chamaeleonidae	reptile	Mid Miocene-late Pliocene	MtDNA sequence data revealed that diversification in the genus is linked to the shift from open to closed habitats during the Miocene, and later to changes in the vegetation and the establishment of the current rainfall regimes in the region.	Tolley et al. 2008.
Cape legless skink *Acontias meleagris* species complex	reptile	Pliocene-Pleistocene	MtDNA and protein coding nuclear DNA sequences revealed the presence of five distinct lineages in the *Acontias meleagris* species complex, which likely diversified due to the climatic (increase in aridity) and sea level oscillations during the Pliocene and Pleistocene.	Engelbrecht et al. 2013
Freshwater crab *Potamonautes perlatus sensu lato*	invertebrate	Pleistocene	MtDNA and multiple nuclear genetic markers revealed the presence of two major geographically structured clades in *P. perlatus* (with one clade comprised of two sub-clades) across river networks of the Cape Fold Mountains. Divergence time estimates reveal that diversification was likely driven by drainage contractions due to the increase in aridity in the region during the Pleistocene.	Phiri & Daniels 2014.

Resting frequency variation in *R. capensis* is correlated to the increase in vegetation clutter which characterises the different biomes from west to east across the species' distribution (Chapter 2). Neutral genetic variation is then also expected to broadly reflect habitat discontinuities between biomes. Furthermore, RF divergence is predicted to directly reflect mtDNA structure given that (i) the fine tuning of the echolocation frequency of young horseshoe bats are partly learned from their mothers, (ii) female philopatry and male dispersal characterise other horseshoe bats studied to date (Chen et al. 2008; Yoshino et al. 2008; Flanders et al. 2009; Mao et al. 2010), and (iii) the amount of RF divergence observed among populations (range: 1 – 11 kHz) is similar to that reported for other high duty-cycle bats which show corresponding significant genetic structure among maternal lineages (Yoshino et al. 2008; Clare et al. 2013). Instead, spatially explicit analyses of genetic variation reveal a general pattern of moderate genetic structure among acoustically divergent and geographically proximate, and distant, *R. capensis* populations. A number of mitochondrial haplotypes are shared between geographically distant populations (Figure 3.1); these are neither geographically, nor environmentally structured (Figure 3.2) and may reflect long-distance dispersal events or the retention of common ancestral polymorphisms. A Bayesian clustering analysis identified four dominant genetic clusters (Figure 3.3.) which broadly reflect the areas of high genetic differentiation identified from the genetic landscape interpolation plot (Figure 2.4), suggesting extensive historic regional connectivity among populations. Only LS and TF (populations at the opposite extremes of the distribution where bats echolocate at 75kHz and 86 kHz respectively) are classified as unique genetic clusters (Figure 3.3) with corresponding regions of high genetic distances (Figure 3.4), further supporting the idea that novel environments at range edges can drive local adaptation, potentially leading to phenotypic and genetic divergence of edge populations from those elsewhere across the species' distribution (Smith et al. 1997; Kark & Rensburg 2006; Kawecki 2008). Mitochondrial data clearly reveals a recent evolutionary history of complex reticulations in *R. capensis* suggesting roles for either gene flow or incomplete lineage sorting. The latter scenario is however unlikely given that recent phylogenetic studies exploring the origin and diversification of southern African horseshoe bats found no evidence of cryptic lineages in *R. capensis* (Stoffberg 2007; Jacobs et al.

2013). Thus gene flow and not incomplete lineage sorting is likely responsible for the observed genetic structure or rather lack thereof.

Maternal transmission may influence RF variation

Geographic distance accounted for a minor proportion of the variation in both RF (44 %) and genetic distance (30 %) (Figure 3.6 A and B respectively), suggesting that drift impacts both measures. RF divergence is also positively correlated to some extent with mtDNA genetic distance (Figure 3.6 C). However, mtDNA genetic distance only accounts for a very small proportion of RF variation (only 7.5%) after controlling for the effect of geographic distance. This is in sharp contrast to results reported for *Rhinolophus cornutus pumilus*, where mtDNA (D-loop) distance accounted for 60% of the variation in RF when taking geographic distance into account (Yoshino et al. 2008). The maternal transmission hypothesis also predicts that mtDNA genetic structure is greater than nuclear DNA structure, and that male mediated dispersal characterises the dispersal ecology of the species under study (Yoshino et al. 2008). Exploring patterns of RF variation with the context of neutral genetic variation obtained from both sex-specific and bi-parentally inherited markers such as microsatellites is required if we are to gain a comprehensive understanding of how sex-biased behaviours may influence RF variation in *R. capensis*.

Trait diversification in the presence of gene flow

While phylogeographic patterns of maternally (mtDNA) and bi-parentally (e.g. microsatellites) inherited genetic markers often agree, discordance between phenotypic and genetic structure is also reported (reviewed in: Toews & Brelsford 2012). This is usually attributed to demographic processes that characterise species, such as sex-biased dispersal (Chen et al. 2008; Turmelle et al. 2011) or secondary contact following historical isolation (Armstrong & Coles 2007; Fontenot et al. 2011). Similar discordance has been reported in several European and Asian horseshoe bats either as a result of male-biased dispersal and female philopatry (e.g. *R. monoceros*: Chen et al. 2008, *R. c. pumilus*: Yoshino et al. 2008) or historical introgression of mtDNA (*R. pearsoni*: Mao et al. 2010; *R. sinicus*: Mao et al. 2013a; *R. affinis*: Mao et al.

2013b) or nuclear genomes (*R. yunanensis* to *R. pearsoni*: Mao et al. 2010) between sister lineages. Population genetic structure generally reflects the variation of echolocation frequencies across often widespread high duty-cycle bats (e.g. *Pteronotus parnellii*: Clare et al. 2013; *R. clivosus*: Stoffberg et al. 2012; *R. hildebrandtii*: Taylor et al. 2012; *R. rouxii*: Chattopadhyay et al. 2012). However, estimates of maternal gene flow in this study support significant regional connectivity in the recent past and are at odds with the pattern of structuring in RF observed in *R. capensis*.

Estimates of gene flow based only on mitochondrial sequences probably underestimates the degree to which *R. capensis* populations are connected via past or contemporary gene flow because of the inherent small effective population size of mtDNA genomes (Toews & Brelsford 2012). This highlights the need for using fast evolving nuclear genetic markers such as microsatellites to provide important insight into the dispersal ecology of species'. Nonetheless, the pattern of RF variation in the face of asymmetric gene flow recovered in this study suggests that both selection for increased detection distance in less cluttered habitats and adaptive phenotypic plasticity may have influenced the evolution of matched echolocation frequencies and habitats across different populations of *R. capensis*.

A complementary hypothesis: adaptive phenotypic plasticity

Adaptive trait divergence is influenced by the interaction between diversifying selection and homogenizing gene flow between different environments across a species range. A degree of phenotypic plasticity together with selection on heritable traits, may also account for significant population divergence across different environments (Pigliucci 2001; Crispo 2008; Chapter 1). Phenotypic plasticity can be advantageous if it results in the expression of different phenotypes that increase an individual's fitness in diverse environments (Pfennig et al. 2010; Fitzpatrick 2012; Gomez-Mestre & Jovani 2013). In this way plasticity can minimise the costs incurred from dispersal into environments with different selection regimes, leading to a pattern of phenotypic divergence in the presence of gene flow (Crispo 2008; Jourdan-Pineau et al. 2012).

At a 'local to regional' scale geographic distance is clearly not a significant barrier to gene flow in *R. capensis*. The evolution of sensory divergence in the presence of this gene flow may also reflect a degree of adaptive phenotypic plasticity in RF. Despite the tight coupling between RF and the acoustic fovea in high duty-cycle bats (Neuweiler 1984; Chapter 1), empirical studies have shown that species are able to shift their RFs in response to both neighbouring conspecifics (maximum shift 3.9 kHz: Hiryu et al. 2006) and different ambient noise conditions (maximum shift <0.5 kHz: Hage et al. 2013). Such small shifts in frequency may explain the range of RF variation in the southern and eastern populations of this study (approximately 3 kHz) where plasticity in response to slightly varying degrees of vegetation clutter towards the east might occur. It appears that southern and eastern populations of *R. capensis* use RFs within the best hearing range of the acoustic fovea of their nearest neighbours, possibly facilitating gene flow and promoting relatively flexible RFs in these populations. Adaptive plasticity is however unlikely to explain the 9 kHz shift in bats from LS. The steep habitat gradient between LS (genetic cluster 1) and its nearest neighbours (SKK and ZPK: genetic cluster 2) suggest that selection dominates in this region. Plasticity may be favoured as an explanation for RF variation amongst the other populations because the habitat gradients are not as steep. A notable exception is that of the population at TF. At TF (genetic cluster 4) bats use similar RFs to other populations situated in ecotones (genetic cluster 3), and yet appears to be relatively isolated genetically (Figure 3.5). TF is situated in region at the interface between winter + aseasonal and summer rainfall zones (Chase & Meadows 2007) known as the Bedford Gap (Lawes 1990). The remarkably high genetic distance between TF and its nearest neighbours is surprisingly similar to patterns of genetic structure uncovered for different mammals in this region (e.g. Lawes 1990; Willows-Munro & Matthee 2011; du Toit et al. 2012). The shift in rainfall seasonality regimes together with the intrinsic habitat heterogeneity of ecotones may serve as a significant barrier to gene flow even in vagile species such as *R. capensis* (Figure 3.4).

While small shifts in the acoustic fovea and its corresponding reference frequency appear possible in high duty-cycle bats (Hiryu et al. 2006; Hage et al. 2013), the precise limits of the flexibility of the acoustic fovea is unknown. Long-term experimental

studies evaluating the change in RFs in response to the RFs of bats from acoustically divergent populations may shed light on the degree of plasticity in this system.

Alternatively, the relative influence of plasticity versus selection can be evaluated indirectly by investigating the social life of horseshoe bats. If bats are able to recognise conspecific calls from a range of acoustically divergent populations it may suggest that selection for some degree of plasticity in the trait is also favoured. Classic playback experiments can be used to assess the sensitivity of individuals to the range of frequencies exhibited by a species. Furthermore, recent experimental evidence reveals echolocation calls may play a role in sex recognition in horseshoe bats (Schuchmann et al. 2012). The limited gene flow between LS and other populations may be a consequence of LS bats not effectively recognizing other *R. capensis* as potential mates. Divergence in RF may have under-appreciated consequences for the evolution of reproductive isolation via female preference for male RFs in different populations of horseshoe bats and evaluating female preference in LS bats for local versus allopatric RFs may provide intriguing insights into the causes and consequences of sexual selection in horseshoe bats.

Selection may also better explain the structuring of RF in *R. capensis* if variation in functional genes involved in hearing co-varies with RF variation across populations. Recent studies reveal a wide range of candidate hearing genes which show strong signals of ancestral positive selection in the evolution of echolocation in bats and cetaceans (Li et al. 2010; Davies et al. 2012).

Conclusions

Results presented here reveal significant sensory trait variation in *R. capensis* despite substantial historic gene flow. While genetic and geographic distances do influence sensory variation to some extent, results reported here and in the previous chapter, suggest that differences in habitat complexity across the range of *R. capensis* may be the dominant driver of sensory differentiation in this system. Classical divergent selection together with some degree of phenotypic plasticity may be responsible for RF variation in the presence of gene flow. This will further be explored by assessing the

relationship between RF variation and the gene directly responsible for the amplification of sound in the mammalian cochlea, *Prestin*, among species closely related to *R. capensis* (the *R. capensis* clade: *R. capensis*, *R. denti*, *R. simulator* and *R. swinnyi*: Stoffberg et al. 2010), and among populations of *R. capensis* with divergent RFs.

CHAPTER 4

SENSORY DIVERGENCE IN HORSESHOE BATS IS NOT REFLECTED IN PROTEIN SEQUENCE VARIATION OF THE HEARING GENE *PRESTIN*

INTRODUCTION

Two approaches have traditionally been used to investigate the evolutionary processes responsible for the generation and maintenance of phenotypic diversity; (i) exploring variation at the genotype level by investigating patterns of spatial and temporal distribution of allele and genotype frequencies, and (ii) investigating evolutionary change at the phenotype level by exploring how ecologically important traits vary among individuals across heterogeneous environments (Hoekstra 2006). Combining these approaches however yields extraordinary insights into how diversifying selection, homogenising gene flow and adaptive phenotypic plasticity interact to shape patterns of adaptive trait divergence within and between species (Ghalambor et al. 2007; Crispo 2008; Räsänen & Hendry 2008). To gain a comprehensive understanding of the molecular basis of phenotypic adaptation however also requires knowledge of the genes encoding ecologically important traits of interest (Fitzpatrick et al. 2005; Stinchcombe & Hoekstra 2008). Such a 'candidate gene approach' has provided important insights into evolutionary changes in adaptive traits such as morphology (Abzhanov et al. 2004; Albertson et al. 2005; Raeymaekers et al. 2014), behaviour (Ben-Shahar 2005; Wohlgemuth et al. 2014), venom toxicity (Aminetzach et al. 2009), physiology (McCairns & Bernatchez 2009), cryptic colouration (Mullen et al. 2009; Dobson et al. 2012) and sensory perception (Larmuseau et al. 2010; Shen et al. 2012; Jones et al. 2013; Stathopoulos et al. 2014) in natural populations. Acoustic signals are used by animals to mediate courtship, resource defence and species recognition (Wilkins et al. 2013). The ability to perceive these signals and process the biologically relevant information encrypted in them, is controlled by the auditory system comprised of hearing organs (ears) and the auditory cortex (the region of the brain responsible for processing auditory signals) (Fay & Popper 2000). Exploring the

molecular basis of auditory sensitivity may provide important insights into the evolution of animal acoustic communication systems. A wide range of candidate genes directly involved in acoustic perception and processing in mammals has recently been identified (Zheng et al. 2000; Accetturo et al. 2010; Davies et al. 2012; Jones et al. 2013) and these genes provide an excellent opportunity to investigate the genetic basis of adaptive divergence in mammalian acoustic signals.

