Geographic variation in the phenotype of an African horseshoe bat species, *Rhinolophus damarensis*, (Chiroptera: Rhinolophidae)

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To my late father: J.V. Maluleke, I vividly remember your encouraging words and support. Thanks for everything, and may your soul rest in perfect peace.

To my late mother: T.T Shihlomulu, the completion of this work is a confirmation that your inspiring words and support have kept me going. I will forever cherish you, and may your soul rest in perfect peace.

GENERAL ABSTRACT

Studies involving geographic variation in the phenotypes of bats help scientists to explain why these mammals are the most species rich mammalian order second only to rodents, with well more than 1 300 species occurring worldwide. Such species richness or high diversity is the manifestation of the generation of biodiversity through the splitting of lineages within bat species. A lineage of bat species can diversify into several lineages which then differentiate from each other in allopatry. Thus, the spatial separation of a lineage into several lineages could be attributed to geographical, ecological and environmental factors across the distributional range of the species. Similarly, vicariant events may also play a role in separating lineages within species.

The Damara horseshoe bat species, *Rhinolophus damarensis*, is widely distributed but restricted to the western half of southern Africa, where it occurs across several major biomes. Formerly regarded as the subspecies, *R. darlingi damarensis*, it was elevated to full species status on the basis of genetic and phenotypic differences between it and *R. darlingi darlingi*. *Rhinolophus damarensis* is itself made up of two ecologically separated genetic lineages. A total of 106 individuals of *R. damarensis* were sampled from seven localities across its distributional range, with a view to determining and documenting the extent of geographic variation in body size, echolocation parameters, wing parameters, cranial shape and post-cranial morphology of individuals from populations of *R. damarensis* across the distributional range of the species.

Firstly, an investigation into geographic variation in resting echolocation frequency (RF) of the horseshoe bat species, *R. damarensis* was carried out in the western half of southern Africa (Chapter 2). Three hypotheses were tested. The first one, James'Rule (JR), states that individuals occurring in hot humid environments generally have smaller body sizes than conspecifics that occur in cooler, dryer environments, and the largest are expected to occur in cool, dry areas. On this basis and because of the known relationship between body size and RF, it was predicted that there should be a correlation between body size and climatic factors and between body size and RF. The second hypothesis was Isolation by Environment (IbE) mediated through sensory drive, which proposes that diversification of lineage may be driven by environmentally-mediated differences in sensory systems. Under this hypothesis, it was predicted that call frequency variation should be correlated with climatic variables. The third hypothesis was that Isolation by Distance (IbD) can influence phenotypic trait variation by

restricting gene flow between populations. Under the Isolation by Distance (IbD) Hypothesis, it was predicted that call frequency variation should be partitioned in accordance with geographic distance between populations. To investigate the probability of the JR, IbE and IbD, the Akaike's information criterion AICc candidate models were evaluated with different combinations of environmental (annual mean temperature and relative humidity), spatial (latitude and region) and biological (forearm as a proxy for body size) predictor variables to determine their influence on resting frequency (RF) across the distributional range of *R. damarensis*. Linear mixed effects models (LMEs) were employed to analyse the relationship between the response variable (RF) and the environmental, spatial and biological predictor variables. The influence of prey detection range and atmospheric attenuation was also investigated. The results showed no evidence for JR or for random genetic drift. Body size was neither correlated with RF nor environmental variables, suggesting that variation in RF was not the result of concomitant variation in body size as proposed by JR. Similarly, the Mantel test showed no IbD effect and there was therefore no evidence that genetic drift was responsible for the variation in RFs. In contrast, the LMEs showed that there was support for IbE in the form of an association between RF and region (in the form of the variable "Reg") which was based on the two geographically separated genetic lineages. Furthermore, RF variation was also associated with the climatic variable AMT.

The taxonomic status of *R. damarensis* was investigated using ecological traits and phenotypic characters including skulls, wings and echolocation (Chapter 3) and three dimensional (3D) scanned skulls and mandibles (Chapter 4). The main objective (Chapter 3 and Chapter 4) was to test whether previously reported genetic divergence between the two *R. damarensis* lineages was associated with phenotypic divergence. Morphometric and echolocation measurements were taken from hand held individual bats in the field, and skull measurements were taken from field collected voucher specimens as well as museum specimens. Discriminant Function Analyses (DFA) revealed that there was geographic variation among populations and lineages of *R. damarensis*. Multivariate Linear Regressions (MLV) and Linear models (LM) on the basal parts of bacula revealed significant differences between the southern and northern lineages of *R. damarenis*. The bacula of the two lineages of *R. damarensis* appear to have different shapes. Diversification through shape analyses (Chapter 4) was investigated using three dimensional (3D) geometric morphometric analyses based on X-ray microcomputed tomography (µCT) scanning of dried skulls and mandibles of *R. damarensis.* Procrustes Anova results of both mandibles and skulls indicated that there

were no significant differences between sexes but that the shape of skulls and mandibles varied across different localities (Chapter 4). Canonical Variate Analysis (CVA) suggested that geographic variation in *R. damarensis* mandibles was based on the shape and thickness of the alveolar bone. Geographic variation in the skulls was based on changes in the rostrum, anterior medial swelling and brain case. Some populations had slightly deeper rostra, slightly larger anterior medial swellings and smaller braincases, whilst others had slightly shallower rostra, slightly smaller anterior medial swellings and larger braincases. The northern lineage was found to be separated from the southern lineage based on the changes in skull and mandible shape. Therefore, separation of lineages within *R. damarensis* (Chapter 4) could be associated with the foraging and feeding behaviour of the species under different ecological conditions due to ecological opportunity. Overall, differences in the RF were found to be associated with Isolation by Environment mediated through sensory drive and this has led to the formation of two regional (northern and southern) groupings in RF (Chapter 2). The two lineages were supported by both the phenotypic divergence (Chapter 3) and shape variation within *R. damarensis* skulls and mandibles (Chapter 4). Thus, phenotypic differences corresponded to genetic differences between the two lineages and provide support for IbE.

Komiso hi Xitsonga

Ndzavisiso wa ku hambana ka swiharhi swo fana na vamangedyani hi kuya hi tindzhawu ta laha swi kumekaka eka tona, swi pfuna vanhu va sayense (science) ku hlamusela kuri hikokwalaho kayini swiharhi leswi swa ku mamisa swi tele ngopfu. Hi kuya hi ndzavisiso wa sayense ya ntumbuluko, swiharhi swo fana na makondlo hi swona leswi nga tala ngopfu i vi ku landzela vamangedyana, tinxaka ta swiharhi leswi ti hundza 1 300 laha emisaveni. Matalelo ya swiharhi leswi ya tikombisa hiku hambana ka tinyimba (genetic lineages) hi kuya hi "species." Ku hambana ka tinyimba (genetic lineages) ku vangiwa hi tindzhawu ta laha swiharhi leswi swi kumekaka kona. Tintshava, malwandle, magova, madamu lamakulu, mimpfhuka, kun'we na maxelo na swona swa vanga mahambanelo ya ti tinyimba (genetic lineages) eka swiharhi.

Xivumbiwa lexi xivitaniwaka Damara horseshoe bat species, *Rhinolophus damarensis*, xi kumeka hi xitalo evupela dyambu bya dzonga wa Africa. Khale xiharhi lexi a xi ri "subspecies" leyi a yi ri *R. darlingi damarensis*, kambe xi vuye xi tlakusiwa xi nyikiwa "full species status" hi kuya ka ku hambana ka tinyimba (genetic lineages) na xivumbeko exikarhi ka xiharhi lexi na xin'wana lexi a xi ri *R. darlingi darlingi*. *R. damarensis* xi na tinyimba (genetic lineages) timbirhi hi kuya hi laha xi kumekaka kona. Ndazvisiso lowu wu phasile wu tlhela wu khoma swiharhi swa 106 swa *R. damarensis* eka tindzhawu to ringana nkombo. Xikongomelo a kuri ku lava ku tiva ku ri swiharhi leswi swi hambana njhani hi kuya hi laha swi kumekaka kona. Ndzavisiso wu lavisise ku hambana ka mirhi, mimpfumawulo, swivumbeko swa tipiku na swivumbeko swa marhambu ya tinhloko/swipalapala ta swiharhi lexi xa *R. damarensis*.

Xosungula ndzavisiso wu kongomisiwe eka kuhambana ka mimpfumawulo hi kuya hi tindzhawu ta laha swiharhi swa *R. damarensis* swi kumekaka kona evupela dyambu bya dzonga wa Africa. Ndzavisiso lowu wu kambisise ti "hypotheses" tinharhu. Yo sungula, "James'Rule (JR), states that individuals occurring in hot humid environments generally have smaller body sizes than conspecifics that occur in cooler, dryer environments." Hi kuya hi hypothesis leyi na vuxaka lebyi byi tivekaka exikarhi ka vukulu bya mirhi na mimfumawulo ya *R. damarensis*. Ndzavisiso wu bvumbe ku ri ku ta va na ku kotlana na vuxaka exikarhi ka vukulu bya mirhi na mimpfumawulo ya *R. damarensis.* "Hypothesis" ya vumbirhi, "Isolation by Environment (IbE) mediated through sensory drive, and it was hypothesised that there should be an association between phenotypic differences (i.e resting frequency) and

environmental differences.” Hi kuya hi hypothesis leyi, ndzavusiso wu bvumbe ku ri kuhambana ka mimpfumawulo swi ta kotlanisana na ku hambana ka maxelo ya tindzhawu ta swiharhi leswi. “Hypothesis” ya vunharhu, “the third hypothesis was that Isolation by Distance (IbD) can influence phenotypic trait variation by restricting gene flow between populations.” Hi kuya hi hypothesis leyi, ndzavisiso wu bvumbe ku ri ku hambana ka mimpfumawulo swi fanele ku kotlanisana na mipfhuka ya mitlawa ya swiharhi swa *R. damarensis*. Ku lavisisa vu hlohloteri bya “Jame’s Rule, Isolation by Environment kun’we na Isolation by Distance hypotheses”eka swiharhi leswi swa *R. damarensis*. Ndzavisiso wu lavisise ku ringana ka ti “candidate models” ta “Akaike’s information criteria” kun’we ni mintlawa ya ta maxelo, ndzhawu na mirhi wa swiharhi ku kambisisa kucetelo wa mimpfumawulo ya swiharhi leswi. “Linear Mixed Effects models” ti tirhisiwile eka ndzavisiso lowu ku xopaxopa vuxaka exikarhi ka mimpfumawulo na ndzhawu laha swiharhi swa *R. damarensis* swi kumekaka kona. Vu hlohloteri bya “prey detection range” na “atmospheric attenuation” na byona by lavisisiwile eka ndzavisiso lowu. Mbuyelo wu kombisile ku ri a kuna vumbhoni lebyi kombaka leswaku kuna vuxaka exikarhi ka mimpfumawulo na “James’Rule.” Na kambe ndzavisiso a wu kumanga vuxaka exikarhi ka makulelo ya mirhi na mimpfumawulo ya swiharhi leswi swa *R. damarensis.* Mbuyelo wa “mantel test” na wona wu kombe leswaku a kuna vuxaka exikarhi ka mimpfumawulo ya swiharhi swa *R. damarensis* na mimpfhuka leyi nga kona exikarhi ka mitlawa ya swiharhi leswi. Ehandle ka mimbuyelo leyi, mbuyelo wa “Linear Mixed Effects Models” wona wu kombe leswaku kuna vuxaka exikarhi ka “Isolation by Environment” na mimpfumawulo ya swiharhi leswi swa *R. damarensis*. Leswi swi kombisisa swinene leswaku ku hambana ka maxelo ya tindzhawu ta swiharhi leswi swi hlohlotela mimpfumawulo ya swiharhi leswi.

Ndzavisiso wa “taxonomic status” ya *R. damarensis* wu lavisisiwile hi ku tirhisa ndzavisiso wa ndzhawu leyi swiharhi leswi swi kumekaka kona, swi vumbeko leswi swi katsaka marhambu ya tinhloko/swipalapala, tipiku ta swiharhi leswi na mimpfumawulo ya swona kun’we na marhambu ya tinhloko/swipalapala leswi nga sikeniwa (scanned) na marhambu ya tihlaya leti nga sikeniwa (scanned) ta swiharhi swa *R. damarensis.* Xikongomelo xikulu xa a ku ri ku kambela kuri ku hambanaka ka ti tinyimba (genetic lineages) ta *R. damarensis* swi seketeriwa hi swivumbeko swa mirhi ya swiharhi leswi swa *R. damarensis*. Vukulu na vuvumbeko kun’we na mimpfumawulo ya *R. damarensis* swi kamberiwile swi tlhela swi pimiwa enhoveni laha swiharhi leswi swi khomiweke kona. Marhambu ya tinhloko ta *R. damarensis* ma tekiwe eka ti “voucher specimens” (i.e voucher specimens ensure that the

identity of organisms studied in the field or in the laboratory experiments can be verified). Man'wana marhambu ya tinhloko ta *R. damarensis* ma lombiwile eka ti "museum". Vuxopaxopi bya "Discriminant Function Analyses" byi kombise leswaku swiharhi swa *R. damarensis* swa hambana hi kuya ka tindzhawu ta laha swi kumekaka kona. "Multivariate Linear Regressions" na "Linear Models" swi kombe leswaku ti "bacala" (penile bones) ta swiharhi leswi ta hambana swinene exikarhi ka swiharhi leswi swi kumekaka en'walungwini ni le dzongeni wa xifundza xa vupela dyambu bya le dzongeni wa Africa. Leswi swi tikombe hi ku hambana ka swi vumbeko swa ti "bacula" ta *R. damarensis*. Ku hambana ka mintlawa ya *R. damarensis* swi lavisisiwile na kambe hi ku tirhisa "three dimensional (3D) geometric analyses" laha ndzi nga tirhisa muchini wa "X-ray micromputed tomography" ku kambela marhambu ya tinhloko/swipalapa na ya tihlaya ta mintlawa ya *R. damarensis*. Mbuyelo wa "Procrutes Anova" wu kombe leswaku a kuna ku hambana ka vumbewu exikarhi ka marhambu ya tinhloko kun'we na ya tihlaya ta mintlawa ya *R.damarensis*. Vumbewu byi ti kombe ku hambana hi kuya ka vuvumbeko na tindzhawu ta laha mintlawa ya *R. damarensis* yi kumekaka kona. Mbuyelo wa "Canonical Variate Analyses" wu kombe leswaku ku hambana hi kuya ka swivumbeko swa tihlaya ta mintlawa ya *R. damarensis* swi tikombisile swinene hi ku anama ka marhambu ya alivhiyola (alveolar bone). Ku hambana exikarhi ka mintlawa ya tinhloko/swipalapala swi tikombise hi macincelo yaku anama ka marhambu ya tinhompfu na ya byongo. Ku hambana exikarhi ka mintlawa ya le dzongeni na ya le n'walungwini swi kombisa leswi swiharhi swa *R. damarensis* swi hlotaka ha kona ni leswi swi dyisaka xi swona enhoveni etindzhawini leti hambaneke. Hi ku angarheta, ku hambana ka mimpfumawulo ya swiharhi leswi swi kongomisana na ku hambana ka tindzhawu ta mimbangu ya laha swiharhi leswi swi kumekaka kona. Leswi swi endle leswaku ku va na mintlawa yi mbirihi hi kuya hi mimpfumawulo ya swharhi leswi kun'we ni tindzhawu ta mimbangu ya laha swi kumekaka kona. Mintlawa leyi yi mbirhi ya le n'walungwini na le dzongeni wa ndzhawu ya laha swiharhi leswi swi kumekaka kona yiseketeriwa hi ku hambana ka mipfumawulo, vukulu bya tipiku, vukulu bya marhambu ya tinhloko/swipalapala na hi ti "bacula" kun'we na swivumbeko swa marhambu ya tinhloko/swipalapala na marhambu ya tihlaya ta swiharhi leswi. Mahambanelo ya swivumbeko swa mintlawa bya swiharhi leswi swi kotlanisana ni mahambanelo ya tinyimba (genetic lineages) exikarhi ka mintlawa ya swiharhi swa *R. damarensis*. Mbuyelo lowu wu seketeriwa hi ku hamabana ka mimbago ya tindzhawu leti swiharhi leswi swi kumekaka eka tona.

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DECLARATION

I, Tinyiko G. Maluleke, hereby declare that the work presented in this thesis is based on my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or 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. This thesis was submitted to the turnitin module and I confirm that my supervisors have seen my turnitin report and any concerns revealed by such have been resolved with my supervisors.

Candidate: Tinyiko G. Maluleke

Signature: Signed by candidate

Date: 1/07/2017

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CHAPTER 1

General Introduction

Geographic variation in phenotypic traits of populations within species.

Understanding patterns of geographic variation in phenotypic traits of animal populations may provide insights into the microevolutionary processes which cause phenotypic divergence within a species and ultimately speciation (Mayr, 1963; Yoshino *et al*., 2008; Campbell *et al*., 2010). Phenotypic trait variation can be the result of isolation of populations in different geographic regions that are characterised by different environmental conditions. Such isolation may limit gene exchange. This could be reflected in variation among species and within species distributed over different ecological and environmental regions. Such geographical variation within species has been studied in different animal groups including invertebrates (Stillwell *et al*., 2007), birds (James, 1970; Bowie *et al*., 2006; Shieh and Liang, 2007) and mammals (Monteiro *et al*, 2003; Russo *et al*., 2007; Szuma, 2008). Geographic variation is described as the differences that can be found among the populations of the same species occurring in separate geographical locations (Endler, 1977). It is the first step towards lineage divergence and understanding the causes of geographic variation provides insight into the processes responsible for biodiversity (Endler, 1977).

There is now increasing evidence that signals used in sensory systems are to a certain extent tuned to match environmental characteristics in a wide range of animal species including fish (Endler, 1980), birds (Marchetti, 1993), reptiles (Leal and Fleishman, 2002) and bats (Odendaal *et al*., 2014; Mutumi *et al*., 2016). Therefore, habitat heterogeneity may cause divergent selection on signals associated with mate choice and assortative mating (Coyne and Orr, 2004; Bolnick and Kirkpatrick, 2012; Safran *et al*., 2016). Thus, reproductive isolation could arise when male mating signals and female preferences vary among populations of the same species (Boughman, 2002). There is evidence that natural selection contributes to reproductive isolation in natural populations (Rundle *et al*., 2000). Thus, geographic variation in acoustic signals may lead to lineage diversification (Slabberkoorn and Smith, 2002; Odendaal *et al*., 2014; Mutumi *et al*., 2016), and potentially facilitate speciation (Endler, 1992; Boughman, 2002). Understanding the processes that lead to geographic variation in acoustic signals may provide scientists with knowledge to better characterise biodiversity, and especially uncovering undocumented diversity so that the maximium amount of this

diversity and its ecological roles can be protected. Echolocation is a special type of sensory system that is used in orientation and prey capture, and involves the emission of acoustic signals, and the detection and analysis of the returning echoes reflected by objects in the echolocator's environment (Griffin, 1958). Geographic variation in echolocation and acoustic signals in general may be caused by both stochastic (e.g random genetic drift and founder effect) and deterministic (i.e. natural selection) forces acting independently or together (Endler, 1977; Aspetsberger *et al*., 2003; Bernal *et al*., 2005; Mutumi *et al*., 2016). It is only through an in-depth analysis of the patterns of geographic variation that the various causes of geographic variation can be teased apart.

Stochatistic (genetic drift) and deterministic (selection) influences on variation.

Differences in foraging habitat structure may influence resting frequency variation within echolocating bat species, because of the ‘‘adaptive complexes’’ between wing morphology and echolocation frequency (Jones and Barlow, 2004; Jacobs *et al*., 2007). On the other hand, genetic drift, which is the random fixation of alleles (Munguia-Vega *et al*., 2007) can lead to changes in gene frequency especially in small populations, and could cause phenotypic variation between populations of the same species (Slatkin, 1987). For example, shifts in allele frequency at allozyme loci in small isolated populations of brown kiwis, *Apteryx mantelli*, in New Zealand, were attributed to genetic drift (Wright, 1931; Baker *et al*., 1995; Allendorf *et al*., 2013). Similarly, founder effects can lead to geographic variation either by the splitting of large populations into smaller populations or by a small number of individuals dispersing and becoming established in a different area to the parent population (Leberg, 1992; Kolbe *et al*., 2012). In each case the smaller populations would carry only a subset of the genetic material of the larger populations. Coupled with genetic drift each founder population would follow a different evolutionary trajectory (Mayr, 1963; Baker *et al*., 2009).

Physical barriers (i.e. mountains, rivers, valleys and harsh climatic conditions) may limit dispersal over the distributional range of a species, and this may lead to the separation of once continuously distributed species (Gascon *et al*., 1996; Toda *et al*, 1998). For example, the ridge of the Cordillera Oriental in Colombia is a barrier to gene flow between populations of the frog, *Colostethus palmatus*, causing calls of males to differ across the ridge (Bernal *et al*, 2005). Similarly, the morphological variation in the arboreal lizard, *Anolis oculatus*, has been attributed to barriers that caused divergence in allopatry (Malhotra and Thorpe, 2000). Furthermore, the central mountain range in New Guinea, acting as a barriers to dispersal for

many taxa with lowland distribution (Deiner *et al*., 2011), may also be implicated in the large amount of phenotypic variation across the range of the little Shrike-thrush, *Colluricincla megarhyncha*, for which there are 30 recognised subspecies (Ford, 1979). In bats, significant isolation by distance was found in both sexes of *Myotis daubentonii* in the Archipelago Sea (Laine *et al*., 2013). This indicates a gradual increase of population differentiation across a large geographic scale (Laine *et al*., 2013). In addition, the strait of Gibraltar, which separates the Iberian Peninsula from the Maghreb by a minimum distance of 14 km of the open sea, acts as a barrier to gene flow in *Myotis myotis* (Castella *et al*., 2000). Results based on nuclear macrosatelite loci revealed no significant population structure within regions, but a complete isolation between bats sampled on each side of the strait (Castella *et al*., 2000). Furthermore, echolocation frequency variation in *Rhinolophus monoceros* was correlated with geographic distance (Chen *et al*., 2009). However, broad differences in constant frequency among populations corresponded to discontinuities in allele frequencies resulting from vicariant events (Chen *et al*., 2009).

Physical barriers may reduce local genetic diversity by preventing gene flow among isolated populations, and thus increasing genetic drift (Slatkin, 1981; Allendorf, 1983). Genetic drift and natural selection are implicated in driving evolutionary changes (Nei, 2005; Prentice *et al*., 2017), and genetic drift may have significant effects on small populations (Nei *et al*., 1975; Forsdick *et al*., 2017). Furthermore, genetic drift has the potential to outweigh the effects of selection in small populations. This in turn, may lead to undesirable or deleterious mutations and adaptive genetic variation (Lynch *et al*., 1995; Hedrick, 2001). However, if strong genetic drift or selections act upon small isolated populations, rapid differentiation among populations may occur (Funk *et al*., 2016). Moreover, genetic drift can influence populations by causing ecological niche divergence (Wang *et al*., 2017). Phenotypic and genetic divergence among populations is also likely to be shaped by a combination of drift, dispersal and selection (Weber *et al*., 2017). Phenotypic divergence among populations may be manifested in particular patterns of divergence where Isolation by Distance (IbD) and Isolation by Environment (IbE) occur. IbD is defined as the tendency for geographically distant populations of the same species to be more genetically differentiated than those that are close to one another (Wright, 1943). Phenotypic or genetic differentiation between populations may be the result of drift acting within populations faster than it is ameliorated by gene flow among populations (Slatkin, 1993). On the other hand, adaptation of populations within species to different habitats may lead to the reduction in the effectiveness

of gene flow, and contribute to a pattern of IbE (Wang and Summers, 2010; Wang and Bradburd, 2014; Weber *et al*., 2017). There is a growing evidence suggesting that IbE may arise through selection of populations to different local habitats (Armsworth and Rhoghgarden, 2008; Bolnick and Otto, 2013) including environmental diffferences (Wang and Summers, 2010; Wang and Bradburd, 2014; Manthey and Moyle, 2015). Acoustic signals among populations of the same species that are geographically isolated may result in geographic divergence in sensory system and eventually lead to speciation. There are few studies that have detected and reported significant differences between geographic distance and acoustic signals among animal populations. For example, vocal differences among populations of male bearded seals, *Erignathus barbatus*, were attributed to geographical isolation (Risch *et al*., 2007). Genetic differences among populations of humpback whale *Megaptera novaeangliae* were also reported to have been caused by geographical distance (Kershaw *et al*., 2017). Moreover, populations isolated for a very long time generally show greatest variation, irrespective of the distance between them (Koetz *et al*., 2007). Furthermore, historically isolated populations of chowchilla, *Orthonyx spaldingii*, were distinguished by the bandwidth and peak frequency of their songs (Koetz *et al*., 2007). In bats, echolocation call variation between allopatric Myanmar and Thai populations of *Craseonycteris thonglongyai*, could result from local adaptation re-inforced by limited gene flow (Puechmaille *et al*., 2011).

Different populations of the same species may also experience natural selection and become adapted to local environments. This could eventually result in populations diverging morphologically and genetically, and it is likely to happen in species with wide geographic distribution that span multiple habitat types. Species with such a wide geographic distribution may be susceptible to divergence through adaptation to different environments (Schluter, 2001). Recent studies on the barn swallow, *Hirundo rustica*, reveal that morphological adaptation plays a crucial role in shaping differentiation of poplations within the species (Safran *et al*., 2016). Furthermore, populations of *Hirundo rustica* from Israel and the continental Europe were found to be fairly divergent in phenotypes, despite being relatively genetically similar (Safran *et al*., 2016). Moreover, the ability of the animal species to interact with their surrounding environments may shape distributions of spatial genetic variation, resulting in patterns of IbE (Wang and Bradburb, 2014). IbE may be initiated when divergent or disruptive selection acts on phenotypes that convey a fitness advantage in one environment but not another (Shafer and Wolf, 2013). Such fitness differentials may bring about a shift in

allele frequencies of the selected loci leading to adaptation to local habitat. Adaptation to local environment will in turn, reduce gene flow between populations through selection against migrants (Thibert-Plante and Hendry, 2009), assortative mating (Coyne and Orr, 2004) and matching habitat choice (Edelaar *et al*., 2008). Therefore, IbE is a pattern in which genetic differences among populations increases with environmental differences, independent of geographic distances (Wang and Bradburd, 2014). When IbE increases with environmental differences, natural selection may generate IbE among populations inhabiting heterogeneous environments (Nosil *et al*., 2005). Therefore, changes in climatic and environmental conditions may create environmental differences and populations may adapt to these novel environments, resulting in phenotypic differentiation among populations. For example, there is an evidence for IbE in white-breasted Nuthatches *Sitta carolinensis* of the Madrean Archipelago (Manthey and Moyle, 2015). IbE has also been investigated and inferred in other vertebrate species, including fish (Cooke *et al*., 2012; Vincent *et al*., 2013), birds (Smith *et al*., 2005; Manthey and Moyle, 2015), mammals (Murray *et al*., 2017) and amphibians (Dudaniec *et al*., 2012; Oyamaguchi *et al*., 2016). Natural selection may drive the origin of species, as it was initially claimed by Charles Darwin. This may occur through ecological speciation, and in general, it might occur in allopatry or sympatry (Schluter, 2001). Ecological speciation involves many agents of natural selection, and result from combination of adaptive processes (Schluter, 2001). Furthermore, ecological speciation may occur when divergent selection on traits between populations or subpopulations in different environments leads directly or indirectly to the evolution of reproductive isolation (Schluter, 2001). However, divergent selection may also occur under uniform selection. For example, if different advantageous (but incompatible) mutations arise in separate populations occupying similar environments (Turelli *et al*., 2001).

Body size may be a confounding factor in investigations of phenotypic traits that are correlated with body size. Changes in body size, under selection for example, could result in correlated changes in other phenotypic traits that are not directly under selection and in such cases it would be inaccurate to conclude that variation in those traits are the result of selection. Echolocation is such a trait and one has to exclude the effects of body size before making conclusions about the processes responsible for variation in echolocation. Body size and echolocation form an adaptive complex in echolocating bats such that the two are inversely correlated (Heller and von Helversen, 1989; Barclay and Brigham, 1991; Jones, 1996; Jacobs *et al*., 2007; Taylor *et al*., 2012). Larger individuals of bats have longer and

thicker vocal chords and larger resonant chambers producing lower frequency sound (Heller and von Helversen, 1989; Barclay and Brigham, 1991; Jacobs *et al*., 2007; Taylor *et al*., 2012). Therefore, changes in body size may result in changes in echolocation frequency (Jacobs *et al*., 2007; Taylor *et al*., 2012). For example, echolocation frequency divergence in the *Rhinolophus hildebrandtii* was associated with an adaptive shift in body size with consequent changes in echolocation frequency. As a result four new rhinolophid species, *R. mabuensis*, *R. mossambicus*, *R. cohenae* and *R. smithersi* were identified within the *Rhinolophus hildebrandtii* complex (Taylor *et al*., 2012). However, in the Rhinolphidae (horseshoe bats) changes in peak echolocation frequency appear to have preceded changes in body size suggesting that variation in echolocation frequency may have been caused through evolutionary processes acting directly on echolocation rather than through changes in body size (Stoffberg *et al*., 2011). In addition, divergent selection may lead to local adaptation, and this in turn may result in the curtailment of gene flow between populations (Schluter, 2000). Moreover, low levels of migration (low gene flow) may also increase the influence of genetic drift, and thus ameliorating phenotypic divergence among populations (Shafer and Wolf, 2013). This may lead to reproductive isolation among populations (Nosil, 2012).

James (1970) found that climatic factors (i.e. moisture and temperature) are correlated with body size variation in the populations of bird species including Downy Woodpecker *Dendrocopos pubescens*, Hairy Woodpecker *Dendrocopos villosus*, Blue Jay *Cyanocitta cristata*, Carolina Chickadee *Parus carolinensis*, White-breasted Nuthatch *Sitta carolinensis*, and Eastern Meadowlark *Sturnella magna*, and concluded that animals occurring in hot humid environments generally have a smaller body size than animals of the same species that occur in cooler, arid environments. Therefore, the differences in body size of bats with wide geographic distributions and the consequent changes in echolocation frequency could be explained in terms of James' Rule, which is a modification of Bergmann's Rule (Blackburn *et al*., 1999). However, James' Rule has not consistently been supported across the few species of bat case studies where it has been investigated. For example, although, skull size variation among populations of the fruit eating bat, *Artibeus lituratus*, was correlated with environmental variables (i.e. precipitation and temperature) (Marchán-Rivadeneira, 2012), supporting James' Rule (James, 1970), contrary to the latter, skull size in North American *Eptesicus fuscus*, is inversely related to temperature but positively related to moisture (Burnett, 1983).

This is reflected in the influence that factors other than body size have on echolocation call variation (Siemers *et al*., 2005). For example, sexual dimorphism exists in certain bat species. Males of *Hipposideros ruber* were found to use higher pitched calls than females in almost all the colonies in the Gulf of Guinea (Guillén *et al*., 2000). Also, female *Hipposideros speoris* produce sound that is 3 KHz lower than males, despite the absence of significant differences in forearm length between males and females (Jones *et al*., 1994). Furthermore, female *Rhinolophus capensis* use higher frequency echolocation calls than the males (Odendaal and Jacobs, 2011), and juveniles of *R. euryale, R. mehelyi* and *R. blasii* often produce lower frequency echolocation calls than adults (Russo *et al*., 2001; Siemers *et al*., 2005). There is a suggestion that the differences in echolocation call frequency of *Rhinolophus cornutus pumilus* could be the result of random cultural drift (Yoshino *et al*., 2008). Moreover, sexual dimorphism in peak echolocation frequencies of bats suggests an essential social function of echolocation, possibly, by signalling the gender of the caller, and this in turn may facilitate selection of mate and reproductive success (Guillén *et al*., 2000; Kazial and Masters, 2004). For example, females of *Rhinolophus mehelyi*, preferentially select males with high frequency calls during the mating season (Puechmaille *et al*., 2014).

Echolocation may be used in communication systems (Jones and Siemers, 2011; Finger *et al*., 2017) and therefore mate choice (Puechmaile *et al*., 2014) and thus implicated in sensory drive. Sensory signals (e.g. acoustic signals) of bats are emitted and perceived under local environmental conditions, and such environmental conditions may influence the evolutionary trajectory of the signalling system. Therefore, acoustic signals are crucial in the context of lineage diversification (Slabberkoorn and Smith, 2002; Odendaal *et al*., 2014; Mutumi *et al*., 2016). This has led to the formulation of the Sensory Drive Hypothesis (Endler, 1992; 1993; Boughman, 2002) which proposes that diversification of lineage may be driven by environmentally-mediated differences in sensory systems.

Rhinolophid bat species, may use echolocation (Siemers *et al*., 2005; Puechmaile *et al*., 2014) or modified echolocation (Finger *et al*., 2017) calls for communication. In addition to echolocation, horseshoe bats also emit a diverse repertoire of social calls. These calls are emitted in different circumstances and may convey specific messages (Andrews and Andrews, 2003; Ma *et al*., 2006). In addition, emitted echolocation calls may act as unambiguous badges of species identity (Russo *et al*., 2007) and provide important information for species recognition at foraging sites (Heller and von Helversen, 1989). Both

echolocation and social calls of horseshoe bats are species specific (Jones and van Parijs, 1993; Kingston *et al*, 2001; Russo *et al*., 2009). Additionally, if horseshoe bats do use echolocation calls for communication purposes, social information may be encoded in small intraspecific differences in frequency and other call parameters, and individuals that use calls differing from those of conspecifics will be unable to communicate effectively (Jacobs *et al*., 2007). Thus, there may be selection for each species in an assemblage to occupy a unique acoustic space so that intraspecific communication is facilitated (Heller and von Helversen, 1989; Guillén *et al*., 2000; Jacobs *et al*., 2007; Bastian and Jacobs, 2015).

Environmental differences could also result in adaptive variation in acoustic signals (Handford and Lougheed, 1991; Tubaro and Segura, 1995; Odendaal and Jacobs, 2011). For example, intraspecific song variation in birds has been correlated with acoustic properties of their habitat (Handford and Lougheed, 1991; Tubaro and Segura, 1995; Slabberkoorn and Smith, 2002; Ruegg *et al*., 2006). Similarly, frequency reduction in the Neotropical frog, *Dendrobates pumilio* might reflect adaptation to cooler environments (Pröhl *et al*., 2007). The propogation of acoustic signals is differentially affected by atmospheric attenuation, the reduction in the energy of sound due to spreading and absorption by the atmosphere. The degree of atmospheric attenuation is dependent on the complex non-linear interaction between frequency of the signal and the temperature and humidity of the air (Lawrence and Simmons, 1982; Armstrong and Kerry, 2011; Luo *et al*., 2014). As a genral rule of thumb, high frequency signals are affected more by atmospheric attenuation than low frequency signals. Species of bats with high peak echolocation frequencies are therefore affected more by climatic conditions through atmospheric attenuation. Humidity and temperature, which are likely to differ appreciably over the distributional range of echolocating bat species, may therefore, cause geographic variation in echolocation call frequency of bats. Atmospheric attenuation is directly related to both humidity and temperature (Lawrence and Simmons, 1982; Hartley, 1989; Armstrong and Kerry, 2011; Luo *et al*., 2014). Therefore, bats are ideal animals for testing the importance climatic factors to the evolution of acoustic signals. Furthermore, bats using higher frequencies to forage in cluttered vegetation and in humid tropical conditions (rain forests) would also encounter more severe atmospheric attenuation than those in dry areas (Lawrence and Simmons, 1982; Hartley, 1989; Guillén *et al*, 2000; Luo *et al*., 2014, Mutumi *et al*., 2016). Within echolocating bats, species from drier climates have on average higher call frequencies than those species from the humid tropics (Heller and von Helversen, 1989; Guillén *et al*, 2000). For example, *Hipposideros caffer*, living in

African dry forest areas use higher echolocation frequency than its sibling species, *H. ruber*, living in more humid areas (Guillén *et al*, 2000). It was also found that for a given set of atmospheric conditions, an increase in call frequency of *Rhinonicteris aurantia* by 6 KHz resulted in a slight decrease in its prey detection range (Armstrong and Kerry, 2011). Furthermore, an increase in relative humidity and temperature reduced detection range significantly, and a similar trend was shown in prey detection volume ratios (Armstrong and Kerry, 2011). Armstorng and Kerry (2011) also argued that any tradeoff between maximising detection range and prey resolution could be swamped by the effects of other factors.

