An investigation of the evolutionary diversification of a recent radiation of dwarf chameleons (*Bradypodion*) from KwaZulu-Natal Province, South Africa

By
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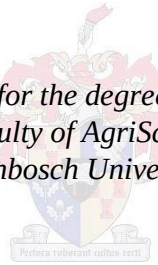

Dissertation presented for the degree of Doctor of Science in the Faculty of AgriSciences at Stellenbosch University

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Dedication

This research is dedicated to
my family without whose support
I could never have gotten this far.

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“It is not the strongest of the species that survives,
nor the most intelligent that survives. It is the one
that is the most adaptable to change.”

Paraphrase of Charles Darwin
by Leon C. Megginson

A ZULU VERSION OF THE LEGEND OF THE "ORIGIN OF DEATH"

“GOD (Unknlunkulu) arose from beneath (the seat of the spiritual world, according to the Zulu idea), and created in the beginning men, animals, and all things. He then sent for the Chameleon, and said,

> Go, Chameleon, and tell Men that they shall not die.

The Chameleon went, but it walked slowly, and loitered on the way, eating of a shrub called Bukwebezane.

When it had been away some time, God sent the Salamander after it, ordering him to make haste and tell Men that they should die. The Salamander went on his way with this message, outran the Chameleon, and, arriving first where the Men were, told them that they must die.”

James A. Honey
South African Folk-tales
Baker & Taylor Company, New York, 1910

Acknowledgements

This thesis represents not only the culmination of my research on KwaZulu-Natal dwarf chameleons, but also the fulfilment of one of my life goals. The five years it has taken to achieve this have been long and full; marked by both personal and professional struggles and, more importantly, successes that I could not have accomplished alone.

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Note to Reviewers

My dissertation is broken down into seven chapters. Chapter 1 is the introduction which explains and provides context to the overall research, Chapters 2-6 comprise the body of the thesis, and Chapter 7 concludes the thesis by summarizing the main findings and providing recommendations. Chapters 2 through 6 are written in the first person plural as they reflect the work conducted by me and my collaborators. They are prepared as a collection of seven research articles, which have been published, are in press, are under review, or are destined to be published in the near future. By preparing these chapters in this way, considerable repetition is found in Chapter 1 and the introduction of all papers (specifically regarding the study region and animals), which could not be avoided. Even though each article is to be published in different journals, for the purposes of consistency for this thesis, all have been formatted in the same way and share the a single referencing style. Full citations are compiled in a Reference List at the end. A modified version of the 6th edition of the APA style was used to ensure that maximal information is provided for each reference in a clear format. Lastly, the first page of any thesis chapters that have been published are included in an appendix. Please note that page numbers of the appendix are per the original publications.

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Abstract

An important prerequisite for evolutionary change is variability in natural populations; however, when phenotypic and molecular rates of change differ, species delimitation is problematic. Such discordance has been identified in a recent radiation of dwarf chameleons (*Bradypodion*) from KwaZulu-Natal Province, South Africa. This radiation is comprised of several phenotypic forms, two of which have been classified taxonomically – *Bradypodion melanocephalum* and *Bradypodion thamnobates*. Early phylogenetic analysis did not support the forms primarily because geographic sampling and the set of molecular markers used were appropriate for detecting deep divergences and, therefore, less effective for understanding species boundaries within a recent radiation. In this radiation, the forms are allopatric, occupy different habitats, and vary in size and colouration, suggesting local adaptation and ecological speciation. To test this hypothesis, morphometric and habitat data were collected for each form to examine ecologically relevant morphological differences that reflect differential habitat use. Morphological differences were then associated with functional adaptations by testing locomotor performance and bite force. Next, fine-scale genetic sampling was used to examine lineage diversification using a combination of mitochondrial DNA and microsatellites. Spatial information was incorporated into these analyses to quantify the genetic effects of landscape barriers on genetic structure. Finally, ecological niche modelling was used to examine the abiotic factors involved in shaping the climatic niches of these chameleons, and to gain insight into their biogeographic history. Results show morphological distinctions between phenotypic forms, with corresponding differences in performance, indicating functional adaptations to habitats, which can be broadly classified as either open- or closed-canopy vegetation. Specifically, chameleons in open-canopy habitats have proportionally smaller