Mammalian hearing

Among the various novel features that characterise mammals, few are as exceptional as the mammalian auditory system (Fritzsch et al. 2013). Although the use of acoustic signals for communication is not a mammalian novelty, the ability to perceive high (> 10 kHz) and ultrasonic (> 20 kHz) frequencies is (Manley 2012). Compared to the extraordinary frequency sensitivity of the mammalian cochlea, hearing in most non-mammal vertebrates is limited to frequencies below 10 kHz (reviewed in Heffner & Heffner 2008). Three key innovations during the course of mammalian evolution contributed to the evolution of high frequency hearing in mammals (Vater & Kössl 2011; Manley 2012). The first was the evolution of the three-ossicle middle ear in the ancestral synapsid reptilian lineage, where the articular and quadrate bones of the primary jaw-joint was combined with the ancestral single-ossicle (stapes) middle ear (reviewed in: Manley 2010; Takechi & Kuratani 2010). The derived three-ossicle middle ear is more effective at transmitting higher frequencies, and therefore served as a pre-adaptation facilitating the subsequent evolution of high frequency hearing limits in the inner ear of mammals (Manley 2010; Vater & Kössl 2011). Another key innovation was the elongation of the auditory sensory membrane (the basilar membrane) of the cochlea which was accompanied by an increase in both lower and upper hearing limits of mammals (LePage 2003). Furthermore, the cochlea of therian mammals (marsupials and placental mammals) are characterised by spiral coils which may have evolved to accommodate the elongated basilar membrane (Manley 2012, but see West 1985) as well as to play a role in improving sensitivity to lower frequencies (Manoussaki et al. 2008). Lastly, the mammalian Organ of Corti is characterised by two unique, spatially separated and morphologically distinct types of sensory receptor cells, the inner and

outer hair cells (IHCs and OHCs respectively), which differ in innervation and function (reviewed in Fettiplace & Hackney 2006). The IHCs are the actual sensory receptors which relay signals to the auditory processing centre of the brain. OHCs are directly responsible for creating the high sensitivity and sharp frequency tuning of the cochlea by substantially increasing the amplitude of incoming sound waves, i.e. it serves as a cochlear amplifier (Davis 1983). Amplification is achieved by the elongation and contraction of the OHC cylindrical cell body in response to changes in membrane polarisation (Mellado Lagarde et al. 2008) and this so-called somatic electromotility of OHCs is the mammalian innovation responsible for the wide range of frequencies mammals are able to perceive (Mellado Lagarde et al. 2008; Tan et al. 2011; Tang et al. 2013). Fourteen years ago Zheng et al. (2000) discovered the unique motor protein prestin, encoded by the gene *Prestin* that mediates the somatic electromotility of mammalian OHCs. *Prestin* is responsible for the exceptional sensitivity and frequency selectivity of the mammalian cochlea (Zheng et al. 2000). In the years following its discovery, a number of other genes involved in auditory perception and processing have been reported (Accetturo et al. 2010; Jones et al. 2013). Although many more genes are required for normal hearing in mammals, the structural and functional properties of the prestin protein, as well as the evolutionary history of the gene has been extensively studied (reviewed in He et al. 2014). Prestin is a member of the solute carrier anion transport family 26 (SLC26A5) which functions in the transfer of anions across the cell membrane. Unlike other well-known cellular motor proteins that require ATP-hydrolysis e.g. myosin, prestin-mediated somatic motility is voltage dependent, and therefore prestin acts several orders of magnitude faster than other motor proteins (Zheng et al. 2000; Ashmore et al. 2010). Knock-out experiments in mice revealed that deletion of *Prestin* results in the loss of OHC motility, leading to a 40 – 60 dB loss in cochlea sensitivity (Liberman et al. 2002; Cheatham et al. 2004) and mutations in the gene are associated with nonsyndromic hearing loss in humans (Liu et al. 2003). Expression of the gene is therefore crucial for normal hearing in mammals (Dallos et al. 2006; Dallos 2008).

Positive selection characterises the evolutionary history of *Prestin* in echolocating mammals

The evolutionary history of *Prestin* in mammals is characterised by episodes of substantial positive selection associated with the evolution of increasingly high frequency hearing (Franchini & Elgoyhen 2006; Elgoyhen & Franchini 2011). Positive selection in *Prestin* has been detected in the ancestral lineage of all bats using laryngeal echolocation (Li et al. 2008) as well as in the lineages leading to horseshoe (Rhinolophidae) and Old World leaf-nosed (Hipposideridae) bats (Li et al. 2008). Remarkably, echolocating dolphins and whales share many amino acid mutations in *Prestin* with echolocating bats and the majority of these are shared with horseshoe bats (Li et al. 2010; Liu et al. 2010). Furthermore, the number of amino acid changes in *Prestin* along the evolutionary pathways leading to both echolocating and non-echolocating mammals broadly correlate to the frequencies of maximum hearing sensitivity (best-hearing frequencies) of different species (Li et al. 2010; Liu et al. 2010; Rossiter et al. 2011). This suggests that amino acid changes in *Prestin* may be driven by diversifying selection for greater auditory sensitivity to higher frequencies in mammals (Rossiter et al. 2011).

Exploring variation in *Prestin* within and among acoustically divergent southern African horseshoe bats

Horseshoe bats display an extraordinary diversity in echolocation frequencies ranging from 23.7 kHz in *Rhinolophus rex* (China: Huihua et al. 2003) to 121 kHz in *Rhinolophus landeri landeri* (Nigeria: Novick 1977). Even closely related species may show substantial differences in their echolocation calls and often the detection of different phonic types within widespread high duty-cycle bats has led to the discovery of morphologically cryptic species complexes (e.g. *Hipposideros larvatus* 85 and 98 kHz phonic types: Thabah et al. 2006; *R. rouxi* 80 and 90 kHz phonic types: Chattopadhyay et al. 2012; *R. hildebrandtii* 32 – 46 kHz: Taylor et al. 2012; but see Sedlock & Weyandt 2009). Given that the evolution of *Prestin* in the lineages leading to rhinolophid and hipposiderid bats and echolocating cetaceans is characterised by convergent

signatures of adaptive and accelerated evolution (Li et al. 2008; Li et al. 2010; Lui et al. 2010), it is reasonable to expect that echolocation diversity among extant horseshoe bat species may also be associated with similar signatures of evolution. The four members of the African *Rhinolophus capensis* clade (*R. capensis*, *R. denti*, *R. simulator* and *R. swinnyi*: Stoffberg et al. 2010) display substantial differences in their echolocation frequencies. *R. denti* is widely but sparsely distributed in the western regions of southern Africa (Monadjem et al. 2010) and emits echolocation calls at 111.2 ± 1.8 kHz (Schoeman & Jacobs 2008). *R. simulator* is widespread in the eastern regions of southern Africa (Monadjem et al. 2010) and produces echolocation calls of 80.1 ± 1.2 kHz (Schoeman & Jacobs 2008). The distribution of *R. swinnyi* largely overlaps with that of *R. simulator* in the eastern region of southern Africa, although it extends further south along the east coast than *R. simulator* (Monadjem et al. 2010), and they produce echolocation calls of 106.6 ± 0.4 kHz (Schoeman & Jacobs 2008). Whether significant geographic variation in echolocation frequencies also characterise the distributions of these three species, remains to be investigated. Nonetheless, echolocation diversity within this clade may be reflected in significant signatures of adaptive evolution in *Prestin*.

Both genetic drift and natural selection can promote the evolution of geographic variation in RF among populations of horseshoe bats (Chen et al. 2009; Stoffberg et al. 2012; Taylor et al. 2012; Sun et al. 2013). Results in this study indicate that the evolution of geographic variation in RF within *R. capensis* is consistent with a model of 'divergence-with-gene flow' (Nosil 2008; Pinho & Hey 2010); where ecological (habitat structure) and morphological correlates (nasal chamber size) explain the greatest proportion of variation in RFs, independent of geographic distance and in the presence of substantial gene flow among populations (Chapter 2 and 3). Sensory divergence in *R. capensis* may therefore be mediated by divergent selection for lower frequencies in xeric habitats and/or some degree of phenotypic plasticity (Chapter 2 and 3). Studies have found a significant positive correlation between the frequency of best-hearing (estimated from spectrograms) and the number non-synonymous amino acid substitutions in *Prestin* in the ancestral lineages of mammals (Rossiter et al. 2011) and echolocating bats (including horseshoe bats) and cetaceans (Liu et al. 2010). *Prestin*

evolution in lineages leading to species with higher best-hearing frequencies is generally characterised by a greater number of non-synonymous substitutions than species with lower best-hearing frequencies. Although these correlations are no longer significant after phylogenetic correction (Liu et al. 2010; Rossiter et al. 2011) sequence variation in *Prestin* may nonetheless reflect intra and interspecific variation in best-hearing frequencies. This is particularly relevant for horseshoe bats because of the tight coupling between call frequency and the frequency of best-hearing of their acoustic fovea, called the reference frequency (Schuller & Pollak 1979).

Horseshoe bats compensate for both positive and negative Doppler shifts induced by their own flight speed with extreme precision to ensure that returning echoes always return at their individual reference frequencies of their acoustic fovea (Metzner et al. 2002). The complex audio-vocal feedback mechanism controlling Doppler shift compensation behaviour allows horseshoe bats to detect fluttering insects in cluttered habitats (Neuweiler 1989). Furthermore, the reference frequency is always 150 – 200 Hz higher than the RF, and thus RF is considered a reliable indicator of the reference frequency of the acoustic fovea (Siemers et al. 2005; Schnitzler & Denzinger 2011). Given the tight coupling between RF and best-hearing frequency in horseshoe bats, it is reasonable to predict that coding sequence variation in *Prestin* may reflect changes in best-hearing frequencies and therefore also echolocation frequencies within and among horseshoe bat species.

An emerging theme in evolutionary ecology is the study of variation in both the phenotype and its underlying genes within an ecological context (e.g. Hoekstra et al. 2004; Mullen & Hoekstra 2008). Here, DNA sequencing of a candidate gene is used to (i) determine whether members of the *R. capensis* clade groups with other rhinolophid and hipposiderid species by reconstructing the evolutionary relationships between *Prestin* coding sequences in these and other mammalian species; (ii) explore functional variation in *Prestin* among the four members of the *R. capensis* clade as well as between acoustically divergent populations of *R. capensis* (LS: 75 kHz, genetic cluster 1; SKK and DHC: 80 kHz and 84 kHz respectively, genetic cluster 2; and TF: 86 kHz, genetic cluster 4: Chapter 2 and 3) to determine whether functional variation in *Prestin* is associated with RF differences within and between species; and (iii) test whether the

selective regime of *Prestin* within *R. capensis* and among the members of the *R. capensis* clade is characterised by signatures of adaptive evolution as might be expected if functional variation in *Prestin* is related to RF divergence within and among horseshoe bat species.

MATERIALS AND METHODS

Selected samples and taxonomic coverage

To investigate within species sequence variation, forty individuals of *R. capensis* were selected from four populations representing the gradient of increasing RF from west to east across the distribution of the species. These included 10 individuals each from LS (Desert Biome), SKK (Succulent Karoo Biome), DHC (Fynbos Biome) and TF (ecotone between multiple biomes). Total genomic DNA was also extracted from six individuals each of *R. denti*, *R. simulator* and *R. swinnyi* using standard protocols of the Qiagen DNeasy Blood and Tissue Kit. Published *Prestin* sequences for 20 eutherian mammals were also analysed for comparison (GenBank accession numbers provided in Appendix 4) including: three horseshoe bats (*R. luctus*, *R. ferrumequinum* and *R. pusillus*), three Old World leaf-nosed bats (*Hipposideros armiger, H. larvatus* and *H. pratti*), four FM-echolocating bats (*Megaderma spasma*, *Myotis ricketti*, *Pteronotus davyi* and *Murina leucogaster*), two non-echolocating fruit bats (*Rousettus leschenaultia* and *Cynopterus sphinx*), three echolocating cetaceans (bottlenose dolphin: *Tursiops truncatus*, common dolphin: *Delphinus delphis* and harbour porpoise *Phocoena phocoena*), two non-echolocating whales (fin whale: *Balaenoptera physalus* and humpback whale: *Megaptera novaeangliae*), and the mouse (*Mus musculus*), cow (*Bos taurus*) and dog (*Canis familiaris*).

Amplification and alignment of *Prestin*

Ten out of the 18 exons that encode mammalian *Prestin* was amplified from genomic DNA using standard PCR methods. A suite of internal primers were designed to target regions of the gene identified by Li et al. (2008) as being under strong positive selection in the ancestral lineages of rhinolophid and hipposiderid bats; these included

exons 4, 5, 9 – 11 and 15 – 18. These exons were mainly situated in the functionally important exposed domains of the protein, including the extracellular loops, coil domains and the STAS (sulphate transporters and antisigma factor antagonists: Navaratnam et al. 2005) domain (Figure 4.1). The seminal literature on *Prestin* evolution was based on cDNA synthesised from mRNA obtained from the brain tissues from only a few individuals representing phylogenetically diverse echolocating and non-echolocating mammalian lineages. Sequencing those exons characterised by strong signatures of positive selection allows a greater number of individuals with diverse echolocation frequencies to be sampled, thereby enabling a more robust comparison of *Prestin* sequence variation within and between closely related species.