There are also several other factors that can affect variation in echolocation such as unique foraging styles of bats, habitat (Jones and Barlow, 2004), diet (Houston *et al*, 2004), inaudibility to eared insects (Fenton and Fullard, 1979), acoustic resource partitioning (Duellman and Pyles, 1983), background noise (Russo *et al*., 2017) as well as humidity and temperature (Lawrence and Simmons, 1982). However, with the exception of humidity and temperature, these factors will not be entertained here because at the high frequencies at which *R. damarensis* echolocate differences in wavelength are unlikely to be large and the ecological consequence of frequency differences are likely to be small. The relationship between frequency and wavelength is non-linear and differences in wavelength associated with high frequencies are too small to result in ecological differences (Jacobs *et al*., 2007). Similarly, selection pressure from eared insects is unlikely to differ geographically and the echolocation calls of *R. damarensis* are likely to be audible to eared insects in any case (Jacobs *et al*. 2007).

Diversity and distribution of bats.

Bats have occupied most terrestrial habitats and climatic zones, and they are widely distributed mammals with high potential vagility (Burnett, 1983), and are one of the most successful mammalian orders (Altringham, 1996). At present, there are more than 1 300 recognized species of bats in the world (Simmons, 2005) and more than 100 species have been recorded in southern Africa (Monadjem *et al*., 2010). Many species of bats have wide geographical ranges e.g *Rhinolophus clivosus* (Jacobs *et al*., 2007), *Lasiurus cinereus* (O'Farrell *et al*., 2000), *Rhinolophus swinyi* and *R. simulator* (Mutumi *et al*., 2016). Such wide geographic distribution might lead to geographic variation within species (Endler, 1977; Aspetsberger *et al*., 2003; Jacobs *et al*., 2007), making bats ideal for studying geographic variation. Furthermore, bats also show a high degree of variety of adaptations to their

environment varying in size (Jones, 1996; Heller and von Helversen, 1989), wing parameters (Jones and Barlow, 2004; Jacobs *et al.*, 2007), echolocation (Jacobs *et al*., 2013) and skull morphology (Stoffberg *et al*., 2004; Jacobs *et al*., 2014).

The family Rhinolophidae (horseshoe bats).

The family Rhinolophidae (horseshoe bats) is one of the most diverse families of bats, currently numbering 77 species (Simmons, 2005). Studies based on fossil and molecular data (Teeling *et al*., 2005; Eick *et al*., 2005) suggest that horseshoe bats underwent adaptive radiation during the middle Eocene. Horseshoe bats are insectivorous bats that occur throughout the tropical and temperate regions of the world (Csorba *et al*., 2003). The Indomalayan region has the most diversity with 42 horseshoe bat species (Csorba *et al*, 2003). The African region has about 27 horseshoe bat species (Happold and Cotterill, 2013). Horseshoe bats are divided into two main *Rhinolophus* clades, namely: Afro-palaearctic and Asian (Stoffberg *et al*, 2010; Dool *et al*, 2016). The split between hipposiderids (the leaf nosed bats) and rhinolophids (the horseshoe bats) is estimated to have occurred between 39 and 55 million years ago (Mckenna and Bell, 1997; Teeling *et al*., 2003) with other studies estimating 39 million years ago (Eick *et al*., 2005; Foley *et al*., 2014). Rhinolophids are likely to have originated in the tropical rain forest of Asia during the Eocene (37-33MYA) and dispersed to Europe and North Africa (Stoffberg *et al*., 2010), resulting in speciation in Africa (Maree and Grant, 1997). The radiation of rhinolophids into the many extant forms may have started around 17-15 MYA, with the African clade radiating between 16-14 MYA (Csorba *et al*., 2003). The phylogenetic relationship between rhinolophid species of southern Africa indicates that the diversity of habitats throughout the large continent of Africa could probably be due to climatic changes and vicariance in the Plio-Pleistocene (Maree and Grant, 1997). The shift in vegetation might have been influenced by rainfall and temperature which could have experienced great changes during the Pliocene-Pleistocene. The uplift of Africa and the resultant change in climate through the late Neogene might have contributed to the radiation of many species in the region (Patridge, 2010; Spiegel *et al*., 2007).

Rhinolophid bat species produce medium to long echolocation calls with a high duty cycle (HDC). Duty cycle is the ratio of call duration to call period and is usually expressed as a percentage (Fenton, 1994). Most energy is concentrated in the second harmonic. Their calls (Fig. S1) consist of an important constant frequency (CF) component preceded and followed by a frequency modulated (FM) component (Neuweiler, 1984). The constant frequency (CF)

calls of horseshoe bats have higher peak frequencies for a given body size than the frequency modulated (FM) calls of other bats (Francis and Habersetzer, 1998). They compensate for flight induced Doppler shifts by lowering by a few kHz the frequency of their emitted echolocation calls to ensure that the returning echo falls within the narrow frequency range of the acoustic fovea (from which we can derive the reference frequency), a region of the cochlea which has an over-representation of neurons sensitive to this narrow range of frequencies (Neuweiler, 1984; 1989). The calls emitted by bats in motion are very similar in frequency to the peak frequency (frequency of maximum amplitude) of the CF component of a call they emit when they are stationary, which is commonly known as the resting frequency (RF) (Schuller and Pollak, 1979). This RF is 100 Hz to 300 Hz lower than the reference frequency (Schuller and Pollak, 1979). RF can therefore be recorded from hand-held bats which eliminates variance in frequency caused by horseshoe bats compensating for Doppler shifts in the returning echo during flight (Schnitzler and Denzinger, 2011). Therefore, rhinolophids are ideal for studying variation in echolocation frequency because their resting frequency calls are easily recorded from the individuals held in the hand.

Damara horseshoe bat (*Rhinolophus damarensis*).

Rhinolophus damarenesis (Jacobs *et al*., 2013), is a medium-sized insectivorous bat with a relatively high echolocation frequency (85.4 ± 1.4 kHz) and a mean forearm length of 49.5 ± 1.7 mm (Jacobs *et al*., 2013). Like other horseshoe bats, *R. damarenesis* uses high duty cycle (HDC) echolocation. Rhinolophids have several harmonics in their echolocation calls but concentrates call energy in the second harmonic (Neuweiler, 1989; Schnitzler *et al*., 2003). Most horseshoe bats hunt from a perch (i.e perch hunters) and are able to echolocate from a resting position while scanning the environment for prey (Jacobs *et al*., 2007). *R. damarensis* is a member of the *maclaudi* subclade (Stoffberg *et al*., 2010). Recent genetic analysis suggests that *R. damarensis* and *R. darlingi* diverged from each other about ~9.2 Ma. Furthermore, *R. damarensis* consists of two (north-south) lineages which diverged from each other 5.3 Ma with a percentage sequence divergence of 4.1 % (Jacobs *et al*., 2013). In addition, the percentage sequence divergence within the northern and southern lineages was 0.7% and 0.5%, respectively (Jacobs *et al*., 2013).

R. damarensis is ideal for studying geographic variation because it is distributed widely in the more arid western half of southern Africa, with its distribution stretching from western South Africa through Namibia to south-west Angola (Jacobs *et al*., 2013; Chapter 2). This region is

characterised by a wide range of arid and mesic climatic conditions. Therefore, the research presented here used phenotypic, ecological and enivironmental data to document phenotypic variation amongst the populations of *R. damarensis* in greater detail, and then to investigate the factors that led to phenotypic divergence within *R. damarensis* and the observations made were then used to resolve prevailing taxonomic issues that arose as a result of the different lineages uncovered within *R. damarensis*. The following hypotheses were tested: (1) James'Rule, (2) Isolation by Environment; (3) Random genetic drift (IbD).

Under James'Rule, it was predicted that there should be an inverse relationship between body size, and temperature and humidity. Larger individuals of *R. damarensis* should occur in cool but wet environments and smaller individuals in hot, humid environments. Body size should also increase with elevation, because climate changes with altitude and conditions are cooler and dryer at higher altitude.

Under random genetic drift (IbD), it was predicted that phenotypic variation in *R. damarensis* should be correlated with distance between populations and there should be no association with environmental gradients (i.e. annual mean temperature and relative humidity).

Isolation by Environment mediated by sensory drive (Endler, 1992; Boughman, 2002; Wang and Bradburd, 2014) proposes that geographic variation in echolocation frequency is caused by differences in environmental factors across the habitats of the various populations of *R. damarensis* i.e each population has become adapted to the environmental conditions that prevail in their habitats. Such adaptation allows optimal prey detection ranges under different atmospheric conditions. There should therefore be a correlation between echolocation frequency, temperature and humidity across the habitats of the various populations within each species.

Thesis outline

The main objective of this study was to test the two categories of hypotheses: adaptation and random genetic drift, and their predictions by comparing the phenotypes (i.e. morphology and resting frequency) of populations of *R. damarensis* across its geographic range. Geographic variation in the phenotype of the species was related to the historical, environmental and ecological factors of the region where the populations were sampled. The four main questions of this study were as follows: (1) Is there phenotypic variation evident in *R. damarensis* according to regional environmental conditions that would suggest adaptation and the

potential for ecological speciation? (2) Is random genetic drift responsible for phenotypic variation among the populations of *R. damarensis*? (3) Are the two genetic lineages in *R. damarensis* associated with a particular pattern of phenotypic variation within the species? (4) Are the two genetic lineages in *R. damarensis* representing one species or two different species?

The three main aims were as follows:

- To investigate the extent of geographic variation in *R. damarensis* by measuring phenotypic parameters (body size, echolocation parameters, wing parameters, cranial shape and post-cranial morphology) of individuals from populations of *R. damarensis* across the distribution range of the species.
- To collect and analyse environmental data including vegetation/ land use cover, humidity, altitude and temperature for each population of *R. damarensis* sampled to explain echolocation frequency divergence.
- To observe and resolve the prevailing taxonomic status of *R. damarensis* by collecting taxonomically informative phenotypic data and tissue samples for genetic analyses.

Chapter 2 investigated geographic divergence in the resting frequency of *R. damarensis* using spatial (i.e region and latitude), environmental (i.e relative humidity and annual mean temperature) and biological (i.e. body size) variables. All these were used to test James' rule, IbE and IbD.

The investigation into phenotypic divergence was extended to include other echolocation parameters besides resting frequency as well as skull and wing morphology (Chapter 3). For the skull analyses, both traditional (Chapter 3) and 3-D geometric morphometrics (Chapter 4) to consider differences in size and shape in the context of the two regional groupings in Chapter 2. In Chapter 3, three ecologically informative parameters including (echolocation, skulls and wings) were analysed using Principal component analysis (PCA) and Discriminant function analysis (DFA) to determine morphological variation and lineage divergence within the species. Chapter 4 was based on shape analyses and used geometric morphometrics on three dimensional (3D) images of scanned skulls of *R. damarensis* to determine morphological divergence within the species. Procrustes anova (PA) and Canonical variate analysis (CVA) were used to analyse the shapes of *R. damarensis* skull and mandibles (Chapter 4).

The general synthesis and conclusion (Chapter 5) summarises the whole thesis and gives an overview of the study as well as recommendations for future studies on this species.

Ethical Statement

The sampling methods used in capturing and handling species of horseshoe bats in this research were carried out in accordance with guidelines of the American Society of Mammalogists (Animal Care and Use Committee 1998). This current study followed the sampling techniques/guidelines outlined by Kunz and Parsons (2009) and Sikes *et al* (2011). These techniques/guidelines were approved by the Science Faculty Animal Ethics Committee of the University of Cape Town (clearance number 2013/2012v6/DJ) and conducted under permits from the permitting authorities in the respective southern African countries (Namibia – 1429/2010; South Africa – AAA003-00030-0035; 1197/2008). The places accessed for sampling horseshoe bats were publicly or privately owned. Protected areas were not accessed. I obtained the necessary permission to carry out this research at all times. Neither protected nor endangered species were sampled in this research.

CHAPTER 2

Isolation by environment mediated by sensory drive partly explains geographic divergence in a phenotypic trait: a case study using bat echolocation

Abstract

Divergent selection on sensory systems may result in lineage diversification within species driven by sensory traits. Such diversification could be largely influenced by selection in ecologically different environments. Processes driving acoustic divergence have been well documented in many examples but the origins of the divergence have been largely unexplored in bats. Here, an investigation into geographic variation in resting echolocation frequency of the horseshoe bat, *Rhinolophus damarensis* was carried out in the arid western half of southern Africa. Bats were sampled from seven localities along a latitudinal gradient ranging from 16˚S to 32˚S. Body size (as estimated by FA) and peak resting frequencies (RF) were measured from hand held individual bats. Three hypotheses were tested (1) James' Rule (2) Isolation by Environment (IbE) mediated through sensory drive and (3) Isolation by Distance (IbD) to isolate the effects of body size, local climatic conditions and geographic distance on the resting frequency of *R. damarensis.* The results were not consistent with an explanation of IbD or James' Rule. There was no significant relationship between (i) geographic distances and RF, (ii) body size and RF or (iii) body size and climatic variables. Instead, the results were consistent with an explanation of IbE as local climatic conditions explained a significant proportion of variation in observed RF of *R. damarensis.* This finding was linked to the Sensory Drive Hypothesis as a likely driver for IbE in *R. damarensis*. Variation in RF was best explained by IbE, mediated through differences in climatic conditions across the habitats of the populations within *R. damarensis*. RF variation is concordant with genetic variation in *R. damarensis*.

Key words: acoustic signals, climate, horseshoe bats, resting frequency.

Introduction

Every region has its own geological past, with a unique flora and fauna, as a result of environmental variation and barriers to gene flow that exist between geographic areas (Neuweiler, 2000). This is reflected in variation not only between species but also within species distributed over different environmental regions. Although stochastic factors such as random genetic drift can exert an influence on variation in phenotypic traits, traits that are heritable and have a profound impact on fitness (e.g. sensory traits) are more likely to be impacted by natural selection resulting in adaptation to novel environments (Endler, 1992; Boughman, 2002; Campbell *et al*., 2010).

Sensory signals (e.g. acoustic signals) are important in the context of lineage diversification (Slabberkoorn and Smith, 2002; Odendaal *et al*., 2014; Mutumi *et al*., 2016) and the generation of biodiversity because they have been implicated in mate choice and assortative mating (Coyne and Orr, 2004; Bolnick and Kirkpatrick, 2012). These signals are propagated through the environment and specific environmental conditions can influence the evolutionary trajectory of the signalling system. This has led to the formulation of the Sensory Drive Hypothesis (Endler, 1992; 1993; Boughman, 2002) which proposes that lineage diversification may be driven by environmentally-mediated differences in sensory systems. Acoustic signals, in particular, are part of a sensory system that relies on audition and the propagation of sound through the atmosphere.

Atmospheric conditions (i.e. climate) can exert a strong influence on geographic variation of complex signals, such as bird song (Lengagne and Slater, 2002), frog mating calls (Lingnau and Bastos, 2007) and the echolocation calls of bats (Lawrence and Simmons, 1982). These acoustic signals may diverge along climatic gradients because of variation in atmospheric attenuation of sound. Atmospheric attenuation, caused by the scattering and absorption of sound by the atmosphere, is the result of a complex interaction between the humidity and temperature of the air as well as the frequency of the sound (Lawrence and Simmons, 1982; Hartley, 1989; Luo *et al*., 2014; Mutumi *et al*., 2016). Therefore, climate could play a pivotal role in driving the evolution of signalling systems through its effect on atmospheric sound attenuation (Griffin, 1971; Richards and Willey, 1980). The echolocation call frequency of *Hipposideros ruber* (Guillén *et al*., 2000) and some horseshoe bat species (Mutumi *et al*., 2016) were correlated with climatic variables as a result of atmospheric attenuation of

acoustic signals. Furthermore, other studies have suggested that the frequency of bat echolocation calls diverge along climatic gradients (Snell-Rood, 2012).

Environmentally-mediated differences in sensory systems leading to lineage diversification and speciation can arise as a result of geographic or ecological isolation of populations (Schluter, 2001). Isolation by geographic barriers and distance can influence trait variation by restricting gene flow between populations. This is usually manifested by an association between genetic or phenotypic differences and geographic distances and referred to as Isolation by Distance (IbD; Wright, 1943). Alternatively, when a species has a distribution across several biomes, discontinuities in habitat can also result in environmentally-mediated ecological speciation (Dobzhansky, 1937; Schluter 2001) of local populations and a restriction of gene flow amongst them. Gene flow can be restricted in such situations either by selection against dispersers moving between populations in different habitats or by individual preference to remain in a particular habitat (Nosil, 2004; Nosil *et al*., 2005). This is usually manifested by an association between genetic or phenotypic differences and environmental differences and referred to as Isolation by Environment (IbE, Wang and Summers, 2010; Shafer and Wolf, 2013). Isolation by Environment through local adaption may be evident even in the absence of isolation by distance. In the absence of an ecological cline amongst habitats, IbD may be manifested as a break in the distribution of phenotypic or genetic variation (Meirmans, 2012).

Bat echolocation has evolved primarily for orientation (Simmons and Stein, 1980; Schnitzler *et al*., 2003; Schuchmann and Siemers, 2010) and food acquisition (Heller and von Helversen, 1989) and there is some, but not conclusive evidence for communication (Siemers *et al*., 2005; Knörnschild *et al*., 2012; Puechmaille *et al*., 2014; Bastian and Jacobs, 2015). There is ample evidence that the frequency of bat echolocation is adapted to the bats' habitat and foraging mode (Aldridge and Rautenbach, 1987; Jones and Holderied, 2007). Echolocation thus provides an opportunity to investigate the role of adaptation in the geographic variation of a phenotypic trait that is essential to survival and reproduction. However, only a few studies have investigated geographic variation in resting frequency and the environmental factors (humidity and temperature) responsible for it (Armstrong and Kerry, 2011; Luo *et al*., 2014; Mutumi *et al*., 2016).

A potential confounding factor in understanding the effect of climate on geographic variation of resting frequency is the inverse correlation between body size and echolocation frequency

in bats (Jones, 1996; 1999). Larger bats tend to have lower frequencies, because they have longer and thicker vocal chords as well as larger resonant chambers (Jacobs *et al*., 2007). James' Rule (JR) proposes that individuals occurring in hot humid environments generally have smaller body sizes than conspecifics that occur in cooler, drier environments (James, 1970). This is because selection in cooler environments would favour larger individuals with lower surface area to volume ratio, enabling individuals to conserve heat in cooler environments (Meiri and Dayan, 2003). In addition, hot humid environments may favour smaller individuals with higher surface area to volume ratio, enabling them to dissipate heat in hot humid environments (Ralls and Harvey, 1985). The variation in echolocation frequency might therefore simply be the result of the response of body size to different temperatures and humidity. This may also suggest that animals in cooler habitats do not enter torpor.

The Damara horseshoe bat, *Rhinolophus damarensis* Roberts, 1946, has a wide distribution in southern Africa which stretches from the more arid regions in north-western South Africa and southern Namibia to the more mesic regions of northern Namibia and southern Angola (Jacobs *et al*., 2013). Genetic analyses indicated that this species consists of two lineages, a northern lineage restricted to the more mesic regions of northern Namibia and a southern lineage restricted to the more arid regions of central and north-western South Africa, extending into central Namibia (Jacobs *et al*., 2013). The situation of *R. damarensis* therefore provides an ideal opportunity for testing the Sensory Drive Hypothesis.

Under this hypothesis it was predicted that: 1) call frequency variation should correspond with climatic variables i.e resting frequency should have a linear relationship with temperature but it should be inversely related to humidity; 2) call frequency variation should be partitioned in accordance with the genetic groupings, i.e. there should be a strong signal of IbE, and 3) if call frequency is the result of selection rather than genetic drift, call frequency variation should not be correlated with geographic distance, i.e. there should be no signal for IbD. Under JR it was predicted that 1) there should be a correlation between body size and climatic factors and between body size and call frequency.

Materials and Methods

Study area and species

Rhinolophus damarensis has a wide distribution which stretches from the more arid regions in north western South Africa and southern Namibia to the more mesic regions of northern Namibia and southern Angola (Jacobs *et al*., 2013; Maluleke *et al*., 2017). *R. damarensis* occurs across several biomes (i.e. Namib Desert, Savanna, Succulent Karoo and Nama Karoo) in the western half of southern Africa (Jacobs *et al*., 2013; Maluleke *et al*., 2017). The northernmost populations occur within the Nama Karoo and Savanna biomes in the northern Namibia. The central Namibian population occurs within an ecotone of the Nama Karoo and Savanna biomes. The southernmost localities are within the Savanna, Nama Karoo, Succulent and Namib Desert biomes in the Northern Cape Province of South Africa. The Nama Karoo and Succulent Karoo biomes differ significantly in their climatic regimes. The Nama Karoo, which is arid has a more continental climate, characterised by hot summers with low rainfalls, and very dry, mild to cool winters (Russell, 1987; Cowling *et al*., 1998). An extremely arid Succulent Karoo has a warm temperate and oceanic climate characterized by relatively mild winters with low winter rainfall, and relatively mild summers (Cowling *et al*., 1998; Cowling *et al*., 1999). The hyper-arid Namib Desert biome covers 15% of Namibia's land area (Foissner *et al*., 2002) and it is characterised by extreme climatic conditions (Burke, 2001; Foden *et al*., 2007). In contrast, the Savanna biome, which covers the largest area in the region, is more mesic (Russel, 1986). Studies on geographic variation of mean annual and winter temperature in Namibia showed a south-north gradient (Thuiller *et al*., 2006). In addition, the average maximum temperatures during the hottest month increase from 20 ºC at the coast to about 30–34 ºC in central Namibia and are higher than 36 ºC in the southern part of Namibia (Mendelsohn *et al*., 2002). The northern Namibia receives approximately 140 mm of rainfall per annum, supporting the Savannah Woodland vegetation (Barnard, 1998). The southern part of the region lies largely in the Northern Cape Province of South Africa, which is characterised by a harsh climate with minimal rainfall and prolonged droughts. The climate in this area is generally dry and the rainfall varies from less than 100 mm to more than 400 mm per annum, with a gradient of increasing rainfall from west to east of the Province (Tyson, 1987; Jacobs *et al*., 2013).

Sampling

Data were collected across the known distributional range of *R. damarensis*, in southern Africa from 7 localities along a latitudinal gradient ranging from 16˚S to 32˚S (Fig. 2.1). The geographic coordinates (latitude and longitude) of each locality at which bats were sampled were recorded in the field, using a Garmin GPS unit (Colorado, Garmin International Inc, Kansas). Bats were captured from their roosting sites such as mines, caves, abandoned buildings, hollow trees and culverts under roads using hand nets during the day, or mist nets and harp traps as bats emerged from the roosts at dusk. Harp traps and mist nets were checked regularly throughout the trapping period to ensure that bats were not trapped in these for too long. All captured horseshoe bats were transferred directly into soft cotton holding bags.

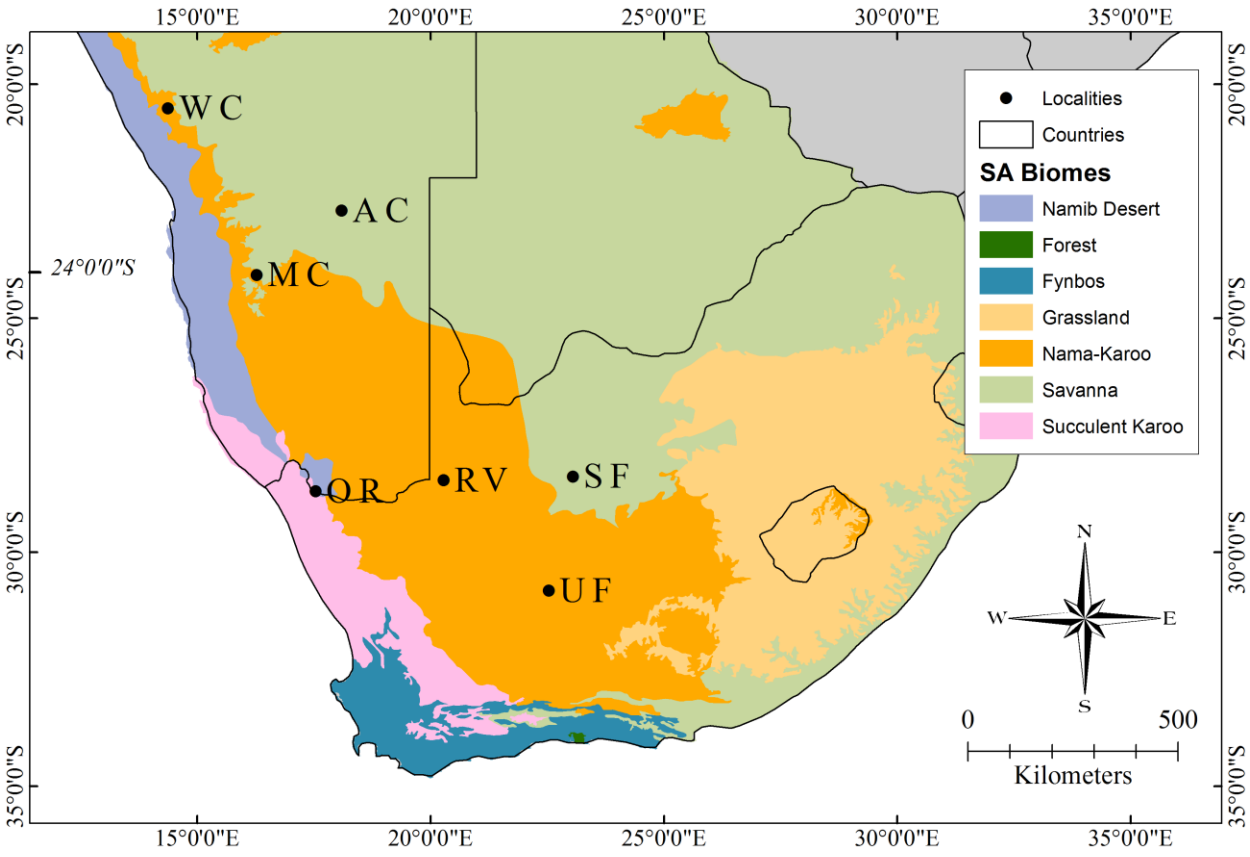

Figure 2.1: Sampling localities of *R. damarensis* in the western half of southern Africa. Wondergat Cave (WC), Arnhem Cave (AC), Märcker Cave (MC), Orange River (OR), Riemvasmaak (RV), Soetfontein (SF) Untjiesburg Farm (UF).

Morphology

The body mass of each bat was measured using a portable electronic balance (Scout Pro SPU402, Ohaus Corporation, Pine Brook, NJ USA) to the nearest 0.1g, and forearm length (FA), was measured to the nearest 0.1 mm using (VITA, TA) dial calipers. Juveniles were excluded from this study and were identified by the presence of epiphyseal plates in their finger bones. The plates were detected by trans-illuminating the bats' wings (Anthony, 1988). Forearm length instead of mass was used as an indicator of body size because mass varies diurnally, seasonally and with life-history stage (Jacobs *et al*., 2013; Mutumi *et al*., 2016). The sex of each bat was also recorded.

Echolocation

We recorded echolocation calls in the field from hand-held bats positioned 10cm in front of an Avisoft Ultrasound Gate 416 (Avisoft Bioacoustics, Berlin, Germany) microphone connected to a HP Compaq nx7010 notebook computer running Avisoft SasLab Pro software (Avisoft Bioacoustics Schönfließer, Germany) with the sampling rate set at 500 kHz. I measured 10 high quality calls (high signal to noise ratio) that occurred after the first 10 calls in each recording because horseshoe bats tune into their peak resting frequency after a period of silence (Schuller and Suga, 1976; Siemers *et al*., 2005). Calls were analysed with BatSound Pro (version 3.20; Pettersson Elektronik AB, Uppsala, Sweden) using a sampling rate of 44 100 Hz (16 bits, mono). The peak resting frequency was measured (kHz; the frequency of maximum intensity of the constant component) from the Fast Fourier Transformation (FFT) power spectrum (size set at 1024 samples) of the dominant 2nd harmonic in each call for 10 high quality (high signal to noise ratio) calls. The average RF over the 10 calls for each bat was used in the analyses.

Environmental Variables

ArcGIS shape files for climate data were downloaded from the following BIOCLIM websites: (http://www.worldclim.org/bioclim) and OEI (www.en.openei.org). The shape files were analysed in ArcGIS v.10 to extract data on Relative Humidity (RH) and Annual Mean Temperature (AMT). RH was based on data taken at 10 m above the surface of the earth by National Aeronautics and Space Administration (NASA), Surface Meteorology and Solar Energy (SSE Release 6.0, Data Set, November 2007), a 22 year (1983-2005) monthly and annual average dataset (http://eosweb.larc.nasa.gov/sse/). AMT shape files were based on

monthly temperature values that were averaged over 50 years (1950-2000). All environmental data were extracted for a radius of 10km around each locality at which *R. damarensis* was sampled.

Atmospheric Attenuation and Detection Range

Atmospheric attenuation and detection distances of prey for each population of *R. damarensis* were calculated using the web calculator developed and described in Stilz and Schnitzler (2012). These two ranges were calculated to determine the impacts of ecological constraints shaping the echolocation behaviour of *R. damarensis* (Neuweiler, 1990; Fenton *et al*., 1995; Denzinger and Schnitzler, 2004). The calculations required the following information (a) climatic conditions (e.g AMT, RH and atmospheric pressure) at each sampled site. Atmospheric pressure was kept as that for normal atmospheric conditions, taken as 101. 325 pascal; (b) resting frequency (Hz) of each individual bat; (c) the dynamic range, which is the difference between peak intensity (dB SPL) measured at 1m and the auditory threshold of the bat, assumed to be 0 dB SPL for horseshoe bats (Long and Schnitzler, 1975; Kingston and Rossiter, 2004); (d) reflection loss, C_1, accounts for the fraction of the energy reflected and; (e) geometric spreading, C_2, quantifies the loss of energy due to spreading multiplied by 2 for both outgoing emitted call and the returning echo. The values of the latter two factors are dependent on the geometry of the reflected wave. Although evidence suggests that detection distance changes with prey size (Armstrong and Kerry, 2011), the aim here was to determine the influence of climatic conditions on the detection range of *R. damarenis* and for this we used the prey size category on the online calculator that corresponded closely to the prey size range of bats (Stilz and Schnitzler 2012). The web calculator generates C_1 and C_2 depending on target selected. The point function reflector which resembles the insect prey of bats was chosen. A call intensity of 123.7dB SPL was used for (c) above, measured by Fawcett *et al*. (2015) for a horseshoe bat of similar size (*R. capensis*). The actual call intensity of *R. damarensis* is currently unknown.

Statistical Analysis

This study recorded and analysed 1 060 RF individual pulses from 106 adult *R. damarensis* from 7 localities, taking 10 calls per bat (Table B2). To investigate the plausibility of the JR, IbE and IbD, candidate models with different combinations of environmental, spatial and

biological predictor variables were evaluated to determine their influence on RF across the distributional range of *R. damarensis*.

Biological predictor variables included forearm length (FA), which was used as a proxy for body size (James' Rules) and sex, which was used to determine if there was sexual dimorphism in RF within *R. damarensis*.

The two alternative environmental variables considered were annual mean temperature (AMT) and relative humidity (RH), which have previously been suggested as factors largely responsible for atmospheric attenuation of sound (Lawrence and Simmons, 1982; Hartley, 1989, Luo *et al*., 2014). To avoid collinearity between AMT and RH, these two environmental variables were fitted into separate models and excluded candidate models with combinations of RH and AMT.

Spatial predictors included region (Reg) and latitude (Lat) based on the two genetically differentiated lineages within *R. damarensis* (Jacobs *et al*., 2013). The split between the two *R. damarensis* lineages provided insight in determining the spatial variables responsible for RF divergence in *R. damarensis*. One lineage included populations that were located north of latitude (-24ºS) and was designated the northern lineage. The other lineage included populations further south than latitude -24ºS and was designated the southern lineage (Fig. 2.1).

Statistical Procedure

Geographic variation in RF may be the result of stochastic processes (e.g. genetic drift). One way in which such stochastic processes would be manifested is in a positive relationship between the RF of populations and the geographic distance between them. This current study therefore also determined whether differences in RF were associated with geographic distances by calculating a dissimilarity matrix of RF (kHz) differences amongst localities and Euclidean distances among populations from their geographic coordinates (longitude and latitude). A simple pairwise Mantel test was employed to analyse associations between RF differences and geographic distances (Legendre and Fortin, 2010). For this purpose, 10 000 permutations based on Monte-Carlo simulation tests were used.

Linear Mixed Effects models (LMEs) were applied to analyse the relationship between the response variable (RF) and the environmental (AMT or RH), spatial (Lat or Reg) and

biological (FA, sex) predictor variables. LMEs incorporate both fixed and random effects (Verbeke and Lesaffre, 1996; Zuur *et al*., 2009), where the fixed effects quantify the overall effects across the different localities and the random effects quantify variation of the fixed effect parameters across localities (Bolker *et al*., 2008). The random effect for sampling was included to account for the nested sampling design as a result of sampling several individuals from a single location. In addition, LMEs can incorporate for autocorrelation in the data, which was considered here to account for potential spatial dependencies among sampled localities (Bjørnstad and Falck, 2001) to ensure that IbE is not being driven by spatial autocorrelation (Shafer and Wolf, 2013). IbD was investigated for this reason (Shafer and Wolf, 2013).

A reasonably complex set of variables (AMT, Reg and Sex with resting frequency as the response variable) was first used to determine the best mixed effects structure (Zuur *et al*. 2009). LMEs were fitted with and without spatial correlation structures and a random effect for sampling locality. The candidate LMEs were fitted with various spatial autocorrelation functions on longitude and latitude coordinates, including corGaus, corExp, corRatioa corSpher and AR1. However, the most parsimonious error structure, as judged by the Akaike's information criterion (AICc), was a linear mixed model (LME) that only included a random effect for locality. This was also supported by non-parametric spatial spline correlograms based on the model residuals, which showed that spatial autocorrelation evident for a fixed-effects linear model, could be effectively accounted for by including the random effect for locality (Fig. S2.1). Inspection of residuals from the random effects LME showed that the residuals closely approximated a normal distribution and provided no evidence for violation of the assumption of homogenous variance (Fig. S2.2). The LME with a random effect for locality was then selected as most parsimonious error model for analysing the variation in RF.