heads and feet than their closed-canopy counterparts, and correspondingly weaker bite forces and forefoot grip strengths. Varying degrees of sexual dimorphism were detected, with the closed-canopy forms being more dimorphic than the open-canopy forms. This suggests that sexual selection is the predominant force within the closed-canopy habitat, which are more protected from aerial predators, thereby enabling them to invest in dimorphic traits for communication; while, in open-canopy habitats, natural selection is the predominant force, ultimately enforcing their overall diminutive body size and constraining performance. Genetic structure was observed, with the mitochondrial DNA revealing three genetic clusters and the microsatellites revealing seven. This likely reflects the different mutation rates and modes of inheritance between these two markers. Three of the microsatellite clusters were supported by morphological and ecological data and should, therefore, be recognised as separate species. The remaining microsatellite clusters showed discordance with the ecomorphological data; however, given their genetic distinctiveness, they should be recognized as separate conservation units. The climatic niches of the three proposed species showed high to moderate levels of climatic stability, while the four proposed conservation units showed low climatic stability. These results indicate that this species complex is affected by both climatic niche conservatism and lability, which could explain the observed patterns of morphological and genetic diversity. In summary, these results support the hypothesis of ecological speciation within this radiation.

Opsomming

'n Belangrike voorvereiste vir evolusionêre verandering is variasie in natuurlike bevolkings, maar wanneer fenotipiese en molekulêre tempo van verandering verskil, is spesies definieering problematies. Sulke onenigheid is geïdentifiseer in ‘n onlangse radiasie van dwerg verkleurmannetjies (Bradypodion) van die KwaZulu-Natal Provinsie, Suid-Afrika. Hierdie radiasie bestaan uit verskeie fenotipiese vorms, waarvan twee taksonomies geklassifiseer is – Bradypodion melanocephalum en Bradypodion thamnobates. Vroeë filogenetiese analise het nie die vorms ondersteun nie, hoofsaaklik omdat geografiese steekproefneming en die stel van molekulêre merkers gebruik geskik was vir die opsporing van diep afwykings, en dus minder effektief is vir die begrip van spesies grense binne 'n onlangse radiasie. In hierdie radiasie is die vorms allopatries, beset verskillende habitatte, en wissel in grootte en kleur, wat dui op plaaslike aanpassing en ekologiese spesiasie. Om hierdie hipotese te toets, is morfometriese en habitat gegewens ingesamel vir elke vorm om sodoende ekologies relevante morfologiese verskille te ondersoek wat verskil in habitat gebruik reflekteer. Morfologiese verskille is geassosieer met funksionele aanpassings deur lokomotoriese prestasie en byt krag te toets. Volgende is fyn-skaal genetiese steekproefneming gebruik om afkoms diversifikasie met behulp van 'n kombinasie van mitochondriale DNS en mikrosatelliete ondersoek. Ruimtelike inligting is geinkorporeer in die ontleding om sodoende genetiese gevolge van landskap hindernisse op genetiese struktuur te kwantifiseer. Ten slotte, is ekologiese nis modelle gebruik om die abiotiese faktore wat betrokke is by die vorming van klimaat- nisse van hierdie verkleurmannetjie te ondersoek en om insig te verkry oor hul biografiese geskiedenis. Resultate toon morfologiese onderskeid tussen fenotipiese vorms, met saameenlopende verskille in prestasie, wat dui op funksionele aanpassings tot habitat, wat breedweg as oop-