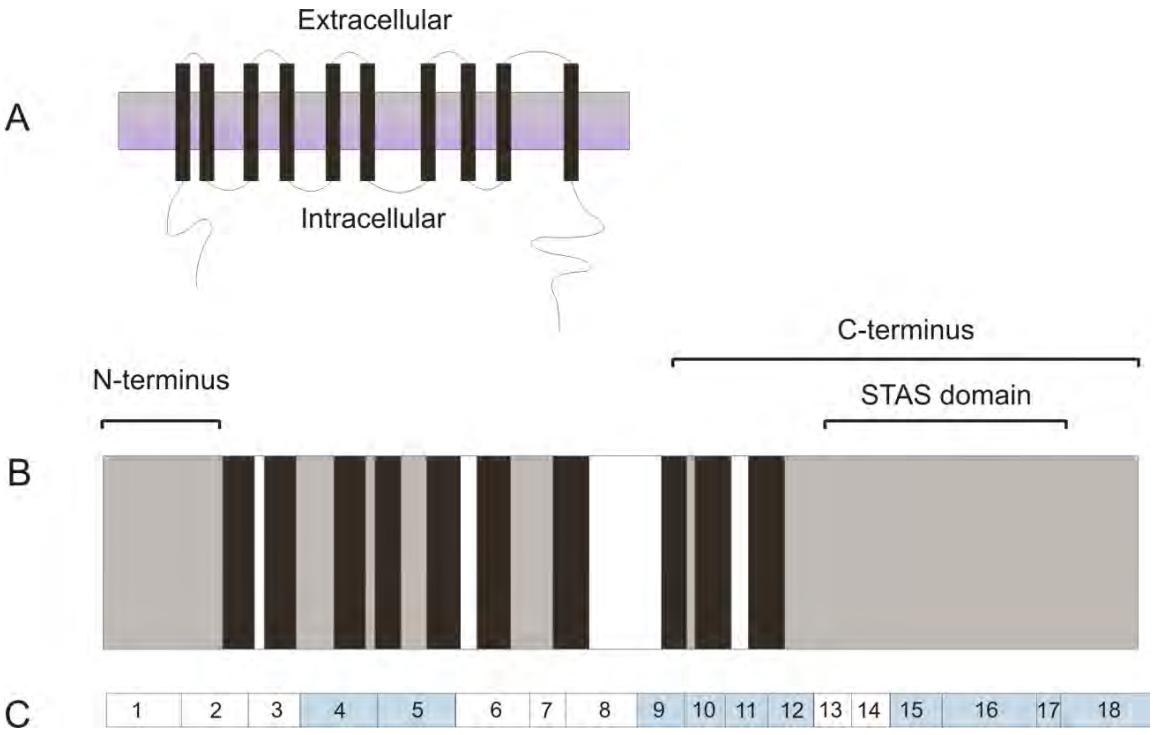

Figure 4.1: (A) Simplified structural model of the prestin protein showing 10 transmembrane domains (black) (modified from Li et al. 2010). (B) Schematic showing the different domains of the protein including the transmembrane (black), extracellular (white) and intracellular (grey) domains with (C) the corresponding positions of the 18 exons of the *Prestin* gene (modified from Li et al. 2008). Exons highlighted in blue were amplified in this study.

Primers were designed from conserved intron or exon regions of the gene using Primer3Plus (Untergasser et al. 2007) (see Table 4.1 and Figure 4.2 for details). PCR conditions consisted of an initial cycle of 94°C for 5 minutes, followed by 40 cycles of 94°C, 50 – 55°C and 72°C each for one min and a final step of 72°C for 20 minutes. All PCR reactions included a negative control consisting of all reagents except DNA to check for contamination. PCR products were separated by electrophoresis in a 2% agrose gel with ethidium bromide and gel purified using a Wizard SV Gel and PCR Clean-up System (Promega).

Table 4.1: Primer sequences used to amplify the different exons of the *Prestin* gene used in this study.

Primer name		**Primer sequence**	**Product size**
		5' **3'**	
Pres EX 4/5		F: GTCCGTTTGCTGTTATTAGCC	655 bp
		R: CACTGAAAAGATCCCACTGTAC	
Internal primers	PR Ex4	R: GCCAAGCGTGCTCAATAGAC	
	PR Ex5	F: CTCACAGAGCCCCTGGTG	
Pres EX 9/10		F: CTCATTGCCCTGGGACTG	985 bp
		R: GGGTAATGATTCAAAGAGGAATC	
Internal primers	PR Ex9	R: CCTGAGAGGCCAGTTGTAC	
	PR Ex10	F: CGTCTCCTAAAAGCCCCTTC	
Pres EX 11/12		F: GCTGTCGGCCATTGTTATC	401 bp
		R: GTTCTGTAAATCACAGTCATCAG	
Internal primers	PR Ex11	R: GCACTACTCCAGGTGTTGC	
	PR Ex 12	F: GGTCATCTGGCTTAGCAC	
Pres EX 15 – 17		F: CTGGAGTGAACCCAGCATTC	1849 bp
		R: CACTGCARCCTGCTAAATACA	
Internal primers	PR Ex15	R: GAGAAGGAGGTGACAATGAAGG	
	PR Ex16	R: CAACACTGGGCACAGAGC	
	PR INT	F: GCAGAAGTAGATGCAGAAGATGG	
Pres EX 18		F: CAAGTTATAAGTGACCTCACTC	184 bp
		R: CTAGACGTCGGGAGTGGC	

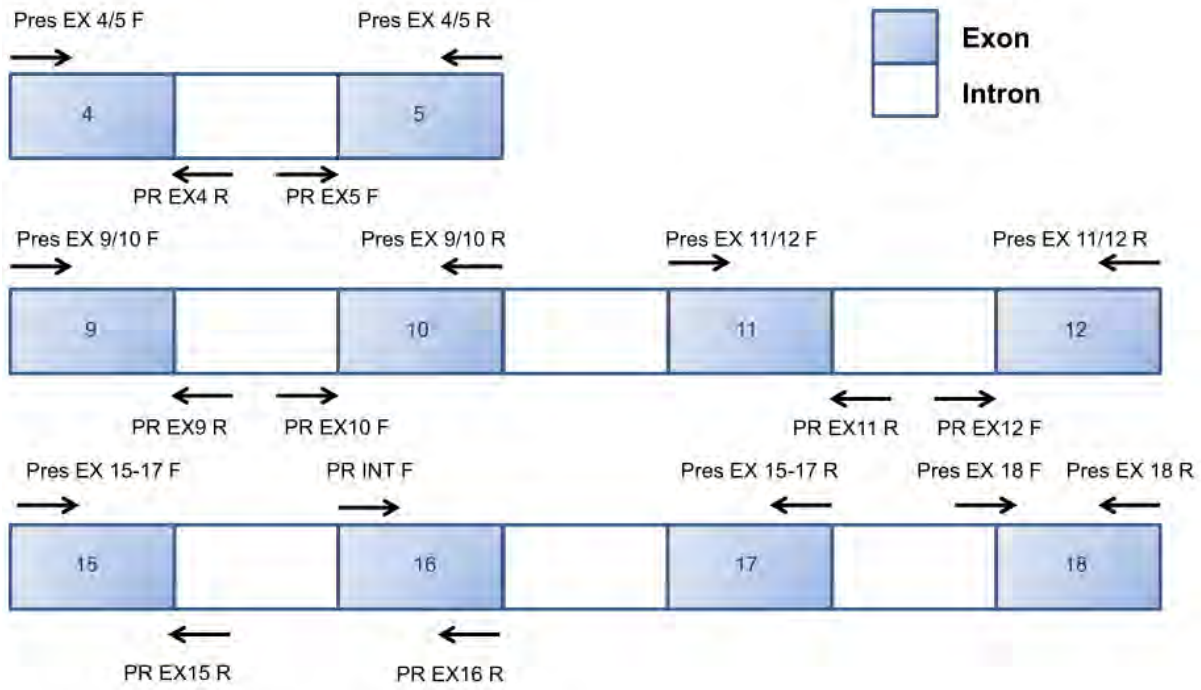

Figure 4.2: Schematic diagram showing the location of each primer designed to amplify the 10 different exons of the *Prestin* gene used in this study.

Exons were sequenced using BigDye 3.1 chemistry on an ABI 3730 XL DNA Analyser (Applied Biosystems). The exon-intron boundaries were verified using the complete coding sequence (from genomic DNA) of *Prestin* from the bottlenose dolphin (*Tursiops truncatus*) to ensure the correct reading-frame was used when converting the nucleotide sequences to amino acid sequences. The alignments of the ten exons were combined to create a 1272 bp fragment (= 424 amino acids) of the coding region of *Prestin*, and the in-frame protein coding sequences were edited, aligned and translated into amino acid sequences using BioEdit version 7.1.3.0 (Hall 1999). The amino acid alignment of all taxa used in this study is provided in Appendix 5.

Data analysis

(i) Reconstructing the evolutionary relationships in the *Prestin* gene tree

A Bayesian phylogenetic reconstruction based on amino acid sequences was implemented in MrBayes 3.2 (Ronquist et al. 2012) to determine whether (i) the phylogeny based on the partial *Prestin* coding sequence was concordant with the

previously published *Prestin* trees based on the entire coding sequence of the gene where echolocating dolphins and porpoises form a sister group to rhinolophid and hipposiderid bats; and (ii) to determine whether members of the *R. capensis* clade groups with other horseshoe bats. The dataset for this analysis included the 20 eutherian mammal sequences and one randomly selected representative of *R. capensis*, *R. swinnyi*, *R. denti* and *R. simulator*.

Following Lui et al. (2010) Bayesian phylogeny reconstruction of amino acid sequences was performed in MrBayes using the JTT + I + Γ + F substitution model. The amino acid substitution model was determined using ProtTest (Abascal et al. 2005) using Akaike information criterion (Akaike 1974). Phylogeny reconstruction was based on 5 million generations with a sampling frequency of 100 and a burn-in of 25%. Markov chain convergence after burn-in was checked in Tracer v1.6 (Rambaut et al. 2014).

(ii) Functional variation in *Prestin* within the *R. capensis* clade

Divergent selection can act against maladaptive genes that specifically encode a trait under selection while at the same time allowing gene flow between neutral alleles (Hey 2006). Under this scenario adaptive loci are expected to covary with trait divergence among divergent populations even in the presence of gene flow (Nosil et al. 2009; Freeland et al. 2010). To explore whether RF divergence among *R. capensis* populations and echolocation diversity between species of the *R. capensis* clade is reflected in coding sequence variation in *Prestin*, the number of amino acid substitutions in the protein sequences were determined. Variable amino acid sites were mapped onto the simplified structural model of prestin to determine whether these replacements occurred in functionally important domains of the protein. The frequencies of occurrence of the variable amino acid sites were calculated (i) across populations of *R. capensis* to determine whether the spatial distribution of functional variation in *Prestin* covaries with acoustic divergence among *R. capensis* populations and (ii) across members of the *R. capensis* clade with divergent echolocation frequencies to identify potential species-specific and/or clade-specific variable amino sites.

(iii) Tests of molecular evolution

Previous studies have shown that *Prestin* evolution in ancestral lineages of rhinolophid and hipposiderid bats and echolocating cetaceans are characterised by signatures of adaptive and accelerated evolution (Li et al. 2008, 2010; Liu et al. 2010; Rossiter et al. 2011). SELECTON 2.2 (http://selecton.bioinfo.tau.ac.il) (Stern et al. 2007) was used to determine whether protein sequences in the extant lineages used in this study are also shaped by signatures of adaptive evolution. Highly variable sites may indicate positive Darwinian selection, which can be interpreted as the consequence of adaptation at the molecular level. In contrast, evolutionary conserved sites may indicate functionally important amino acids and therefore mutations at these sites could be deleterious. Conserved sites are therefore likely to represent sites under purifying selection. SELECTON detects the level of selection operating on different amino acid sites by computing the ratio, ω, between non-synonymous substitutions (K_a: amino acid changing) and synonymous substitutions (K_s: silent changes). Generally a K_a:K_s ratio (ω) > 1 indicates positive selection at that amino acid site, whereas a ω < 1 suggests purifying selection (Nielsen 2005). To test the hypothesis that positive selection may influence the evolution of RF divergence among populations of *R. capensis* and among species of the *R. capensis* clade, ω was calculated for each of the 424 amino acid sites of *Prestin*. The dataset included five individuals (randomly selected) from each *R. capensis* population, and five each from *R. denti*, *R. simulator* and *R. swinnyi*. The significance of these selection estimates were tested by comparing the second order Akaike Information Criterion (AIC_C) scores between the null M8a model (which does not allow for positive selection) and the MEC model (which does allow for positive selection); the lower the AIC_C score, the better the fit of the model to the data. The MEC model using the JTT amino acid replacement matrix was implemented to account for different replacement probabilities between different amino acids. For comparative purposes, this analysis was repeated with a the dataset comprised of horseshoe bats, hipposiderid bats and echolocating cetaceans to confirm whether protein sequences in these extant lineages are also characterised by positive selection, as has previously been found for their ancestral lineages.

RESULTS

(i) Phylogenetic reconstruction of *Prestin* gene tree

The Bayesian gene tree based on the partial *Prestin* sequence recovered a similar topology to previously published results for *Prestin*; horseshoe and hipposiderid bats grouped with echolocating toothed whales and all other echolocating bats and fruit bats formed a monophyletic clade (Figure 4.3 A). This result, as others have also found, is in conflict with the currently accepted species tree (Figure 4.3 B) where laryngeal echolocators are paraphyletic with one clade including non-echolocating fruit bats as sister to horseshoe bats (Yinpterochiroptera), and another comprised of all other bats that use laryngeal echolocation (Yangochiroptera) (Li et al. 2008; Teeling et al. 2012). As expected the members of the *R. capensis* clade grouped together with other rhinolophid and hipposiderid bats in the *Prestin* gene tree.

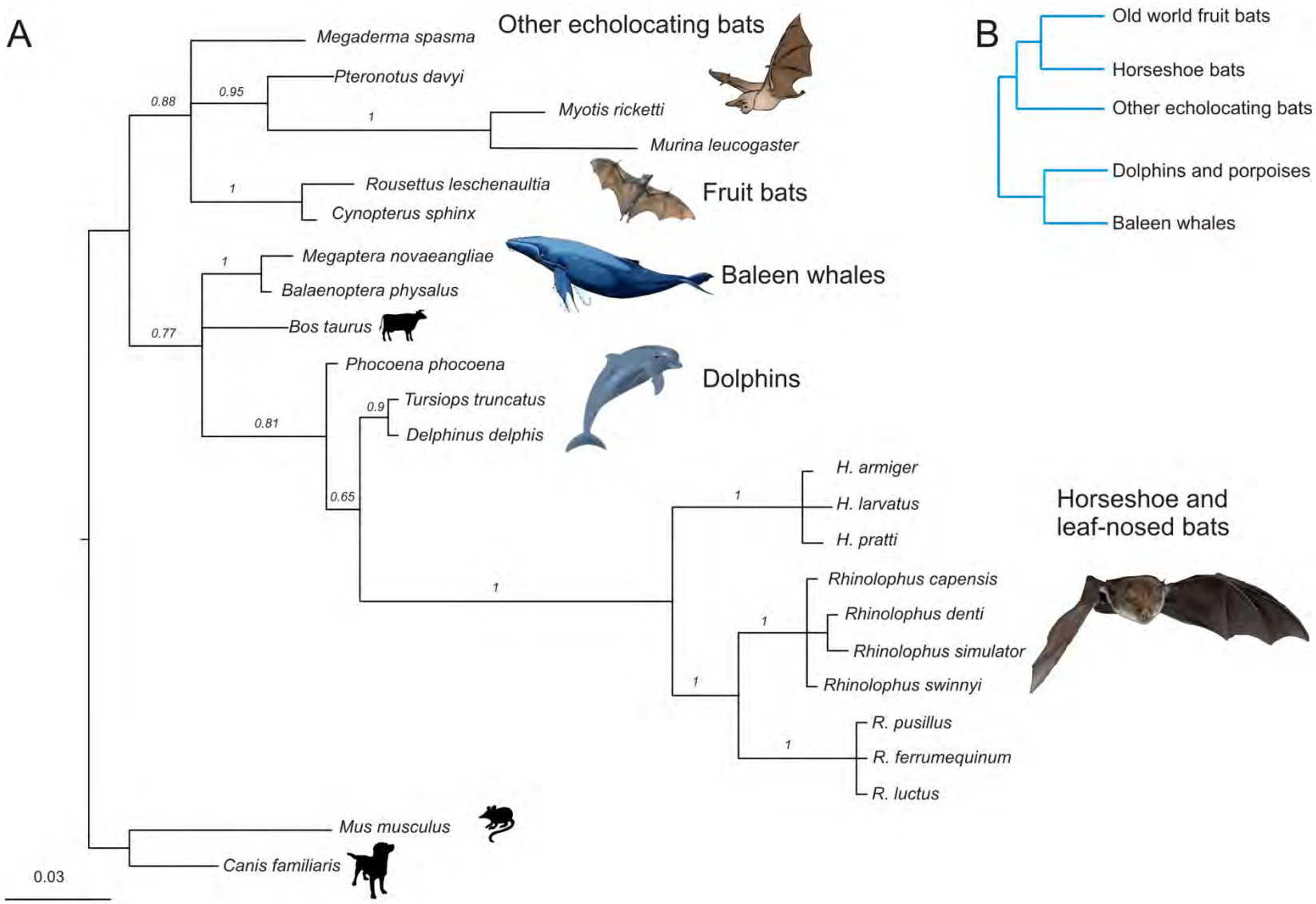

Figure 4.3: (A) A Bayesian *Prestin* protein tree with posterior probability values reported and (B) simplified currently accepted species topology tree adapted from Jones 2010.