To determine the optimal combination of covariate, competing models were ranked based on their Akaike's information criterion (AICc), which is the AIC corrected for a small sample size (n) relative to the number of parameters being estimated (K). A total of 22 model permutations were conducted to examine which model subsets best explained the variation in our data. AICc differences (Δ_i) and Akaike weights (w_i) were used to identify the best candidate models (Amar *et al*., 2014; Odendaal *et al*., 2014). Models with the lowest AICc and highest Akaike weights values were considered the most parsimonious and the

differences in AIC scores (Δ_i) were calculated to determine the likelihood that a given model was the best approximating model relative to other candidate models (Symonds and Moussalli, 2011; Amar *et al*., 2014; Odendaal *et al*., 2014). A model with a (Δ_i) value of zero was considered the best approximating model, and those with values of up to 2 were regarded as good as the best model (Symonds and Moussalli, 2011). Evidence ratios of the best fitting models were calculated relative to the other subsequent candidate models to determine the relative evidence for the best approximating model in relation to the other candidate models (Amar *et al*., 2014).

The predictions of James' Rule were tested by incorporating forearm in the model because body size can influence resting frequency variation in echolocating bats due to allometric relationship between body size (e.g forearm) and resting frequency (Jones, 1996; Jones, 1999; Jacobs *et al*., 2007).

All the analyses were conducted in R version 3.1.2 and the following packages were used: 'AICcmodavg' for model selection and multi model inference to compare and rank multiple competing models and to estimate those that best approximate the true processes underlying the biological phenomenon under study (Symonds and Moussalli, 2011), 'lme' for fitting the linear mixed effects model and to incorporate both fixed and random effects in a linear predictor expression from which the conditional mean of the response was evaluated (Bates *et al*., 2015), 'effects' for displaying the effect sizes of linear, generalised linear and other models (Fox and Anderson, 2006), 'car' a companion to applied regression for regression diagnostics and other regression related tasks (Fox, 2002), 'ncf' a spatial nonparametric covariance function to make correlograms and to check for spatial autocorrelation (Bjørnstad and Falck, 2001), 'Ade4' for estimating geographical distances between sites and response variable (Thioulouse *et al*., 1997; Legendre and Fortin, 2010).

Results

The Damara horseshoe bat, *R. damarensis* had an average RF of 85.43±1.3 kHz and average call duration of 31.14±5.9ms. Mean RF ranged from 84.4±0.7 kHz to 87.6±1.1 kHz across localities. Females had a mean RF ranging from 84.5±0.4 kHz to 87.8±1.0 kHz, whilst the RF of males ranged from 84.4 kHz±0.9 to 86.9±1.1 kHz.

Differences in RF were not associated with geographic distances. A simple pairwise Mantel test revealed that there was no significant association between RF differences and geographic distances (Monte-Carlo test Observation: 0.084, 10 000 replicates, $p > 0.05$).

For the LMEs model selection, only models with an accumulative weight (Cum.wt) up to 95% were considered. This resulted in three most supported models (Table 2.1). In order of highest ranking based on Akaike information criterion weight (highest AICwt) these three models were Reg+AMT+Sex, Reg+AMT+Sex+FA and Lat+AMT+Sex. However, the evidence ratio indicated that the first model (Reg+AMT+Sex) was 3 and 7 times stronger than the second and third models, respectively (Table 2.1) and each of the three variables from the first best model had a $p < 0.05$ (Table 2.2).

Table 2.1: Results from linear mixed effects models (LMEs) testing for association between the resting frequency of *R. damarensis* and environmental variables.

Model Candidate	K	AICc	ΔAICc	AICcWt	Cum.Wt	LL	ER
Reg+AMT+Sex	**6**	**294.38**	**0.00**	**0.65**	**0.65**	**-140.76**	
Reg+AMT+Sex+FA	**7**	**296.62**	**2.24**	**0.21**	**0.86**	**-140.74**	**3.07**
Lat+AMT+Sex	**6**	**298.58**	**4.20**	**0.08**	**0.94**	**-142.87**	**7.71**
Lat+AMT+Sex+FA	7	300.87	6.49	0.03	0.97	-142.86	24.8
Sex	4	302.58	8.21	0.01	0.98	-147.09	60.5
AMT+Sex	5	303.43	9.06	0.01	0.98	-146.42	92.6
Lat+RH+Sex	6	304.47	10.09	0.00	0.99	-145.81	>100
RH+Sex	5	304.48	10.10	0.00	0.99	-146.94	>100
Reg+RH+Sex	6	305.33	10.95	0.00	0.99	-146.24	>100
AMT+Sex+FA	6	305.68	11.30	0.00	1.00	-146.42	>100
RH+Sex+FA	6	306.73	12.35	0.00	1.00	-146.94	>100
Lat+RH+Sex+FA	7	306.76	12.39	0.00	1.00	-145.81	>100
Reg+RH+Sex+FA	7	307.62	13.25	0.00	1.00	-146.24	>100
Reg+AMT	5	317.31	22.93	0.00	1.00	-153.35	>100
Lat+AMT	5	317.44	23.06	0.00	1.00	-153.42	>100
FA	4	320.32	25.94	0.00	1.00	-155.96	>100
Lat	4	322.64	28.26	0.00	1.00	-157.12	>100
Reg	4	322.95	28.58	0.00	1.00	-157.28	>100
AMT	4	323.79	29.41	0.00	1.00	-157.70	>100
Lat+RH	5	323.82	29.44	0.00	1.00	-156.61	>100
RH	3	324.57	30.20	0.00	1.00	-158.09	>100
Reg+RH	5	325.13	30.75	0.00	1.00	-157.27	>100

(AMT) Annual Mean Temperature, (RH) Relative Humidity, (FA) Forearm, (Lat) Latitude, (Reg) Region, (K) Number of parameters, (AICc) Akaike information criterion scores, (ΔAICc) Change in AICc relative to the highest ranked model, (AICwt) Akaike information criterion weight, (Cum.wt) Cumulative weight, (LL) Log likelihood and (ER) Evidence Ratio.

Table 2.2: Summary statistics for the most parsimonious linear mixed-effects models (LMEs) fitted by REML on the RF of *R. damarensis*.

Variable	**Value**	**SE**	**DF**	***t*-value**	***p*-value**
Intercept	74.733	2.464	98	30.328	0.001
AMT	0.468	0.120	4	3.873	0.017
Reg	2.198	0.551	4	3.989	0.016
Sex	1.039	0.200	98	5.187	0.001

(SE) Standard error, (DF) Degree of freedom, (Reg) Region, (FA) Forearm and (AMT) Annual Mean Temperature.

All LMEs models indicated that forearm (FA), being a proxy for body size, did not strongly influence resting frequency (RF) variation in *R. damarensis*. This was further supported by regression analysis which showed no correlation between FA and RF (R^2=0.02, $p > 0.05$). There was a positive correlation between RF and AMT (Fig 2.2a). Comparison of spatial (latitude and region) and environmental (RH and AMT) factors using linear mixed effects models revealed that AMT and region had the lowest AIC value. This indicated that environmentally mediated sensory drive (including AMT and IbE) was strongly associated with resting frequency variation in *R. damarensis*. RH did not appear to be a good predictor of RF variation (Fig. 2.2b). There was also sexual dimorphism in the RF of *R. damarensis*, females having higher RF than males (Fig 2.2c). The very same results were found when the southern populations were analysed without the northern lineage population. However, only AMT and sex were responsible for RF variation in southern lineage populations.

The inclusion of Reg in the model, indicated that the northern and southern regional groups based on genetic lineages of *R. damarensis*, had distinct RFs; the southern region had higher RF than the northern region (Fig. 2.2d). However, the RFs of the two regions overlapped; the southern region had RFs which ranged from 82.8 to 88.9 kHz and that of the northern region ranged from 82.9 to 86.1 kHz (Table B2; Fig. S2.3 and Fig. S2.4).

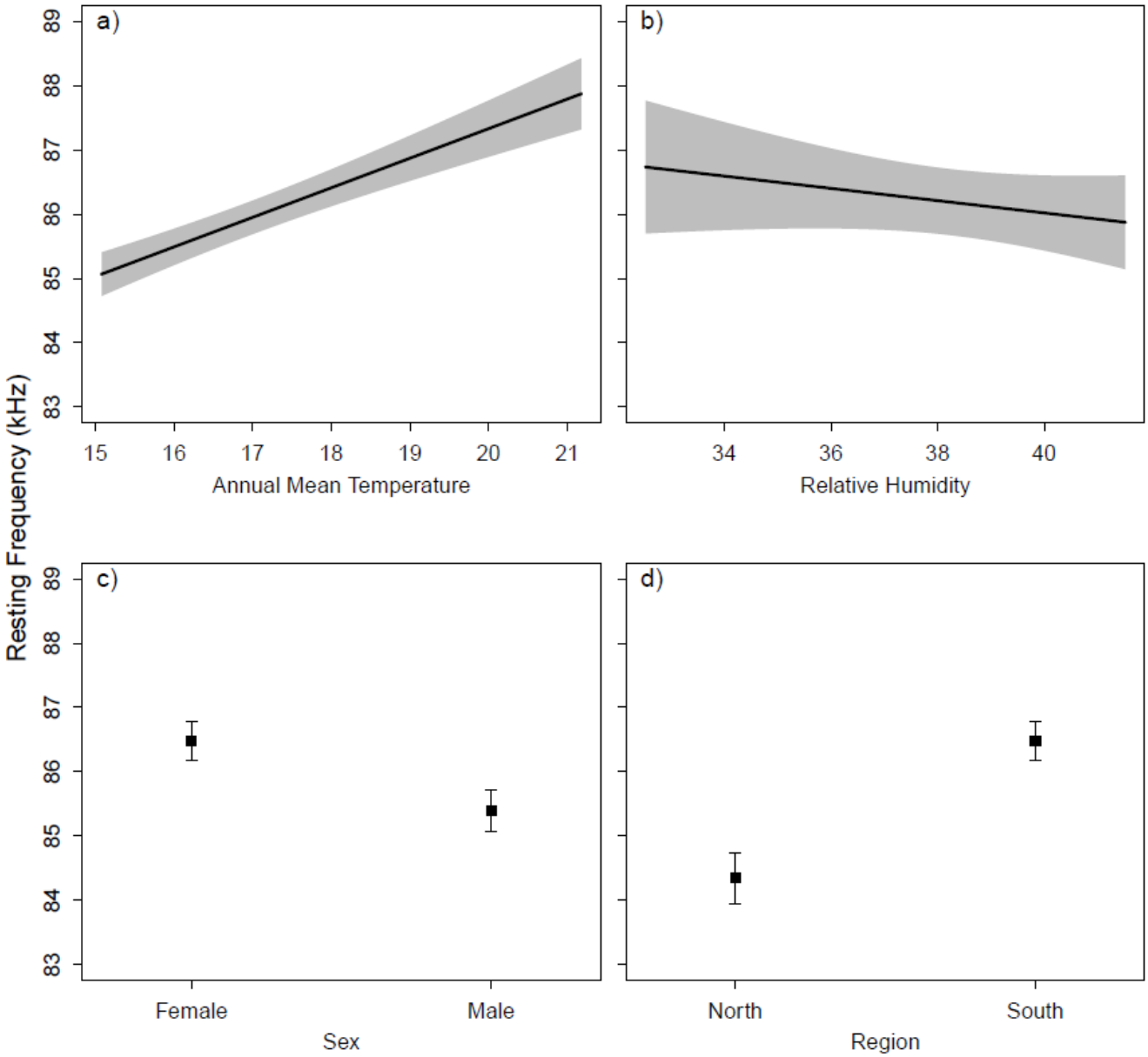

Figure 2.2: Predicted effects of a) annual mean temperature, b) relative humidity, c) sexual dimorphism and d) region cause RF variation in *R. damarensis*.

The mean detection range and atmospheric attenuation of echolocation signals across populations of *R. damarensis* were 9.84±0.8 m and 2.24±0.2 dB per meter, respectively. Across populations of the northern lineage the mean atmospheric attenuation was 2.42±0.1 dB/m and the mean detection range was 9.24±0.2 m, whilst across populations of the southern lineage the mean atmospheric attenuation and mean detection range were (2.18±0.1 dB/m and 9.99±0.6 m respectively). However, these differences were not statistically significant (Mann-Whitney *U* Test: $p > 0.05$). Changes in atmospheric attenuation were most

pronounced in the population of the southern lineages (e.g. Märcker) that was closest to populations from the northern lineage.

Discussion

The study found no evidence that variation in RF is explained by James' Rule or for random genetic drift. Body size was not correlated with RF or climatic variables, suggesting that variation in RF was not the result of concomitant variation in body size as proposed by James' Rule. Similarly the Mantel test showed no IbD effect and there was therefore no evidence that genetic drift was responsible for the variation in RFs. In contrast, the LMEs indicated that there was support for IbE in the form of an association between RF and region (in the form of the variable "Reg") which was based on two geographically separated genetic lineages. Furthermore, RF variation was also associated with the climatic variable AMT. Thus all three predictions of the Sensory Drive Hypothesis were supported. This was so because, 1) call frequency variation corresponded with climatic variables; 2) call frequency variation partitioned in accordance with the genetic groupings, i.e. there was a strong signal of IbE; and 3) call frequency differences was the result of selection rather than genetic drift, call frequency variation was not correlated with geographic distance, i.e. there was no signal for IbD.

Most studies on geographic variation in RF have focused on humidity as the main predictor of such variation (for review see Jiang *et al*., 2015), providing evidence that echolocating bats that are found in humid areas will experience severe atmospheric attenuation resulting in lower RF than those that are found in dry areas (Lawrence and Simmons 1982; Heller and von Helversen, 1989). In addition, echolocating bats adapt call frequency to the relative humidity conditions of their particular habitat, making a very fine adjustment in the trade off point between increasing frequencies to enhance prey resolution and decreasing it to maximize prey detection range (Guillen *et al*., 2000). For example, geographic variation in the RF of *R. pusillus* was positively correlated with mean annual rainfall (Jiang *et al*., 2010). However, the propogation of echolocation calls and acoustic signals in general, is influenced by both temperature and relative humidity (Lawrence and Simmons 1982; Luo *et al.* 2014) through their effect on atmospheric attenuation. Atmospheric attenuation is the result of a complex interaction between temperature, humidity and the frequency of the sound being propagated (Luo *et al*. 2014). For example, in two species of horseshoe bats, *R. simulator* and *R. swinnyi,* distributed in the more mesic eastern half of southern Africa, acoustic divergence

in RF was influenced by the interaction between temperature and relative humidity and the degree of influence was higher in *R. swinnyi* than in *R. simulator* because *R. swinnyi* echolocated at higher frequencies (103.77 ± 1.70 kHz and 80.32 ± 2.20 kHz, respectively; Mutumi *et al*., 2016). The results for *R. damarensis* indicated that temperature (AMT) was the predominant climatic factor that corresponded with the divergence in echolocation RF. This was also the case in the geographic divergence of echolocation calls in *R. ferrumequinum* (Jiang *et al*., 2015). The reason why RH did not significantly influence RF variation in *R. damarensis* (Fig. 2.2b), despite it being a major factor in other species and areas, is possibly because the distribution of *R. damarensis* encompasses the more arid western half of southern Africa, indicated by the low RH values (Table B2; Fig. S2.5) at the sampled localities relative to those reported for the localities at which *R. simulator* and *R. swinnyi* were sampled (Mutumi *et al*., 2016). This is supported by studies on, *R. capensis*, which also has a distribution that extends into the more arid western and north-western regions of South Africa (Odendaal *et al*., 2014; Neumann and Bamford, 2015). Here too there was no correlation between RF and RH (Odendaal *et al*., 2014).

There was no clear pattern of temperature that separated the northern and southern regions of the distribution of *R. damarensis*. This was probably because the effect of temperature is mediated by its interaction with RH (see Luo *et al*., 2014; Mutumi *et al*., 2016), despite the low RH reported here. The interaction between temperature and humidity may vary from one region to another such that one or the other may exert the predominant influence on atmospheric attenuation and the propogation of acoustic signals. The results of this current study and those of Mutumi *et al*. (2016) suggest that in warm mesic regions both temperature and relative humidity are likely to influence the propogation of acoustic signals but in warm arid regions the predominant climatic factor that defines atmospheric attenuation may be temperature. The interaction between temperature and humidity may also explain the paradoxical results obtained by studies on the climatic influence on geographic variation in acoustic signals. For example, geographic variation in RF was positively correlated with mean annual rainfall in *R. pusillus* (Jiang *et al*., 2010) but negatively correlated with mean annual rainfall in *Hipposideros ruber* (Guillén *et al*., 2000). These contrasting findings are often attributed to the complexity of natural selection (Jiang *et al*., 2015) but may instead result from the non-linear effects of temperature and humidity on atmospheric attenuation (Luo *et al*. 2014). Neither Jiang *et al*., (2010) nor Guillén *et al*., (2000) considered the interactive effects of temperature and humidity on atmospheric attenuation.

In the case of *R. damarensis*, divergence in RFs may be the result of ecological selection for optimal detection ranges within the respective habitats of the populations in the two regions. Divergence in acoustic signals may arise from the influence of various evolutionary forces, among which ecological selection is the most common (Podos and Warren, 2007; Wilkins *et al*., 2013). Populations of *R. damarensis* in the northern region have RF on the lower end of the range of that for populations in the southern region. The lower RF of populations from the northern region could be the result of the less arid conditions in northern Namibia, differences in temperatures (Table B2; Fig. S2.6) and the effects of interaction between temperature and humidity across the region. Acoustic divergence within species may occur when signals undergo selection for optimal propagation of acoustic signals in the local acoustic environment (Wiley and Richards, 1982) leading to populations occupying different ecological niches (Nosil, 2012). The mean atmospheric attenuation was higher amongst populations in the northern region (2.51±0.1dB/m) than that amongst populations of the southern region (1.83±0.1dB/m). This resulted in a lower mean detection range (8.97 ± 0.0 m) amongst the northern populations compared to the southern populations (11.3 ± 0.1 m). Amongst populations of the southern region, atmospheric attenuation was also particularly low at the southern (Märcker) and (Uintjiesberg) distributional limits of the southern region leading to the longest detection ranges in these two populations. These changes in atmospheric attenuation and detection ranges between the southern and northern lineages as a result of habitat structure and climate induced differences, suggest that lineage divergence in *R. damarensis* is the result of isolation by environment (IbE).

The requisite combination of genetic and phenotypic divergence which is an indicator of IbE (Nosil, 2012) is present in *R. damarenssis*. The genetic differentiation reported by Jacobs *et al*. (2013) between the northern and southern regions of the species'distribution is supported by the RF differences between the two regions (this study). There is therefore both genetic and phenotypic evidence suggesting that populations within the two regions represent diverging lineages. The two regional groupings were revealed in genetic analyses which suggested that *R. damarensis*, consists of two (north-south) lineages which diverged 5.3 Ma with a percentage sequence divergence of 4.1 % (Jacobs *et al*., 2013). The northern part of the region is comprised of populations of *R. damarensis,* which are found in the northern part of Namibia, whilst the southern region has populations with distribution stretching from central Namibia to north-western and central South Africa. Besides difference in atmospheric

attenuation, there are other ecological differences between the two regions occupied by the diverging lineages and it is likely that other factors contribute to their ecological isolation.

The northern and southern regions coincide with the north-south climatic gradient of the region. The northern part of Namibia is a more mesic region and characterised by woodland vegetation (White, 1983; Simmons *et al*., 1998, Hoetzel *et al*., 2015), whilst central Namibia and the Karoo ecoregions (Succulent and Nama) of South Africa are semi-arid (Okitsu, 2005; Thuiller *et al*., 2006; Neumann and Bamford, 2015). The Succulent Karoo biome of southern Africa stretches from southern Namibia, along the western side of the South African escarpment to the eastern border of the Western Cape Province (Lombard *et al*., 1999). The eastern part of the Northern Cape Province is largely dominated by the Nama Karoo biome (Hoetzel *et al*., 2015). The Desert biome is in the western side of South Africa, and it covers a strip along the Orange River and extends to the Namib Desert (Neumann and Bamford, 2015). The importance of region as a predictor of RF variation may be indicative of the environmental discontinuities described here.

This coincidence of genetic and phenotypic difference with environmental discontinuities strongly suggests that divergence in this species is the result of IbE, possibly facilitated by sensory drive. Such environmental discontinuities in the context of the propagation of acoustic signals may be defined by atmospheric conditions. Furthermore, other climatic variables (associated with latitude and altitude) also influenced RF (Mutumi *et al*., 2016) and this too may be suggestive of the influence of ecological discontinuities on phenotypic traits. Geographic variation in RF between populations of *R. damarensis* in the two regions may have evolved as a consequence of local adaptation to climatic conditions. The data also support IbE in the form of an association between RF and Reg (Fig. 2.2d), which was based on two geographically separated genetic lineages (Jacobs *et al*., 2013). Thus, ecologically mediated selection could also have led to regional RF variation in *R. damarensis* as a result of populations adapting to different local ecological conditions. This could impede gene flow between the northern and southern regions occupied by *R. damarensis* (Rundle and Nosil, 2005; Gillam and McCracken, 2007) through several processes, including selection against migrants (Hendry, 2004; Thibert-Plante and Hendry, 2009) or matching habitat choice (Edelaar *et al*., 2008). The latter process is likely if bats, especially at the margins of suitable habitat (e.g. Märcker and Uintjiesberg populations in this currrent study) choose habitats on the basis of maximizing their detection ranges. Such choice would result in a reduction of gene flow to habitats with decreased detection distances (e.g. the northern region) and

possibly reduce gene flow between the two regions. Evidence suggests that bats may alter the structure of their calls when foraging in different habitats to efficiently hunt prey (Schnitzler *et al*., 2003). This may result in bats adapting call frequency to the environmental conditions of their particular habitat, making very fine adjustments in the trade-off between increasing frequency to enhance prey resolution and decreasing it to maximize prey detection range (Guillén *et al*., 2000). Bats may therefore be reluctant to move into habitats where this trade-off is not optimized or where very large adjustments in frequency are required to obtain optimal detection distances, particularly in rhinolophids whose echolocation is based on an acoustic fovea.

The genetic difference between the two lineages of *R. damarensi*s (Jacobs *et al*., 2013), and statistical support for differences between RF for the two regions is an indication that populations of *R. damarensis* may be isolated by environment. This is supported by other studies which revealed lineage diversification within species in the western half of southern Africa as a result of climate induced changes in biomes (Matthee and Flemming, 2002; Bauer and Lamb, 2005). Comparative studies in the whole region of southern Africa revealed lineage diversification and clades within species in a wide range of animal taxa including reptiles (da Silva and Tolley, 2013), insects (Pitzalis and Bologna, 2010) and mammals (Matthee and Robinson., 1999; Willows-Munro and Matthee, 2011; du Toit *et al*, 2012) including horseshoe bats (Taylor *et al*., 2012; Jacobs *et al*., 2013; Odendaal *et al*., 2014). Resting frequency variation resulting from adaptation to local environments might lead to pre-mating isolation among populations of the same species occupying different habitats if calls from immigrants are detected towards the margins of the acoustic fovea (Armstrong and Kerry, 2011). It appears therefore that sensory drive may have at least in part mediated divergence through an interruption of gene flow resulting from IbE. The genetic differences beween the two lineages of *R. damarensis* are not as pronounced as that between *R. damarenis* and other recognized species in the same genus (Jacobs *et al*., 2013) and does not support the conclusion that speciation has occurred.

Recent studies suggest that climate change may have a severe effect on the sensory ecology of sound-mediated behaviours (Luo *et al*., 2014). An increase in temperature and/or relative humidity combined with the high RF used by *R. damarensis*, is likely to result in a decrease in the detection volume of *R. damarensis* under a global warming scenario (Luo *et al*., 2014). Global warming may therefore directly impact the sensory ecology of organisms reliant on sound mediated behaviours (Møller, 2010; Luo *et al*., 2014). If changing ambient temperature

increases atmospheric attenuation, then prey detection distances of echolocating bats may be reduced accordingly (Luo *et al*., 2014). Reduction in prey detection distances could lead to ineffective foraging and an increase in the costs benefits ratio of foraging which could lead to local extinction of populations. However, in this current study, RH did not explain the differences in resting frequency of *R. damarensis* and the suggestions of Luo *et al* (2014) will not be relevant if climate change resulted in different prey availability because of changes in habitat productivity.

Sex in *R. damarensis* was a significant predictor of RF with females generally echolocating at slightly higher RF than males. Similarly, in several other horseshoe bat species, females emit echolocation calls of higher frequencies than males (Siemers *et al*., 2005; Yoshino *et al*., 2006; Chen *et al*., 2009) but this may not be a pattern common to all populations, because sexual dimorphism in RF may change across populations. In addition, males in *Rhinolophus swinnyi* produce higher RFs than females (Mutumi *et al*., 2016). Sexual dimorphism difference in RF of *R. damarensis* was 0.7 kHz. Other echolocation parameters (e.g inter pulse interval, duration of call, minimum frequency and bandwidth of the FM component, slope of the FM component, duration of FM component) need to be incorporated to determine the true extent of sexual dimorphism in calls. Bats appear to have a wide diversity of mating systems but at the moment there is no much information about the mechanisms involved in courtship and mating (Grilliot *et al*., 2009). Evidence suggests that echolocation calls of a bat may serve as a source of information for individual recognition (Siemers *et al*., 2005; Siemers and Kerth, 2006). Sexual dimorphism in RF of *R. damarensis* may have an essential social function by signaling the sex of the caller (Kazial and Masters, 2004), promoting mate recognition (Guillén *et al*., 2000) and potential reproductive success (Grilliot *et al*., 2009; Puechmaille *et al*., 2009). It has been shown for some species that males and females do not overlap in their call frequency, and frequency may encode the sex of the signaller reliably (Neuweiler *et al*., 1987). This may imply that female and male individuals of *R. damarensis* might keep their frequencies within a narrow range for reliable sex recognition. Sexual dimorphism in RF of *R. damarensis* may benefit both signallers and receivers in mate selection and listening to echolocation calls may probably allow mate location at distances greater than is possible through olfaction (Jones and Siemers, 2011).

Climate-induced variation in acoustic signals may not be restricted to bats. There is also some support for climate-induced geographic variation in bird song (Snell-Rood, 2012) suggesting that lineage diversification in general may be driven by habitat mediated differences in

acoustic signals, especially when such differences are accompanied by genetic differentiation. Climatic gradients in conjunction with other ecological discontinuities could lead to local adaptation and directed dispersal (Edelaar *et al*. 2008) such that gene flow is restricted, allowing lineages to diverge. In addition, call frequency of a bat can change significantly with only a relatively small effect on detection range (Armstrong and Kerry, 2011). However, it is also argued that any trade-off maximising detection range and prey resolution may be swamped by the effects of other factors (Armstrong and Kerry, 2011). This current study, assumes that the size range of the diet of the different regional populations of *R. damarensis*, could be similar, and that the change in emitted resting frequency could be a response to maintain prey detection ranges of different taxa but probably similarly-sized insects.

CHAPTER 3

Regional phenotypic divergence partially explains lineage diversification within a southern African horseshoe bat species, *Rhinolophus damarensis*, (Chiroptera: Rhinolophidae)

Abstract

The Damara horseshoe bat species, *Rhinolophus damarensis*, is restricted to the western half of southern Africa, where it is widely distributed and occurs across several major biomes. Formerly regarded as the subspecies *R. darlingi damarensis*, it was elevated to full species status on the basis of genetic and phenotypic differences between it and *R. darlingi darlingi*. *Rhinolophus damarensis* is itself made up of two ecologically separated genetic clades. This current study therefore investigated whether there is sufficient corroborating evidence that the two genetic groups of *R. damarensis* are sufficiently divergent to consider them as full, reporductively isolated species, using ecological traits and phenotypic data (skulls, bacula, wings and echolocation). The main objective of this study was to test whether previously reported genetic divergence between the two *R. damarensis* lineages is associated with phenotypic divergence. Here, a total of 106 individuals of *R. damarensis* were sampled from seven localities across its distributional range. Morphometric and echolocation measurements were taken from hand-held individual bats in the field, and skull measurements were taken from field collected voucher specimens as well as museum specimens. Discriminant function analyses (DFA) were carried out on each set of *R. damarensis* data (echolocation, wing and skull parameters) to find a set of phenotypic data that maximises differences among populations. DFA analyses revealed that there were separations of populations and lineages within each of the three data sets, and Multivariate analysis of variances (MANOVA) and Linear models (LM) on some parts of bacula revealed significant differences between the southern and northern lineages of *R. damarenis*. Furthermore, the shapes of the bacula of the two lineages of *R. damarensis* differed between the two lineages.

Key words: Echolocation, lineage diversification, skull, bacula, wings.

Introduction

The continent of Africa has a diverse biota (Vences *et al*., 2009; Lotz *et al*., 2013), and southern Africa is characterised by ecologically, regionally and locally steep environmental gradients (Andrews and O'Brien, 2000; Taylor *et al*., 2012; Jacobs *et al*., 2013). Amongst African biota, bats are one of the most ecologically and morphologically diverse mammalian clades (Simmons and Conway, 2003). This diversity in bats is associated with the diversification of forest savanna, desert biomes (Bogdanowics, 1992; Koopman, 1993) and flowering plants (Jones *et al*., 2005). Strong climatic oscillations during the middle Miocene resulted in warm periods and ice ages, and affected and changed faunal and floral distribution patterns. This in turn led to geographic separation of lineages and many speciation events (Datzmann *et al*., 2012) as the biota became adapted to local habitats. Therefore, there is a close association between the phenotypes of organisms and aspects of their life history, e.g. diet and foraging strategies (Freeman, 1979; Neuweiler, 1984; Grant, 1986; Aldridge and Rautenbach, 1987; Losos, 1990). There is evidence of relationship between ecology and phenotype in various species (Neuweiler, 1984; Aldridge and Rautenbach, 1987; Losos, 1990). This correlation is so strong that phenotypic characteristics are frequently used to infer ecology in studies of community structure and the evolutionary processes responsible for such structure. A corollary to this is that patterns of geographic phenotypic variation within and among species are reflections of the different selective pressures that have been exerted on populations across the geographic ranges of species, as a result of the different environments they have experienced over their evolutionary history. Such variation, particularly amongst isolated populations may subsequently lead to lineage divergence and speciation. Studies of phenotypic variation can therefore uncover lineage divergence and elucidate the evolutionary processes responsible for such divergence.

The phenotype of animals, including bats, appears to be a strong determining factor on how bat species function in their natural environments (Norberg and Rayner, 1987; Aldridge and Rautenbachs, 1987; Dietz *et al*., 2006). Species of bats with low frequency calls often have high wing loading, pointed wingtips and high aspect ratio (long narrow wings) and they fly rapidly in open space (Norberg and Rayner, 1987), whilst those that have higher frequency calls have low wing loading, rounded wingtips and low aspect ratio (short broad wings) can manoeuvre and fly slowly in or close to dense vegetation (Norberg and Rayner, 1987; Aldridge and Rautenbach, 1987; Jones, 1996; Jones, 1999; Jacobs *et al*., 2007). Therefore, differences in echolocation, wing shape and body size may influence flight behaviour

(Noberg and Rayner, 1987) and restrict some populations or species to certain foraging habitats (Noberg and Rayner, 1987, Saunders and Barclay, 1992). As a result, this could lead to a spatial partitioning of foraging habitats in insectivorous bats (Aldridge and Rautenbach, 1987). Body size of insectivorous bat species generally has an inverse relationship with peak echolocation frequency (Heller and von Helversen, 1989; Jones, 1996; Jones, 1999). Studies on horseshoe bats have also shown an inverse relationship between echolocation calls and body size (Heller and van Helversen, 1989; Thabah *et al*., 2006; Stoffberg *et al*., 2007; Taylor *et al*., 2012). In most cases, larger bat species tend to have lower echolocation call frequencies, because they have longer, thicker vocal chords and larger resonant chambers (Jacobs *et al*., 2007). In addition, larger bat species use lower echolocation frequencies to increase the detection range for targets, and this may potentially give them enough time to manoeuvre and capture prey (Heller and von Helversen, 1989; Barclay and Brigham, 1991). Variation in echolocation calls (Guillén *et al*., 2000; Odendaal *et al*., 2014; Mutumi *et al*., 2016) and body size (Kryštufek, 1993; Dietz *et al*., 2006; Yoshino *et al*., 2006) within horsehoe bat species has also been documented. Echolocating bats emit echolocation calls for orientation and prey detection, and echolocation calls have features that are related to the ecological niches of bats and may therefore be shaped by natural selection (Schnitzler *et al*., 2003; Jones and Holderied, 2007).

High duty cycle bats are good focal animals for studies of lineage divergence, because they are widely distributed (Csorba *et al*., 2005; Monadjem *et al*., 2010) and their echolocation calls, can be easily recorded from hand held individuals. Furthermore, bat species have a variety of different adaptations to their environment (Guillén *et al*., 2000; Jiang *et al*., 2010). They also have the highest frequency calls in the animal kingdom. Therefore, atmospheric attenuation will be more likely pronounced in calls with the highest echolocation frequencies (Griffin, 1971; Lawrence and Simmons, 1982; Hartley, 1989; Luo *et al*., 2014). Through atmospheric attenuation, the environment, particularly its climatic component, influences how acoustic signals, including echolocation calls of bats, travel from signaller to receiver (Richards and Wiley, 1980; Endler, 2000). This could cause acoustic signals, like the echolocation calls of bats, to diverge along climatic gradients (Guillén *et al*., 2000; Jiang *et al*., 2010; Snell-Rood, 2012; Mutumi *et al*., 2016; Chapter 2). Climatic gradients could also influence anatomical features. For example, skull size variation among populations of the fruit eating bat, *Artibeus lituratus*, was correlated with environmental variables such as precipitation and temperature (Marchán-Rivadeneira, 2012). On the other hand, skull size in

North American *Eptesicus fuscus*, was found to be inversely related to temperature but positively related to moisture (Burnett, 1983). Responses of bat species to climatic gradients could be an indication of phenotypic variation driving adaptive processes (Steven *et al*., 2016). Populations within species that experience different selective pressures across the species' range may have different phenotypic variation, and this may subsequently lead to lineage divergence. Although there is evidence of correlations between climate and echolocation frequency (Mutumi *et al*., 2016, Jacobs *et al*., 2017) in the context of atmospheric attenuations as a causal factor, the general correlations between morphology and climatic factors on these bats are inconclusive and remain elusive. Bats may respond to a different food items in a particular environment and changes in morphometric variables within species may more likely represent adaptations associated with dietary change (Eastman *et al*., 2012).

However, in many bat groups phenotypic traits, particularly morphological ones, may be highly convergent (Ruedi and Mayer, 2001; Jiang *et al*., 2008; Jacobs *et al*., 2013) even amongst genetically distinct lineages, leading to the erroneous classification of several distinct lineages into one. For example, recent studies on *R. darlingi* revealed two genetically distinct lineages within the species but the phenotypes (echolocation frequencies, skull and post cranial morphology) were highly convergent (Jacobs *et al*., 2013). Furthermore, some bat species in different biogeographic regions showed convergent adaptive radiations but genetic analyses revealed divergent clades (Ruedi and Mayer, 2001; Kingston and Rossiter, 2004).