(ii) Functional variation in *Prestin* within the *R. capensis* clade

Prestin variation within the *R. capensis* clade is characterised by highly conserved gene sequences. Only one point mutation in the nucleotide sequences among populations of *R. capensis* with divergent RFs was found. This was a non-synonymous substitution resulting in an amino acid change from glycine (Gly: symbol G) to glutamic acid (Glu: symbol E) at amino acid site 361 (G361E) (Figure 4.4 A). While this point mutation did not occur among individuals from LS (75 kHz), a large proportion of individuals from different *R. capensis* populations displayed the change from Gly to Glu at this site (Figure 4.4 C). Furthermore, the same amino acid replacement was also found in some individuals from different species in the *R. capensis* clade (Figure 4.4 C), and only *R. simulator* sequences had an additional amino acid substitution from glutamine (Gln: symbol Q) to arginine (Arg: symbol R) at position 374 (Q374R) (Figure 4.4 A). Both positions are situated in the functionally important STAS domain of the protein (Figure 4.4 B). The paucity of substitutions and low number of variable amino acid sites across species and among populations with highly divergent call frequencies (Fig 4.4 A), and therefore frequencies of best-hearing, precludes any correlation between the amino acid replacements discovered and the frequency of best-hearing within *R. capensis*, and among its close relatives.

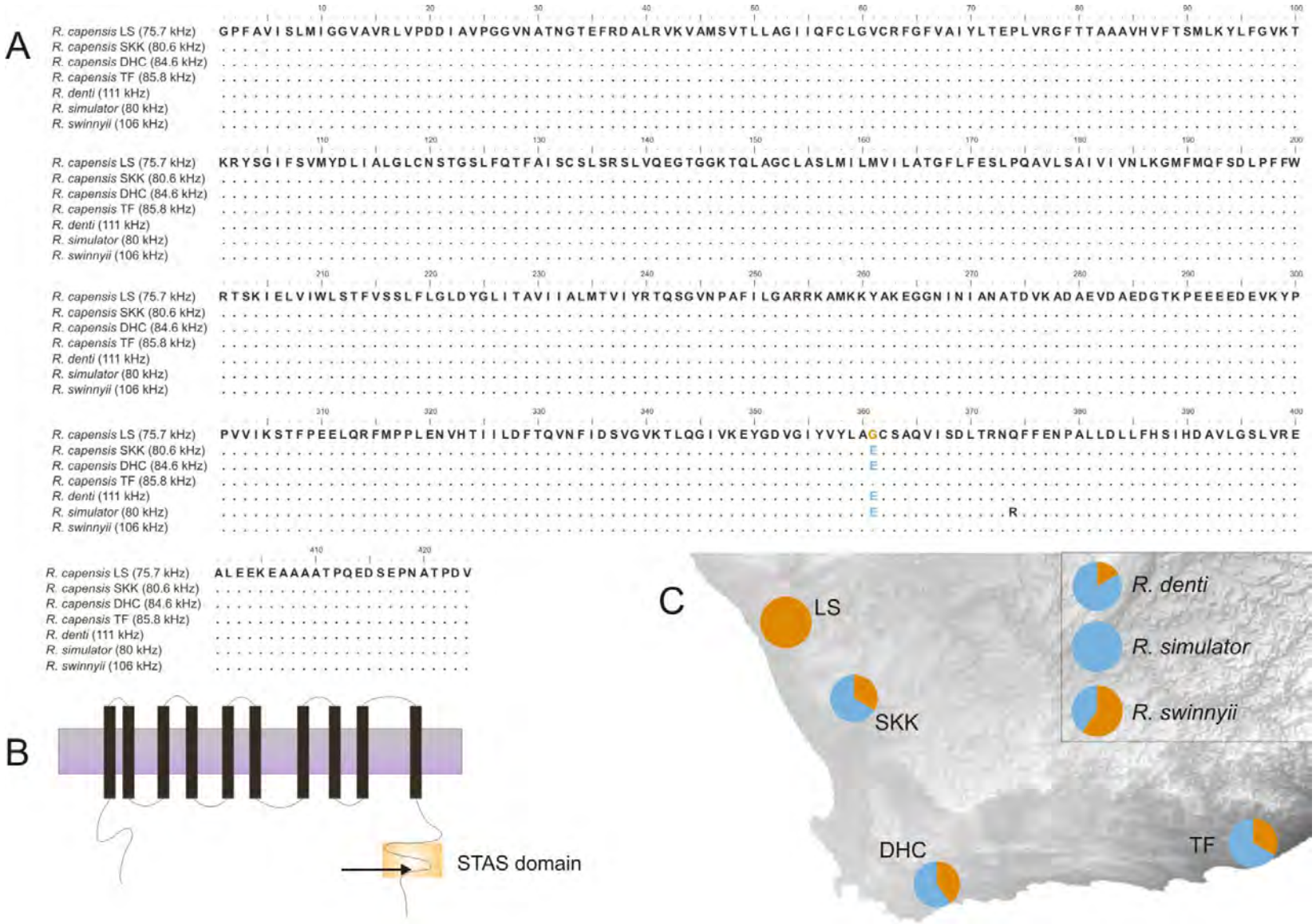

Figure 4.4: (A) Functional variation in *Prestin* amino acids among *R. capensis* populations with divergent RFs and among members of the *R. capensis* clade. (B) Relative location of the two variable amino acid sites (G361E and Q374R) in the structural model of prestin. (C) Frequency distribution of the amino acid replacement at position G361E (orange: G; blue E) within each *R. capensis* population and within other members of the *R. capensis* clade.

(iii) Tests for selection

The analysis based on the dataset containing horseshoe bats, hipposiderid bats and cetacean species indicated that most amino acid sites in the *Prestin* sequences were characterised by signatures of purifying selection, but there was also evidence of strong positive selection at seven amino acid sites, and moderate positive selection at 24 sites (Figure 4.5 A). Furthermore, both variable amino acid sites identified within the *R. capensis* clade (G361E and Q374R) are characterised by signatures of strong positive selection in rhinolophid and hipposiderid bats and echolocating cetaceans (Figure 4.5 A). When comparing the MEC model which does allow for positive selection with the M8a model which does not, the AIC_C scores indicated that the MEC model was a better fit to the data (AIC_C score for MEC: 5440.5 and AIC_C score for M8a: 5468.6). In contrast, the analysis based on the dataset containing only the four populations of *R. capensis* with divergent RFs and other members of the *R. capensis* clade found no positively selected sites in the protein sequence (Figure 4.5 B). *Prestin* within the *R. capensis* clade is therefore highly conserved and is characterised by strong signatures of purifying selection, even though the two amino acid substitutions detected occur in a functionally important region of the protein.

Figure 4.5: Colour-coded SELECTON results for *Prestin* run with (A) 15 sequences for rhinolophid and hipposiderid bats, and echolocating cetaceans and (B) 35 sequences for species of the *R. capensis* clade, including four populations of *R. capensis* with divergent RFs. Warm colours indicate $\omega > 1$ (positive selection) and cool colours indicate $\omega < 1$ (purifying selection).

DISCUSSION

A candidate gene approach was used to explore whether sensory divergence within *R. capensis* and among species of the *R. capensis* clade is reflected in functional sequence variation in *Prestin*, the gene responsible for the remarkable frequency selectivity and sensitivity of the mammalian cochlear. Members of the *R. capensis* clade group with other rhinolophid and hipposiderid bats in the *Prestin* gene tree and, consistent with results from previous studies, the clade is sister to echolocating cetaceans (Figure 4.3). Only two variable amino acid sites situated in the STAS domain was found in the *Prestin* coding sequences among members of the *R. capensis* clade; one is restricted to *R. simulator* (Q374R), and the other (G361E) occurs across acoustically divergent *R. capensis* populations as well as across species in the clade (Figure 4.4). Functional variation in *Prestin* is therefore unlikely to be associated with RF differences either among populations of *R. capensis* (range: 75.7 – 85.8 kHz) or among its closely related species (range 75.7 (*R. capensis* at LS) – 111 kHz (*R. denti*)). In contrast to the significant signatures of positive selection reported in ancestral high duty-cycle echolocating bats and echolocating cetaceans, *Prestin* evolution within the *R. capensis* clade is characterised by signatures of purifying selection (Figure 4.5 A and B). Indeed, *Prestin* appears to be highly conserved within and between species of the *R. capensis* clade. It is possible that differences in *Prestin* gene expression may instead contribute to RF variation in *R. capensis* and if so, it may suggest that selection for gene expression mediated phenotypic plasticity may play an underappreciated role in the evolution of sensory divergence, at least in horseshoe bats. However, echolocation is a complex trait that relies on the interaction of many genes involved in different aspects of the echolocation system of bats (Teeling 2009; Jones et al. 2013; Table 4.1). Therefore the role of different genes such as those involved in the production of high frequency calls and/or the detection of the echoes from the calls and/or the interpretation of these echoes cannot be discounted.

Discordance between RF divergence and functional variation in *Prestin*

Because functional genes are directly responsible for generating adaptive phenotypes, a candidate gene approach can provide important insights into the molecular basis of

evolutionary adaptation (Fitzpatrick et al. 2005). Studies investigating genes involved in foraging (e.g. venom protein genes: Binford et al. 2009, Gibbs et al. 2009; craniofacial morphology: Abzhanov et al. 2004, Parsons & Albertson 2009) and sensory ecology (e.g. visual genes: Larmuseau et al. 2010; olfactory genes: Stathopoulos et al. 2014; pigmentation genes: Hubbard et al. 2010, Manceau et al. 2010; auditory genes: Shen et al. 2012; vocalisation genes: Wohlgemuth et al. 2014) have been very valuable in expanding our understanding of adaptive evolution because the phenotypic traits they encode directly influence fitness. Many studies report a strong link between the origin and maintenance of adaptive phenotypes and functional variation in protein coding sequences and/or differential gene expression (e.g. Hoekstra et al. 2004; Steiner et al. 2007; Larsen et al. 2008; Mullen et al. 2009; Manceau et al. 2010; Pavey et al. 2010; Hunt et al. 2013; Schneider et al. 2014). Sensory divergence among *R. capensis* populations is characterised by a clinal increase in RFs from west to east across the distribution of the species, likely due to an increase in habitat complexity across biomes (Chapter 2). Clinal variation in adaptive traits can provide strong evidence for local adaptation across heterogeneous environments (Mullen & Hoekstra 2008; Antoniazza et al. 2010) even in the presence of significant gene flow (reviewed in: Hey 2006; Nosil 2008; Pinho & Hey 2010). Indeed, the differential exchange of genes that do and do not encode the traits under selection is central to 'divergence-with-gene' flow models (Pinho & Hey 2010). If selection drives sensory divergence among populations of *R. capensis* in the presence of substantial gene flow, it's reasonable to predict that RF differences will be associated with functional variation in associated coding gene sequences. Instead, results from this study indicate that *Prestin* coding sequences are highly conserved within the *R. capensis* clade, and only two variable amino acid sites were found, both situated in the STAS domain. This domain is situated in the cytoplasmic C-terminus of the protein known to play a role in anion binding, membrane targeting and the voltage-dependent conformational changes in the protein (Zheng et al. 2005). Furthermore, the STAS domain functions in both intramolecular (e.g. lipid bilayer, transmembrane domains) and intermolecular (e.g. other proteins) interactions (Pasqualetto et al. 2010) and therefore the two variable amino acid sites identified likely have functional consequences for the protein. However, these sites do not appear to be associated with any particular RF either

among *R. capensis* populations, or between members of the clade. For example, three *R. capensis* individuals from TF (85.8 kHz) share an amino acid sequence with *R. capensis* from LS (75.6 kHz) and the amino acid replacement at position 361 (from G to E) is also shared across individuals from acoustically divergent populations of the species.

Although only 60% of the coding region of *Prestin* was sequenced and analysed in this study, it is unlikely that amino acid substitutions in other regions of the protein not sequenced would reveal different associations between coding sequence variation and RF variation because only sequences for the functionally important exposed domains of the protein (the cytoplasmic and extracellular loops including the coil domains and STAS domain) under strong positive selection in horseshoe bat lineages were analysed (Li et al. 2008). Furthermore, phylogenetic reconstructions based on nucleotide sequences corresponding to synonymous substitutions or transmembrane domains of the protein (not sequenced in this study) both recovered the currently accepted species topology where laryngeal echolocators were paraphyletic (Li et al. 2008). The results presented here therefore indicate that coding sequence variation in *Prestin* does not reflect divergent selection for different frequencies of best-hearing of the acoustic fovea, and corresponding RFs within the *R. capensis* clade.

A few amino acid substitutions in protein sequences can result in large differences in adaptive phenotypes (Carroll 2005; Hoekstra & Coyne 2007). For example, a single non-synonymous substitution in the coding region of the pigmentation melanocortin-1-receptor gene (*Mc1r*) correlates with adaptive pigmentation variation in rodents (Hoekstra et al. 2006; Mullen et al. 2009; but see Mullen & Hoekstra 2008) and birds (Uy et al. 2009). In contrast, the amino acid substitution in *Prestin* at position G361E is not fixed in any particular *R. capensis* population, and therefore is not associated with any particular RF. Remarkably, the same amino acid substitution is also found among different individuals of *R. swinnyi*, *R. simulator* and *R. denti*. Given that RF is tightly coupled to the frequency of best-hearing of the acoustic fovea in horseshoe bats (reviewed in Schnitzler & Denzinger 2011), and that bats with more than a 30 kHz difference in RF share *Prestin* coding sequences, it is unlikely that the variable amino sites identified are associated with sensory divergence within and between species of

the *R. capensis* clade. Instead, *Prestin* sequences in the clade reflect phylogeny rather than RF differences within and between species of the clade.

Recently Liu et al. (2014) used functional assays to determine whether parallel amino acid substitutions in *Prestin* identified in the ancestral branches of all echolocating mammals and branches leading to high duty-cycle bats and echolocating cetaceans, resulted in parallel functional convergence in the protein. Somatic motility of OHCs is accompanied by voltage-mediated charge movement which is reflected in nonlinear capacitance (NLC) (Ashmore 1989). Because NLC can easily be measured, it is often used to evaluate the function of the protein using functional assays. Liu et al. (2014) found that the parallel substitution at position N7T (situated in exon 1, not sequenced in this study) resulted in the convergence in a functional parameter, $1/\alpha$, of prestin NLC in all echolocating mammals. A second parallel substitution at position I384T (corresponding to position 122 in this study) occurring only in lineages leading to echolocating cetaceans and high duty-cycle bats was responsible for the convergence in $V_{1/2}$, another functional parameter of prestin NLC (Liu et al. 2014). Moreover, these functional parameters were significantly correlated to the best-hearing frequencies measured from published audiograms of mammals used in their analysis even after correcting for phylogeny. This showed for the first time that parallel amino acid replacements at the protein sequence level may underlie convergent functional changes in prestin in echolocating mammals (Liu et al. 2014). Interestingly, in this study amino acid position 122 which corresponds to position I384T of Liu et al. (2014) is conserved across acoustically divergent *R. capensis* populations and among species of the clade (Figure 4.4). This further supports the idea that *Prestin* sequences are highly conserved within the clade, and does not reflect echolocation frequency variation neither among *R. capensis* populations nor among its close relatives. Similarly, Audzijonyt et al. (2012) found no correlation between amino acid changes in opsin gene sequences and the spectral absorbance of visual pigments within and among *Mysis* (Crustacea: Mysida) species living under different light environments. Taken together, this suggests that sequence variation in ecologically important genes may not always contribute to the evolution of local adaptation (e.g. Audzijonyt et al. 2012; Dobson et al. 2012; Raeymaekers et al. 2014).

Selective regime shifts in the evolution of *Prestin*

In sharp contrast to the significant signatures of positive selection detected in the ancestral lineages of echolocating mammals (Figure 4.5 A; Li et al. 2008, 2010; Liu et al. 2010; Rossiter et al. 2011; Liu et al. 2014), *Prestin* evolution within the *R. capensis* clade is characterised by substantial purifying selection (Figure 4.5 B). This is perhaps unsurprising since non-synonymous mutations in coding regions are usually deleterious and therefore, as predicted by neutral evolutionary theory (Kimura 1983), purifying selection is usually the dominant force acting on functionally important coding regions (Kimura 1977; Hughes 2007). Although adaptive changes in *Prestin* were crucial for the evolution of echolocation in general (Liu et al. 2014), results reported here suggest that once the ability for sophisticated high frequency hearing evolved in horseshoe bats further amino acid changes were not favoured, leading to prestin becoming functionally constrained.

Intraspecific and interspecific variation in RFs in the *R. capensis* clade may be influenced by changes in *Prestin* gene expression patterns rather than by changes at the coding sequence level (Hughes 2007). Gene expression patterns are shaped by genotype (and are therefore partly heritable) and by the environment (Pavey et al. 2010) and therefore both natural selection and genetic drift can influence its evolution (Whitehead & Crawford 2006). Studies have shown that changes in gene regulation and expression regularly underlie adaptive phenotypic differences within (Oleksiak et al. 2002; Fangue et al. 2006; Badyaev et al. 2008; Larsen et al. 2008; Gibbs et al. 2009; McCairns & Bernatchez 2009; Scoville & Pfrender 2010; Granados-Cifuentes et al. 2013; Morris et al. 2014) and between (Oleksiak et al. 2002; Abzhanov et al. 2004, 2006) species (reviewed in: Gilad et al. 2006; Whitehead & Crawford 2006; Fay & Wittkopp 2008; Pavey et al. 2010). For example, differences in the gene expression patterns of the bone morphometric protein 4 (*Bmp4*) and calmodulin gene (*CaM*) underlie adaptive divergence in craniofacial morphology in Darwin's finches (Abzhanov et al. 2004, 2006) and African cichlids (Albertson et al. 2005; Parsons & Albertson 2009), highlighting the importance of gene regulation and expression in ecological speciation (Parsons & Albertson 2009; Pavey et al. 2010). Furthermore,

environmentally induced changes in gene expression can facilitate the evolution of adaptive phenotypic plasticity in populations colonising new environments (Aubin-Horth & Renn 2009; Pavey et al. 2010). Gene expression mediated plasticity may therefore promote population persistence (Pavey et al. 2010; Gomez-Mestre & Jovani 2013), thereby increasing the potential for future adaptive genetic divergence (Schlichting & Pigliucci 1993; Schlichting & Smith 2002; Scoville & Pfrender 2010; Morris et al. 2014; Schneider et al. 2014). Comparisons of gene expression patterns of candidate hearing genes among populations of horseshoe bats with divergent RFs may provide important insights the role of plasticity in the evolution of RF divergence within and between species. However, because echolocation is a highly complex trait that requires the integration of various morphological and neurophysiological adaptations to facilitate the emission of calls and the reception and interpretation of their echoes (Teeling 2009; Moss & Surlykke 2010), the influence of many other genes on the evolution of sensory divergence (summarised in Table 4.2) cannot be discounted.

Conclusions

This chapter explored whether intraspecific and interspecific variation in RFs was associated with protein coding sequence differences in the hearing gene *Prestin*. Results suggest that coding sequence variation in *Prestin* is not associated with sensory divergence within *R. capensis* or among species of the *R. capensis* clade and that *Prestin* is functionally constrained within the clade. Environmentally induced differences in *Prestin* gene regulation and expression patterns may instead influence RF divergence within and between species of the clade and if so, selection for gene expression mediated phenotypic plasticity may play an underappreciated role in the evolution of sensory divergence in horseshoe bats. However, because echolocation is a highly complex trait controlled by many other genes in addition to *Prestin*, rather an in-depth analysis of variation in coding sequences and gene expression patterns of multiple candidate genes involved in echolocation is likely to provide novel insight into the evolution of sensory divergence within and between species.

Table 4.1: A brief review of selected auditory genes with known function, and which are suggested to have played a role in the evolution of echolocation. Genome scans have identified a large number of other genes possibly involved in hearing in echolocating bats (e.g. Parker et al. 2013), but the exact functional role of these genes have yet to be determined, and therefore are not included here.

Echolocation genes	Known functional role	Key findings related to echolocation	References
FoxP2	Involved in the development of orofacial coordination related to vocalisation as well as sensory-motor coordination in mammals and birds	*FoxP2* evolution shows signals of accelerated and divergent selection among echolocating lineages, possibly related to the evolution of different echolocation strategies and their integration with motor behaviours such as flight.	Fisher &Marcus 2006; Li et al. 2007; Wohlgemuth et al. 2014.
Kcnq4	Encodes a protein that forms a voltage-gated potassium channel which plays a key role in the regulation of electrical signalling in OHCs.	Phylogenetic reconstructions based on this gene group all laryngeal echolocating bats into a monophyletic clade, and several amino acid substitutions are shared between echolocating bats in the Yinpterochiroptera and Yangochiroptera suborders.	Kharkovets et al. 2000; Liu et al. 2011; Liu et al. 2012.
Tmc1	Encodes a transmembrane protein of both IHCs and OHCs that may play roles in transporting molecules to the plasma membrane and in hair cell maturation.	Similar to results for *Prestin* and *Kcnq4*, phylogenetic reconstructions based on *Tmc1* and *Pjvk* group all laryngeal echolocating bats into a monophyletic clade. Also, signals of positive selection in *Tmc1* were found in some bat lineages and echolocating cetaceans, indicating that the gene may be involved in the evolution of high frequency hearing.	Marcotti et al. 2006; Delmaghani et al. 2006; Schwander et al. 2007; Davies et al. 2012.
Pjvk	Encodes a protein called pejvakin and mutations in this gene has been linked auditory neuropathy in humans and the disruption of hair cell activity in mice.		
Cdh23 and its ligand *Pcdh15*	The proteins encoded by these genes are required for efficient hair bundle motility because they form the upper and lower parts of tip-links which lie between the stereocilia of the hair bundle.	Evidence of parallel evolution in *Cdh23*, *Pcdh15* and *Otof* was found in both bat suborders and echolocating cetaceans. Significant signals of positive selection in *Cdh23* and *Pcdh15* were also found in different echolocating lineages. Furthermore, higher levels of *Otof* expression were found in the auditory cortex of adult echolocating bats than their embryo's or non-echolocating bats. These results indicate that genes which play different roles in the echolocation system of different echolocating lineages co-evolved.	Di Palma et al. 2001; Alagramam et al. 2001; Roux et al. 2006; Schug et al. 2006; Shen et al. 2012.
Otof	Encodes a protein that triggers membrane fusion at the ribbon synapse of IHC, as well as potentially playing a role in transmitting auditory signals to the brain		

CHAPTER 5

SYNTHESIS & CONCLUSIONS

In species distributed across heterogeneous environments, spatially varying selection pressures can promote local adaptation (Williams 1966; Kawecki & Ebert 2004; Nosil 2012; Blanquart et al. 2013). The degree to which this occurs depends not only on the complex interactions between selection and gene flow, but also on their relative strengths within single populations across a species distribution (Chapter 1; Lenormand 2002; Kawecki & Ebert 2004; Garant et al. 2007; Cheviron & Brumfield 2009; Blanquart et al. 2013). A comprehensive understanding of the evolution of local adaptation requires both an investigation of the relationship between traits and their associated environments, and the quantification of gene flow between populations occupying different environments (Garant et al. 2007; Räsänen & Hendry 2008; Cheviron & Brumfield 2009).

This study explored the relative roles of neutral and adaptive processes in the evolution of sensory divergence in Cape horseshoe bats, *Rhinolophus capensis.* The species demonstrates remarkable ecological success across a wide range of habitats and is characterised by extensive geographical variation in the resting frequency (RF) of its echolocation calls. Population variation in RF follows a strong gradient of increasing frequency from west (75.7 kHz: open and sparse habitats) to east (86 kHz: more cluttered habitats) across the range of the species (Chapter 2). The aims of this study were to determine (i) how the interplay between spatially varying selection and genetic drift shape patterns of trait divergence in a species that is distributed across a heterogeneous landscape; (ii) whether ecologically mediated sensory divergence among populations is associated with reduced gene flow among divergent populations; and (iii) whether sensory divergence is reflected in protein-coding sequence differences in a functional gene associated with the trait. To do this a range of hypotheses were explored to understand the relative influence of body size, functional morphology, habitat structure, humidity, gene flow, and functional gene variation on the observed pattern of RF variation in *R. capensis*. The findings presented

here reveal that sensory divergence in echolocation frequencies is largely driven by a distinct habitat gradient which characterises the distribution of this species, and that both divergent selection and adaptive phenotypic plasticity may promote the evolution of local adaptation of RFs despite the presence of considerable gene flow. Furthermore, protein-coding sequences of a candidate gene associated with the trait are highly conserved, suggesting that the evolution of local adaptation may not always be reflected in coding sequence variation in ecologically important genes.

An emerging paradigm: gene flow can promote, rather than constrain, the evolution of local adaptation

Adaptive divergence among populations within a species is generally thought to reflect a balance between the diversifying effects of selection and the homogenising effects of gene flow (Haldane 1948). Indeed, an inverse relationship between gene flow and adaptive divergence characterises population differentiation in many organisms (e.g. Nosil & Crespi 2004; Crispo et al. 2006), which led to the prevailing view that gene flow plays a constraining rather than diversifying evolutionary role (Räsänen & Hendry 2008). Recent studies however also report that gene flow among populations may be an important source of novelty that can introduce mutations on which local selection can act (Swindell & Bouzat 2006; Sexton et al. 2011; Fitzpatrick et al. 2015). Gene flow can therefore also promote the evolution of local adaptation (Garant et al. 2005, 2007; Räsänen & Hendry 2008; Edelaar & Bolnick 2012). Moreover, dispersal and gene flow can promote local adaptation via selection for phenotypic plasticity (Lind et al. 2010; Jourdan-Pineau et al. 2012), where high dispersal rates across heterogeneous environments might favour the evolution of plasticity over genetically-based adaptive divergence (Scheiner 1998; Sultan & Spencer 2002; Scheiner et al. 2012). Although much is now known about the complex and often opposing evolutionary roles of gene flow, remarkably little is known about how patterns of gene flow vary across species' distributions, and how these patterns relate to adaptive divergence of traits across environmental gradients (Sexton et al. 2014).

This study used an integrative framework combining ecology and neutral evolutionary theory to elucidate the complex interactions between selection and gene flow in

shaping patterns of sensory divergence across the distinct habitat gradient that characterises the distribution of *R. capensis*. While a number of recent studies have explored the link between neutral population structure and sensory divergence in bats (e.g. Chen et al. 2009; Sun et al. 2013) this study specifically quantifies the strength and direction of maternal gene flow among acoustically divergent populations across the range of a species, revealing for the first time that locally adapted echolocation frequencies can be maintained in the presence of substantial historic gene flow. Furthermore, the high levels of gene flow detected among populations in the centre of the species' distribution versus those at the periphery invokes the hitherto unexplored role of adaptive plasticity in shaping patterns of sensory divergence in horseshoe bats.