Many of Africa's rhinolophid species, such as *R. hildebrandtii* (Taylor *et al*., 2012), *R. darlingi* (Jacobs *et al*, 2013) and *R. simulator* (Mutumi *et al*., 2016), have wide geographic distributions. Species with such wide geographic distributions are likely to contain cryptic species (Jacobs *et al*., 2006; Stoffberg *et al*., 2007; Morateli *et al*., 2011; Datzmann *et al*., 2012). There are several morphologically cryptic species, as confirmed by recent molecular analyses (Jacobs *et al*., 2006; Taylor *et al*., 2012; Soisook *et al*., 2016). Morphologically cryptic species exhibit stronger differences in ecological traits than would be predicted from their morphological or phylogenetic similarity. Some cryptic bat species have been discovered as a result of differences in their echolocation call frequencies. For example, the differences in echolocation calls of *Pipistrellus pipistrellus* despite striking similarity in morphology suggested that it consisted of two distinct species (Jones and van Parijs, 1993). The existence of two distinct species was later confirmed through molecular analyses (Baratt

et al., 1997), behavioural tests (Barlow and Jones, 1997) and ecological modelling (Barlow, 1997). Furthermore, analyses of molecular and morphological characters in *Hipposideros commersoni*, revealed the existence of cryptic species (Rakotoarivelo *et al*., 2015). Therefore, cryptic species are likely to occur in widely distributed taxa, currently described as single species, which vary geographically either in echolocation, morphology, genetics, or a combination of all of these (Ramasindrazana *et al*., 2011; Taylor *et al*., 2012). However, it is often difficult to uncover cryptic bat species by using external morphology only. Instead cryptic species have been identified largely by either echolocation parameters (Jones and van Parijs, 1993; Jones, 1997; Thabah *et al*., 2006; Taylor *et al*., 2012) or very specific morphological characters (Francis *et al*., 2007; Goodman *et al*., 2011) and confirmed with genetic analyses (Hulva *et al*., 2004; Jacobs *et al*., 2013; Soisook *et al*., 2016).

Rhinolophidae Gray, 1825 are represented by a single genus *Rhinolophus* Lacépéde, 1799 which is divided into two clades, an Asian and an Afro-Palaearctic' clade (Stoffberg *et al*., 2010; Dool *et al*., 2016). *Rhinolophus damarensis*, a member of the Afro-Palaearctic clade, was formerly the sub-species, *R. darlingi damarensis* but was elevated to full species status on the basis of genetic and phenotypic analyses (Jacobs *et al.*, 2013). *R. damarensis* was separated from *R. darlingi* on the basis that *R. darlingi* was found to be a paraphyletic species (Jacobs *et al*., 2013), with the disjunct eastern and western parts of its distribution forming two genetically distinct non-sister lineages with sequence divergence of 8.1% with divergence estimated at ~9.7 Mya (Jacobs *et al*., 2013).

The aim of this chapter was to further assess the IbE hypothesis by investigating divergence in other phenotypic traits that are known to be adaptive in bats e.g. wing and skull parameters and whether such divergence is associated with the two regions identified in Chapter 2. It was therefore hypothesized that selection could result in phenotypic divergence among populations of *R. damarensis* inhabiting different environments. Under this hypothesis it was predicted that: 1) there should be phenotypic divergence among populations of *R. damarensis* occupying different environments; 2) phenotypic divergence should be partitioned in accordance with the two RF groupings identified in Chapter 2. For this purpose acoustic, skull and wing parameters as well as bacular morphology were collected from across the distribution range of *R. damarensis* in the western half of southern Africa to investigate divergences both among populations and between the northern and southern genetic lineages identified by (Jacobs *et al*., 2013).

Materials and Methods

Study Species

Rhinolophus damarenesis is a medium-sized insectivorous horseshoe bat with a relatively high echolocation frequency (85.4 ± 1.4 kHz) and a long duration of the constant frequency component (31.15±5.9 ms), as well as a mean forearm length of 49.5 ± 1.7 mm (Jacobs *et al*., 2013; Chapter 2). *R. damarenesis* has a wide geographical distribution in the western half of southern Africa stretching from western South Africa through Namibia to south-west Angola (Jacobs *et al*., 2013, Chapter 2). This region is characterised by a wide range of arid and mesic climatic conditions.

Horseshoe bats (Rhinolophidae) are high duty cycle eholocators (i.e. duty cycle is the ratio of the call duration to call period) that concentrate echolocation call energy in the second harmonic (Hartley and Suthers, 1988; Fenton *et al*., 1995; Schnitzler and Denzinger, 2011). Their calls are comprised of an important constant frequency (CF) component preceded and followed by a frequency modulated (FM) component (Neuweiler, 1984). The CF component is mainly used to detect temporal, frequency and amplitude modulations (collectively called acoutic glints) in the echoes reflected off the beating wings of insect prey and to evaluate Doppler-shifted echo frequency. The FM components are used for echo ranging and fine target feature analysis (Neuweiler, 1984; 1989; Kober and Schnitzler, 1990; Schnitzler and Denzinger, 2011). Horseshoe bats are perch hunters that are able to echolocate from a resting position while scanning the environment for prey (Csorba *et al*., 2003; Jacobs *et al*., 2007). The peak frequency (frequency of maximum amplitude) of the CF component of a call emitted while at rest is called the resting frequency (RF). This RF is 100 Hz to 300 Hz lower than the reference frequency, a narrow range of frequencies to which the acoustic fovea (a region of over-representation of neurons in the auditory cortex) is tuned (Schuller and Pollak, 1979). Resting frequencies can therefore be recorded from hand-held bats which eliminate variance in frequency caused by bats compensating for Doppler shifts during flight (Neuweiler, 1984).

Collection of samples

Data were collected across the known distribution range of *R. damarensis*, in southern Africa from 7 localities along a latitudinal gradient ranging from 16˚S to 32˚S (see Fig. 2.1). The geographical coordinates (longitude and latitude) of each locality at which *R. damarensis* was

sampled (together with other rhinolophid species) were recorded in the field after capturing bats, using a Garmin GPS unit (model Colorado, Garmin International Inc, Kansas). All bats were captured from their roosting areas such as hollow trees, caves, abandoned buildings, old mine shafts and culverts under roads using hand nets during the day, or mist nets and harp traps as bats emerged from the roosts at dusk. Harp traps and mist nets were checked regularly throughout the trapping period to ensure that bats were not trapped in these for too long. All captured horseshoe bats were transferred directly into soft cotton holding bags, and kept individually until measured.

Morphology

Juveniles were excluded from this study to avoid bias as a result of differences in measurements between juveniles and adults. Juveniles were recognized by the presence of cartilaginous epiphyseal plates in their finger bones which were detected by trans-illuminating the bat wings (Anthony, 1998). The sex of each captured bat was recorded and only non-pregnant females were measured to avoid differences in body mass that are due to pregnancy. The body mass of each bat was measured using a portable electronic balance (Scout Pro SPU402, Ohaus Corporation, Pine Brook, NJ USA) to the nearest 0.1g, and the external morphological measurements (i.e forearm and noseleaf width) were measured using (VITA, TA) dial calipers to the nearest 0.1 mm. A photograph of the extended right wing (as per Saunders and Barclay, 1992), including the tail and half the body was taken with a Cannon Powershot S60 PC1088 digital camera (Cannon Inc, Japan) positioned 90º above the bat and parallel to the flat table top on which the bat wing was extended to prevent angular distortion (Jacobs *et al*., 2007). From each bat image, wing parameters including wing area, wingspan and length measurements were taken using SigmaScan Pro 5 software (version 3.20 SPSS Inc, Cary, NC, USA). Wing area and wingspan were then used to calculate wing loading (weight divided by wing area) and aspect ratio (wingspan squared divided by wing area) as described by Norberg and Rayner (1987). The wing was extended on graph paper which was used to calibrate Sigma Scan. All bats, with the exception of taking one male and one female voucher from each site, where possible, were safely returned to their roosting areas immediately after taking all the morphometric and acoustic measurements. One or two voucher species were humanely sacrificed in the field by placing them in the tightly closed bottle with cotton wool damped with a few drops of halothane.

Skull extraction and measurements

Skulls were extracted from the voucher specimens in the laboratory following the method described by Dejtaradol (2009). This was done by peeling back the facial skin from the mandible and rostrum using either a blunt scalpel or forceps. Caution was taken not to damage the skull. The upper cervical spine was then cut at the base of the skull to free the skull. It was then placed in a small container with meal worms so that the meal worms could clean all the remaining flesh from the skull. The skull was removed from the container and cleaned thoroughly under a dissecting microscope. Lastly, the label was attached to the cranium and mandible and placed in a small plastic vial with a secure lid and supported on cotton wool to minimise damage during storage. In addition to skulls extracted from voucher specimens, this current study also used *R. damarensis* skulls from museum specimens in the collections of two South African museums (Ditsong Natural History Musem, Pretoria and Amathole Natural and Cultural Museum, King Williams Town). Skulls of other rhinolophid species (*R. capensis, R. clivosus, R. blasii and R. darlingi*) loaned from Ditsong Natural History Museum, which are similar in size and echolocation calls to *R. damarensis*, and likely to be confused with it, were also included in this study. Only adult *R. damarensis* and other rhinolophid species'skulls were used. Adults in museum collections were identified by the degree of tooth wear. This study also recorded the sex and locality for each museum specimen from the specimen labels and museum data provided. The following linear cranial and dental measurements were taken to the nearest 0.01mm using precision digital callipers (0-150 mm, 0-6 inch) with accuracy of 0.01mm: mandible length, distance from the most posterior point of the mandibular condyle to the most anterior point of the dentary (ML); maxillary toothrow length, distance from the most posterior point of the last upper molar to the most anterior point of the upper canine (UCM^3L); mandibular toothrow length, distance from the most posterior point of the last lower molar to the most anterior point of the lower canine. (LCM_3L); greatest skull length measured dorsally from occiput to anterior point of skull (GSL); zygomatic width, the greatest distance across the zygoma (ZW); braincase width measured at dorsal surface of posterior root of zygomatic arches (BW); and posterior palatal width, distance between outermost points of the last upper molar (M^3M^3W). The procedure followed the method described by Freeman (1984), Bates *et al*., (1997) and Taylor *et al*., (2012).

Echolocation

All echolocation calls used in this study were recorded in the field from hand-held bats held 10cm from the microphone of an Avisoft Ultrasound Gate 416 (Avisoft Bioacoustics, Berlin, Germany). The microphone was connected to a HP Compaq nx7010 notebook computer running Avisoft SasLab Pro software (Avisoft Bioacoustics Schönfließer, Germany) with the sampling rate set at 500 kHz. 10 high quality echolocation calls (i.e high signal to noise ratio) that occurred after the first 10 calls in each recording were measured, because horseshoe bats tune into their peak resting frequency after a period of silence (Schuller and Suga, 1976; Siemers *et al*., 2005). Echolocation calls were analysed in BatSound Pro (version 3.20; Pettersson Elektronik AB, Uppsala, Sweden) using a sampling rate of 44 100 Hz (16 bits, mono). The peak resting frequency was measured in kHz and the RF was averaged over the 10 calls taken for each bat. The following parameters were measured from the dominant 2nd harmonic in each call: peak resting frequency (RF, kHz, the frequency of maximum intensity of the constant component of the call determined from Fast Fourier Transformation (FFT) power spectrum with a size of 1024); duration (ms, time from beginning to end of a call, determined from the oscillogram); minimum frequency (kHz) of the terminal frequency modulated (FM) component, determined from the spectrogram; duration of the FM component (ms, time from beginning to end of FM call, determined from oscillogram) and the inter-pulse interval (ms, time between adjacent calls). The minimum frequency of the FM component was subtracted from the peak resting frequency to derive the bandwith. Duty cycle was obtained by dividing the duration of the whole call by the duration of the whole call plus inter pulse interval, and multiplying by 100, while sweep rate was calculated by dividing the bandwidth by duration of the frequency modulated component. Prey detection range was estimated using the method described in Chapter 2. The mean value of each echolocation parameter was then calculated from the selected 10 calls for each bat. The echolocation parameters of an original call that most closely resembled the calculated mean parameters were selected for all subsequent analyses (Jacobs *et al*., 2007; Odendaal *et al*., 2014).

Baculum extraction and preparations

The rapid divergence of the male baculum, a bone that is found in the penis of most mammalian species, is a recurring evolutionary pattern (Schultz *et al*., 2016). Bacula

divergence can occur even when divergence in other morphological traits is not evident, probably because baculum divergence is based on relatively few mutations (Schultz *et al*., 2016). In groups of organisms, where gross morphology is often convergent, such as in bats, the baculum may be used as a taxonomic character to distinguish species that are otherwise morphologically indistinguishable (Herdina *et al*., 2014). It can also be used as an indicator of lineage diversification within species (Ramm *et al*., 2010; Klaczko *et al*., 2015). This study used bacula extracted from voucher specimens of *R. damarensis* captured in northern Namibia (Wondergat cave and Arnhem cave), southern Namibia (Märcker cave) and north western South Africa (Orange River and Augrabies) to determine if divergence in baculum shape corresponded with the two genetic clades identified in *R. damarensis*.

All bacula were extracted in the laboratory following methods described in Lidicker (1968), Thomas *et al*., (1994), Kearney *et al*., (2002), Jacobs *et al*., (2013). A total of 5 bacula from male voucher specimens of *R. damarensis* were prepared and penile tissue was submerged in distilled water for 12 to 15 hours. Bacula were then macerated in 5% KOH solution stained with alizarin red for one hour. Fine forceps were used to tease off the flesh from around the baculum, under a dissecting microscope of 3X magnification. All bacula were then placed in glycerol solutions with concentrations ranging from 20% to 80% for 24 hours to clear them. They were then stored in vials with 100% glycerine and a crystal of thymol to prevent fungal growth (Goodman *et al*., 2012). Bacula were later dried and photographed using a Nikon Digital Stereo Microscope (WD54 Nikon, Japan) with the scale set at 1mm. An annotation and measurements software program (NIS Elements D) was used to measure the following 10 dimensions of each baculum: Greatest width of the baculum base viewed from the dorsal position (GWB-d); greatest width of the baculum base viewed from the lateral position (GWB-l); greatest length of the baculum (GBL); greatest width of the baculum shaft (GWS); baculum length from the tip to the point of greatest shaft width (BLTW); narrowest width of the baculum shaft (NW); baculum length from the tip to the point of narrowest shaft width (BLT-n); greatest length of the baculum incision on the dorsal side of the base (GLI); the height of the base, from the midpoint in the dorsal plane of the upper edge of the base to the most extended point at the edge of the base (BH) and the greatest length of the shaft (GLS).

Statistical analyses of morphometric and echolocation data

Multivariate statistics were employed using principal component analysis and discriminant function analysis. These analyses were done to identify parameters that are efficient in

maximising separation among populations. The two lineages of *R. damarensis* were divided at latitude 24ºS (see Fig. 2.1) based on the genetic lineages in the two regions (Jacobs *et al*., 2013). The northern lineage of *R. damarensis* included bats caught from Wondergat and Arnhem caves in northern Namibia, whilst the southern lineage included bats caught from central Namibia and central, north-western and south-western South Africa. All the extracted and loaned museum skulls of *R. damarensis* were grouped in accordance with these two lineages to pool biogeographically similar localities into Operational Taxonomic Units (OTUs) and skulls were coded from the northern and southern populations differently to investigate the degree of differentiation. Other rhinolophid species included in this study were *R. blasii*, *R. darlingi*, *R. clivosus* and *R. capensis*. Data sets were divided into three categories i.e wing, skull and acoustic parameters. All variables from the three datasets were standardized using the z-transformation in Statistica (computed as Standardized Score = (raw score – mean)/Std. deviation. Analyses using multivariate linear regressions (MVLR) were performed on each dataset with measured parameters skulls (n = 100) and wings (n = 62) for echolocation (n = 62), as multivariate dependent variables, and locality and sex as categorical predictors to test the main effects of sex and locality and their interaction. This was followed by a principal component analysis (PCA) and discriminant function analysis (DFA) on each dataset to find out which phenotypic dataset maximises differences among populations. Each of the separate principal component analyses (PCA) on wing, skull and acoustic parameters extracted its independent and uncorrelated factors from its original set of variables. For *R. damerensis*, skulls from the northern and southern populations were coded differently to investigate the degree of differentiation. DFA on each dataset was employed on the factor scores of principal components with an Eigenvalue ≥ 1 (Kaiser's criterion, 1960) to examine multivariate morphometric and acoustic differences between the populations of *R. damarensis*. To test if there was an adaptive complex between echolocation and wings, the two parameters (RF and wing loading) were regressed using log-tranformed data from individual bats of *R. damarensis* across localities. It is sensible to do this, because of the negative relationship between wing loading and echolocation (Heller and von Helversen, 1989; Jacobs *et al*., 2007). In addition, evidence suggests that wing morphology and echolocation call design play important roles in the foraging ecology of insectivorous bats (Aldridge and Rautenbach, 1987; Noberg and Rayner, 1987; Fenton, 1990). All morphometric and acoustic analyses were done in Statistica version 13.0.

Linear models (LM) were employed on the ten measurements of 5 bacula from voucher specimens of *R. damarensis*. Bacula were classified into two northern and southern lineages depending on where the individual bat was caught. Shape of each baculum was also physically examined to determine if there were shape differences among bacula of *R. damarensis*.

Results

Sexual dimorphism

Results from the multivariate linear regressions on wing ($F = 0.57$, df = 14, $p > 0.05$), and skull parameters ($F = 0.71$, df = 7, $p > 0.05$) indicated that there was no morphological sexual dimorphism within *R. damarensis* (Tables B3.1 and B3.2). However, there was sexual dimorphism in the echolocation parameters of *R. damarensis* ($F = 2.3$, df = 9, $p < 0.05$) (Table B3.3).

Wing parameters

Populations of *R. damarensis* were differentiated on the basis of their wing parameters. The first two roots (Root 1 and 2) extracted by discriminant function analysis on the four PCs extracted by PCA had Eigenvalues of < 1 and explained 81% of the variance. Total classification success was 87.72% across the populations, and ranged from 25% to 100%. PC 1 was associated mainly with size (wing area, arm area, hand area, body area, armwing length, handwing length, tip area ratio and tip shape index) which loaded highest on Root 1, and PC 2 was associated with wingspan, aspect ratio, wing loading which loaded highest on Root 2 (Tables 3.1 and B3.4). The Orange River population from the southern lineage, placed on the extreme right of Root 1, was clearly separated from all other populations, which overlapped with each other, along Root 1. Size related variables (e.g. wing area, arm area, hand area, body area, arm wing length and hand wing length) as well as tip area ratio and tip shape index were good discriminators between populations (Soetfontein, Riemvasmaak, Wondergat, Arnhem, Märcker and Orange River) of *R. damarensis* (Root 1; Fig. 3.1). Bats from the Orange River population had the largest mean measurements for (wing length, wingspan, wing loading, aspect ratio, tip length ratio, tip area ratio and tip shape index (Fig. 3.1; Table 3.1), whilst bats from the Wondergat population, which is part of the northern lineage had the smallest mean measurements and were placed on the left side of Root 1 (Fig. 3.1; Table 3.1). Although Soetfontein overlapped with the northern lineage (e.g. Wondergat

and Arnhem populations), there was separation between Wondergat and Riemvasmaak populations as well as Arnhem and Märcker populations along Root 2 (Fig. 3.1). The squared Mahalanobis distance was largest (D^2 = 16.46) between the centroids of Orange River and Wondergat populations (F = 23.24, df = 4.48; $p < 0.05$), followed by the Mahalanobis distance (D^2 = 13.41) between Orange River and Soetfontein (F = 10.82, df = 4.48; $p < 0.05$) as well as Orange River and Märcker caves (D^2 = 11.61; F = 9.37, df = 4.48; $p < 0.05$) (Table B3.5). Orange River and Arnhem populations had the lowest squared Mahalanobis distance (D^2 = 9.06; F = 16.08, df = 4.48; $p < 0.05$) (Table B3.5). There were significant differences among the populations of *R. damarensis* (Table B3.6). However, there was no differentiation in wing parameters between the northern (Wondergat and Arnhem) and southern (Soetfontein, Riemvasmaak, Märcker and Orange River) genetic lineages of *R. damarensis* (Fig. 3.1). For example, wing parameters of bats from Soetfontein (southern genetic lineage) overlapped with those from the northern lineage (Wondergat and Arnhem). Similarly, those from Riemvasmaak, a southern population overlapped with those from the Arnhem population along Root 2 (Fig. 3.1). Regression analyses between wing loading and RF revealed no correlation between the two parameters (R^2=0.01; $p > 0.05$).

Table 3.1: Mean±SD of wing parameters for each population of *Rhinolophus damarensis*. Number of individuals per population is shown in parentheses. Samples size are given in parentheses below each locality and ranges are given below the means and standard deviations for each parameter.

	Soetfontein (6)	Riemvasmaak (6)	Wondergat (8)	Arnhem (12)	Märcker (6)	Orange River (24)
AWL (cm)	6.22±0.35	6.14±0.58	6.15±0.36	6.32±0.51	6.14±0.34	6.13±0.47
	(5.94-6.47)	(5.44-7.21)	(5.84-7.15)	(5.89-7.49)	(5.92-7.10)	(5.74-7.05)
HWL (cm)	7.13±0.37	6.81±0.42	7.14±0.43	7.02±0.41	7.12±0.40	6.94±0.53
	(6.98-7.48)	(6.63-7.92)	(6.90-7.58)	(6.24-8.10)	(6.71-7.99)	(6.41-8.28)
AAR (S/m^2)	0.005±0.00	0.004±0.00	0.004±0.00	0.004±0.00	0.004±0.00	0.004±0.00
	(0.004-0.005)	(0.003-0.005)	(0.003-0.005)	(0.003-0.005)	(0.003-0.005)	(0.003-0.005)
HAR(S/m^2)	0.002±0.00	0.002±0.00	0.002±0.00	0.002±0.00	0.002±0.00	0.002±0.00
	(0.002-0.003)	(0.002-0.003)	(0.002-0.003)	(0.002-0.003)	(0.002-0.003)	(0.002-0.003)
BAR(S/m^2)	0.001±0.00	0.001±0.00	0.001±0.00	0.001±0.00	0.001±0.00	0.001±0.00
	(0.001-0.002)	(0.001-0.001)	(0.001-0.002)	(0.001-0.002)	(0.001-0.002)	(0.001-0.002)
WAR (S/m^2)	0.009±0.00	0.008±0.00	0.008±0.00	0.008±0.00	0.008±0.00	0.008±0.00
	(0.007-0.01)	(0.007-0.01)	(0.007-0.01)	(0.007-0.01)	(0.007-0.01)	(0.006-0.01)
WLT (cm)	15.10±0.58	14.73±0.88	14.95±0.68	15.22±0.75	15.10±0.72	15.23±0.91
	(14.86-17.72)	(13.50-15.49)	(13.26-15.48)	(13.13-16.87)	(14.74-16.67)	(14.21-18.42)
WSP (B/m)	0.300±0.02	0.295±0.01	0.301±0.01	0.304±0.01	0.301±0.01	0.305±0.02
	(0.29-0.34)	(0.28-0.31)	(0.27-0.32)	(0.28-0.33)	(0.29-0.32)	(0.26-0.37)
WLD (N/m^{-2})	6.212±0.60	6.465±0.38	5.375±0.81	6.464±0.61	6.168±0.65	7.011±1.36
	(5.54-7.04)	(5.97-7.04)	(4.62-7.78)	(5.47-7.68)	(5.36-6.99)	(4.48-10.31)
ART (A)	5.248±0.30	5.458±0.34	5.193±0.22	5.466±0.27	5.168±0.23	6.014±0.48
	(4.86-5.60)	(4.86-6.04)	(4.71-5.59)	(4.87-7.76)	(4.92-5.49)	(5.05-10.24)
TLR (T_l)	1.148±0.04	1.118±0.05	1.132±0.07	1.113±0.05	1.153±0.04	1.151±0.06
	(1.09-1.19)	(1.04-1.19)	(0.99-1.27)	(0.99-1.18)	(1.10-1.20)	(1.02-1.28)
TAR (T_s)	0.645±0.02	0.651±0.06	0.629±0.03	0.622±0.03	0.661±0.03	0.689±0.06
	(0.61-0.68)	(0.58-0.76)	(0.57-0.68)	(0.59-0.67)	(0.62-0.70)	(0.60-0.82)
TSI (I)	1.313±0.08	1.448±0.39	1.295±0.30	1.246±0.17	1.356±0.17	1.575±0.44
	(1.21-1.45)	(1.01-2.40)	(0.96-1.73)	(0.96-1.52)	(1.22-1.52)	(0.98-2.97)

Arm wing length (AWL), Hand wing length(HWL), Arm area (AAR), Hand area (HAR), Body area (BAR), Wing area (WAR), Wing length (WLT), Wing span (WSP), Wing loading

(WLD), Aspect ratio (ART), Tip length ratio (TLR), Tip area ratio (TAR) and Tip shape index (TSI).

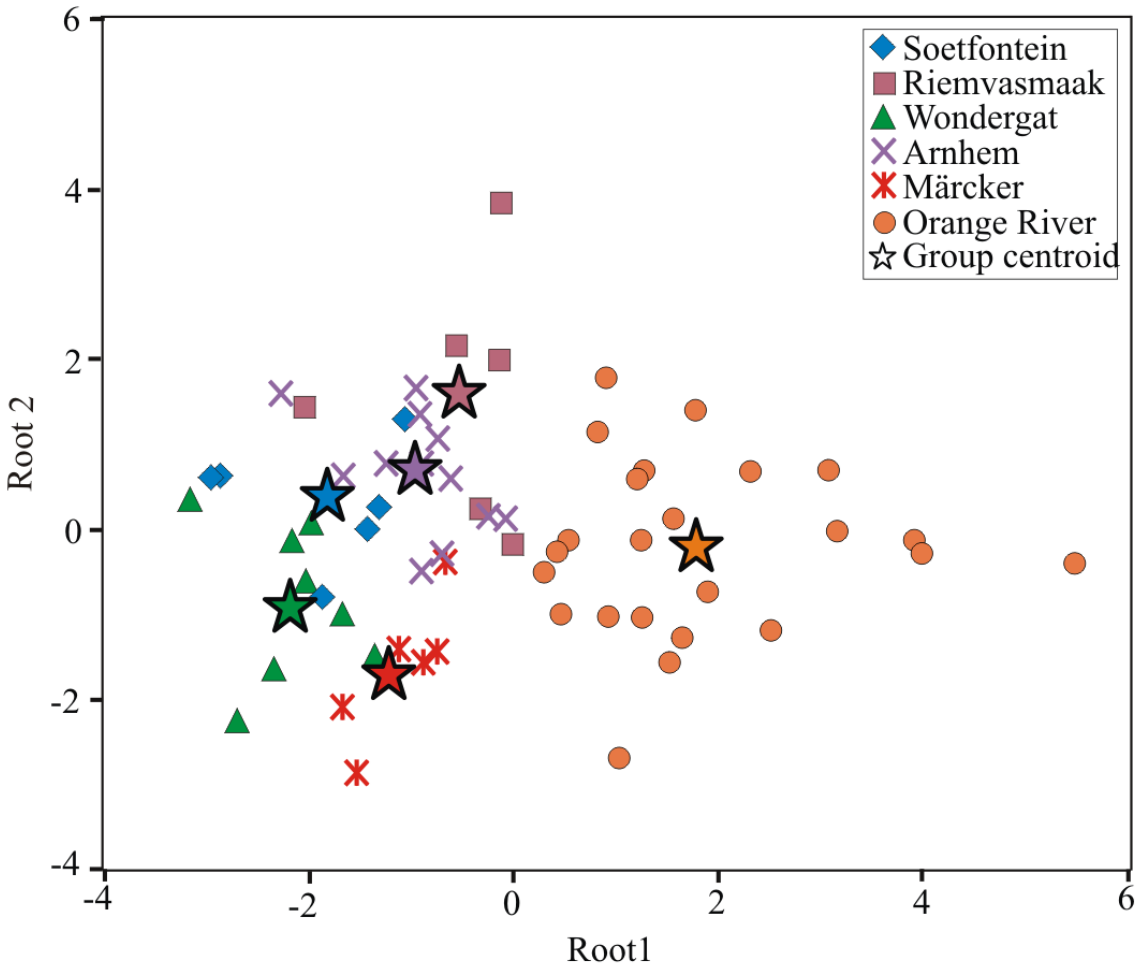

Figure 3.1: Plot of canonical scores extracted by Discriminant Function Analysis from wing parameters of *R. damarensis*. Group centroids are shown as stars.

Skull morphometrics

The analyses of skull parameters showed separation between the five species of *Rhinolophus*, included in the analyses, as well as between the northern and southern lineages of *R. damarensis* along both Root 1 (mainly) and Root 2. However, the two *R. damarensis* lineages overlapped along both roots but separation was more pronounced along Root 2 than Root 1 (Fig. 3.2). The first two roots (Root 1 and 2) extracted by discriminant function analysis on the three principal components (PCs) extracted by PCA, had Eigenvalues of < 1 and explained 93% of the variance. Total classification success was 93.45% across the populations and it ranged from 71.42% to 100%. Furthermore, the classification success of

the two northern and southern lineages was 71.42% and 90.0% respectively. PC 1 was associated mainly with size (upper toothrow length, lower toothrow length, zygomatic width, mandibular length, rostral width) which loaded highest on Root 1, and PC 2 was associated with braincase width and greatest skull length which loaded highest on Root 2 (Table 3.2 and Table B3.7). The mean morphological measurements suggest that size related variables (e.g. upper toothrow length, lower toothrow length, zygomatic width, mandibular length and) were main discriminators among species (*R. clivosus, R. capensis, R. darlingi and R. blasii*) as well as between these species and the northern and southern lineages of *R. damarensis* (Root 1; Fig. 3.2). *R. clivosus* had the largest mean measurements for these variables and was placed on the extreme right of Root 1 (Fig. 3.2; Table 3.2), whilst *R. blasii* with the smallest mean measurements was placed on the extreme left of Root 1 (Fig. 3.2; Table 3.2). The squared Mahalanobis distance was lowest ($D^{2\ =}$ 6.52, F = 13.10, df = 3.99; $p < 0.05$) between the centroids of northern *R. damarensis* and southern *R. damarensis* (Table B3.8) but this distance was nevertheless statistically significant (Table B3.9). However, the means of upper toothrow length, braincase width, zygomatic width and mandibular length of the northern lineage were slightly higher than the southern lineage of *R. damarensis*, but their ranges overlapped (Table 3.2).

Table 3.2: Mean±SD of skull parameters of five *Rhinolophus* species found in southern Africa. Number of individuals per population is shown in parentheses. Samples size are given in parentheses below each species name and ranges are given below the means and standard deviations for each parameter.

	R. blasii (22)	*R. darlingi* (10)	*R. clivosus* (14)	*R. capensis* (8)	*R. dam*-Northern (6)	*R. dam*-Southern (40)
UCM^3L (mm)	6.66±0.11 (6.48-6.79)	7.08±0.05 (7.03-7.18)	8.13±0.13 (8.01-8.38)	7.58±0.17 (7.41-7.76)	7.50±0.02 (7.45-7.55)	7.32±0.23 (6.9-7.72)
LCM_3L (mm)	6.89±0.10 (6.7-7.04)	7.53±0.17 (7.25-7.84)	8.69±0.29 (8.55-9.04)	8.06±0.17 (7.85-8.25)	7.54±0.31 (7.50-8.00)	7.81±0.33 (6.90-8.22)
M^3M^3W (mm)	6.34±0.12 (6.06-6.52)	7.25±0.07 (7.11-7.36)	8.32±0.32 (7.55-8.46)	7.51±0.11 (7.34-7.65)	7.57±0.35 (7.50-8.00)	7.55±0.24 (7.00-8.00)
BW (mm)	9.10±0.15 (8.89-9.27)	9.19±0.13 (9.02-9.36)	10.12±0.15 (9.93-10.29)	9.99±0.19 (9.88-10.35)	9.59±0.14 (9.50-9.75)	9.28±0.33 (8.50-10.0)
GSL (mm)	18.82±0.26 (18.46-19.22)	19.21±0.38 (18.61-19.63)	21.58±0.47 (20.55-22.43)	20.57±0.41 (20.13-21.16)	19.85±0.24 (19.50-20.00)	20.72±0.53 (19.72-22.0)
ZW (mm)	8.91±0.18 (8.60-9.21)	10.02±0.25 (9.79-10.42)	11.51±0.29 (11.12-11.66)	10.37±0.04 (10.29-10.43)	10.39±0.28 (10.0-10.75)	10.27±0.33 (10.0-11.0)
ML (mm)	11.89±0.17 (11.62-12.19)	12.76±0.31 (12.36-13.13)	14.71±0.32 (14.32-14.97)	13.45±0.30 (13.04-13.97)	13.85±0.37 (13.50-14.50)	13.14±0.48 (12.00-14.1)

Maxillary toothrow length (UCM^3L), Mandibular toothrow length (LCM_3L), Posterior palatal width (M^3M^3W), Braincase width (BW), Greatest skull length (GSL), Zygomatic width (ZW) and Mandible length (ML).