Both divergent selection and adaptive plasticity can influence trait divergence in the face of gene flow

The close association between RF variation and differences in habitat clutter in the presence of asymmetric gene flow among populations of *R. capensis* may be a consequence of both (i) divergent selection and (ii) adaptive phenotypic plasticity. Classical divergent selection i.e. RF changes due to increased fitness of heritable RFs, may be the dominant driver of RFs emitted by edge populations (LS and TF), leading to a pattern of reduced gene flow and greater genetic differentiation between edge and central populations (Chapter 3). On the other hand, selection for adaptive plasticity i.e. the ability of individuals to modify their RFs to some degree to better match their environment, in the high gene flow area of the central area of the species' distribution may better explain the relatively small difference in RF (3 kHz) across a moderate clutter gradient (Chapter 3). Some plasticity in echolocation calls may enable dispersing individuals to subtly modify their RFs to better match new habitats characterised by slightly different degrees of clutter.

Irrespective of the roles of divergent selection and/or phenotypic plasticity in RF variation among populations of *R. capensis*, the clear match between RFs and the degree of habitat clutter remains to be explicitly tested; the relationship reported in this study is correlative but nevertheless suggests a close association between RF variation and the degree of habitat clutter among populations (Chapter 2). A

systematic comparison of foraging behaviours of bats between different habitats across the distribution of the species is required to better understand the link between RF variation, flexibility in RF, and foraging behaviour in response to habitat differences in *R. capensis*. This requires knowledge of the various dimensions of the acoustic window of different habitats, which is responsible for delimiting the range of frequencies that function effectively in habitats characterised by different degrees of clutter. Empirical studies using playback experiments to test how habitats vary in their sound transmission properties have revealed mixed support for the Sensory Drive Hypothesis, where habitat differences may (e.g. birds: Tobias et al. 2010, Slabbekoorn et al. 2007; anurans: Ryan & Sullivan 1989; insects: McNett & Cocroft 2008) or may not (e.g. birds: Fotheringham et al. 1997; anurans: Malone et al. 2014; insects: Henry & Wells 2004) explain sensory divergence in various organisms (reviewed in: Malone et al. 2014). Within *R. capensis* this approach could be used to directly measure the transmission properties of different biomes and thereby validate the link found between RF variation and habitat differences across the species' distribution. It is also not known how foraging ecologies differ across species' ranges; most studies investigating habitat use and foraging ecology of horseshoe bats are restricted to a small spatial scale usually encompassing the home ranges of specific species (e.g. Goiti et al. 2003, 2008; Lee et al. 2012). Combining experiments exploring the transmission properties of habitats with a thorough investigation of differences in foraging ecologies in species distributed across environmental gradients is a potentially powerful empirical test for the roles of selection versus plasticity in the evolution of sensory divergence in echolocating bats. Even so, recent empirical studies have shown that high duty-cycle bats are indeed able to modify their echolocation frequencies to some degree in response to different habitats (Xu et al. 2008), conspecifics (Hiryu et al. 2006) and local ambient noise conditions (Hage et al. 2013) and this flexibility may, in part, be due to vocal production learning.

Vocal learning during post natal development and/or over an individual's lifetime influences the evolution of acoustic signals

Vocal production learning, the ability to modify or learn new vocalisations based on auditory input (Janik & Slater 1997) is relatively rare and only documented in mammals and birds. Although evidence of vocal production learning is reported in bats (reviewed in: Knörnschild 2014), cetaceans (Janik & Sayigh 2013; Crance et al. 2014), elephants (Poole et al. 2005) and pinnipeds (Reichmuth & Casey 2014), it is best studied in birds. Because of the numerous neurological and developmental parallels between vocal learning in birds and human speech development, the hummingbirds, parrots and songbirds (reviewed in: Nottebohm & Liu 2010) have been the focus of much research (Doupe & Kuhl 1999; Brainard & Doupe 2002). However, the development and flexibility of birdsong also has some parallels with the ontogeny of echolocation behaviour in bats. For example, songbirds learn their song repertoire from the adults they are exposed to during a critical sensitive period in early development (usually before reproductive maturity). During this time they first memorise a template of the adult song, and then use auditory feedback to compare and modify their developing vocalisations to match that of the adult template (Konishi & Nottebohm 1969; Konishi 1985). Similarly, postnatal development of echolocation in juvenile horseshoe bats is also under auditory feedback control (Rübsamen & Schäfer 1990). However, instead of learning from other conspecifics, young horseshoe bats learn their RFs from their mothers (Jones & Ransome 1993); and this may also be a critical developmental stage for learning frequencies best suited to local conditions. Experimental studies show that both deafened juvenile horseshoe bats (Rübsamen & Schäfer 1990) and songbirds (Konishi 1965) display significant abnormalities in their vocalisations, highlighting the importance of auditory feedback in the development of normal vocalisations. Learning has also been found to play an important role in shaping foraging ecologies of both birds (Slagsvold & Wiebe 2011) and bats (Wund 2005). For example, a cross-fostering study found that early learning caused blue tits, *Cyanistes caeruleus*, to shift their foraging niche to match that of their foster species, and this effect lasted for life (Slagsvold & Wiebe 2011). Using a flight arena, Wund (2005) showed that juvenile little brown bats, *Myotis lucifugus*, changed their echolocation behaviour to match habitats

characterised by different degrees of clutter. With experience, the foraging performance of little brown bats improved significantly, suggesting that bats learnt to produce more efficient calls in different habitats. A similar experiment could be used to assess whether adult and juvenile *R. capensis* individuals are able to modify their echolocation frequencies in response to different degrees of clutter over time, and may shed light on the degree of plasticity in the echolocation system of horseshoe bats generally, and whether vocal learning in bats is restricted to early development as in birds (but see Nottebohm et al. 1986). The latter seems unlikely given that the longevity of bats (Wilkinson & South 2002) provides them with abundant opportunities for learning (Page et al. 2012; Knörnschild 2014).

Dispersal ecologies influence levels of gene flow and adaptive divergence among populations

Dispersal is an important life history trait which shapes the genetic and demographic structure of populations and the extent to which populations are genetically linked and locally adapted (Garant et al. 2007). While gene flow (the movement of gametes between populations) is clearly the result of successful dispersal (the permanent movement of individuals from one population to another), not all dispersal events result in gene flow. Where dispersing individuals have lower fitness in their new habitats, estimates of dispersal may exceed gene flow (Hendry 2004; Nosil et al. 2005; Tobler et al. 2009). The timing of dispersal and the spatial scale over which it occurs relative to environmental heterogeneity also determines whether plasticity or diversifying selection is likely to influence trait evolution (Baythavong 2011; Thibert-Plante & Hendry 2011). If the spatial scale of environmental differences is greater than individual dispersal distances, phenotypic plasticity should be favoured (Sultan & Spencer 2002; Scheiner et al. 2012). While if plasticity occurs after dispersal, it might decrease selection against immigrants because plastic individuals are able to match their phenotype to the new habitats (Thibert-Plante & Hendry 2011).

Although the pattern of long-term maternal gene flow detected among populations of *R. capensis* suggests that individuals are able to disperse over great distances, an in-depth analysis of the dispersal ecology using bi-parentally inherited markers is

required to determine whether sex-biased behaviours influence adaptive divergence in RFs in any way. Dispersal in *R. capensis* may be male-biased, as is the norm for horseshoe bats (e.g. Chen et al. 2008; Yoshino et al. 2008; Flanders et al. 2009; Mao et al. 2010), and this could have important implications for immigrant fitness. For example, a recent study found that female *R. mehelyi* prefer males with higher echolocation frequencies, which were associated with higher body condition indices, and that high frequency males also sire more offspring. This showed for the first time that sexual selection influences the evolution of echolocation in horseshoe bats (Puechmaille et al. 2014). One scenario worth testing in *R. capensis* is whether males disperse into regions with generally lower frequencies (from east to west across the distribution); if so, females may find the immigrant males more attractive than local males, potentially increasing the reproductive success of immigrants, and facilitating gene flow among populations.

An important caveat of the current study is the statistical non-independence of populations due to the significant levels of gene flow detected among them (Stone et al. 2011). Irrespective of the local selection pressures they experience, populations that are more closely related to one another or exchange higher numbers of migrants are likely to have similar phenotypic trait values (Stone et al. 2011). Ideally, the inclusion of migration matrices in the ecological models used in this study would control for the effect of gene flow, but the development of the necessary computational methods to include the complex reticulate relationships among population's remain challenging (Felsenstein 2002; Stone et al. 2011). Nevertheless, the spatial distribution of the four dominant genetic lineages of *R. capensis* recovered in this study does not reflect the broad geographic pattern of RF variation in this species, but rather reflects regional patterns of gene flow. This suggests that gene flow is not associated with RF variation among populations of *R. capensis*, although this remains to be explicitly tested.

Adaptive trait divergence is not always dependent on amino acid sequence variation in functional genes

There is considerable debate as to whether divergence in adaptive traits is mediated by functional gene sequence variation or differences in gene expression (reviewed in: Carroll 2005; Hoekstra & Coyne 2007). While studies have shown that a few amino acid substitutions in protein-coding sequences can result in large differences in adaptive phenotypes (Hoekstra et al. 2006; Hoekstra & Coyne 2007; Mullen et al. 2009), this is not always the case (e.g. Audzijonyte et al. 2012; Dobson et al. 2012; Raeymaekers et al. 2014). Differences in gene regulation and expression may also play a major role in the evolution of adaptive trait divergence within and among species (reviewed in: Whitehead & Crawford 2006; Fay & Wittkopp 2008; Pavey et al. 2010). Because the RF of horseshoe bats is tightly coupled with the frequency of best-hearing of their acoustic fovea, functional variation in the hearing gene *Prestin* was explored to determine whether sensory divergence among populations of *R. capensis* and between members of the *R. capensis* clade was in any way associated with sequence variation in the gene. Results revealed that *Prestin* is highly conserved and does not reflect RF differences (and associated best-hearing frequencies) among acoustically divergent populations of *R. capensis* or among its closely related species (Chapter 4).

It is possible that differences in *Prestin* gene expression, rather than differences at the coding sequence level, may influence RF variation in *R. capensis*. If so, selection for some degree of plasticity in RF, via gene expression, may play a role in the evolution of sensory divergence in horseshoe bats. While many studies have found that differences in gene expression promotes the evolution of local adaptation (e.g. Fraser 2013) recent theoretical (Espinosa-Soto et al. 2011) and empirical (e.g. deer mice: Cheviron et al. 2014; cichlids: Schneider et al. 2014; sticklebacks: Morris et al. 2014) studies also document a crucial role of differences in gene expression and regulation in the evolution of plasticity in adaptive traits (reviewed in: Schlichting & Pigliucci 1993; Aubin-Horth & Renn 2009). A recent study exploring the conditions under which gene expression-mediated plasticity influences the evolution of caste polyphenisms in social insects found that differentially expressed genes are characterised by weaker purifying

selection (Hunt et al. 2011). Genes freed from selective constraints may therefore play an important role in the origin of phenotypic plasticity (Hunt et al. 2011; Leichty et al. 2012) and this may explain the variability found in the strength of purifying selection at different amino acid sites in *Prestin* within the *R. capensis* clade, even though the gene appears to be highly conserved. These results highlight the need for studies exploring the link between differential gene expression of hearing genes not only among closely related but acoustically divergent species, but also within species characterised by significant geographic variation in the frequency of their acoustic signals. A comparative approach comparing *Prestin* evolution within bat and cetacean clades may shed light on whether the change in selective regime in *Prestin* evolution uncovered within the *R. capensis* clade is the exception rather than the norm.

Edge populations are natural laboratories for the study of adaptive evolution

A major question still challenging evolutionary biologists in the 21st century is what factors shape species' ranges (Kirkpatrick & Barton 1997; Bridle & Vines 2006). Theoretical advances together with empirical studies have greatly increased our understanding of how adaptation to marginal habitats is influenced by genetic and demographic differences between marginal and central populations (reviewed in: Eckert et al. 2008; Kawecki 2008). Generally, marginal populations display lower genetic diversity and greater genetic differentiation relative to central populations (Eckert et al. 2008), and also act as demographic sinks due to the net influx of immigrants from central populations (Kawecki 2004). Studying populations at range edges can therefore provide insight into how asymmetric gene flow, dispersal and selection gradients interact to shape patterns of local adaptation at range margins in natural populations (Kawecki 2008). The two most geographically divergent 'edge' populations in this study, Table Farm (TF) and Lekkersing (LS), are (i) genetically and ecologically distinct, (ii) characterised by reduced levels of gene flow and (iii) have the most divergent RFs. While plasticity provides a plausible alternative to classic divergent selection in the face of gene flow among central populations of *R. capensis*, it is

unlikely to explain the RFs of populations situated at the edge of the species range and instead, classical divergent selection may be the main driver of RF in these regions.

Interesting variations in the association between skull size and body size characterise edge populations. For example, although LS bats have a similar body size to its nearest neighbours SKK, they also have a significantly larger skull and corresponding larger dorsal nasal chamber and lower RF. TF bats on the other hand have a larger body size, but a similar skull size and corresponding RF to their nearest neighbours situated in the Fynbos Biome. This suggests that RFs of edge populations are by-products of the uncoupling between skull size and body size evolution. In these regions selection may instead act on genes involved in skull size rather than echolocation itself, leading to concomitant changes in dorsal nasal chamber size and RF in edge populations. A candidate gene approach combined with an in-depth dietary analysis of edge populations versus central populations may be particularly useful in exploring the factors influencing skull variation in *R. capensis*. A number of recent studies have identified a range of candidate genes involved in the development of cranial morphology in a wide range of vertebrates (e.g. Abzhanov et al. 2004; Pointer et al. 2012; Schoenebeck & Ostrander 2013) including bats (Phillips et al. 2013). For example, the expression of the paired-domain gene, *PAX9*, is known to influence craniofacial and dental development in bats (Phillips et al. 2013). Similarly the gene *Bmp4* influences skull shape and size differences during craniofacial development in birds and fish (reviewed in: Parsons & Albertson 2009) and differences in expression patterns have been instrumental in generating the dietary and trophic specialisations which characterise the adaptive radiations of Darwin's finches (Abzhanov et al. 2004) and African cichlids (Albertson et al. 2005). These genes may be suitable candidates for exploring the link between skull variation and sensory divergence within an ecological framework at the edge of a species range. Another recent study used a novel molecular genetic approach using next-generation sequencing to investigate dietary differences of *M. lucifugus* at a continental scale, revealing significant seasonal, regional, and inter-annual differences in prey across the distribution of the species which may influence dispersal patterns and local adaptation in this species (Clare et al.

2014). This approach may be particularly useful to elucidate the fine-scale differences in diet which may explain the differences in skull size across populations of *R. capensis*.

Conclusions

By combining analytical tools from a broad range of disciplines including phylogeography, sensory ecology, the study of functional morphology, and functional gene sequencing, a more nuanced understanding of the relative importance of adaptive and neutral evolutionary processes in shaping patterns of trait divergence is possible. Using such a holistic approach not only reveals how complex interactions between these processes can shape patterns of divergence within species, but also highlights how their relative effects may vary across the distribution of species. This study demonstrates the importance of including measures of gene flow in studies of adaptive trait divergence and provides important supporting evidence for the power of population level analyses to elucidate the complex interactions between selection, plasticity and gene flow in the evolution of local adaptation.

CHAPTER 6

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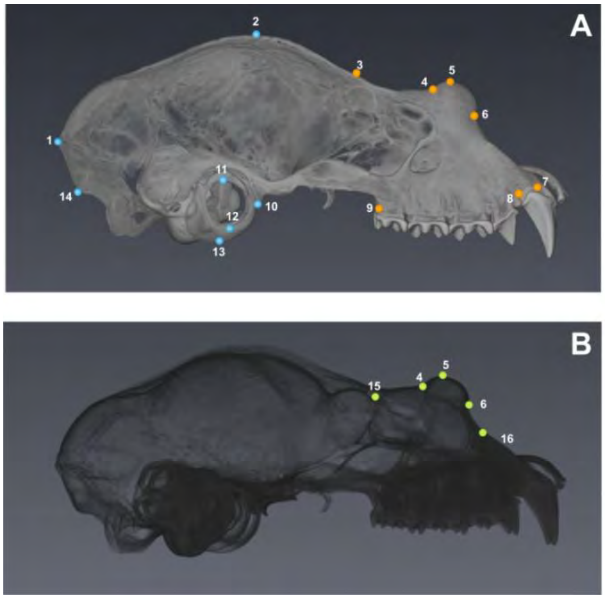
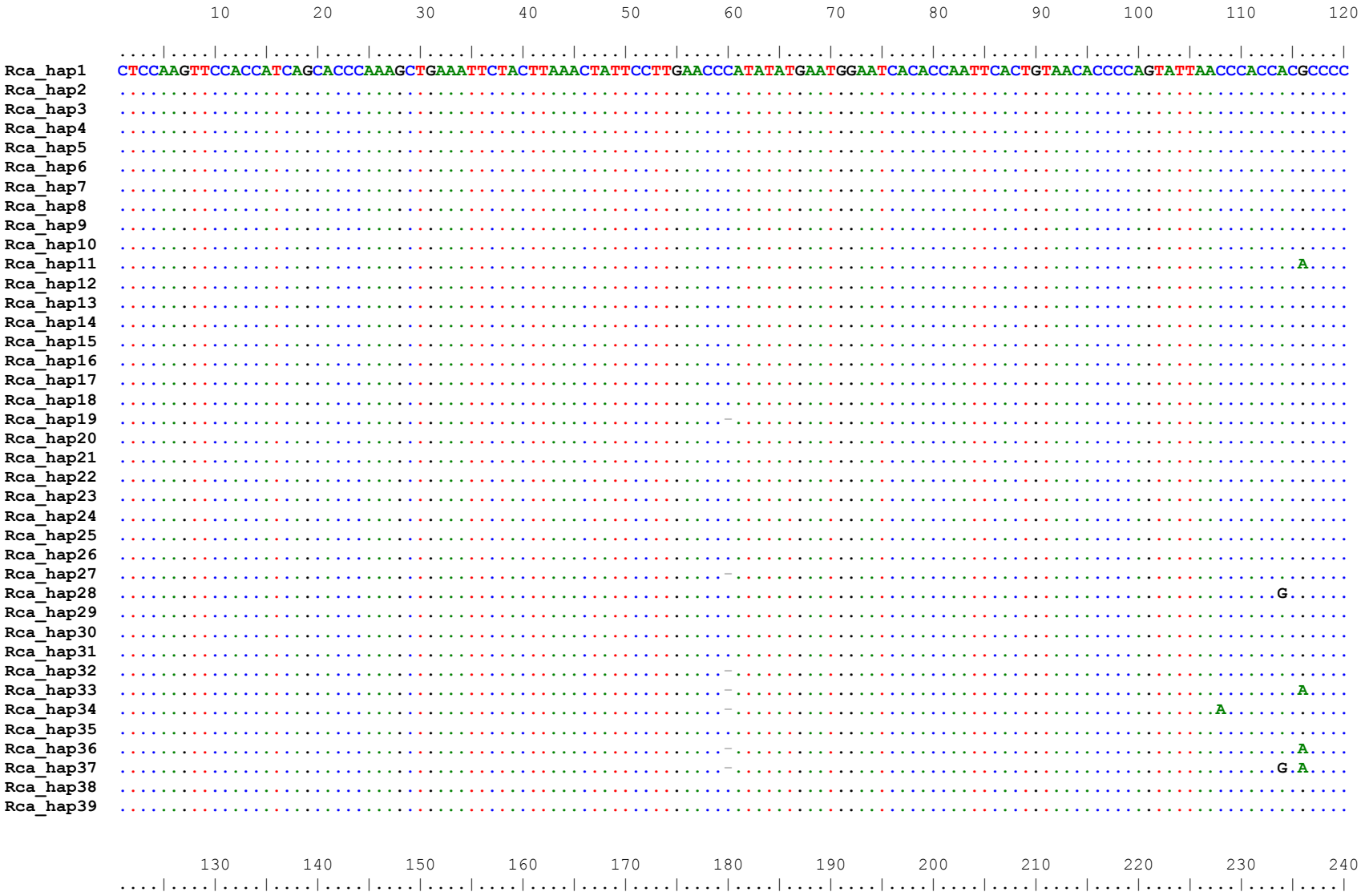
APPENDICES

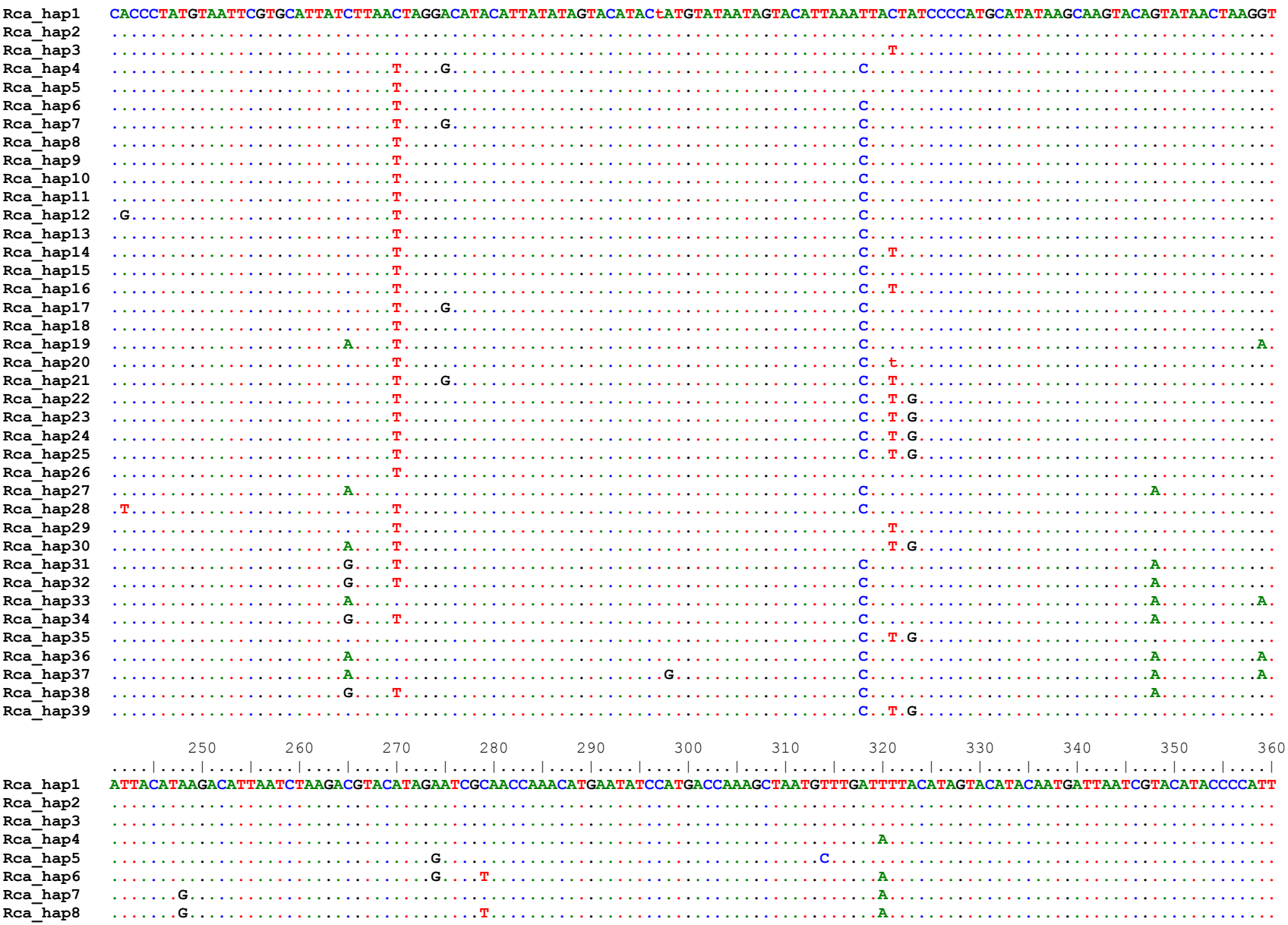
Appendix 1: Description of the cranial landmarks used in geometric morphometric analyses, adapted from Santana and Lofgren 2013 and Stoffberg 2007.



Landmark	Description
1	Most posterior point of the skull at the sagittal and lambdoidal crests
2	Most dorsal point of the sagittal crest
3	Point where the sagittal crest meets the rostral depression
4	Most posterior point of the anterior median swellings of the nasal chamber
5	The highest point of the bulbous nasal chamber
6	Most ventral point of the anterior median swellings of the nasal chamber
7	Most anterior point at the base of the canine
8	Most anterior point at the base of the first premolar
9	End of tooth row at the base of the third molar
10	Most posterior and ventral point of the squamosal
11	Most dorsal point of the external auditory meatus
12	Most ventral point of the external auditory meatus
13	Most ventral point of the auditory bulla
14	Most ventral point of occipital bone
15	Most dorsal and posterior point of the nasal chamber
16	Most ventral and anterior point of the lateral swellings of the nasal chamber
17	Suture between the premaxilla and maxilla at the midline
18	Suture between the palatines at the midline

Appendix 2: Alignment of mtDNA D-loop sequence data of the 39 unique haplotypes found across 11 populations of *R. capensis* sampled in this study.




Rca_hap1 CACCCTATGTAATTCGTGCATTATCTTAACTAGGACATACATTATATAGTACATACtATGTATAATAGTACATTAAATTACTATCCCCATGCATATAAGCAAGTACAGTATAACTAAGGT
Rca_hap2
Rca_hap3
Rca_hap4
Rca_hap5
Rca_hap6
Rca_hap7
Rca_hap8
Rca_hap9
Rca_hap10
Rca_hap11
Rca_hap12
Rca_hap13
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Rca_hap32
Rca_hap33
Rca_hap34
Rca_hap35
Rca_hap36
Rca_hap37
Rca_hap38
Rca_hap39
250 260 270 280 290 300 310 320 330 340 350 360
Rca_hap1 ATTACATAAGACATTAATCTAAGACGTACATAGAATCGCAACCAAACATGAATATCCATGACCAAAGCTAATGTTTGATTTTACATAGTACATACAATGATTAATCGTACATACCCCATT
Rca_hap2
Rca_hap3
Rca_hap4
Rca_hap5
Rca_hap6
Rca_hap7
Rca_hap8


Rca_hap9
Rca_hap10
Rca_hap11
Rca_hap12
Rca_hap13
Rca_hap14
Rca_hap15
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Rca_hap36
Rca_hap37
Rca_hap38
Rca_hap39
370
380
390
400
410
420
430
440
450
460
470
480
Rca_hap1
AAGTCAAATCATTTCCAGACAACACGCATATCACCTCCAATAGGTTATCTCTCGACTACCAACTCACGTGAAACCAGCAACCCTTGCGAGAAGGATCCCTCTTCTCGCCCCGGGCCCATA
Rca_hap2
Rca_hap3
Rca_hap4
Rca_hap5
Rca_hap6
Rca_hap7
Rca_hap8
Rca_hap9
Rca_hap10
Rca_hap11
Rca_hap12
Rca_hap13
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Rca_hap15
Rca_hap16


Rca_hap17 ....C..........
Rca_hap18 ....
Rca_hap19 ....
Rca_hap20 ....g
Rca_hap21 ....G
Rca_hap22 ....G
Rca_hap23 ....G
Rca_hap24 ....G
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Rca_hap30 ....G
Rca_hap31 ....G..........
Rca_hap32 ....
Rca_hap33 ....
Rca_hap34 ....
Rca_hap35 ....G
Rca_hap36 ....
Rca_hap37 ....
Rca_hap38 ....
Rca_hap39 ....G
490
500
510
Rca_hap1 AACCGTGGGGGTTTCTAGTATTGGGGAGTAAACGACATC
Rca_hap2 G...................................
Rca_hap3 G...................................
Rca_hap4 G...................................
Rca_hap5 G...................................
Rca_hap6 G...................................
Rca_hap7 G...................................
Rca_hap8 G...................................
Rca_hap9 G...................................
Rca_hap10 G...................................
Rca_hap11 G...................................
Rca_hap12 G...................................
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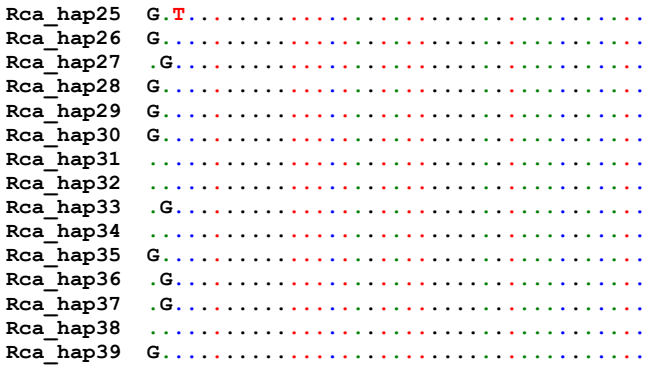
Rca_hap23 G..............A....................
Rca_hap24 G...................................