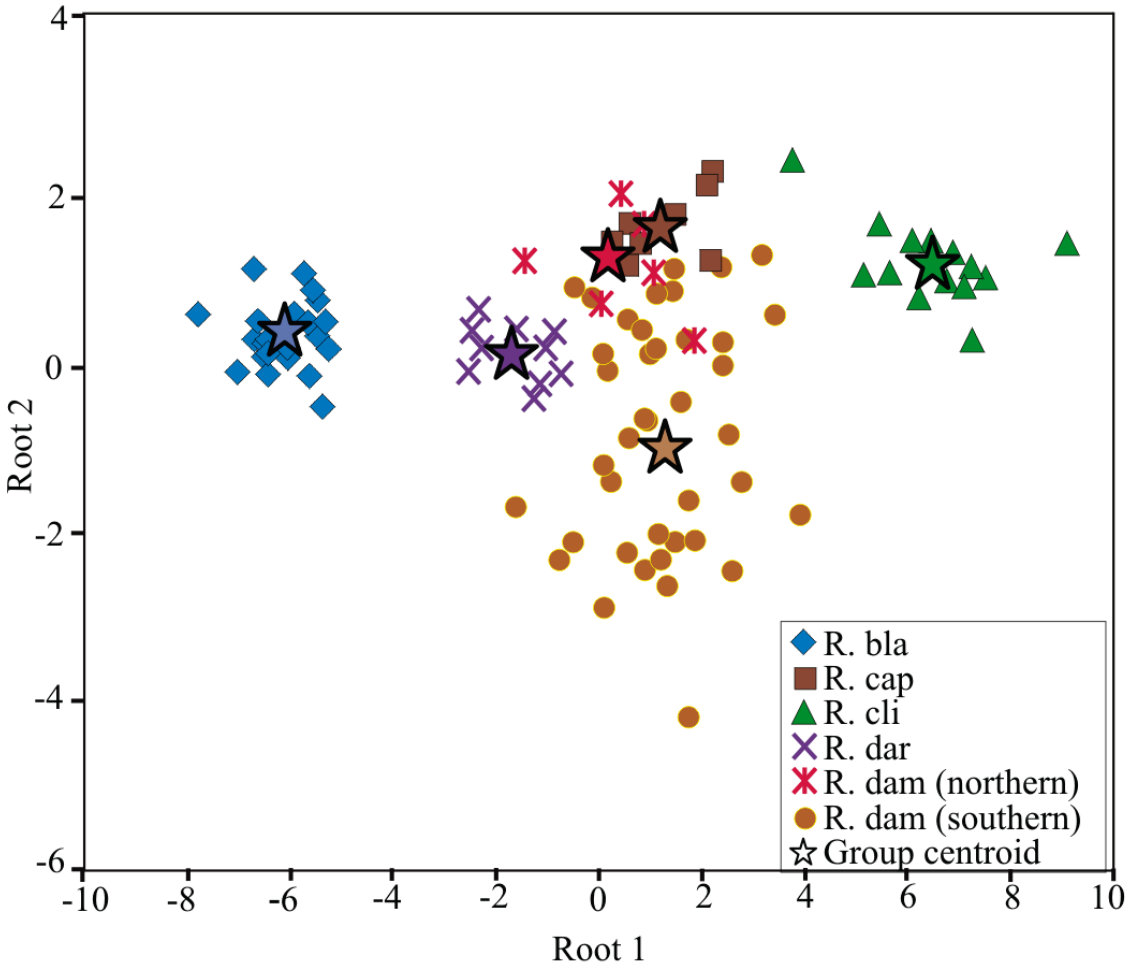

Figure 3.2: Plot of canonical scores extracted by Discriminant Function Analysis from skull parameters of 5 *Rhinolophus* species from southern Africa. Group centroids are shown as stars. R. bla (*R. blasii*), R. cap (*R. capensis*), R. dar (*R. darling*i), R. dam (*R. damerensis*).

Echolocation parameters

The first two roots (Root 1 and 2) extracted by DFA on the four PCs extracted by PCA on echolocation parameters had Eigenvalues of < 1 and explained 79.75% of the variance. The total classification success was 83.87% across the populations and it ranged from 50% to 100%. PC 1, which loaded highest on Root 1 of the DFA, was associated mainly with spectral parameters (inter pulse intervals, frequency modulation minimum frequency, sweep rate, duration of whole call and resting frequency) (Fig. 3.3; Table 3.3 and Table B3.10), and PC 2 which loaded highest on Root 2 of the DFA, was associated with temporal parameters (bandwidth, duty cycle and duration of the frequency modulated component) (Fig. 3.3; Table

B3.10). There was little separation between northern and southern lineages with Riemvasmaak and Soetfontein from the southern lineage overlapping with Arnhem and Wondergat from the northern lineage along both Root 1 and Root 2. Instead, two of southern lineage populations, Märcker and Orange River were separated from each other and the other populations along Root 1 and Root 2, respectively (Fig. 3.3; Table B3.10). The Wondergat population from the northern lineage, placed on the extreme right of Root 1, had slightly smaller mean echolocation parameters for resting frequency, duration of the whole call, frequency modulation minimum frequency and duty cycle than the Märcker population which was placed on the extreme left of Root 1 (Fig. 3.3; Table 3.3). On the other hand, the Arnhem and Wondergat populations from the northern lineage, which together with Märcker from the southern lineage, loaded lowest on Root 2, also had slightly smaller mean measurements for resting frequency, duration of whole call, inter pulse intervals, frequency modulation minimum frequency and duration of the frequency modulated component compared to the Orange River population which loaded highest on Root 2 (Fig. 3.3; Table 3.3). The Mahalanobis differences between the groups were significant. Moreover, the squared Mahalanobis distance was largest (D^2 = 58.25) between the centroids of Orange River and Märcker populations (F = 64.97, df = 4.53; $p < 0.05$), followed by the Mahalanobis distance (D^2 = 14.95) between Orange River and Wondergat populations (F = 30.26, df = 4.53; $p < 0.05$) as well as Orange River and Riemvasmaak populations (D^2 = 11.87; F = 13.25, df = 4.53; $p < 0.05$) (Table B3.11). Orange River and Soetfontein populations had the lowest squared Mahalanobis distance (D^2 = 7.01; F = 7.82, df = 4.53; $p < 0.05$) (Table B3.11). Analysis of squared Mahalanobis distance showed that there were significant differences between localities (Table B3.12).

Table 3.3: Mean±SD of echolocation parameters of *Rhinolophus damarensis*. Number of individuals per population is shown in parentheses. Samples size are given in parentheses below each locality and ranges are given below the means and standard deviations for each parameter.

	Wondergat (14)	Arnhem (8)	Märcker (6)	Soetfontein (6)	Riemvasmaak (6)	Orange River (22)
RF (kHz)	84.4±0.7 (82.8-85.3)	85.1±0.5 (84.4-86.1)	84.7±1.1 (82.8-85.5)	85.8±0.7 (84.7-86.8)	87.6±1.1 (84.7-88.9)	86.2±1.4 (83.4-88.3)
DWC(ms)	31.7±4.3 (27.8-40.5)	30.7±3.2 (27.4-34.7)	33.2±5.2 (29.0-43.4)	36.6±5.3 (30.6-43.1)	30.3±4.7 (25.6-37.8)	32.5±4.7 (26.1-42.3)
IPI (ms)	8.7±1.4 (6.2-11.4)	7.4±1.9 (5.5-10.4)	7.8±1.6 (5.5-10.2)	9.5±2.0 (7.2-9.6)	8.8±2.2 (5.7-11.6)	10.7±3.2 (6.8-18.6)
FMMF(kHz)	67.9±3.1 (62.8-72.9)	67.7±1.5 (64.9-68.8)	68.2±4.5 (65.1-77.4)	70.2±1.3 (68.5-71.7)	71.8±2.2 (69.2-75)	71.0±3.2 (66.4-76.8)
FMD (kHz)	1.1±0.1 (0.9-1.2)	1.1±0.1 (0.9-1.2)	1.1±0.2 (0.9-1.3)	1.1±0.2 (0.7-1.1)	1.2±0.1 (1.0-1.6)	1.6±0.4 (0.9-2.3)
BW(kHz)	16.5±2.8 (12.3-20.5)	17.3±1.5 (15.7-20.3)	16.3±3.9 (8.34-18.5)	15.6±1.9 (13.3-17.8)	15.7±1.4 (13.5-17.7)	15.1±3.3 (9.7-20.8)
DC(kHz/s)	79.1±2.6 (75.2-83.5)	80.8±2.5 (77.2-83.2)	80.3±2.6 (76.6-84.1)	74.9±3.1 (72.7-83.2)	77.4±5.1 (71.5-82.7)	75.4±5.3 (62.4-84.7)
PDR(m)	8.9±0.1 (8.8-9)	9.5±0.0 (9.4-9.6)	11.3±0.2 (11.1-11.5)	9.8±0.1 (9.7-9.9)	9.2±0.1 (9.1-9.3)	9.6±0.2 (9.4-9.8)
SR(kHz/s)	15.2±1.9 (11.6-18.1)	15.6±1.7 (12.8-17.8)	14.9±2.8 (10.4-17.8)	14.4±3.6 (11.7-21.0)	13.0±1.9 (11.2-15.3)	9.9±2.2 7.1-16.7

Resting frequency (RF), Duration of whole call (DWC), Inter pulse interval (IPI), Frequency modulation minimum frequency (FMMF), Duration of frequency modulated component (FMD), Bandwith (BW), Prey detection range (PDR), Sweep rate (SR).

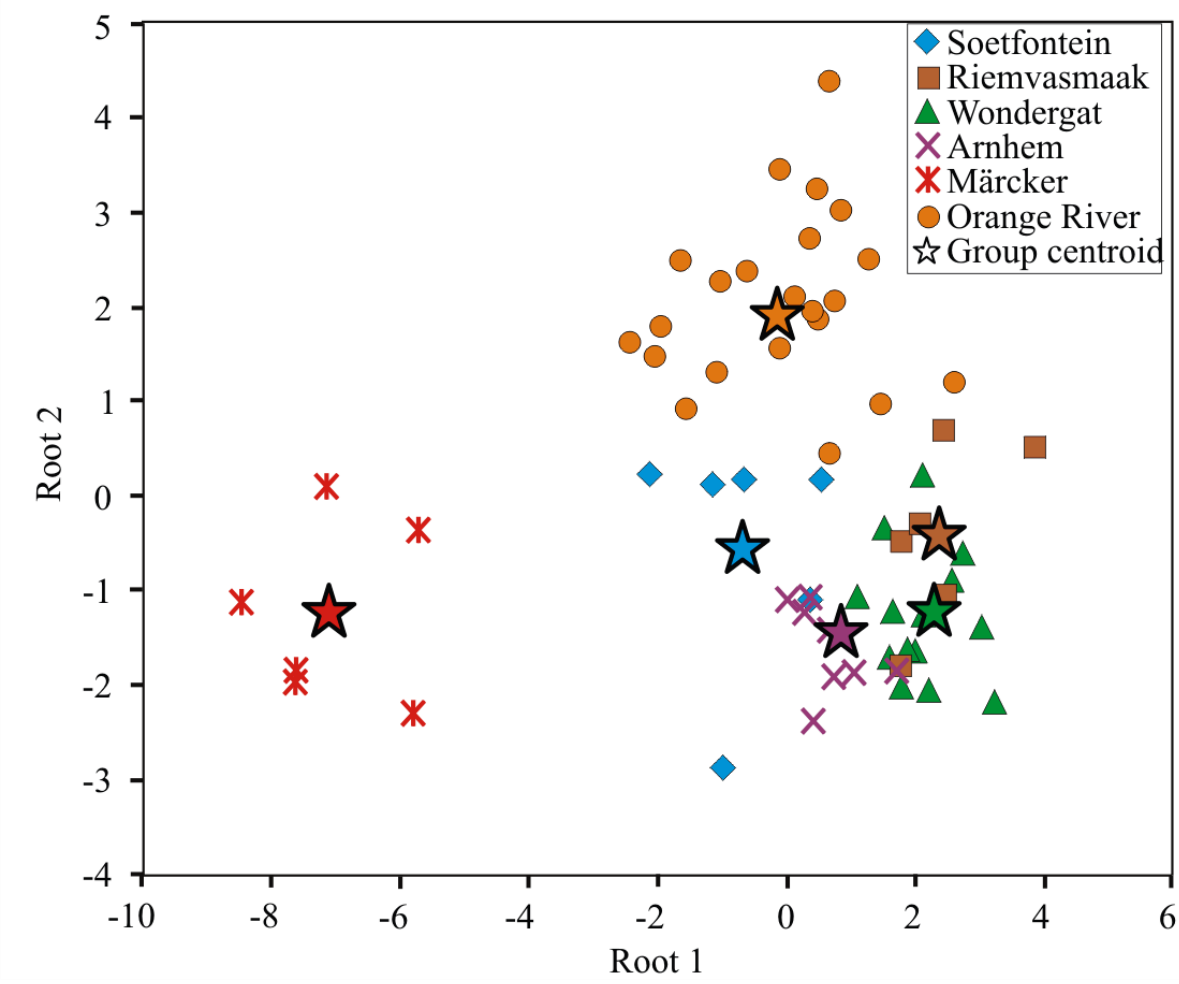

Figure 3.3: Plot of canonical scores extracted by Discriminant Function Analysis from echolocation parameters of *R. damarensis*. Group centroids are shown as stars.

Bacula parameters

The shape and size of the bacula largely supported the two regional groupings of *R. damarensis* in the western half of southern Africa (Fig. 3.4). The baculum of individuals from the northern part of the region had a rounded apical tip, whilst those from the southern part of the region had a flattened apical tip. The shafts of bacula from the northern lineages appeared to be dorsoventrally flattened while those from the southern lineage were more cylindrical. Furthermore, the Arnhem population from the northern lineage appeared to have a slightly deeper incision than the southern lineage populations, and the basal part was the widest part of the bone in both lineages. LM results showed that there were significant differences in greatest length of the incision (GLI) and height of the base (BH) between the two clades of *R. damarensis* (Table B3.13). GLI differed between the two clades (LM: $F_{(1, 4)} = 13.14$, $p < 0.05$; post-hoc Turkey HSD tests $p < 0.05$). Bacula specimens from the southern clade had

the lower mean GLI of 0.32±0.05 mm than that of northern clade which was 0.53±0.08 mm. BH ($F_{(1, 4)} = 10.15$, $p < 0.05$; post-hoc Turkey HSD tests $p < 0.05$). Again, bacula from the southern clade had a lower mean BH of 0.59±0.22 mm than that of northern clade which was 1.12±0.01 mm. All other bacula measurements of *R. damarensis* were not significantly different (all $p > 0.05$). However, the mean greatest bacula length (GBL) of the northern group (3.64±0.13mm) was at least 0.63mm greater than the mean (GBL) of the southern group (3.01±0.61mm).

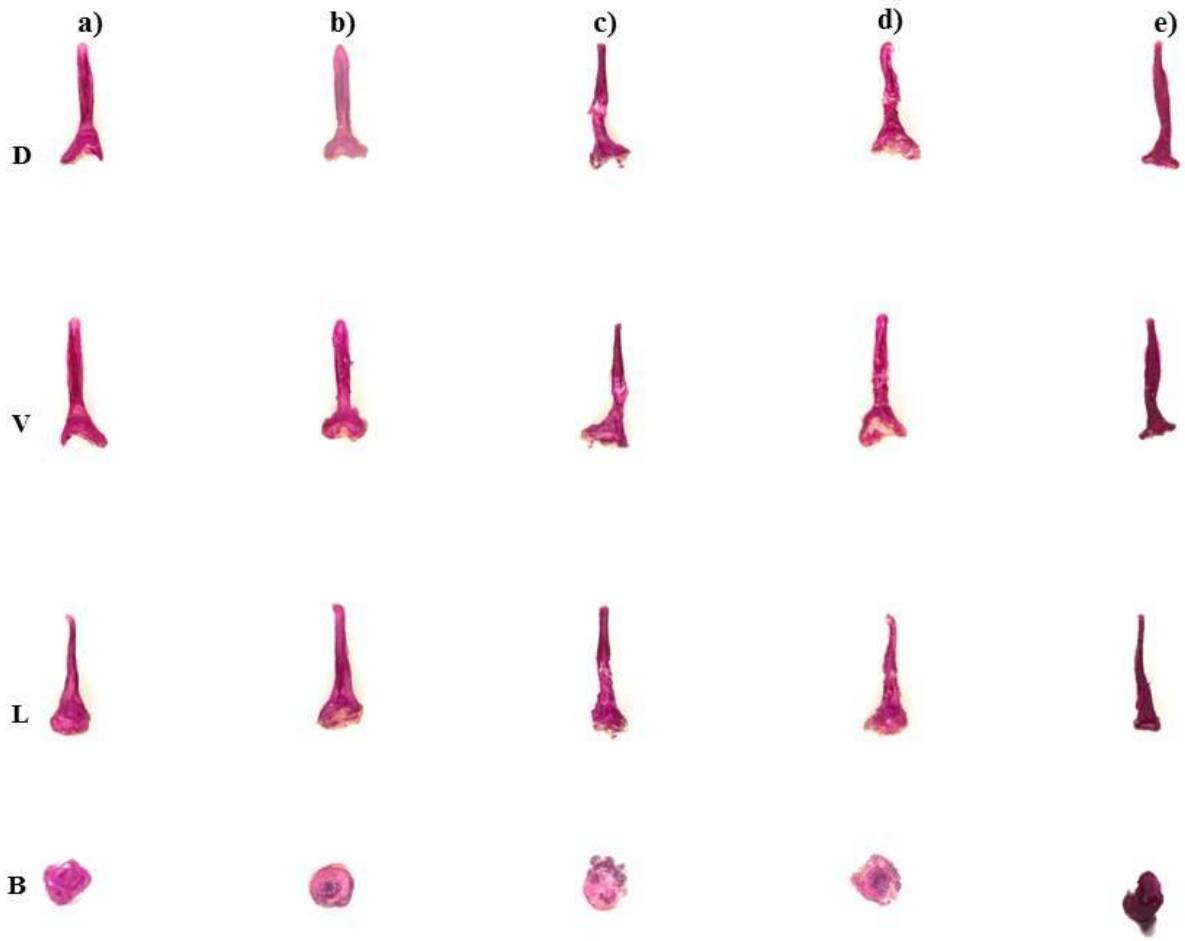

Figure 3.4: Bacula of five individuals taken from each population of *R. damarensis*. The populations are as follows: (a) Arnhem, (b) Wondergat, (c) Augrabies (d) Märcker (e) Orange River. Arnhem and Wondergat populations are from the northern lineage, whilst Augrabies, Märcker and Orange River are from the southern lineage. Dorsal (D), Ventral (V), Lateral (L) and Base (B).

Discussion

This current study showed that divergence in phenotypic traits (i.e echolocation, wings, bacula and skulls) within *R. damarensis*, partially explains lineage diversification within the species. The study revealed that there was separation of populations and lineages within *R. damarensis* as a result of populations occupying different environments. There were phenotypic differences in morphological (mainly skulls) and echolocation parameters between the two lineages of *R. damarensis*. In addition, bacula divergence between the southern and northern lineages of *R. damarenis* was also supported by their shape differences. Thus, divergence in phenotypic traits appears to be associated with the two regions identified in Chapter 2 and this provides support for IbE.

Populations within *R. damarensis* were divergent in wing traits but this divergence was not between regions. There was also separation between the skulls of the five rhinolophid species (*R. blasii, R. capensis, R. clivosus, R. darlingi* and *R. damarensis*) investigated in this study. The Mahalanobis distance between the skulls of *R. damarensis* from the two southern and northern lineages were significant, indicating that there is some separation. However, the magnitude of this difference is much less than that amongst other rhinolophid species and the overlap in some measurements indicates that the difference is not at the level of that between currently recognized species.

This is confirmed by the only slight differences in baculum size and shape, relative to those between recognised species (Jacobs *et al*. 2013). This is supported by the much lower genetic differences between the two lineages of *R. damarensis* (4.1 %, Jacobs *et al*., 2013) compared to those of recognized species that ranged from 3.3 - 27 % (Jacobs *et al*., 2006; Zhou *et al*., 2009; Taylor *et al*., 2012; Jacobs *et al*., 2013). However, the results of this study support the regional division in resting frequency of *R. damarensis* reported in Chapter 2. Furthermore, although phenotypic separation was not as clear as genetic (Jacobs *et al*., 2013) and resting frequency (Chapter 2, this study) separation, there is some phenotypic support for lineage diversification within the species mainly in echolocation, skull parameters and partly in bacula morphology.

The differences in skulls among rhinolophid species and between the two lineages of *R. damarensis* studied here appeared to be the results of selection. Skull morphology can be influenced by selection on diet and call frequency. In echolocating bats, the skull, and the nasal cavity in particular, has been co-opted to function in echolocation. The size of the nasal

cavity may influence the acoustic characteristics of the propagated sound (Armstrong and Coles, 2007; Odendaal and Jacobs, 2011; Jacobs *et al*., 2014). On the other hand, differences in diet may be reflected in the size of the mandible and its articulation with the skull providing increased mechanical advantage (Dumont *et al*., 2014). The northern lineage of *R. damarensis* had a longer mean maxillary toothrow than the southern lineage. The northern lineage appears to have longer snouts and therefore possibly larger nasal cavities allowing the emission of lower echolocation call frequencies. The northern lineage also had a larger mean mandible than the southern lineage of *R. damarensis*. This suggests dietary differences among populations and between lineages. Although there were differences in wing parameters among populations, differences between the two lineages and regions were less pronounced.

Environmental heterogeneity could lead to different populations occupying different habitats and result in the evolution of diverse ways of exploiting environmental resources within those habitats. This may in turn cause phenotypic divergence amongst populations of the same species, because of adaptation to local habitats. Furthermore, phenotypic variation within species is likely to be greater amongst populations that are geographically more distant, because gene flow amongst such populations may be reduced (Mayr and Diamond, 2001). Populations that are geographically isolated may become more genetically isolated overtime, and as such selection may yield to drift as the predominant mechanism shaping population structure (Clegg and Philimore, 2017)

Many African horseshoe bats have wide geographic distributions, e.g. *R. hildebrandtii* (Taylor *et al*., 2012), *R. darlingi* (Monadjem *et al*., 2010; Jacobs *et al*., 2013), *R. simulator* and *R. swinnyi* (Mutumi *et al*., 2016). Such wide geographic distribution may lead to geographic variation within species (Endler, 1977; Aspertsberger *et al*., 2003; Mutumi *et al*., 2016). This is so in *R. damarensis.* It also has a wide distribution across different biomes in southern Africa (Jacobs *et al.*, 2013; Chapter 2), and there is evidence of both genetic (Jacobs *et al*., 2013) and phenotypic divergence (Chapter 2; this chapter). Morphological characters are traditionally used, sometimes exclusively, to ascertain lineage diversification within species. In recent times, both molecular markers and ecology are being used in conjuction with morphology (Crawford, 2000) to investigate lineage diversification.

Divergence is evident in several phenotypic traits but the divergence is not as pronounced as that amongst recognized species, suggesting that the two lineages do not represent different

species. For example, the sequence divergence of 4.1% within the two lineages in *R. damarensis* (Jacobs *et al*., 2013) was lower than that reported for *Hipposideros larvatus* (Thabah *et al*., 2006), but the mean difference in RF (Chapter 2, this study) between the two lineages of *R. damarensis* was 3.2 kHz with overlapping ranges compared to the two forms (85KHz and 98kHz) in *H. larvatus*, that differed by about 13kHz (Thabah *et al*., 2006). Furthermore, the genetic divergence between the two clades of *R. damarensis* was smaller than those reported in several studies (Zhou *et al*., 2009; Jacobs *et al*., 2013; Soisook *et al*., 2015). Similarly, morphological divergence was more pronounced in the *R. hildebrandii* complex (Taylor *et al*., 2012) and *R. luctus* (Volleth *et al*., 2015) than in *R. damarensis*, which had some overlap in morphological data. Baculum morphology variation is usually much more pronounced between species than within a species (Patterson and Thaeler, 1982). However, the bacula size and shape may vary geographically within species (Thomas *et al*., 1994) and baculum variation may be variable enough within a species to allow sexual selection to occur (Herdina *et al*., 2014). Similarly, the bacula of *Rhinolophus hildebrandtii* were found to have a well developed bulbous basal portion that was deeply emarginated in dorsal view except for baculum DM 11560 (Taylor *et al*., 2012). The bacula of *R. hildebrandti* had variable degrees of emarginations from the ventral view, and the height of the emarginations (or incision in this study) ranged from 0.72 to 0.79 (Taylor *et al*., 2012). Furthermore, the base of the *R. darlingi* bacula was narrower than that of *R. damarensis*, and the dorsal incision on the base in *R. darlingi* bacula was shorter than that of *R. damarensis.* However, the basal size and shape differences in the bacula of *R. damarensis* from the five localities suggest some diversification among regions and populations within regions. However, this and the genetic, morphological and acoustic differences in *R. damarensis* are not as great as those between recognized species. This suggests that gene flow may be sufficient to limit other evolutionary processes responsible for the divergence. However, more information on the extent of gene flow is required to fully understand the processes responsible for the degree of divergence reported here within *R. damarensis*. The clear separation was evident in echolocation and morphological (mainly skulls and bacula) parameters between the two lineages of *R. damarensis*.

The separation of populations within *R. damarensis* could be the result of direct influence of selective pressure acting on morphology. Such morphological variation within species may result in differences in flight performance (Norberg, 1981; Aldridge, 1986; Adams, 1996), and this may directly affect habitat use (Aldridge and Rautenbach, 1987; Jacobs, 1999;

Stockwell, 2001). In addition, flight performance in bats requires substantial energy demands, and bats will have to optimise energy expenditure by adapting to ecological factors such as habitat type or food resource that they exploit (Norberg, 1981; Salsamendi *et al*., 2006). Wing design influences flight ability (Norberg and Rayner, 1987), and this could cause a significant relationship between wing morphology and feeding ecology of bats (Aldridge and Rautenbach, 1987; Jones *et al*, 1993). Wing loading in particular, is defined as the weight of a bat (mass multiplied by gravitational acceleration) divided by its total wing area (Norberg and Rayner, 1987). It is positively correlated with minimum flight speed and negatively correlated with manoeuvrability, the ability to turn tightly, and agility, the ability to turn quickly (Saunders and Barclay, 1992). The southern lineage population (Orange River) had the highest wing loadings, whilst the northern lineage population (Wondergat) had the smallest wing loadings (Table 3.1). Wing loading has an influence on the flight speed of bats (Norberg and Rayner, 1987). Bat species with higher wing loading turn less tightly and have lower manoeuvrability than bats with lower wing loading (Aldridge, 1986), and thus turning in cluttered environments requires lower wing loading and higher manoeuvrability (Noberg and Rayner, 1987). In addition, the northern populations of *R. damarensis* (Wondergat and Arnhem) had the smallest mean tip area ratio (the ratio of the hand wing area to the arm wing area), whilst Mäcker and Orange River populations from the southern lineage had the highest tip area ratio (Table 3.1). Similarly, wingtip shape index which indicates the relative shape of the bat wing tip (Noberg and Rayner, 1987) were smaller in the northern lineage populations. The northern populations (Wondergat and Arnhem) had lower values of wingtip shape indices, whilst the southern populations had higher values (Table 3.1). High values indicate a pointed wing and low values a rounded wing (Saunders and Barclay, 1992). The differences in wingtip shape index between the populations of *R. damarensis* suggest different flight styles and flight ability within the species as a result of populations occupying different ecological niches. Furthermore, aspect ratio (an index of wingshape) is correlated with flight cost efficiency (Norberg, 1981; Norberg and Rayner, 1987). Aspect ratio of the Wondergat population in the north was lower than that of the Orange River population in the south (Table 3.1). Thus, differences in wing morphology suggest that bats from the Orange River population, which occupies a desert biome with little vegetation, may fly faster in the relatively open areas of their desert habitat than the Wondergat population which has lower wing loading and, may be adapted to flying slowly in or close to the dense vegetation in their more mesic habitat. This is supported by the generally higher temporal components of the

echolocation calls and the longer prey detection ranges for the southern populations, which occupy more arid regions.

Bats from different geographic locations have shown differences in call frequencies (Heller and Helversen, 1989; Kalko and Schnitzler, 1993; Barclay *et al*, 1999) including horseshoe bats (Taylor *et al*., 2012; Odendaal *et al*., 2014; Mutumi *et al*., 2016). Although differences in resting frequency did not equate to large differences in detection range (Chapter 2) this is likely to change when regional call intensities of *R. damarensis* are used to calculate detection ranges rather than the use of the same call intensitiy for both regions (Chapter 2). Using the call intensities of *R. damarensis* rather than that from a similarly sized congeneric may have further enhanced regions differences in detection ranges (Chapter 2). Source level call intensities were not available for *R. damarensis*. The northern populations were found largely in the Savanna Biome in the northern part of Namibia, which is characterised by woodland vegetation (White, 1983; Simmons *et al*., 1998, Hoetzel *et al*., 2015). Echolocation parameters are related to foraging habitat (Neuweiler, 1984; Mutumi *et al*., 2016). Bat species that forage in hot, dry habitat, emit low frequency echolocation calls that will not quickly attenuate, with a long duration and a narrow frequency bandwidth. On the other hand, bats foraging in more mesic habitat will emit short broadband calls (Jones, 2005), adapted to hunting prey in habitats with greater attenuation (Luo *et al*., 2014; Mutumi *et al*., 2016). In addition, bats may adapt call frequency to the environmental conditions of their particular habitat, making very fine adjustments in the trade-off point between increasing frequency to enhance prey resolution and decreasing it to maximize prey detection range (Guillén *et al*., 2000). This may also assume that diets in different regions have the same size distribution of prey.

Echolocation call design and wing morphology are correlated across species in bat families including the Rhinolophidae (Norberg and Rayner, 1987; Jones, 1996; Jacobs *et al*., 2007). However, this correlation does not seem to exist within species because, this current study did not find a correlation between wing loading and resting frequency.

Lastly, mammalian male bacula exhibit a taxonomically widespread pattern of rapid and divergent evolution (Simmons and Firman, 2013). The differences in shape and size of the baculum indicate a great variety in bats, and basic designs of bacula are clade-specific and provide key diagnostic criteria often used in taxonomy, particularly in the speciose groups rich in convergent phenotypes (Herdina *et al*., 2015). Therefore, the differences in shape and

basal parts of *R. damarensis* bacula suggest that the northern and southern lineages are to some extent morphologically distinct. The basal part of baculum plays a role in strengthening the penis during copulation, and if the bacula of the two lineages of *R. damarensis* continue to diverge, it may lead to reproductive isolation between the lineages of *R. damarensis*. Generally, the baculum appears to result from integration of three structural units that differ in shape and functions: a proximal part interfacing with the corpora cavernosa, a distal part related to the urethral opening, and a medial part responding to prolongation of the structure (Kelly, 2000). Thus, the baculum has different functions across species including mechanical support, protection of the urethra from compression, enabling protracted copulations, stimulation of female reproductive tract, provision of information about male size or quality during intromission and reproductive isolation (Patterson and Thaeler, 1982; Dixson, 1987; Larivière and ferguson, 2002; Baryshnikov *et al*., 2003; Herdina *et al*., 2015).

In conclusion, this study has revealed phenotypic differences in morphological (mainly skulls) and echolocation parameters between the two lineages of *R. damarensis*. These differences support ecological selection (as suggested in Chapter 2) as evidenced by among population phenotypic divergence. Although there was separation of lineages between the two lineages, their phenotypic dimensions slightly overlapped, and the magnitude of the phenotypic difference between the two clades is much less than that amongst other rhinolophid species. Moreover, there are some telling differences between populations that appear to be related to their ecology. A more detailed study of the phenotypes, ecology and genetic population structure within and between lineages, including the quantification of gene flow between populations is required to understand the intraspecific diversification identified here.

CHAPTER 4

Cranial and mandibular shape variation of horseshoe bat, *Rhinolophus damarensis*, (Chiroptera: Rhinolophidae) from southern Africa: a case study of geographic variation in bats using geometric morphometrics.

Abstract

Shape variation in the skull morphology of 41 *R. damarensis* skulls and 38 mandibles was tested through the use of geometric morphometrics which allows the detection of more subtler changes in shape than can be detected by traditional morphometrics. Skull and mandible shape were analysed independently of other aspects of the body form using 24 skull landmarks and 15 mandible landmarks. This study investigated cranial and mandibular shape variation by carrying out comparative three dimensional (3D) geometric morphometric analyses based on X-ray microcomputed tomography (µCT) scanning of skulls and mandibles of *R. damarensis* from across its range. Evaluation of the a priori cranial and mandibular hypotheses of modularity in *R. damarensis* was significant only in skulls (i.e. basicranium and rostrum) and not in mandibles. Procrustes Anova results of both mandibles and skulls showed that there were no significant differences between sexes across different localities. However, there were significant differences in the shape of skulls and mandibles across different localities. Canonical Variate Analysis (CVA) showed that there was geographic variation in both the mandibles and skulls of *R. damarensis.* Population groups from the southern lineage, i.e Southern Namibia (SN) and Central South Africa (CSA) had thinner alveolar bones, whilst the Northern Namibia (NN) had a slightly thinner mandibular corpus relative to the average. In skulls, The NN group had an outline implying slightly deeper rostra, slightly larger anterior medial swellings, smaller braincase relative to the average, whilst the other groups had a slightly narrower rostrum and larger braincase than average. The pattern of geographic variation in *R. damarensis* mandibles and skulls revealed in this study could be associated with differences in the foraging and feeding behaviour of the species under different ecological conditions due to ecological opportunity.

Key words: geometric morphometric, *R. damarensis*, skulls, mandibles and geographic shape variation.

Introduction

Morphological differences within species may indicate speciation or at least diversification of animal lineages (Dumont *et al*., 2011; Taylor *et al*., 2012). Lineage diversification within species has enabled mammals to develop vast ecomorphological diversity, making them one of the most efficient vertebrate groups in terms of both colonising and adapting to new environments (Venditti, *et al*., 2011). This may have led to lineage diversification within species (Wellborn and Langerhans, 2015). Thus, variation within species is mainly associated with the effects of biotic (availability of food resources and competition) and abiotic (climate and geographic position) factors and reflects the adaptation of organisms to local environmental conditions (Burnett, 1983; Tobler, 2008; Huston and Wolverton, 2011; Budinski *et al*., 2015). Furthermore, it could also reflect stochastic evolutionary patterns because of population fragmentation (Straney and Patton, 1980; Malhotra and Thorpe, 2000). Studies on geographic variation within species revealed that the association between local environmental conditions and phenotypic variation within species may provide essential information into the evolutionary history and ecological dynamics within a particular species (Marchán-Rivadeneira *et al*., 2012; Mutumi *et al*., 2016, Chapter 2; Chapter 3). This is so, because local environmental conditions can induce marked phenotypic differences among populations of a species. Therefore, the evolution and diversity of mammals, including bats, may have been largely facilitated by the expansion of vegetation (Koopman, 1993; Maree and Grant,1997), response to climatic gradient conditions, as well as historical and ecological factors (Baryshnikov and Puzachenko, 2012; Hernández-Romero *et al*., 2015). Such ecological factors could include prey availability. In bats, dietary differences have a marked influence on skull morphology (Santana and Lofgren, 2013; Ospina-Garcés *et al*., 2016). Morphological variation associated with diet in animals is largely reflected in the craniofacial skeleton (Hospitaleche and Tambussi, 2006; Flores *et al*., 2010; Nguyen *et al*., 2016; Ospina-Garcés *et al*., 2016). The skull is a source of morphological characters resulting from its complicated structure from both the ontogenetic and functional roles (Hanken and Hall, 1993). It is also influenced by several factors because of its specific functions which involve protection of the brain, smell, vision, hearing, breathing, mastication (Marroig and Cheverud, 2001, 2004) and the production of acoustic signals for orientation (echolocation) and/or communication. Therefore, skull size and shape variation is a common component of the geographic variation in mammals (Puzachenko *et al*., 2017) such as bats (Bornholdt *et al*., 2008; Chapter 3).

Variation in the skulls of bats may reflect their colonization history, their response to a gradient of environmental conditions, their genetic diversity, or a combination of historical and ecological factors (Baryshnikov and Puzachenko, 2012; Hernández-Romero *et al*., 2015; Korablev *et al*., 2015) as well as urbanization (Tomassini *et al*., 2013). It has been observed that cranial shape in horseshoe bats follows two major divisions that may reflect adaptation to environmental and, in particular, dietary differences (Santana and Lofgren, 2013). For example, in some species of bats environmental factors related to precipitation and temperature could influence skull size variation (Burnett, 1983; Marchán-Rivadeneira *et al*., 2012). Larger skull sizes could be an adaptive response to higher levels of food productivity in moist environments (Marchán-Rivadeneira *et al*., 2012). Moreover, there is an interrelationship between cranial morphology and feeding behaviors of bats (Freeman, 1981; Dumont, 2004; Nogueira *et al*., 2005; Valdez and Bogan, 2009). Skull variation in bats may also result in echolocation call differencies. For example, shorter rostra are associated with increased echolocation frequency (Bogdanowicz *et al*., 1999). Nasal capsule dimensions in several species of rhinolophid bats and *Rhinonicteris aurantia* were negatively correlated with echolocation call frequency, but not with body size (Armstrong and Coles, 2007; Odendaal and Jacobs, 2011; Jacobs *et al*., 2014). Therefore, the changes in nasal capsule dimensions may be the result of natural selection acting on skull parameters associated with echolocation calls emission (Armstrong and Coles, 2007; Odendaal and Jacobs, 2011). Similarly, species of bats that consume predominantly hard bodied insects have thicker jaws and more developed cranial crests than those that feed predominantly on soft bodied insects (Freeman, 1979; 1981).

The skull is both a highly integrated and a highly modular structure (Lieberman *et al*., 2008), and the skull of mammals has been traditionally divided into three major regions namely: the face, neurocranium and basicranium (each part is comprised of further sub-modules). These major regions of the skull are known to be relatively independent due to their distinct embryonic origins, different processes of development, and disparate functional roles (Cheverud, 1982, 1996; Lieberman *et al*., 2000). For example, morphological integration and modularity may influence the structuring of morphological variation between and within natural species and generation of morphological diversity (Jarrín *et al*., 2010; Jarrín and Menendez-Guerrero, 2011). Morphological integration is described as the tendency in certain features or traits within a structure to be correlated in their variation, and as a result they may covary (Klingenberg, 2014). On the other hand, modularity is related to integration as it

describes subsets of traits that are highly connected (strongly integrated) to one another as compared to connections between other traits (Klingenberg, 2014). There is a general pattern of cranial modularity based on functional traits that is accepted for many mammal species. This pattern differentiates two different traits one at the facial region (i.e. splanchnocranium) and the other at the posterior region of the skull (Hallgrimsson *et al*., 2004; Koyabu *et al*., 2014). These two traits have different functions, neurocranium (posterior region of the skull) is associated with brain developmental processes and muscle insertion (Reep and Bhatnagar, 2000; Pitnick *et al*., 2006), whilst splachnocranium (facial region of the skull) is associated with the biomechanics of biting behavior (Goswami and Polly, 2010; Wellens *et al*., 2013). Additionally, studies on mammalian mandible have described patterns of mandibular modularity in mammals as a response to functional differences between the two regions in the mandible namely: the ascending ramus and the alveolar region (Klingenberg and Mebus, 2003; Jojić *et al*., 2007; Zelditch *et al*., 2008; Jojić *et al*., 2015). The two regions in the mandible have different functions, the ascending ramus is relevant for muscle insertion and articulation with the skull (Herring *et al*., 2001), whilst the alveolar region of the mandible supports the dentition and is associated with food loading and processing (Cox, 2008). For example, integration patterns of the mandibles in leaf-nosed bats have evolved during relatively recent ecomorphological transitions related to diet (Monteiro and Nogueira, 2010). However, some mammals appear to show relatively few, highly integrated and evolutionary stable cranial and mandibular modules while still achieving a high diversity in morphology and ecology (Goswami, 2006; Porto *et al*., 2009). In bats, and genus *Carollia* in particular, cranial and mandibular modularity revealed different independent patterns (Lopez-Aguirre *et al*., 2015). Mandibular modularity was the same for all species, whilst cranial modularity patterns were species-specific (Lopez-Aguirre *et al*., 2015). This suggests that cranial modularity shifts in relation to selective pressure (Beldade *et al*., 2002; Monteiro and Nogueira, 2010).

Geometric morphometrics is a powerful tool that is widely used to investigate the evolution of forms within and between species (Monteiro, 1999; Cordeiro-Estrela *et al*., 2006; Gannon and Rácz, 2006; Jacobs *et al*., 2014; Tokita *et al*., 2017) and it also explains the problems of functional morphology and ecological divergence (Adams and Rohlf, 2000; McKinnon *et al*., 2004). Moreover, geometric morphometrics has been been applied mostly in wide variety of mammals including rodents (Dos Reis *et al*., 2002; Monteiro *et al*., 2003; Fornel *et al*., 2010), primates (Singleton, 2002; Lieberman *et al*., 2007), and carnivores (Wroe and Milne, 2007;

Meloro *et al*., 2008). Recent studies on bats have used geometric morphometrics to describe cranial size and shape variation in bats (Gannon and Rácz, 2006; Evin *et al*., 2008; Jacobs *et al*., 2014; Ospina-Garcés *et al*., 2016). Geometric morphometrics has also been used to analyse cranial modularity in 22 species of horseshoe bats, as well as skull shape variation in relation to habitat across horseshoe bat species (Santana and Lofgren, 2013). The use of geometric morphometric analyses based on comparisons of biologically definable homologous structures, allows one to describe and interpret changes in the shape of different anatomical structures of organisms (Adams *et al*., 2004).

Horseshoe bats are widely studied as a result of their abundance, distribution and biological sensitivity to ecosystems. Geometric morphometrics allows us to consider aspects of the skull in the context of adaptation and in greater detail than traditional approaches of morphometrics. In this study, patterns of skull and mandibular shape variation were determined across the distributional range of an African horseshoe bat, *Rhinolophus damarensis*. This is an insectivorous bat that inhabits a largely arid region in the western half of southern Africa and has a wide distributional range across different biomes (Jacobs *et al.*, 2013; see Fig 2.1). The investigation of mandible and skull variation in *R. damarensis* using geometric morphometrics allows one to uncover minute differences in shape of the skulls and mandibles of bats that can be interpreted in the context of the known influences of dietary and sensory systems on skull and mandible variation (Santana *et al*., 2010; Jacobs *et al*., 2014).

The aim of this chapter was therefore to study geographic shape variation in the skull and to relate it to potential influences of diet and echolocation to help explain among populations and regional differences uncovered in previous chapters. It was therefore hypothesized that the dual function of echolocation and diet could result in shape variation among *R. damarensis* skulls. Under this hypothesis it was predicted that: 1) there should be skull shape differences among populations of *R. damarensis* occupying different environments; 2) geographic shape differences should be partitioned in accordance with the two RF groupings identified in Chapter 2.

Material and Methods

Sample

R. damarensis were captured throughout their distributional ranges (Monadjem *et al*., 2010; Jacobs *et al*., 2013; Fig. 2.1) in the western half of southern Africa using mist nets, harp traps

and hand held nets. At each site, one female and one male were taken as voucher specimens to support ongoing projects that involve genetic sequencing. A total sample of 41 skulls and 38 mandibles of *R. damarensis* were used in this study (Table B4.1). These specimens are currently in the mammal collections of the following institutions: Animal Evolution and Systematics (AES), University of Cape Town in South Africa; Ditsong Natural History Museum, Pretoria and Amathole Natural and Cultural Museum, King Williams Town. *R. damarensis* skulls extracted from voucher specimens collected from some of the sampled localities (Fig. 2.1) and skulls that were loaned from the two South African museums (Ditsong Natural History Musem and Amathole Natural and Cultural Museum) were used in this study. Precaution was taken to avoid bias because of differences in measurements between adults and juveniles by selecting only adult *R. damarensis* skulls. This selection was based on the degree of tooth wear, and the presence of epiphyseal plates in the finger bones which were detected by trans-illuminating the bat wings (Anthony, 1988; Chapter 3). The same approach as in (Chapter 3) was used to record the locality and gender for each museum specimen from the specimen label and museum database provided. The specimens were grouped into Northern Namibia (NN), Central Namibia (CN), Southern Namibia (SN), Orange River (OR) and Central South Africa (CSA) (Fig. 4.1) based on the northern and southern *R. damarensis* genetic and morphological lineages (Jacobs *et al*., 2013; Chapters 2 and 3) and biogeographically similar localities.

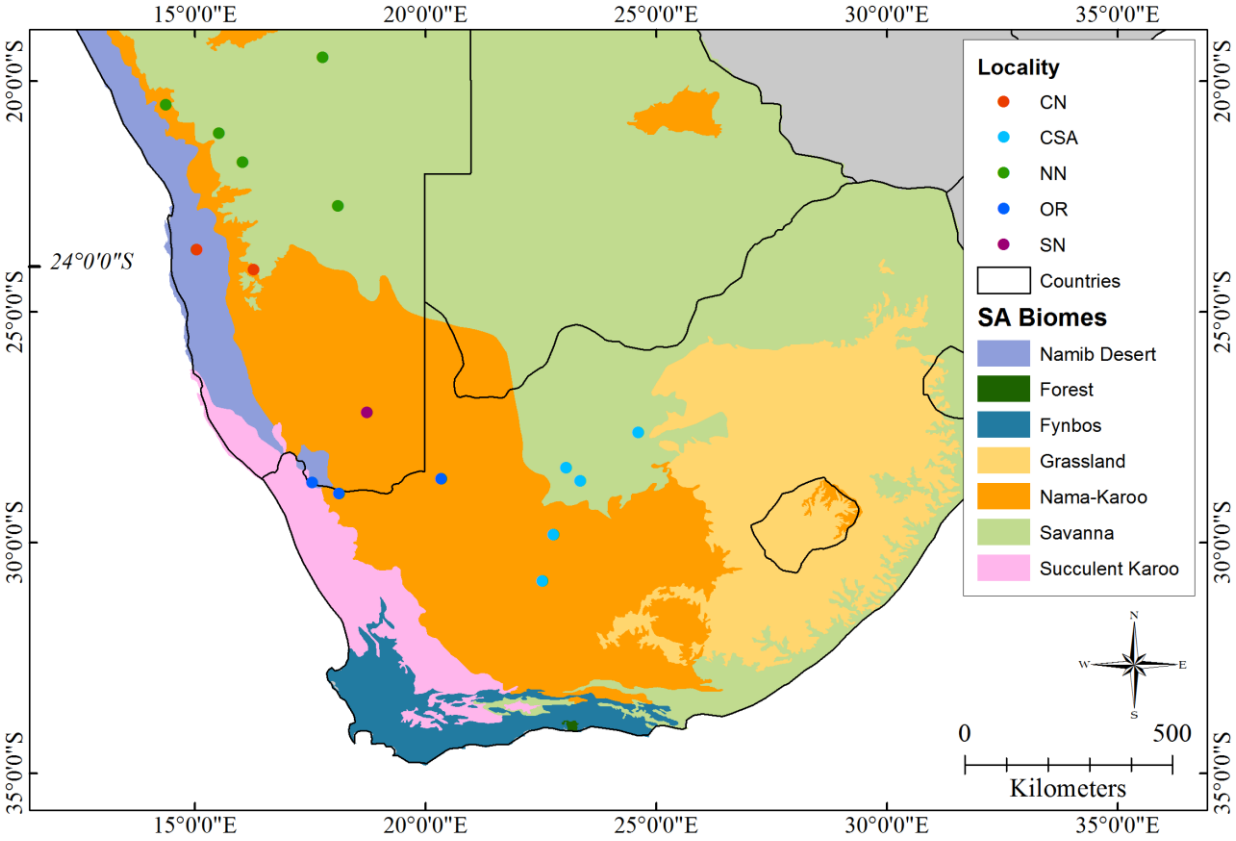

Figure 4.1: Biomes and localities at which *R. damarensis* populations were sampled in the western half of southern Africa. Cental Namibia (CN), Central South Africa (CSA), Northern Namibia (NN), Orange River (OR), Southern Namibia (SN).

Three dimensional (3D) digital images of each adult *R. damarensis* skull were obtained through micro-focus X-ray tomography using the Nikon XTH 225 L micro-focus X-ray tomography system housed at the South African Nuclear Energy Corporation (Necsa, Pretoria, South Africa). The system has a voltage setting ranging between 30 and 225 kV, and its beam current ranges from 0 to 1 mA. The system also has a maximum power rating of 225 W, and this ensures that a wide variety of samples can be investigated, even when the sample density is relatively high. The size of the spot is 0.0037–0.004 mm, and therefore, the geometric blurriness of the object edges associated with cone beam enlargement is minimized. Each skull of *R. damarensis* 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. Reconstruction of the image was done using CT-Pro-reconstruction software, a product of NIKON (www.nikon.com). All the reconstructed 3D images of the skulls and mandibles were loaded into the 3D imaging and analysis software, Avizo (version 9.0; Visualization Sciences Working Group, Merignac, France) as volume files to create iso-surfaces. All

created iso-surfaces from the volume files in Avizo were saved in standard ply format and loaded into Meshlab (64 bit version 1.3.3, Visual Computing Lab of CNR-ISTR, Italy) for placing homologous landmarks. A mandible and a skull were first used to test the accuracy and precision of placing landmarks by repeating the process five times. For the skull, 24 most precise homologous landmarks (Table B4.2; Fig. S4.1) were selected and 15 for the mandible samples (Table B4.3; Fig. S4.2). Homologous landmarks were placed only on the right half of the skull and right half of the mandible to control for the possible asymmetry (Jacobs *et al*., 2014). Three dimensional coordinates (x, y and z) of biological definable homologous landmarks were generated on the lateral, ventral and dorsal projections from each landmark in the 3D space. These homologous landmark co-ordinates were imported into MorphoJ (version 1.7.0_45; Klingenberg, 2011) and used to analyse shape variation in mandibles and skulls of *R. damarensis* across different localities and between the northern and southern lineage of *R. damarensis* (Jacobs *et al*., 2013; Chapter 2; Chapter 3).

Analyses of data

The analyses of homologous landmark co-ordinates were carried out by first conducting a Procrustes superimposition on the homologous landmark coordinates to remove variation amongst the landmarks in location, orientation and scale and to superimpose the landmarks in a common coordinate system (Adams *et al*., 2004). Outliers were thoroughly checked during analyses of data and in some cases landmarks were re-inserted on some volume images. A covariance matrix was generated from the Procrustes coordinates on which a Principal Components Analysis (PCA) was performed to explore variation in skull and mandible shape amongst the populations of *R. damarensis*. The differences in skull and mandible shapes across localities and between sexes were statistically tested using a Procrustes Anova. Canonical Variate Analysis (CVA) was used to visualise the skull and mandible shape differences amongst populations of *R. damarensis*. All shape variables were regressed onto the first two Canonical Variate axes and a scatter plot was displayed. Shape changes in the skulls and mandibles of *R. damarensis* were visually displayed using the warped outline graphs in MorphoJ and compared the average skull and mandible shape along each Canonical Variate (CV) with the warped outlines at the extremes of each CV.

A priori hypothesis of modularity was also tested according to a method outlined by Klingenbeg (2009). It has been shown that modularity is a widespread main attribute of biological systems that explains both the integration within and the autonomy among features

of organisms (Goswami, 2007). Integration maintains certain relationships that are necessary for proper functioning and performance of structures (Cheverud, 1996), whilst autonomy among parts allows for components to change independently (Santana and Lofgren, 2013). Investigating patterns of cranial modularity may reveal processes involved in morphological modularity as well as factors responsible for morphological diversification within and between species (Jojić *et al*., 2015). Therefore, mandible configuration was separated into two subsets of 8 (alveolar region) and 7 (ascending ramus) landmarks and the same approach was done on the cranium into two subsets of 10 (basicranium) and 8 (face or rostrum) landmarks according to a method described by Jojic *et al*. (2015). The RV coefficient in MorphoJ was employed to test the strength of association between hypothesised modules and all other alternative partitions. The RV coefficient, a multivariate analogue of the squared correlation is an overall measure of association between the two blocks (i.e the two modules using covariance matrices of the landmark co-ordinates placed on the hypothesised modules (Robert and Escoufier, 1976). The RV coefficient takes values from 0.0 (completely uncorrelated data) to 1.0 (correlated data). Spatial contiguity of the alternative partitions was displayed using adjacency graphs. The support of modularity is stronger if the RV coefficient between hypothesised modules appears in the lower range of RV distribution for alternative partitions. This means that if the distribution graph shows a significant number of partitions (such that Partitions – $p < 0.05$) appear before the point where the RV value appears (i.e. to the left of the RV value).

Results

Biological definable 3D co-ordinates of 38 mandibles and 41 skulls of *R. damarensis* were analysed using MorphoJ software. Analyses of sexual dimorphism using Procrustes Anova in MorphoJ revealed no statistical significant differences between males and females both in centroid size ($F_{(1,\ 30)} = 0.63,\ p > 0.05$) and shape ($F_{(38,\ 1178)} = 1.28,\ p > 0.05$) of *R. damarensis* mandibles. Similarly, there were also no significant differences between males and females both in centroid size ($F_{(1,\ 37)} = 1.43,\ p > 0.05$) and shape ($F_{(65,\ 2405)} = 0.96,\ p > 0.05$) of the skulls of *R. damarensis*. Therefore, sexes in both skulls and mandibles of *R. damarensis* were pooled for all subsequent analyses. Procrustes Anova showed that there were significant differences across different localities in the shape ($F_{(152,\ 1064)} = 1.23,\ p < 0.05$) but not in centroid size ($F_{(4,\ 28)} = 0.82,\ p > 0.05$) of *R. damarensis* mandibles. Similarly, skull shape ($F_{(260,\ 2210)} = 1.32,\ p < 0.05$) but not centroid size ($F_{(4,\ 34)} = 2.03,\ p > 0.05$) showed significant differences across different localities.

Mandible

The analyses of the mandibles of *R. damarensis* across different localities using canonical variate analysis (CVA) revealed separation of groups within the species. The four canonical variates (CVs) of the CVA had Eigenvalues of 1.1 and the first two CVs (CV 1 and CV 2) of the CVA of shape variation amongst the localities of *R. damarensis* mandible explained (86%) of the variation (Fig. 4.2). CV1 was found to be associated mainly with the thickness of the mandible and the alveolar process. The groups from the southern lineage, i.e Southern Namibia (SN) and Central South Africa (CSA) had an outline implying thinner alveolar bone relative to the average and were placed on the extreme right of CV1, whilst the Northern Namibia (NN) with slightly thinner mandibular corpora relative to average was placed on the left of CV1 (Fig. 4.2). The distance between the mental foramen and the most anterior point of the lower edge of madnidble corpus where the two halves of the mandible join was slightly longer in the SN and CSA groups. The two SN and CSA groups also had a higher coronoid process and lower condylar process (internal edge) as compared to the average. All other dimensions appeared to be consistent with the average shape (Fig. 4.2). The NN, CN and CSA and to a lesser extent SN (with thinner than average alveolar processes) were separated from the OR population which had a slightly thicker than average alveolar process along CV2 (Fig. 4.2). The main separation along CV3 was between SN and the (NN, CN, CSA, OR) populations (Fig. 4.3). SN had slightly thinner mandibular corpora than average and the other populations have slightly thicker mandibular corpora than average (Fig. 4.3).

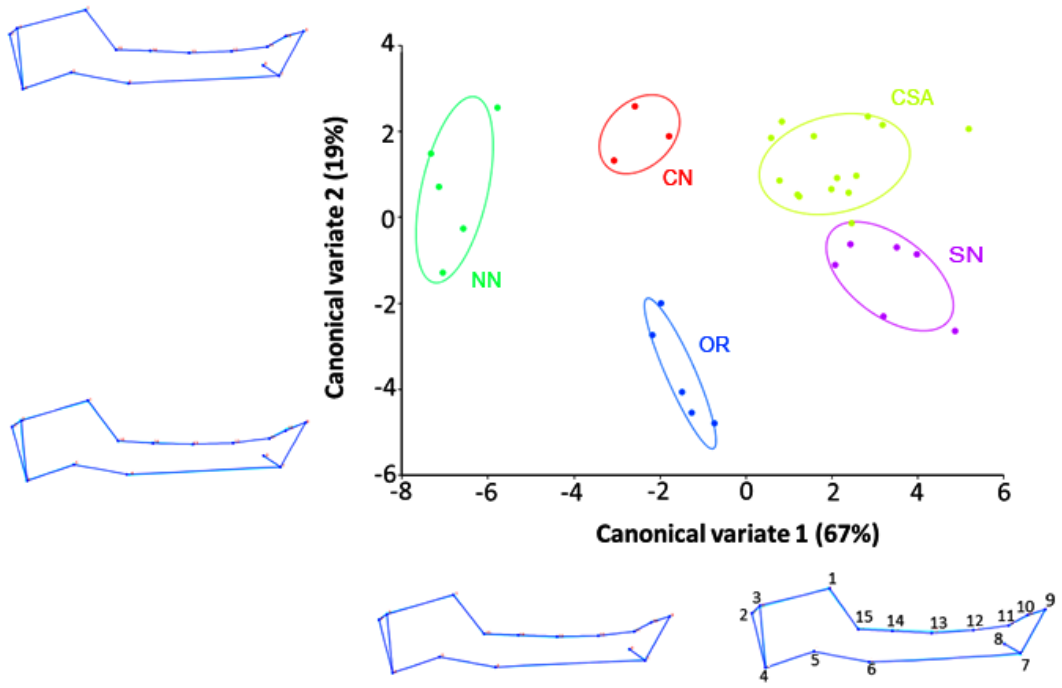

Figure 4.2: The first two canonical variates (CV1 and CV2) of mandible shape variation amongst the populations of *R. damarensis*. The light blue outline indicates the average shape and the dark blue outline indicates variation of shape from the average. Northern Namibia (NN), Central Namibia (CN), Central South Africa (CSA), Orange River (OR), Southern Namibia (SN).

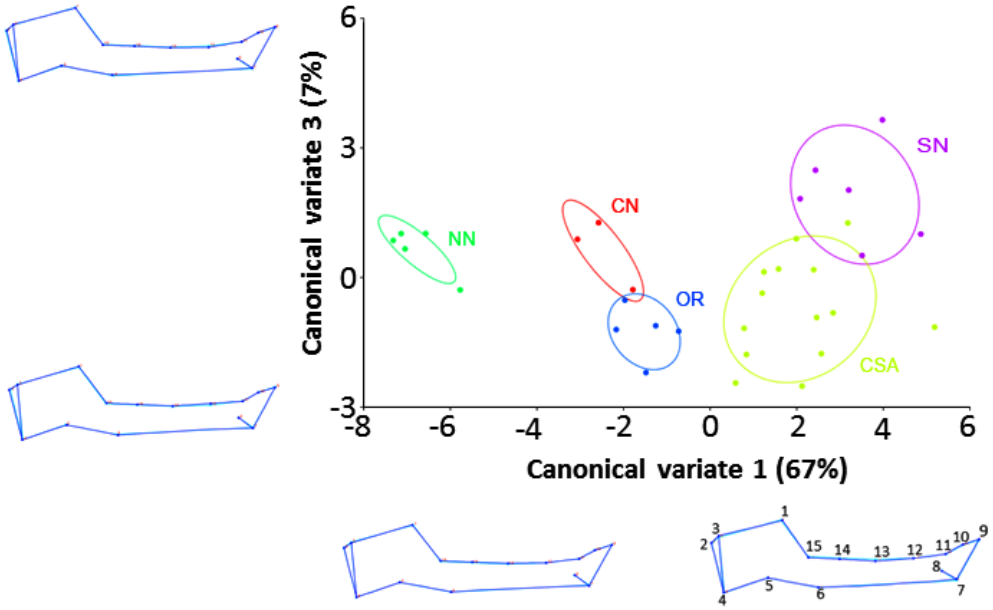

Figure 4.3: The first and third canonical variates (CV1 and CV3) of mandible shape variation amongst the populations of *R. damarensis*. The light blue outline indicates the average shape and the dark blue outline indicates variation of shape from the average. Northern Namibia (NN), Central Namibia (CN), Central South Africa (CSA), Orange River (OR), Southern Namibia (SN).

Skull

The analyses of the skulls of *R. damarensis* across different localities using CVA also revealed separation of groups within the species. The four canonical variates (CVs) of the CVA had Eigenvalues of 0.88 and the first two CVs (CV 1 and CV 2) of the CVA of shape variation amongst the different localities of *R. damarensis* mandible explained (70%) of the variation (Fig. 4.4). CV1 was associated with changes in the rostrum, braincase and basicranium of the skull. The CN group is separated from all other populations along CV 1 (Fig. 4.4). Bats from the CN group have larger than average anterior medial swellings and smaller auditory bulla than the average whereas in the other populations there is very little difference (Fig. 4.4). The only other differences along CV1 are (i) that the foramen ovale is slightly lower and more forward and (ii) the end of the toothrow is higher in the CN population. The NN population is separated from all other populations along CV2 (Fig. 4.4). Along CV2, the NN group, placed at the negative end of CV2 was separated from all other

groups which loaded low on CV2 (Fig. 4.4). The NN group had an outline implying slightly deeper rostra, slightly larger anterior medial swellings, smaller braincase and a higher foramen ovale relative to the average, whilst the other groups had a slightly narrower rostrum and larger braincase and a slightly lower foramen ovale than average (Fig. 4.4). CV3 (Fig. 4.5) only contributed 20% of the shape variation and it showed changes in foramen ovale, at the base of third molar and between the base of first molar and second premolar.

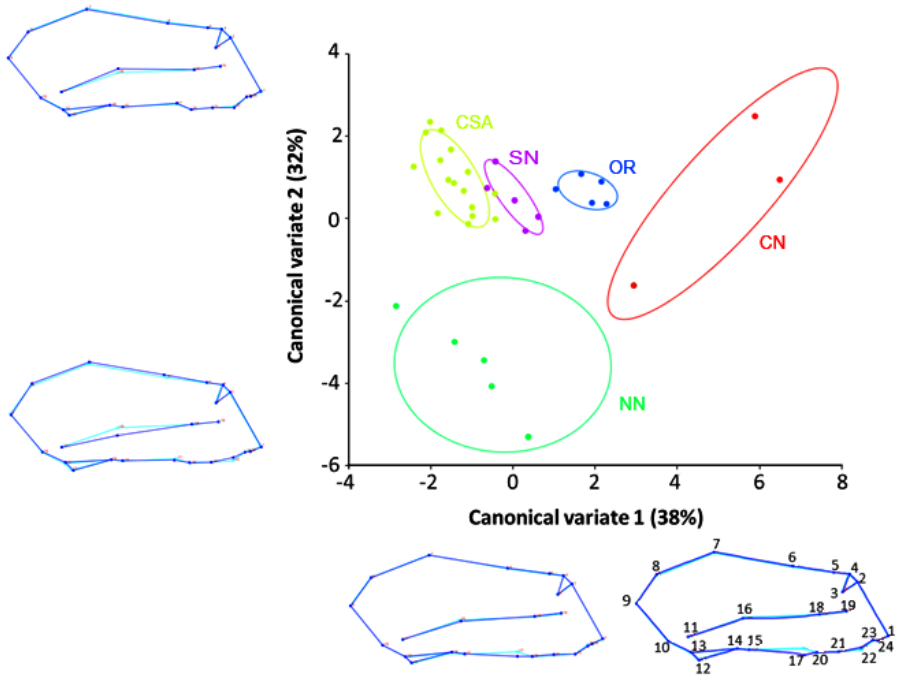

Figure 4.4: The first two canonical variates (CV1 and CV2) of skull shape variation amongst the populations of *R. damarensis*. The light blue outline indicates the average shape and the dark blue outline indicates variation of shape from the average. Northern Namibia (NN), Central Namibia (CN), Central South Africa (CSA), Orange River (OR), Southern Namibia (SN).

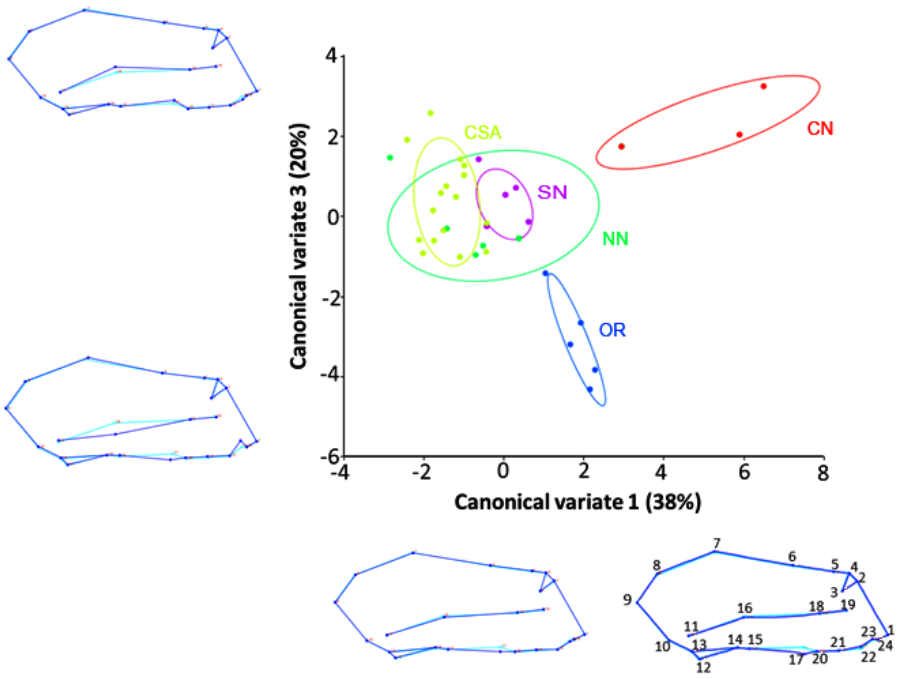

Figure 4.5: The first and third canonical variates (CV1 and CV3) of skull shape variation amongst the populations of *R. damarensis*. The light blue outline indicates the average shape and the dark blue outline indicates variation of shape from the average. Northern Namibia (NN), Central Namibia (CN), Central South Africa (CSA), Orange River (OR), Southern Namibia (SN).

Mandibular and skull modularity

The results showed strong cranial modularity in *R. damarensis*, and in contrast to the skull, the mandible did not show strong modularity. Thus, cranial modularity results indicated that the modules (basicranium and rostrum) in skull have evolved separately. For the skull (Fig. 4.6), the value of RV coefficient observed for the partition into hypothesised skull modules (basicranium and rostrum) was 0.048, and proportions (P) of partitions with RV lower than or equal *a priori* hypothesis was 0.001. For the mandible (Fig. 4.7), the value of RV coefficient observed for the partition into hypothesised mandible modules (alveolar region and ascending ramus) was 0.345, and proportions (P) of partitions with RV lower than or equal *a priori* hypothesis was 0.085.

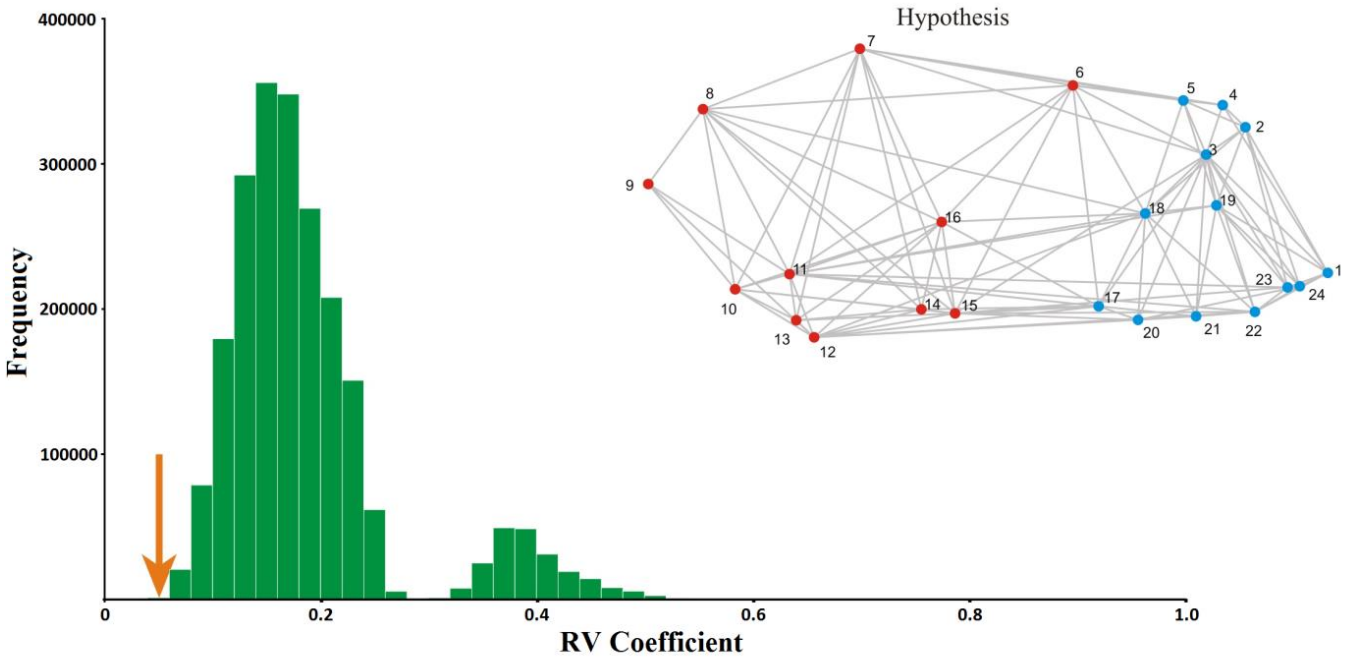

Figure 4.6: Evaluation of the *a priori* cranial hypothesis of modularity in *Rhinolophus damarensis*. The graph shows the separations of the cranium configuration into basicranium (red dots) and rostrum (blue dots) regions (including the adjacency graph). The values of RV coefficients observed for the partition into hypothesised module, cranium: RV = 0.048, and proportions (P) of partitions with RV lower than or equal a priori hypothesis (cranium: p = 0.001 as indicated by the arrow).

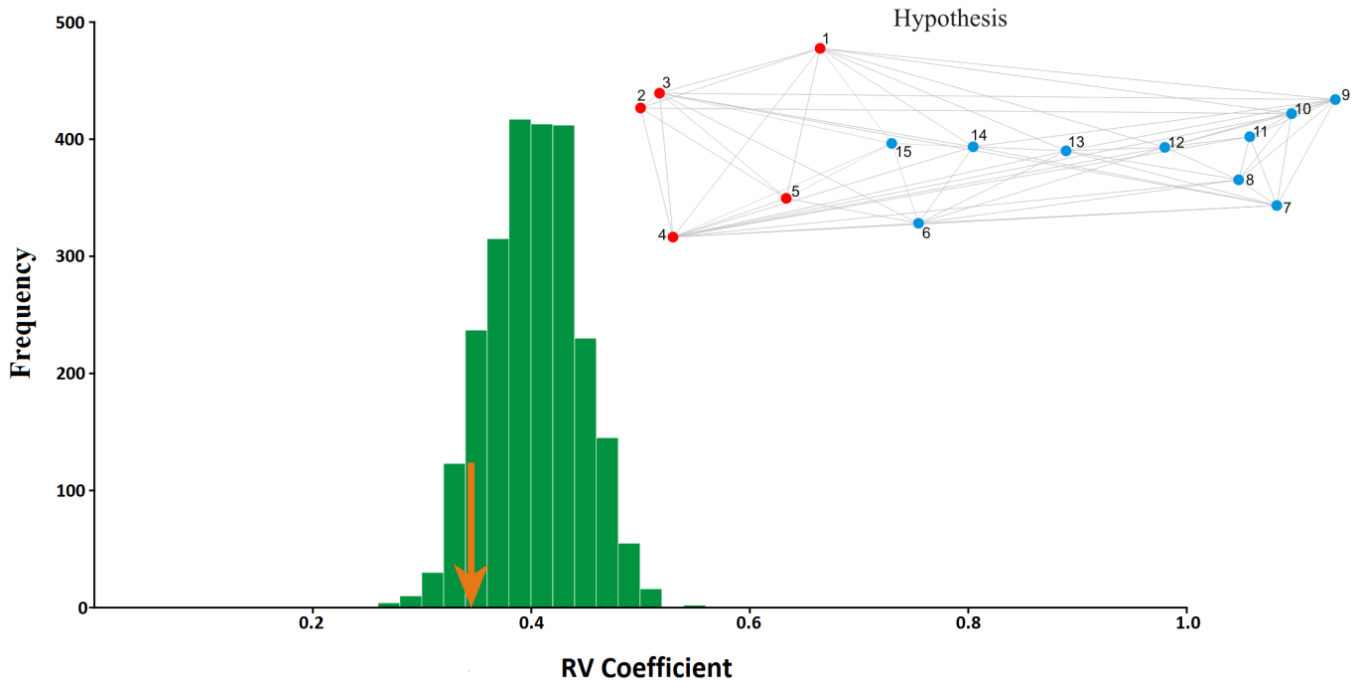

Figure 4.7: Evaluation of the *a priori* mandible hypothesis of modularity in *Rhinolophus damarensis*. The graph shows the separations of the mandible configuration into ascending ramus (red dots) and alveolar bone (blue dots) regions (including the adjacency graph). The values of RV coefficients observed for the partition into hypothesised module RV coefficients, mandible: RV = 0.345, and proportions (P) of partitions with RV lower than or equal *a priori* hypothesis (cranium: $p = 0.085$ as indicated by the arrow).