Rca_hap25 G.T
Rca_hap26 G
Rca_hap27 .G
Rca_hap28 G
Rca_hap29 G
Rca_hap30 G
Rca_hap31
Rca_hap32
Rca_hap33 .G
Rca_hap34
Rca_hap35 G
Rca_hap36 .G
Rca_hap37 .G
Rca_hap38
Rca_hap39 G

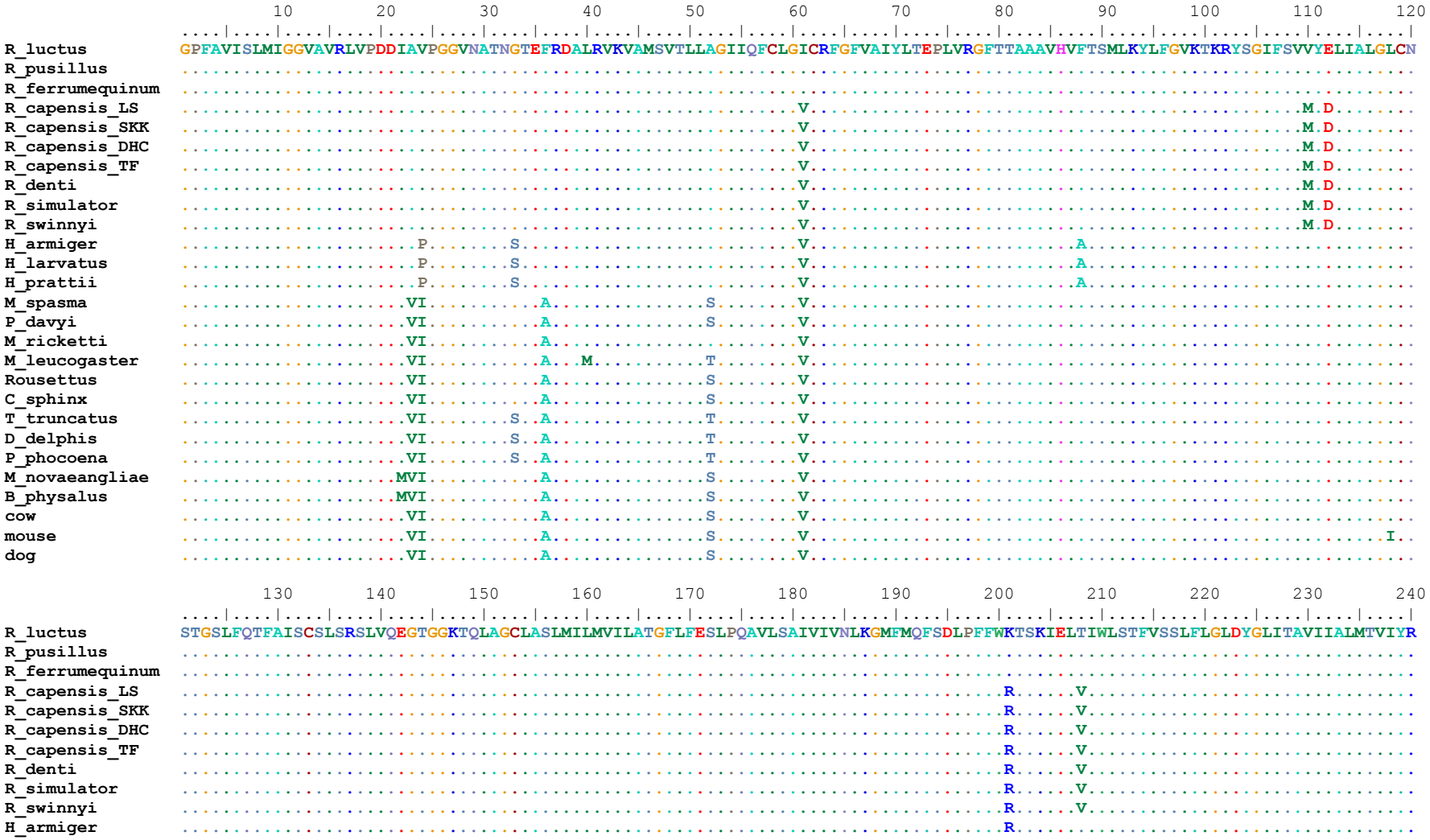
Appendix 3: Lower and upper profile likelihood percentiles of *M*, the number of immigrants per generation scaled by mutation rate, calculated in Migrate-N (Beerli 2009).

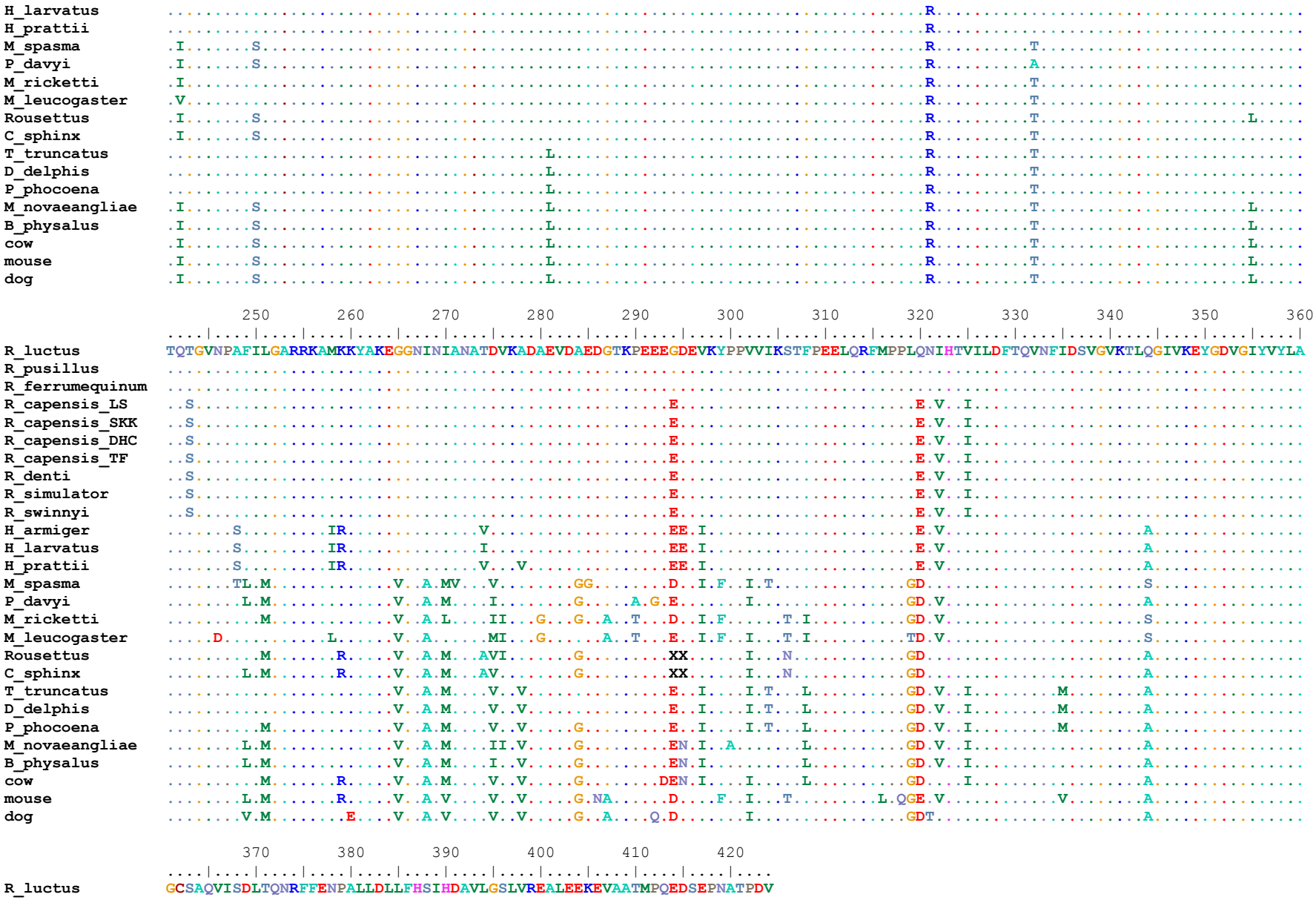
Source population	Receiving population	Lower percentile (0.05)	Upper percentile (0.95)
SKK	LS	8.78E-08	134.25
ZPK	SKK	1725.14	4921.60
DHL	ZPK	70.95	379.21
ZPK	DHL	1840.58	5953.23
BKL	DHL	1531.97	5384.67
BAV	BKL	97.47	434.03
BKL	HDH	1075.43	3250.20
BKL	DHC	104.18	464
BAV	KNY	44.12	493.25
DHC	BAV	917.74	2097.03
KNY	BAV	86.68	607.70
BAV	SPH	68.31	763.70
BAV	TF	115.85	515.85

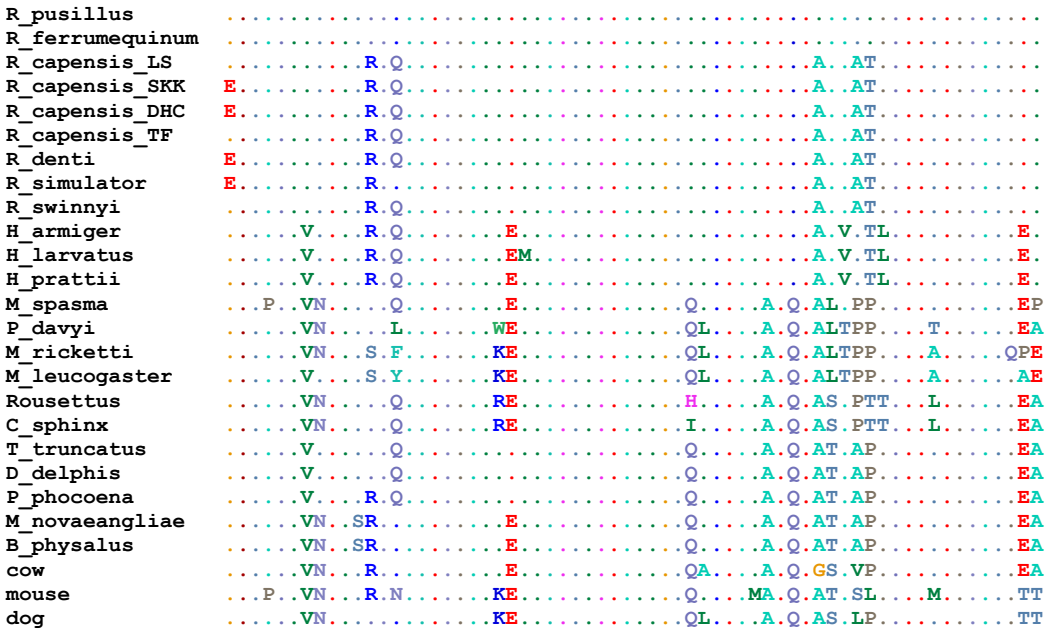
Appendix 4: Genbank accession numbers of *Prestin* sequences for the 20 eutherian mammals used in this study.

Group	Species	Genbank accession number
High duty-cycle bats	*Rhinolophus luctus*	EU914933
	Rhinolophus ferrumequinum	EU914925
	Rhinolophus pusillus	EU914936
	Hipposideros armiger	EU914928
	Hipposideros larvatus	EU914934
	Hipposideros pratti	EU914937
Low duty-cycle bats	*Megaderma spasma*	EU914926
	Myotis ricketti	EU914924
	Pteronotus davyi	JN315990
	Murina leucogaster	GU219836
Non-echolocating fruit bats	*Rousettus leschenaultia*	EU914930
	Cynopterus sphinx	EU914931
Echolocating cetaceans	*Tursiops truncatus*	GU217587
	Delphinus delphis	GU219839
	Phocoena phocoena	GU219842
Non-echolocating whales	*Balaenoptera physalus*	GU219838
	Megaptera novaeangliae	GU219841
Other non-echolocating mammals	*Mus musculus*	NM030727
	Bos taurus	NM001192878
	Canis familiaris	XM540393

Appendix 5: Amino acid alignment of ten exons (= 424 amino acids) of the coding region of *Prestin* for 20 eutherian mammals together with *Rhinolophus capensis* from four populations with divergent resting frequencies, and other species of the *R. capensis* clade.




H_larvatus
H_prattii
M_spasma
P_davyi
M_ricketti
M_leucogaster
Rousettus
C_sphinx
T_truncatus
D_delphis
P_phocoena
M_novaeangliae
B_physalus
cow
mouse
dog
250 260 270 280 290 300 310 320 330 340 350 360
R_luctus TQTGVNPAFILGARRKAMKKYAKEGGNINIANATDVKADAEVDAEDGTKPEEEGDEVKYPPVVIKSTFPEELQRFMPPLQNIHTVILDFTQVNFIDSVGVKTLQGIVKEYGDVGIYVYLA
R_pusillus
R_ferrumequinum
R_capensis_LS
R_capensis_SKK
R_capensis_DHC
R_capensis_TF
R_denti
R_simulator
R_swinnyi
H_armiger
H_larvatus
H_prattii
M_spasma
P_davyi
M_ricketti
M_leucogaster
Rousettus
C_sphinx
T_truncatus
D_delphis
P_phocoena
M_novaeangliae
B_physalus
cow
mouse
dog
370 380 390 400 410 420
R_luctus GCSAQVISDLTQNRFFENPALLDLLFHSIHDAVLGSLVREALEEKEVAATMPQEDSEPNATPDV


R_pusillus
R_ferrumequinum
R_capensis_LS
R_capensis_SKK
R_capensis_DHC
R_capensis_TF
R_denti
R_simulator
R_swinnyi
H_armiger
H_larvatus
H_prattii
M_spasma
P_davyi
M_ricketti
M_leucogaster
Rousettus
C_sphinx
T_truncatus
D_delphis
P_phocoena
M_novaeangliae
B_physalus
cow
mouse
dog