Discussion

Modularity was strongly supported in skulls but not in mandibles of *R. damarensis*. This indicates that the modules (i.e. basicranium and rostrum) in skulls evolve independently. Furthermore, there was evidence of geographic shape variation in both the mandibles and skulls of *R. damarensis*. This shape variation was meaningful, because some of the groups were well separated across different localities in both *R. damarensis* mandibles and skulls. The separation of the mandibles was based on the thickness and shape of the alveolar bone, the distance between mental foramen and the most anterior point of the lower edge of mandidble corpus where the two halves of the mandible join, shape of the mandibula corpora and the length of the coronoid process and condylar process (internal edge). In skulls, the separation was based on the changes in shape and volume or broadness of the rostrum, anterior medial swelling and the braincase of the skull. Changes in the shape and thickness of alveolar bone of the mandible suggested that the NN group from the northern lineage is separated from the other southern lineage groups (i.e. SN and CSA). The OR population also appeared to have a slightly thicker than average alveolar process than the NN, CN and CSA and to a lesser extent SN. Although the CN population from the southern lineage had larger anterior medial swellings and smaller auditory bulla than all other populations, the deeper rostra, slightly larger medial swellings, smaller braincase and a higher foramen ovale of the skulls from northern Namibia has again demonstrated that the NN group from the northern lineage is separated from the other southern lineage groups (i.e. SN, OR, CSA, CN). The NN group differs from the other groups largely in mandible morphology i.e. it seems that diet is the main driver of this difference. In contrast, the CN (along CV1) and NN (along CV2) groups differ from the other groups in size of the anterior medial swelling and rostrum. The NN and CN populations had a larger anterior medial swelling and a broader rostrum, which could be associated with a lower resting frequency.

This suggests that selection for echolocation frequency may have caused the variation in skulls. These findings provide phenotypic support for the existence of two genetic lineages in *R. damarensis* (Jacobs *et al*., 2013). However, morphology of the CN group as found in Chapter 3 suggests that the CN is closer to the northern lineage groups than the southern groups despite individuals from this region being genetically more similar to the southern than the northern group.

There are few studies that have evaluated and investigated cranial and mandibular modularity in bats. For example, recent studies investigated different modularity hypotheses regarding the cranium of horseshoe bats (Santana and Lofgren, 2013) and the palate in pteropodids and phyllostomids (Sorensen *et al*., 2014). In this study, the strong cranial modularity in skulls of *R. damarensis* indicates that the basicranium and rostrum may evolve independently. Similarly, other studies found that the trait integration in *R. ferrumequinum* was not uniform throughout the mandible and the cranium, but structured into modules (i.e. basicranium and rostrum) that were distinct (Jojić *et al*., 2015). Modularity facilitates the responses of organisms to the varied selection pressures exerted by their environment because, it allows evolutionary changes in one module, without the need for much change or trade-offs in another module as result of interaction with traits in other modules (Marroig *et al*., 2009). Moreover, modules allow a certain degree of independence and integration, which promotes quasi-independent or coordinated responses to selection (Marroig *et al*., 2009). Therefore, the differences in cranial and mandibular shape within *R. damarensis* could be related to the foraging and dietary behaviour as a result of ecological differences within this species. Bats show high levels of morphological and ecological diversity (Findley, 1993; Swartz *et al*., 2003). Morphological variation in bat skulls is largely associated with feeding specialisation (Freeman, 1984; Griffiths *et al*., 1992; Dumont, 1997; Freeman, 2000; Dumont, 2007) and echolocation (Jacobs *et al*., 2014). Other studies on bats and phyllostomids in particular found correlations between diet and cranial structure (Nogueira *et al*., 2009; Santana *et al*., 2012). This has been achieved by using deductive models (i.e biomechanics) to show association between cranial shape and dietary habits (Freeman, 1984; Dumont, 1997; Freeman, 2000). In feeding ecology, the most pronounced morphological variation in insectivorous bat species is correlated with the degree to which soft versus hard food items occur in the diet (Freeman, 1981). This has been supported by the association between robust skulls in bats and diets composed largely of harder bodied prey such as hard shelled insects (Husar, 1976; Findley and Black, 1983; Strait, 1993). Some of the characteristics of bats with robust skulls include large masseter muscle volume, stout dentaries, toothrows with fewer but larger teeth, longer canines relative to maxillary toothrow length, wide skull widths relative to skull lengths and the development of cranial crests (Freeman, 2000). However, these features are likely to be expressed in different ways depending on how (i.e. nasally or orally) the echolocation of a bat is emitted (Freeman, 2000). Nevertheless, insectivorous bat species preying on hard shelled insects (i.e. beetles) require higher bite forces, robust cranial structure and strong mandibles (Freeman, 1979; 1981). In contrast, phyllostomid bat species that feed

on nectar tend to have long narrow snouts, whist those that feed on hard fruits have short broad skulls (Nogueira *et al*., 2009; Santana *et al*., 2010; Dumont *et al*., 2011). Furthermore, species that feed on less resistant food items have smaller temporalis muscles with lower bite force than those that feed on increasingly harder food items (Santana *et al*., 2010).

In other studies of bats, it was observed that the cranial shape in horseshoe bats could reflect adaptations to dietary and environmental differences (Santana and Lofgren, 2013). Similarly, the differences in mandible shapes of *Pipistrellus pipistrellus* and *P. pygmaeus* suggested dietary differences between the two species (Sztencel-Jablonka *et al*., 2009). *Pipistrellus pipistrellus* has longer and more upright canines which allow the species to pierce harder prey. The shorter mandible and more developed coronoid process might allow this species to generate a stronger bite (Sztencel-Jablonka *et al*., 2009).

Variation in the shape of *R. damarensis* mandibles across different localities, reported in this chapter, could suggest dietary differences in different habitats. The NN group from the northern lineage differed from the other groups largely in mandible morphology, it therefore, seems that diet is the main driver of this difference. This study showed that the NN group had a slightly thinner mandibular corpus, and was separated from the CSA and SN groups. The OR population which had a slightly thicker alveolar process was also separated from the NN, CN and CSA groups. Thicker mandible or alveolar bone could be associated with stronger bite force. Investigation of morphological variation within and between bat species serve as an example of successfully applied ecomorphology. Size and shape variation are often used to infer ecological features (Swartz *et al*., 2003) and it has been shown that the form of the mandible in each bat species reflects its diet (Freemen and Weins, 1997; Freemen, 2000; Tschapka *et al*., 2008). This would obviously depend on the size and shape of the mandible in relation to the food item the species consumes. Therefore, insectivorous bat species that feed on larger prey tend to have larger heads, larger dentary apparatus and larger gapes than bats that feed on smaller prey (Freeman, 1979). Thus, the differences in shape of *R. damarensis* mandibles could be attributed to dietary differences within the species. This could imply that the slightly thinner mandibular corpora of the NN and thicker alveolar process of the OR groups could be associated with preying largely on either hard bodied or larger insects, whilst the CSA, CN and SN prey largely on either smaller or softer insects. Furthermore, the OR group did not only have thicker alveolar process but also appeared to have higher coronoid process, suggesting that it consumes larger and/or and harder prey. Bats that consume such prey tend to have higher coronoid process (Freeman, 1979; Jacobs, 1996). It is also worth

mentioning that measurements of shape reflect relative changes of one portion of the mandible or skull to another, therefore, relative shortening could reflect a change in the ratio of the length to width (Sztencel-Jabłonka *et al*., 2009). Therefore, shape differences in *R. damarensis* mandibles are highly suggestive of differences in bite force and worth highlighting because the bite force and food processing ability in relation to muscle size may be strongly correlated with diet (Sztencel-Jabłonka *et al*., 2009). For example, bite force differences may be the result of variation in the range of food items a species can consume (Santana and Dumont, 2009). Biting of food items could put pressure on both the mandible and cranial structure of a species, and therefore, the degree of hardness in the food items that a species can comfortably handle may be limited by the strength of its bite and the loads that its cranial structure can withstand (Santana *et al*., 2010). Other studies have used morphological characteristics that included among others size, width of face, rostral length, thickness of dentary and relative size of molars to differentiate the feeding ecology on 85 species of bats (Freeman, 2000). In this study, *R. damarensis* mandibles differed significantly in terms of the shape of mandibular corpora and alveolar process. Thus, the shape of the mandible appears be a good discriminating factor for the separation of groups which may relate to dietary differences. The result of the shape variation in mandibles suggests that there may be separation of the northern and the southern lineages of *R. damarensis.* The NN from the northern lineage and OR from the southern lineage (appeared to have slightly thinner mandibular corpora and thicker alveolar process respectively) and were different from other southern lineage groups. However, the NN and OR groups also differed in size of the coronoid process and condylar process. The NN group had lower coronoid process and higher condylar process, whilst the OR group had a higher coronoid process and condylar process.

The skull of a vertebrate is a complex anatomical system that is comprised of many highly integrated units (Cheverud, 1982; Klingenburg, 2008), and its functional morphology appears to be generally under strong selection. This is so, because it has several functions vital to the survival of the animals such as the acquisition of food (Santana *et al*., 2012). One of the factors that can influence and even mitigate against the response of skulls to selection associated with bite force and diet is echolocation. The evolution of echolocation frequency can impact on skull shape. For example, echolocation call frequencies have been found to be negatively correlated with nasal capsule size (Bogdanowicz *et al*., 1999; Armstrong and Coles, 2007; Odendaal and Jacobs, 2011; Jacobs *et al*., 2014; Wu *et al*., 2015). The mammalian skull is partitioned into two modules, the basicranium and rostrum (Porto *et al*.,

2009; Jojić *et al*., 2015). The rostrum performs several functions including processing of food, emission of acoustic signals, either through the nostrils or the mouth. If the rostrum and the basicranium evolve separately as modules, the rostrum as a separate module might indicate the presence of an evolutionary trade-off between structures associated with emission of acoustic signals and feeding (Jacobs *et al*., 2014). For example, an increase in size of the rostrum could affect the size of the nasal dome through which emission of echolocation frequencies occurs. This in turn might influence the acoustic properties of the emitted echolocation frequencies (Odendaal and Jacobs, 2011; Jacobs *et al*., 2014). The current study has found that *R. damarensis* skulls differed significantly across different localities. The groups contributed 70% of shape variation in *R. damarensis* skulls. The northern lineage NN group which had broader rostrum, anterior medial swelling, braincase, foramen magnum and foramen ovale was separated from the southern CSA, SN and OR groups which had a narrower rostrum, anterior medial swelling, braincase, foramen avale along CV2. CN also had a broader rostrum and anterior medial swelling. Differences in skull traits of the two northern and southern lineages appear to correspond with differences in resting frequency. This current study revealed that bats from the northern lineage (i.e. NN) emit lower echolocation frequencies than those from the southern lineage (Chapter 2) which is supported by the morphology e.g. relatively larger anterior medial swelling and broader rostrum, reported here. The CN population was also found to have a slightly lower echolocation frequency (Chapter 2).

Therefore, the differences in the broadness of the rostrum and anterior medial swelling from the southern and northern lineages of *R. damarensis* skulls could be related to the emission of acoustic signals. Recent studies have shown that there is allometric relationship between peak echolocation calls and morphological traits (i.e. nasal capsule size and volume) through which echolocation calls are emitted (Jacobs *et al*., 2014; Wu *et al*., 2015). Therefore, the broader rostrum and anterior medial swelling in *R. damarensis* skulls from the northern lineage could be correlated with the emission of lower peak echolocation frequencies, whilst the narrower rostrum and anterior medial swelling in *R. damarensis* skulls from the southern lineage could be correlated with emission of higher peak echolocation frequencies. The northern group of *R. damarensis* had lower resting frequencies, whilst the southern group had higher resting frequencies (Chapter 2; Table B2). Body size will not confound this association beween skull morphology and resting frequency, because in Chapter 2 it has already been shown that body size is not a factor in the variation in resting frequency.

Instead, it appears that climate selects for call frequency and this has resulted in variation in parts of the skull associated with the production and emission of echolocation calls.

In conclusion, the differences in the shape of *R. damarensis* mandibles between *R. damarensis* from the northern and southern regions may be related to dietary differences. Thus, the thicker alveolar process from the OR population and a slightly thinner mandibular corpora in NN group may be associated with consuming hard bodied prey, whilst the other groups which had thinner alveolar process (i.e CSA, SN and CN) are presumably thought to consume soft bodied prey. On the other hand, the CN and NN groups had broader anterior medial swellings than other the groups, and this suggests that selection for echolocation frequencies may have caused the variation in skulls of *R. damarensis*. Thus, *R. damarensis* appeared to have evolved some feeding strategies and associated morphological and behavioural adaptations. The regional resting frequency differences (Chapter 2), population phenotypic (Chapter 3) and skull and mandibular shape variation (Chapter 4) in *R. damarensis* suggest that *R. damarensis* has undergone divergence through adaptation to local environments.

CHAPTER 5

General Synthesis and conclusion

This research documented geographic phenotypic divergence in mammals using an African bat as a test case and to elucidate the processes responsible for such divergence. Quantifying geographic phenotypic divergence within a species gives insight into the processes that are responsible for the divergence and the influence that population isolation, through reduced gene flow, has on such divergence. The focal species was the horseshoe bat, *Rhinolophus damarensis*, which has a wide distribution covering several biomes in the more arid western half of southern Africa where it experiences conditions ranging from desert to dry savanna. Populations of species with such a wide geographic distribution are likely to differ phenotypically and genetically as result of populations adapting to local habitats.

Chapter 2 focused on the influence of several factors on geographic variation in acoustic signals (in this case echolocation) and attempted to elucidate the various factors responsible for such variation within the context of the existence of two genetic lineages occupying different geographic regions. Three hypotheses were therefore considered, Isolation by Environment (IbE), Isolation by Distance (IbD) and James' rule. These three hypotheses were tested using a multivariate approach in which the effects of climatic (i.e relative humidity and annual mean temperature; IbE), spatial (region and latitude IbD), biological (body size; James' Rule) on echolocation resting frequencies of *R. damarensis* were investigated using linear mixed effects models. Inter-locality differences in the resting frequency of *R. damarensis* did not follow either Isolation by Distance or James'Rule Hypothesis as per the results of linear mixed effects models. Instead, the differences in resting frequency were best explained by IbE mediated through differences in temperature across the habitats of the populations within *R. damarensis* (Chapter 2).

Populations of *R. damarensis* appeared to have become adapted to different ecological and climatic conditions that prevail in their habitats, and such adaptation may have allowed optimal prey detection ranges under different levels of ecological and climatic conditions (Chapter 2). Furthermore, differences in resting frequency corresponded to the genetic variation uncovered in *R. damarensis* by Jacobs *et al*., (2013) and both genetic variation (Jacobs *et al*., 2013) and variation in resting frequency (this study) appeared to be partitioned regionally. Similarly, other studies also found that climatic conditions are responsible for

resting frequency variation in other bat species (Guillén *et al*., 2000; Jiang *et al*., 2010; Mutumi *et al*., 2016). Geographical variation in resting frequency between the northern and southern lineages of *R. damarensis* might lead to assortative mating if the variation affects recognition between acoustically divergent lineages. This research also showed that female individuals of *R. damarensis* echolocate at higher frequencies than the males. This appears to be a general trend in rhinolophids, because similar findings have been reported in many other horseshoe bats (Neuweiler *et al*., 1987; Guillén *et al*., 2000; Yoshino *et al*, 2008). One of the possible explanations for sexual dimorphism in the resting frequency of *R. damarensis* could be that males and females keep their resting frequencies within a narrow range for sex recognition (Kazial and Masters, 2004; Jiang *et al*., 2010).

Considering the above results, the investigation into phenotypic divergence was extended into other aspects of the phenotypes of *R. damarensis* including bacula and skulls, using traditional linear measurements, as well as wings and echolocation parameters (Chapter 3). All these phenotypic traits were considered because they are known to be adaptive in bats (Heller and Helversen, 1989; Taylor *et al*., 2012; Simmons and Firman, 2013; Mutumi *et al*., 2016). There were significant differences between the southern and northern lineages of *R. damarensis* in the basal parts of the bacula (i.e height of the base and greatest length of incision) as well as in their shapes. The bacula of individuals from the northern part of the region had rounded apical tips, slightly deeper incisions and dorsoventrally flattened shafts than those from the southern part of the range of *R. damarensis*. Bacula variation within species was also reported in other African horseshoe bat species including *R. darlingi* (Jacobs *et al*., 2013) and *R. hildebrandti* (Taylor *et al*., 2012). Phenotypic divergence was evident at the regional, population and sex levels. There was significant variation in skulls, echolocation and, to a lesser extent, wing parameters between the northern and southern lineages. There were also differences among populations in skulls, echolocation and wings. Additionally, there were significant differences in skulls of other rhinolophid species (i.e *R. capensis, R. blasii*, *R. clivosus and R. darlingi*). These other rhinolophids are similar in size and echolocation calls to *R. damarensis*, and likely to be confused with it. This current study revealed that all these other rhinolophid species were significantly separated from the *R. damarensis*. Sexual dimorphism was evident in echolocation parameters but not in wings and skulls suggesting that *R. damarensis* may be using echolocation calls for sex recognition. Behavioural research on bats showed that bats can tell the sex of a caller from peak echolocation frequencies (Kazial and Masters, 2004). Furthermore, the existence of divergent

sexual selection among populations of the same species inhabiting different environments may also generate IbE (Nosil *et al*., 2005; Safran *et al*., 2013; Safran *et al*., 2016). This is so because, divergence in mate choice or sexual selection within a species may reduce reproductive success of dispersers moving between the populations (Nosil, *et al*., 2005; Safran *et al*., 2016).

Chapter four focused on investigating shape variation in skulls and mandibles of *R. damarensis* using comparative three dimensional (3D) geometric morphometric analyses based on X-ray microcomputed tomography (µCT) of scanned *R. damarensis* skulls and mandibles. Modularity was more pronounced and supported in skulls and not in mandibles of *R. damarensis*, suggesting that the modules (i.e. basicranium and rostrum) in *R. damarensis* skulls evolved separately (Chapter 4). Procrustes Anova did not detect sex differences in *R. damaresis* mandibles and skulls across different localities. Differences were only detected in the shapes of skulls and mandibles. Cananonical variate analyse revealed the separation of populations or groups and lineage divergence in both *R. damarensis* skulls and mandibles. *R. damarensis* mandibles appeared to be separated based on the thickness of the alveolar process and mandibular corpora, the groups from the southern lineage (i.e SN and CSA) had an outline implying thinner alveolar process relative to the average, whilst the northern lineage group (i.e NN) had a slightly thinner mandibular corpora (Chapter 4). On the other hand, the NN, CN and CSA and to a lesser extent SN were separated from the OR population which had a slightly thicker alveolar process (Chapter 4). *R. damarensis* skulls differed on the basis of anterior medial swelling and rostrum, the NN group from the northern lineage appeared to have slightly deeper rostra, slightly larger anterior medial swellings, smaller braincase and a higher foramen ovale, whilst the other southern lineage groups had a slightly narrower rostrum and larger braincase and a slightly lower foramen ovale than average (Chapter 4). Furthermore, the CN group which had larger than average anterior medial swellings and smaller auditory bulla than average appeared to be also separated from all other populations whereas in the other populations there was very little difference (Chapter 4). The findings of regional groupings in the resting frequency (Chapter 2), phenotypic variation or divergence (Chapter 3) and the differences in the shapes of skulls and mandibles (Chapter 4) of *R. damarensis* corresponded to the two genetic lineages within the species (Jacobs *et al*., 2013). Differences in skull and mandible shape (Chapter 4) were to some extent supported by the findings in Chapter 3. Although the the magnitude of differences in traditional measurement of skulls were less than that amongst other rhinolophid species and overlap in some

measurements, the Mahalanobis distance between the skulls of *R. damarensis* from the southern and northern lineages were significantly different, this indicates that there is some separation between the two lineages (Chapter 3). Similarly, geometric morphometrics supported such differentiation because the skulls of the NN and CN groups had slightly deeper rostra and slightly larger anterior medial swellings respectively (Chapter 4). These differences in the shape of *R. damarensis* mandibles and skulls between the northern and southern regions appear to be related to dietary differences and emission of resting frequency respectively.

Phenotypic divergence in *R. damarensis* could be the result of stochatistic (genetic drift) or deterministic factors (i.e selection) but the IbD analyses in Chapter 2 indicated that variation in RF was not correlated with distance between populations and that genetic drift was not therefore the cause. This is supported by the study on two other species of rhinolophids which indicated that selection is the major cause of geographic variation and that drift plays a relatively minor role if any at all (Mutumi *et al*., 2017). Instead, it seems that adataption to local habitats was a better explanation for the variation in RF. Furthermore, size differences were not responsible for the diffference in RF because there was no support for James Rule (Chapter 2).

Selective forces often act on phenotypic characters including RF and morphology of an animal species. Geographic variation in the phenotypes of the horseshoe bat, *Rhinolophus damarensis* appeared to be the result of adaptation to local habitats. Therefore, the differences in the mandible and skull shape (Chapter 4), in linear measurements of skulls and wings as well as in echolocation parameters (Chapters 2 and 3) among populations of *R. damarensis* appear to be the result of selection optimizing foraging and feeding behaviour of the species in the different habitats it occupies. This is supported by the correspondence between genetic and phenotypic variation. Populations of *R. damarensis* representing the northern genetic lineage had lower resting frequencies than those from the southern genetic lineage (Chapter 2; Chapter 3). Interestingly, most populations from the southern lineage had smaller anterior medial swelling and rostrum than those from the northern lineage (Chapter 4). This explains why the southern populations had higher call echolocation frequency. Nasal capsule size is negatively correlated with echolocation call frequencies among horseshoe bats (Jacobs *et al*., 2014; Wu *et al*., 2015). It appears that the ecolological and environmental conditions might have played a role in shaping both the morphology of the skulls and resting frequencies of *R. damarensis*.

The skull performs several functions including those related to diet and sensory systems, and if one function is optimised, another function might be less optimised because of evolutionary trade-offs between masticatory and sensory functions (Jacobs *et al*., 2014). For example, selection for higher bite forces could result in a shorter rostrum (Freeman, 2000; Santana *et al*., 2012; Jacobs *et al*., 2014) but this would also result in higher echolocation frequency as a result of the decrease in volume of the nasal capsules (Jacobs *et al*., 2014). Such evolutionary trade-offs may be ameliorated by the evolution of modularity. The modularity in the skulls of *R. damarensis* may allow selection for increased bite force through an increase in the size of the cranium and or the mandibles for the attachment of larger masseter muscles with a consequent increase in bite force, without affecting the rostrum which would then be free to respond to selection pressure for a particular echolocation frequency. The existence of modularity could therefore facilitate adaptive responses to conflicting selective pressures (Esteve-Altava *et al*., 2013; Tokita *et al*., 2017). Therefore, geographic variation in the skull of *R. damarensis* (Chapters 3 and 4) might reflect adaptation for the optimization of feeding, orientation and prey detection across the different habitats occupied by this species. If so, call frequency of *R. damarensis* might also have changed as a result of feeding on different insects in different environments. This could have resulted in a complex relationship between the relative influence of diet, how prey is captured (i.e. wing shape differences between the lineages) and relative humidity in different regions. Wing and skull shape differences (Chapters 3 and 4) between the two lineages of *R. damarensis* suggest different flight styles and feeding ecology in the different regions. However, at the high echolocation frequencies at which *R. damarensis* operates, these small differences in frequency represent minute differences in wavelength and may not therefore have any ecological implications. Nevertheless, a combination of small frequency differences with large intensity differences may have an impact on the distances at which preys are detected in different habitats and this may have habitat-use and dietary consequences. This current study showed that the interactions between organisms and their environments could have shaped distributions of spatial genetic and phenotypic variation, resulting in the patterns of IBE. Thus, phenotypic variation within *R. damarensis* could have been facilitated by both paleoclimatic change and subsequent vegetation shifts including contraction of forests, and the expansion of grasslands, woodlands and savanna during the end of Miocene (Zachos *et al*., 2001; deMenacol, 2004; 2011) as well as the uplift of southern Africa's great escarpment and interior plateau during the Plio-Pleistocene (Baker and Wohlenberg, 1971). Moreover, climate change may have resulted in the expansion of arid and semi arid conditions and the contraction of mesic

environment in the western half of southern Africa. This in turn, could have structured the biogeography of populations within *R. damarensis*. Other subpopulations or lineages within species including birds (Ribeiro *et al*., 2014), mammals (Maswanganye *et al*., 2017) and reptiles (Barlow *et al*., 2013; da Silva and Tolley, 2017) have recently been uncovered in the southern African region.

To totally understand phenotypic variation within *R. damarensis*, future studies should investigate bite force in conjuction with the diets among populations of this species across its range. Geomorphometric analyses of wing shape to determine minute differences could provide more information on differences in flight performance in the different habitats. Furthermore, more criteria are needed to further identify phenotypic divergence through isolation by environment. Future studies should consider determining the relative influences of sexual and natural selection on genetic structure. It is therefore, imperative to disentangle other processes influencing population patterns, and this requires an integrative analytical approach. Additional research would need to be done using several genetic markers, both mitochondrial and nuclear, to further quantify genetic diversity and to determine the levels of gene flow amongst populations. In addition, other genetic methods which could be implemented in BAYES ASS+ may also be incorporated to estimate gene flow between populations. Such estimates may help to determine if gene flow is consistent with population structure. Other ecological processes that need to be considered for future studies under IbE Hypothesis may include (i) dispersal of individuals, because changes in climatic conditions may regulate dispersal of individuals among populations (ii) selection against immigrants and (iii) reduced hybrid fitness.

In conclusion, genetic and phenotypic divergence within and among species may be influenced among others by several factors including genetic drift, geographic isolation, sexual and natural selection. This study showed that phenotypic divergence within *R. damarensis* is allopatric, and that neither James' Rule nor random genetic drift played a role in phenotypic divergence within this species. However, this current study showed that selection is responsible for phenotypic divergence between the populations of *R. damarensis* inhabiting different ecological environments. Although there were differences between the populations within and between lineages of *R. damarensis*, the two lineages of *R. damarensis* appear to represent within species diversification.

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APPENDICES

CHAPTER 1

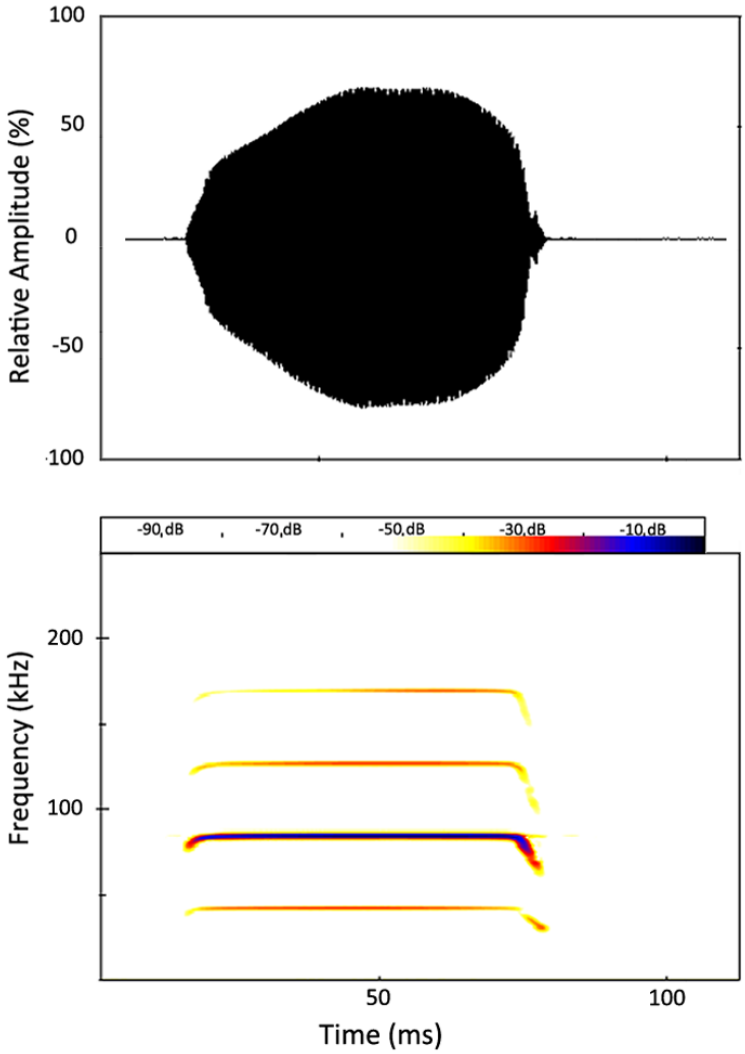

Figure S1: Echolocation calls of *R. damarensis* have a prominent constant frequency (CF) component preceded and followed by a frequency modulated (FM) component. The species has several harmonics in its echolocation calls but invariable concentrate call energy in the second harmonic.

APPENDICES

CHAPTER 2

Table B2: Mean±SD of forearm and resting echolocation frequency for each population of *Rhinolophus damarensis*. Localities are shown in order of increasing latitude from north to south.

	locality						
Variables	Wondergat	Arnhem	Märcker	Soetfontein	Riemvasmaak	Orange River	Uintjiesberg
FA (mm) Mean ±SD	(15) 50.6±1.5	(17) 51.8±1.9	(6) 50.5±1.9	(7) 48.9±2.8	(10) 49.8±1.2	(29) 49.1±1.6	(22) 52.3±1.1
Range	47.1-51.82	46.7-54.8	48.2-52.8	47.3-51.4	47.6-51.1	45.5-51.6	50.4-54.6
RF (kHz) Mean ±SD	(15) 84.4±0.7	85.0±0.9	84.7±1.1	85.7±0.8	87.6±1.1	85.9±1.3	(22) 84.8±0.7
Range	82.8-85.3	83.6-86.8	82.8-85.74	84.7-86.8	85.8-88.9	83.5-88.3	83.3-86.2
F- RF (kHz) Mean ±SD	(7) 84.5±0.4	(13) 85.1±0.9	(3) 85.2±0.9	(4) 86.1±0.7	(7) 87.8±0.8	(11) 87.1±0.8	(22) 84.8±0.7
Range	84.1-85.3	83.6-86.8	84.4-85.7	85.1-86.8	86.1-88.9	85.8-88.3	83.3-86.2
M- RF (kHz) Mean ±SD	(8) 84.4±0.9	(4) 84.8±0.3	(3) 84.1±1.3	(3) 85.2±0.5	(3) 86.9±0.9	(18) 85.2±0.9	_____
Range	82.8-85.2	84.5-85.2	82.8-85.5	84.7-85.7	85.8-88.1	83.5-87.1	_____
Lat	-20.51	-22.70	-24.08	-28.38	-28.47	-28.70	-30.83
Reg	North	North	South	South	South	South	South
RH	38.25	39.98	32.52	40.39	35.9	39.15	41.54
AMT	21.23	18.8	15.75	17.64	20.45	18.29	15.07
AA (dB/m)	2.51±0.0	2.33±0.0	1.83±0.0	2.24±0.0	2.26± 0.0	2.28±0.0	2.02±0.0
PDR (m)	8.97±0.0	9.5±0.1	11.3±0.1	9.77±0.1	9.70±0.1	9.64±0.1	10.53±0.1

The variables are defined as follows: (FA) Forearm, (RF) Resting frequency, (F-RF) Female resting frequency, (M-RF) Male resting frequency, (Lat) Latitude, (RH) Relative Humidity, (AMT) Annual Mean Temperature, (AA) Atmospheric Attenuation, (PDR) Prey Detection Range and (Reg) Region is divided into two (e.g northern and southern regions). Number of individuals (n) per population is shown in parentheses.

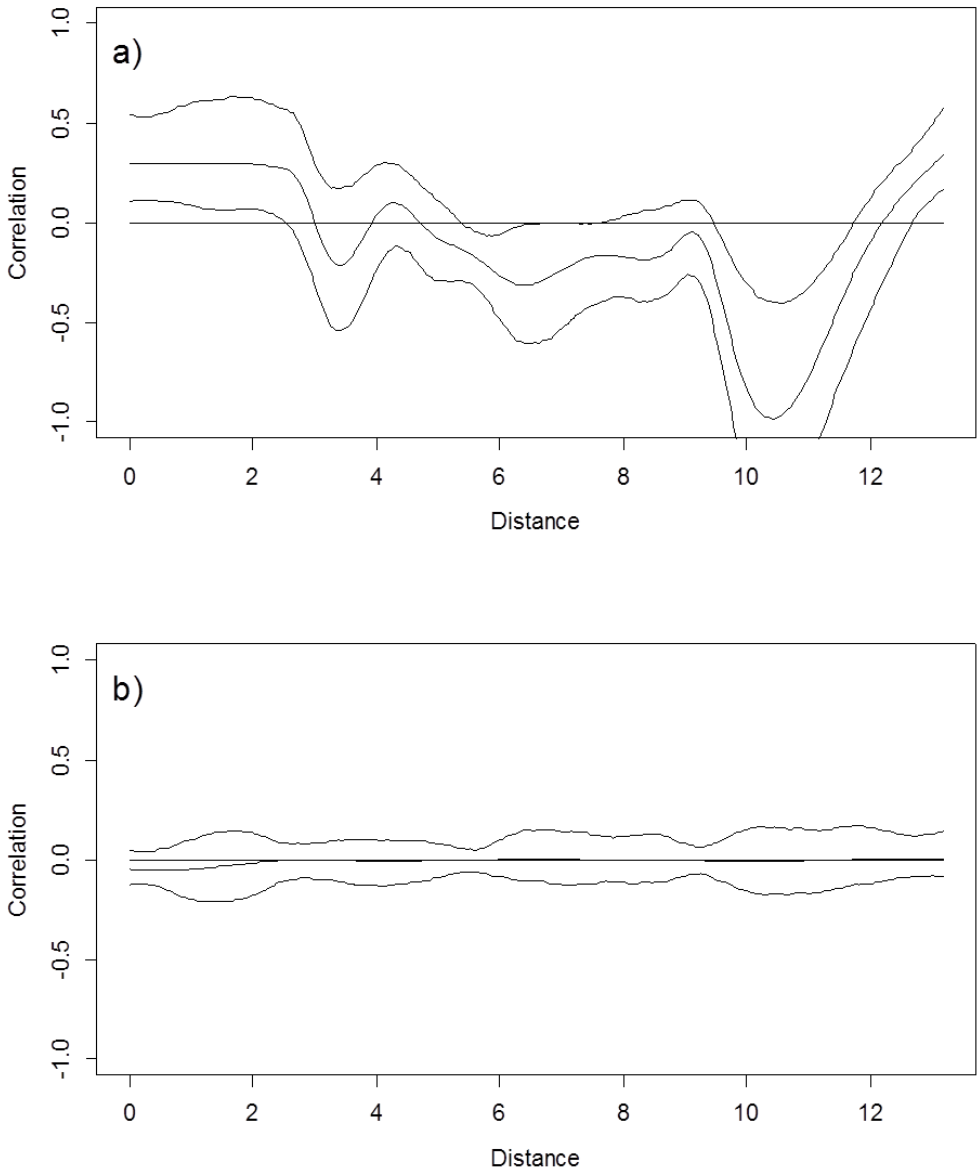

Figure S2.1: Spatial auto correlation due to strong spatial dependencies in observed data (a) and (b) a graph with no spatial auto correlation after spatial dependencies have been accounted for by simple random effects model.

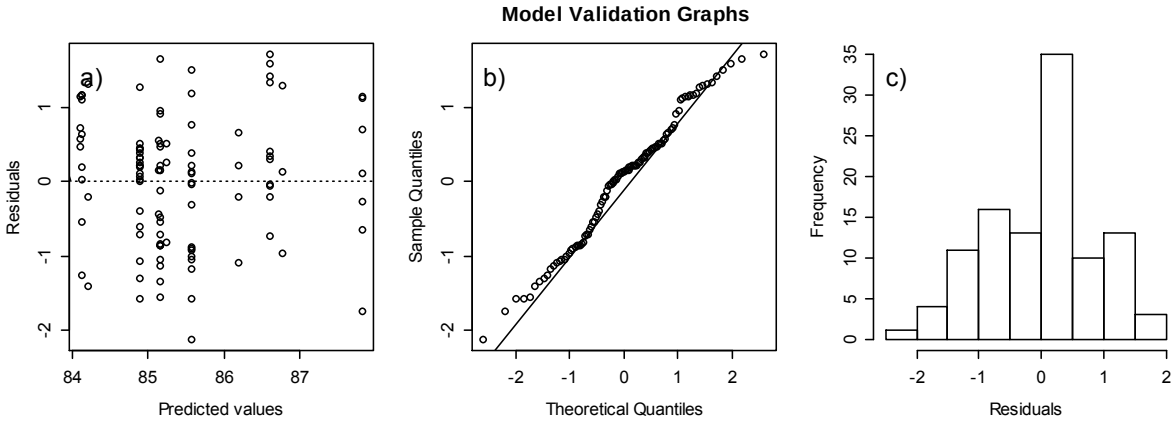

Figure S2.2: Model validation graphs showing residuals closely approximating a normal distribution with no violation of the assumption of homogenous variance. The graphs are a) predicted values against residuals that are clearly spread out, b) a linear relationship between sample quantiles and theoretical quantiles and c) histogram showing normal distribution between frequency and residuals.

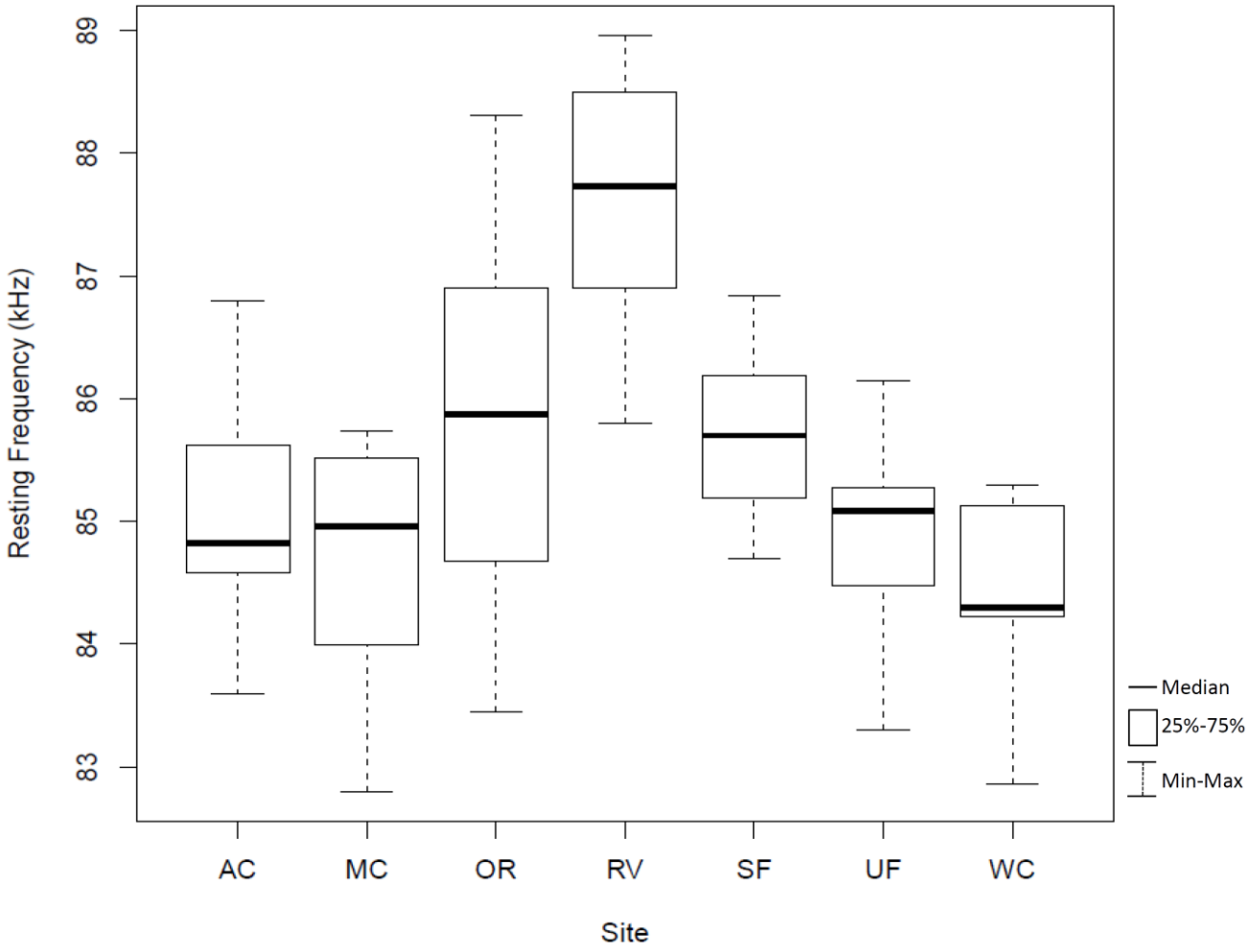

Figure S2.3: Box and whisker plots of median resting frequencies across sites from which *R. damarensis* was captured. Arnhem Cave (AC), Märcker Cave (MC), Orange River (OR), Riemvasmaak (RV), Soetfontein (SF), Untjiesburg Farm (UF) and Wondergat Cave (WC).

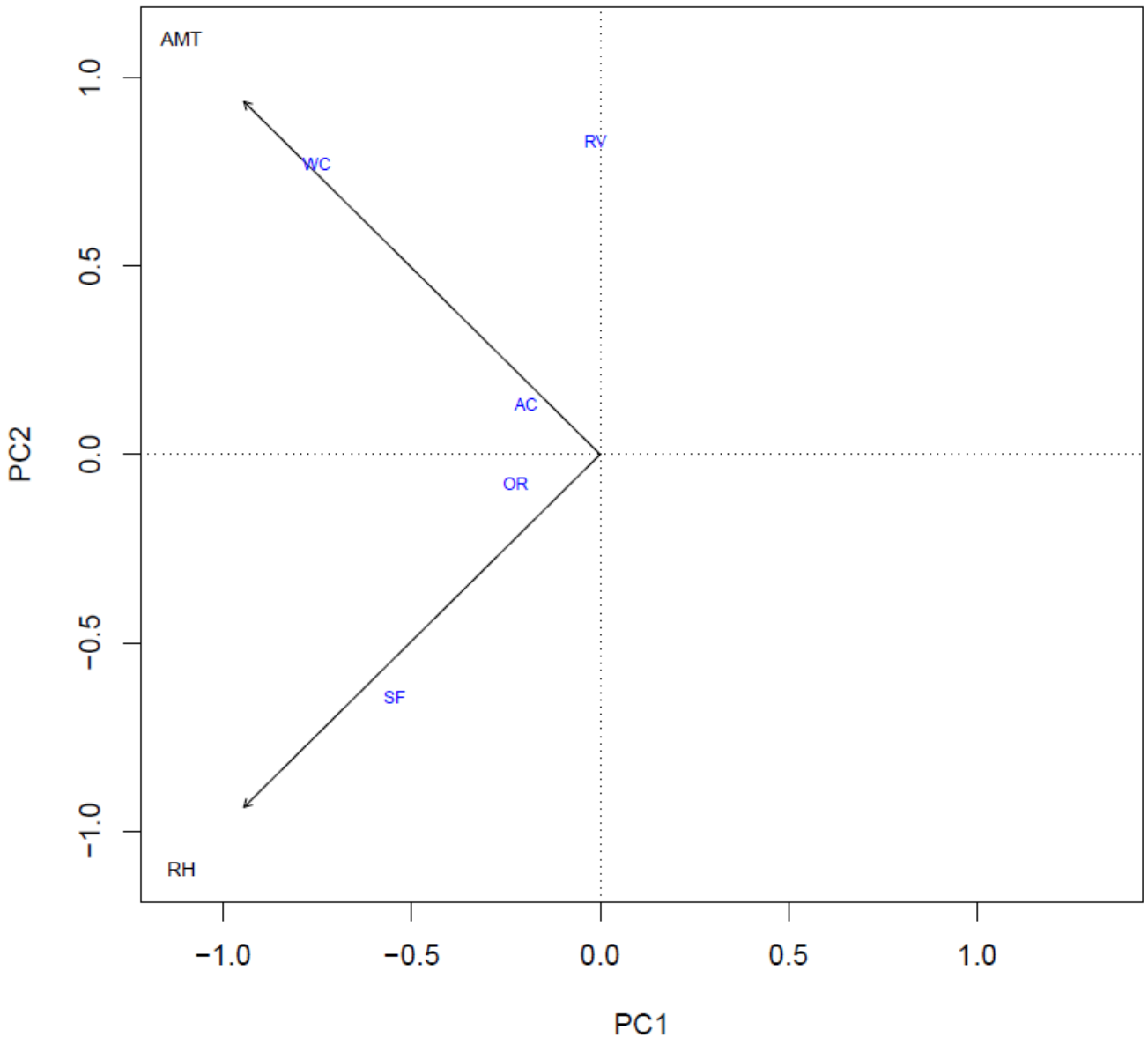

Figure S2.4: Variation in environmental conditions (RH, relative humidity and AMT, annual mean temperature) across sampled populations of *R. damarensis*. Arnhem Cave (AC), Märcker Cave (MC), Orange River (OR), Riemvasmaak (RV), Soetfontein (SF), Untjiesburg Farm (UF) and Wondergat Cave (WC).

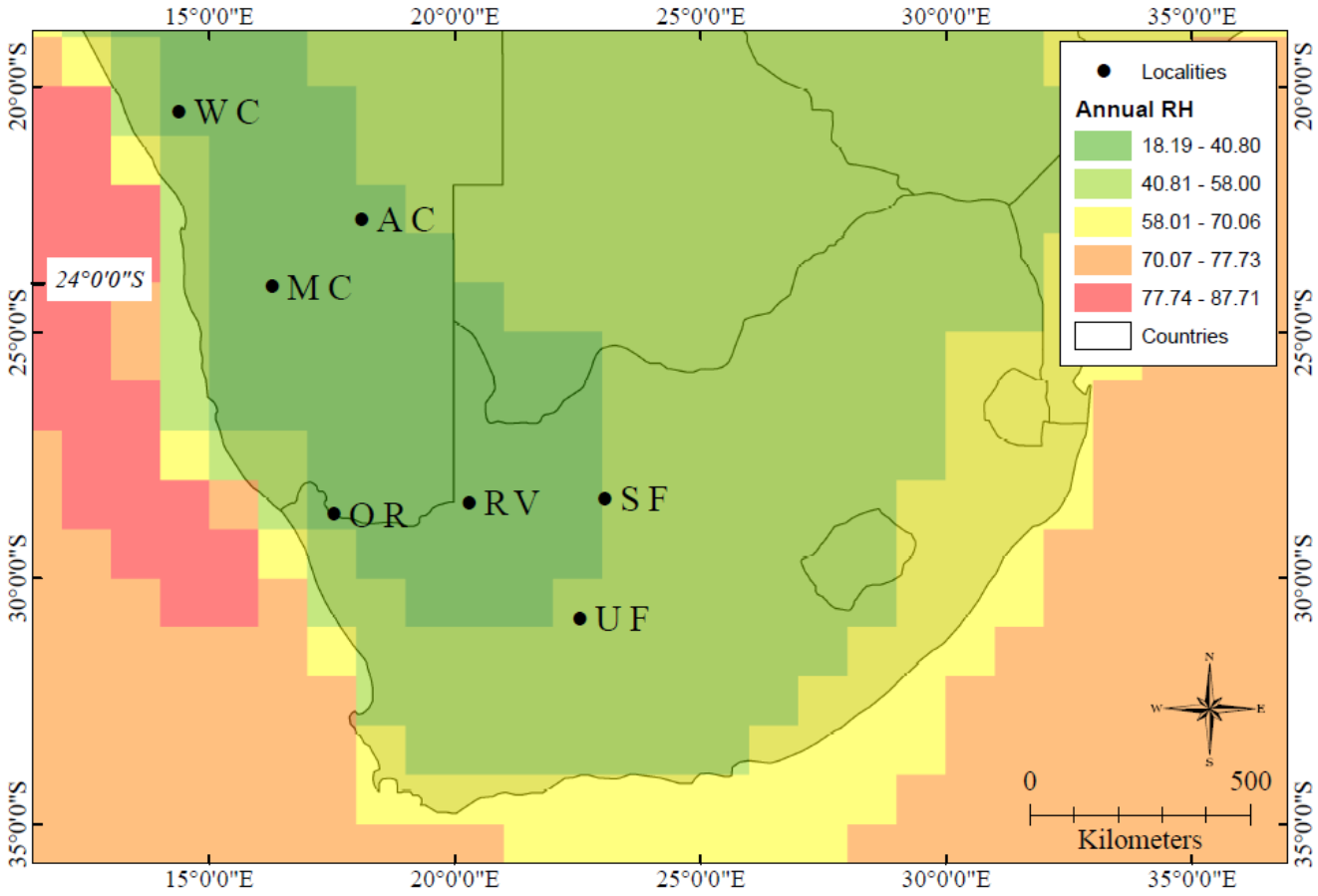

Figure S2.5: Variation in relative humidity across sites from which *R. damarensis* was captured.

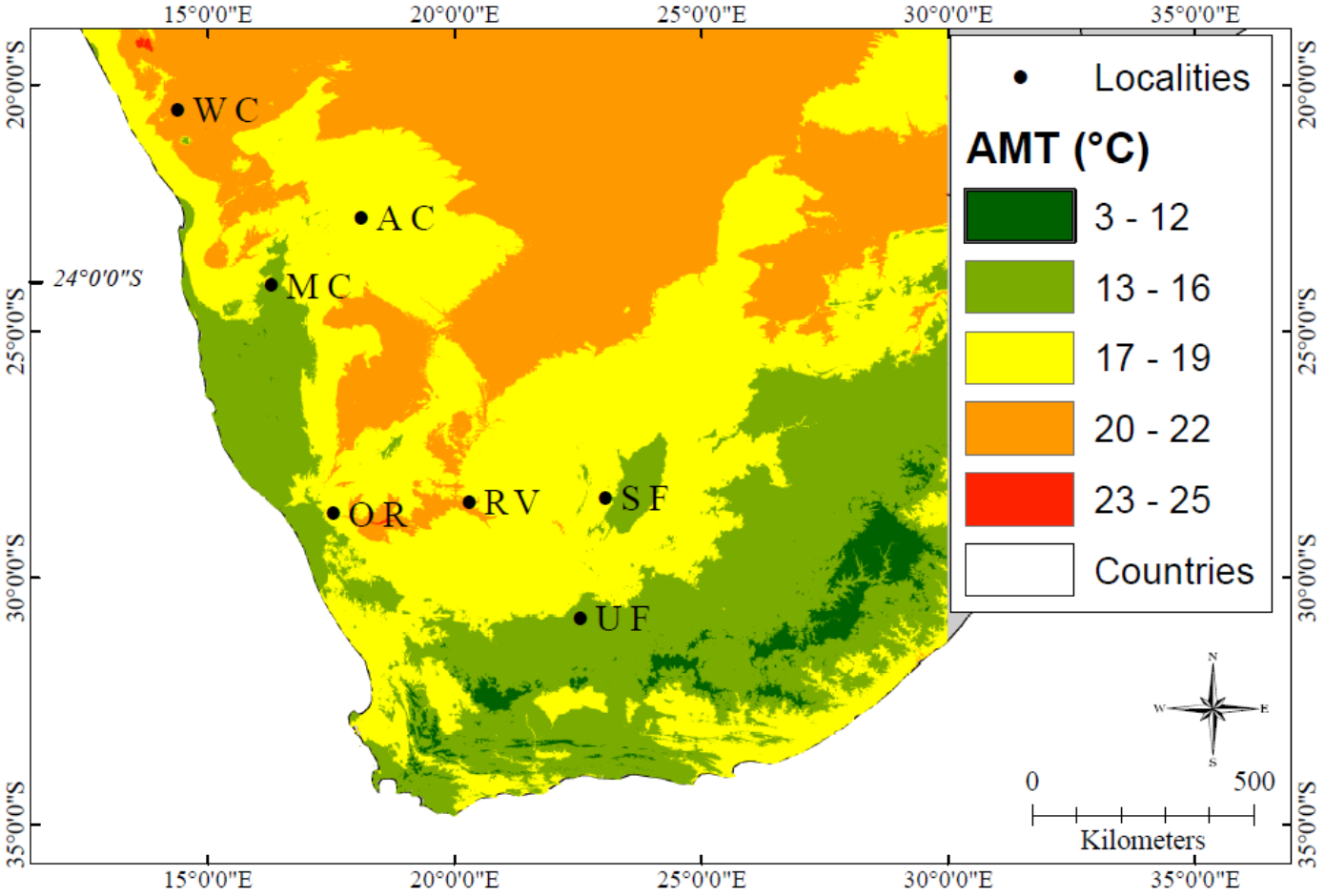

Figure S2.6: Variation in annual mean temperature across sites from which *R. damarensis* was captured.

APPENDICES

CHAPTER 3

Table B3.1: Results from multivariate linear regression analysis of sexual dimorphism in wing parameters of *Rhinolophus damarensis*.

Effect	*F*	Effect df	Error df	*p*
Intercept	23.52	14	37.00	< 0.05
Sex	1.96	14	37.00	0.05
Locality	2.70	70	180.24	< 0.05
Sex*Locality	1.14	70	180.24	0.23

Table B3.2: Results from multivariate linear regression analysis of sexual dimorphism in skull parameters of the 5 rhinolophid species.

Effect	*F*	Effect df	Error df	*p*
Intercept	41078.75	7	89.00	< 0.05
Species	22.74	35	376.82	< 0.05
Sex	0.71	7	89.00	0.66
Species*sex	0.67	35	376.82	0.93

Table B3.3: Results from multivariate linear regression analysis of sexual dimorphism in echolocation parameters of *Rhinolophus damarensis*.

Effect	*F*	Effect df	Error df	*p*
Intercept	3017777.82	9	42.00	< 0.05
Sex	2.31	9	42.00	< 0.05
Locality	26.64	45	190.98	< 0.05
Sex*locality	1.13	45	190.98	0.27

Table B3.4: Results of discriminant function analysis on principal component scores extracted by principal component analyses on wing parameters of *R. damarensis*.

Factor	Root 1	Root 2	Root 3	Root 4	Wilks' λ	$F_{(20,160)}$	*p*
PCA Factor 1	-0.93	-0.33	-0.59	0.01	0.26	12.51	< 0.05
PCA Factor 2	1.06	-0.04	0.50	-0.02	0.31	16.73	< 0.05
PCA Factor 3	0.28	-0.85	-0.20	0.49	0.18	5.41	< 0.05
PCA Factor 4	0.12	-0.67	-0.16	-0.75	0.14	2.74	0.03
Eigenvalues	2.87	0.78	0.19	0.04			
Cumulative %	73.89	94.00	98.99	100			
Wilks' λ	0.11	0.45	0.80	0.96			
X^2	109.66	40.53	11.01	1.96			
df	20	12	6	2			
p	< 0.05	< 0.05	0.08	0.37			

Table B3.5: Squared Mahalanobis distances obtained from Discriminant Function Analysis on wing measurements.

Locality	Soetfontein	Riemvasmaak	Wondergat	Arnhem	Märcker	Orange River
Soetfontein		3.33	1.72	2.05	5.23	13.41
Riemvasmaak	3.33		9.25	3.03	12.01	9.15
Wondergat	1.72	9.25		5.06	2.39	16.46
Arnhem	2.05	3.03	5.06		6.53	9.06
Märcker	5.23	12.01	2.39	6.53		11.61
Orange River	13.41	9.15	2.39	9.06	11.61	

Table B3.6: Squared Mahalanobis distances obtained from Discriminant Function Analysis on wing measurements showing differences between localities.

Locality	Soetfontein	Riemvasmaak	Wondergat	Arnhem	Märcker	Orange River
Soetfontein		0.13	0.38	0.24	0.06	0.01
Riemvasmaak	0.13		0.01	0.04	0.01	0.01
Wondergat	0.38	0.01		0.01	0.22	0.01
Arnhem	0.24	0.04	0.01		0.01	0.01
Märcker	0.06	0.01	0.22	0.01		0.01
Orange River	0.01	0.01	0.01	0.01	0.01	

Table B3.7: Results of discriminant function analysis on principal component scores extracted by principal component analyses on skull parameters of 5 *Rhinolophus* bat species from southern Africa.

Factor	Root 1	Root 2	Root 3	Wilks' λ	$F_{(15, 273)}$	*p*
PCA Factor 1	-1.11	-0.08	-0.01	0.37	265.05	< 0.05
PCA Factor 2	0.56	-0.95	-0.04	0.06	30.78	< 0.05
PCA Factor 3	0.06	0.07	-0.09	0.03	5.57	< 0.05
Eigenvalues	13.96	0.98	0.27			
Cumulative %	91.7	98.19	100			
Wilks' λ	0.02	0.39	0.78			
X^2	368.94	94.34	24.61			
df	15	8	3			
p	< 0.05	< 0.05	< 0.05			

Table B3.8: Squared Mahalanobis distances obtained from Discriminant Function Analysis on skull parameters of five different *Rhinolophus* species from southern Africa.

Species	*R .bla*	*R. cap*	*R. cli*	*R. dar*	*R. dam*-N	*R. dam*-S
R. blas	0.01	56.72	160.47	21.58	46.04	56.16
R. cap	56.72	0.01	28.46	15.58	4.33	7.55
R. cli	160.47	28.46	0.01	70.27	37.14	32.65
R. dar	21.58	15.58	70.27	0.01	6.11	11.62
R. dam-N	46.04	4.33	37.14	6.11	0.01	6.52
R. dam-S	56.16	7.55	32.65	11.62	6.53	0.01

R. blasii (R. bla), *R. capensis* (R. cap), *R. clivosus* (R. cli), *R. darlingi* (R. dar), *R. damarensis* -northern (R. dam-N) and *R. damarensis*-southern (R. dam-S).

Table B3.9: Squared Mahalanobis distances obtained from Discriminant Function Analysis on skull measurements showing the relationship between the northern and southern lineages of *R. damarensis* as well as other *Rhinolophus* species from southern Africa.

Species	*R .bla*	*R. cap*	*R. cli*	*R. dar*	*R. dam*-N	*R. dam*-S
R. bla		0.01	0.01	0.01	0.01	0.01
R. cap	0.01		0.01	0.01	0.002	0.01
R. clivo	0.01	0.01		0.01	0.01	0.01
R. dar	0.01	0.01	0.01		0.01	0.01
R. dam-N	0.01	0.02	0.01	0.01		0.01
R. dam-S	0.01	0.01	0.01	0.01	0.01	

R. blasii (R. bla), *R. capensis* (R. cap), *R. clivosus* (R. cli), *R. darlingi* (R. dar), *R. damarensis* -northern (R. dam-N) and *R. damarensis*-southern (R. dam-S).

Table B3.10: Results of discriminant function analysis on principal component scores extracted by principal component analyses on echolocation parameters of *Rhinolophus damarensis* from southern Africa.

Factor	Root 1	Root 2	Root 3	Root 4	Wilks' λ	$F_{(20,176)}$	*p*
PCA Factor 1	-0.04	-1.05	-0.41	-0.08	0.08	16.83	< 0.05
PCA Factor 2	0.34	0.64	-0.37	-0.77	0.04	5.10	< 0.05
PCA Factor 3	0.77	-0.66	0.64	-0.40	0.07	12.60	< 0.05
PCA Factor 4	-1.25	0.05	-0.09	0.12	0.24	67.43	< 0.05
Eigenvalues	7.18	2.31	0.11	0.01			
Cumulative %	74.66	98.69	99.89	100			
Wilks' λ	0.03	0.26	0.88	0.99			
X^2	191.44	73.72	6.65	0.54			
df	20	12	6	2			
p	< 0.05	< 0.05	0.35	0.76			

Table B3.11: Squared Mahalanobis distances obtained from Discriminant Function Analysis on wing parameters of *R. damarensis*.

Locality	Soetfontein	Riemvasmaak	Wondergat	Arnhem	Märcker	Orange River
Soetfontein		9.59	9.11	3.78	41.95	7.01
Riemvasmaak	9.59		1.33	4.82	90.29	11.87
Wondergat	9.11	1.33		2.25	84.22	14.95
Arnhem	3.79	4.82	2.25		59.50	11.55
Märcker	41.96	90.29	84.22	59.50		58.25
Orange River	7.01	11.87	14.95	11.55	58.25	

Table B3.12: Squared Mahalanobis distances obtained from Discriminant Function Analysis on echolocation parameters of *R. damarensis* showing relationship between localities.

Locality	Soetfontein	Riemvasmaak	Wondergat	Arnhem	Märcker	Orange River
Soetfontein		0.01	0.01	0.02	0.01	0.01
Riemvasmaak	0.01		0.27	0.01	0.01	0.01
Wondergat	0.01	0.27		0.04	0.01	0.01
Arnhem	0.02	0.01	0.04		0.01	0.01
Märcker	0.01	0.01	0.01	0.01		0.01
Orange River	0.01	0.01	0.01	0.01	0.01	

Table B3.13: Results from linear model (LM) testing for relationship between the two northern and southern lineages of *R. damarensis* based on the bacula parameters.

Greatest Length of Incision (GLI) .....

Effect	DF	SS	MS	F	P	DF	SS	MS	F	p
Intercept	1	0.86	0.86	207.01	< 0.05	1	3.47	3.47	105.38	< 0.05
Region	1	0.05	0.05	13.14	< 0.05	1	0.33	0.33	10.16	< 0.05
Error	3	0.01				3	0.09	0.03		
Total	4	0.07				4	0.43			

APPENDICES

CHAPTER 4

Table B4.1: Skulls and mandibles of *R. damarensis* collected from the two South African museums (Ditsong and Amathole) including those from the Animal Evolution and Systematics Lab of the University of Cape Town.

Species	Specimen ID	Sex	Locality	Group	Institution	Lat	Long
R. dam	140410Rda01	F	Ghaub	NN	AES, UCT	-19.48	17.77
R. dam	110410RdWG02	F	Wondergat	NN	AES, UCT	-20.51	14.37
R. dam	110410RdWG01	M	Wondergat	NN	AES, UCT	-20.51	14.37
R. dam	100410Rda01	M	Nooitgedag	NN	AES, UCT	-21.75	16.04
R. dam	070410Rda03	M	Arnhem	NN	AES, UCT	-22.70	18.10
R. dam	070410Rda01	F	Arnhem	NN	AES, UCT	-22.70	18.10
R. dam	KM31805	M	Omaruru	NN	AM	-21.12	15.52
R. dam	080410Rda01	M	Märcker	CN	AES, UCT	-24.08	16.28
R. dam	080410Rda02	F	Märcker	CN	AES, UCT	-24.08	16.28
R. dam	TM12925	M	Gobabeb	CN	DM	-23.65	15.05
R. dam	TM8292	M	Kochina	SN	DM	-27.18	18.73
R. dam	TM8294	M	Kochina	SN	DM	-27.18	18.73
R. dam	TM8293	F	Kochina	SN	DM	-27.18	18.73
R. dam	TM8291	M	Kochina	SN	DM	-27.18	18.73
R. dam	TM8290	F	Kochina	SN	DM	-27.18	18.73
R. dam	161109Rd10	M	Orange River	OR	AES, UCT	-28.70	17.54
R. dam	161109Rd09	M	Orange River	OR	AES, UCT	-28.70	17.54
R. dam	161109Rd02	F	Orange River	OR	AES, UCT	-28.70	17.54
R. dam	290511RdHM02	F	Orange River	OR	AES, UCT	-28.70	17.54
R. dam	290511RdHM01	M	Orange River	OR	AES, UCT	-28.70	17.54
R. dam	181109Rd05	F	Orange River	OR	AES, UCT	-28.94	18.13
R. dam	TM16978	M	Augrabies	OR	DM	-28.61	20.34
R. dam	TM16977	M	Augrabies	OR	DM	-28.61	20.34
R. dam	210711RdaSF05	F	Soetfontein	CSA	AES, UCT	-28.38	23.05
R. dam	210711RdaSF04	M	Soetfontein	CSA	AES, UCT	-28.38	23.05
R. dam	TM36063	F	Wonderfontein	CSA	DM	-28.66	23.36
R. dam	TM36058	F	Wonderfontein	CSA	DM	-28.66	23.36
R. dam	TM35991	F	Wonderfontein	CSA	DM	-28.66	23.36
R. dam	TM35988	F	Wonderfontein	CSA	DM	-28.66	23.36
R. dam	TM35992	F	Wonderfontein	CSA	DM	-28.66	23.36
R. dam	TM35989	M	Wonderfontein	CSA	DM	-28.66	23.36
R. dam	TM27288	F	Keikamspoort	CSA	DM	-29.83	22.78
R. dam	TM27206	M	Keikamspoort	CSA	DM	-29.83	22.78
R. dam	TM27290	M	Keikamspoort	CSA	DM	-29.83	22.78
R. dam	TM27289	M	Keikamspoort	CSA	DM	-29.83	22.78
R. dam	140110R16Un	F	Uintjiesburg	CSA	AES, UCT	-30.83	22.54
R. dam	140110R18Un	F	Uintjiesburg	CSA	AES, UCT	-30.83	22.54
R. dam	TM36096	M	Buxton Mine	CSA	DM	-27.61	24.61
R. dam	TM36094	M	Buxton Mine	CSA	DM	-27.61	24.61
R. dam	TM36093	M	Buxton Mine	CSA	DM	-27.61	24.61
R. dam	TM35878	M	Buxton Mine	CSA	DM	-27.61	24.61

Rhinolophus damarensis (R. dam), Animal Evolution and Systematics, University of Cape Town (AES, UCT), Amathole Museum (AM), Ditsong Museum (DM).

Table B4.2: Definitions of landmarks placed on the cranium in ventral, dorsal and lateral view of of *Rhinolophus damarensis*.

1. Most anterior point at the base of canine.
2. Most anterior point of the anterior medial swelling on the midline between the left and right anterior medial swellings.
3. The widest point of the anterior medial swelling.
4. Most dorsal point of the anterior medial swelling.
5. Most posterior point of the anterior medial swelling on the midline of the skull.
6. Most anterior point of the sagittal crest on the midline of the skull.
7. Most dorsal point of the sagittal crest.
8. Most posterior and lowest point of the sagittal crest above the parietal depression
9. Most posterior point of the skull at the sagittal and lambdoid crests.
10. Most lateral point of the occipital condyle.
11. Most anterior point of the foramen magnum.
12. Most ventral point of the auditory bulla.
13. Most posterior point of the external auditory meatus at the junction with the paraoccipital process.
14. Widest point of the cranium where the zygomatic arch originates from the squamosal.
15. Widest point of the zygomatic arch.
16. Most posterior point of the foramen ovale.
17. End of tooth row at the base of the third Molar (M3).
18. Suture between the palatines at the midline.
19. Suture between the premaxilla and maxilla at the midline
20. Between third Molar (M3) and second Molar (M2) at the base.
21. Between second Molar (M2) and first Molar (M1) at the base.
22. Between first Molar (M1) and second Premolar (PM2) at the base.
23. Between first Premolar (PM1) and second Premolar (PM2) at the base.
24. Between Canine (C) and first Premolar (PM2) at the base.

Table B4.3: Definitions of landmarks placed on the mandible in ventral, dorsal and lateral view of *Rhinolophus damarensis*.

1. Tip of the coronoid process.
2. Tip of the condylar process at external edge.
3. Tip of the condylar process at internal edge.
4. Tip of the angular process midway along the width.
5. Point of extreme curvature at the incisura praemasseterica
6. Point of extreme curvature on the lower edge of mandibular corpus.
7. Most anterior point of the lower edge of mandible corpus at the point where the two mandibles join.
8. Midpoint of the aboral edge of the mental foramen.
9. Most anterior point of the mandible corpus
10. Midpoint at the base, between Incisor 1(I1) and canine 1 (C1).
11. Midpoint at the base, between Canine one (C1) and Premolar one (PM1)
12. Midpoint at the base, between Premolar three (PM3) and Molar one (M1)
13. Midpoint at the base, between Molar one (M1) and Molar two (M2)
14. Midpoint at the base, between Molar two (M2) and Molar three (M3)
15. At the end of Molar three (M3)

Figure S4.1: Landmark coordinates used for the skull of *R. damarensis*

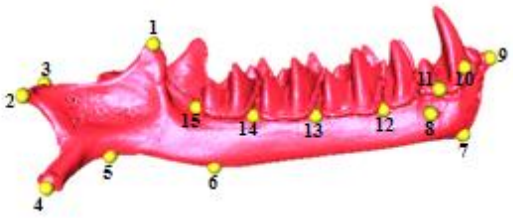

Figure S4.2: Landmark coordinates used for the mandible of *R. damarensis*