of geslote-kap plantegroei geklassifiseer kan word. Spesifiek verkleurmannetjies in oop-kap habitatte het proporsioneel kleiner koppe en voete as hul geslote-kap eweknieë, en ooreenkomstig swakker byt krag en voorvoet greep. Wisselende vlakke van seksuele dimorfisme is vasgestel, met geslote-kap vorms wat meer dimorfies is as oop-kap vorms. Dit dui daarop dat seksuele seleksie die oorheersende krag in geslote-kap habitatte is, wat meer beskerm is teen vlieënde roofdiere, wat hulle in staat stel om te belê in dimorfiese eienskappe vir kommunikasie, terwyl in oop-kap habitatte, is natuurlike seleksie die oorheersende krag, wat uiteindelik kleiner liggaam grootte en beperkte prestasie afdwing. Genetiese struktuur is waargeneem, met die onthulling van drie genetiese groeperings gebasseer op mitochondriale DNS en sewe gebasseer op mikrosatelliete. Dit weerspieël waarskynlik die verskil in mutasie tempo en manier van erfenis tussen hierdie twee merkers. Drie van die mikrosatelliet groeperings is ondersteun deur morfologiese en ekologiese gegewens en moet dus erken word as aparte spesies. Die oorblywende mikrosatelliet groeperings dui op onenigheid met eko-morfologiese data, maar, gegewe hul genetiese eiesoortigheid, moet hulle erken word as afsonderlike bewarings eenhede. Die klimaat-nisse van die drie voorgestelde spesies het hoë tot matige vlakke van die klimaat stabiliteit, terwyl die vier voorgestelde bewarings eenhede lae klimaat stabiliteit het. Hierdie resultate dui daarop dat hierdie spesie kompleks beïnvloed word deur beide klimaat nis konserwatisme en stabiliteit, wat die waargenome patrone van morfologiese en genetiese diversiteit kan verduidelik. In opsomming, hierdie resultate ondersteun die hipotese van ekologiese spesiasie binne hierdie radiasie.

Chapter 1

Introduction

An important prerequisite for evolutionary change is variability in natural populations. Under natural selection this variability must be heritable and lead to differential rates of survival and reproduction (Darwin, 1859). It typically starts with phenotypic adaptation (variation in morphology, anatomy, physiology, and/or behaviour) in response to specific environmental pressures. Thus, individuals within a population are replaced by the progeny of parents that are better adapted to survive and reproduce in the environment in which natural selection took place. This process creates and preserves traits that are seemingly fitted for the functional roles they perform (Mayr, 1942; Simpson, 1944, 1953). In most instances, these phenotypic changes occur alongside genetic changes, allowing the phenotype to easily identify genetically delineated taxa (Alexander, 2006). However, situations exist where the phenotypic rate of change exceeds that of the molecular and vice versa (Bromham *et al.*, 2002) making species delimitation difficult. This is because each of the various species concepts in existence designate species boundaries according to different biological properties (de Queiroz, 2007). Considering the species is the fundamental unit of biodiversity, such ill-defined boundaries have significant consequences for their management and conservation (see Rojas, 1992).

Within the past two decades, DNA sequencing has significantly aided in the identification of morphologically cryptic, yet genetically diverse taxa (Bickford *et al.*, 2007) with species identified as the terminal branches of a phylogenetic tree (following the Phylogenetic Species Concept: Nixon & Wheeler, 1990). However, populations that are morphologically diverse yet seemingly genetically identical have been more difficult to decipher and may cause some to question their validity as species. This is because such variation may simply be a case of phenotypic plasticity or polymorphisms within a given species. However, a population might lack any detectable genetic diversity even if it is evolving separately because it is in the early stages of divergence (de Queiroz, 2007). Such

cases of rapid morphological diversification are commonly observed within adaptive radiations (Schluter, 2000) of which Darwin's finches (Freeland & Boag, 1999; Grant, Grant, & Petren, 2005; Petren *et al.*, 2005), threespine sticklebacks (Kristjánsson, 2005), African cichlids (Seehausen, 2006; Salzburger, 2009), and *Anolis* lizards (Losos *et al.*, 1998) are prime examples. Strong divergent natural selection causes populations to display morphological and behavioural differences that are functionally related to particular microhabitats, making the diversification adaptive (Mayr, 1942; Simpson, 1944, 1953; Givnish & Sytsma, 1997; Schluter, 2000; Salzburger, 2009).

Even though adaptive radiations are characterised by phenotypic divergence, many also incorporate considerable repetition in the form of parallel phenotypic evolution – the independent evolution of the same phenotypic traits in ecologically similar environments amongst distantly related lineages (Futuyma, 1986). Allopatric populations or species displaying parallel evolution are termed ‘ecomorphs’, of which the Greater Antillean anoles are the archetype (Williams, 1972, 1983; Losos, 1990a, b). Each *Anolis* ecomorph is named after the microhabitat they usually occupy, such as grass-bush, trunk-ground, trunk, trunk-crown, crown giant, and twig (Williams, 1972, 1983), and the species that make up each ecomorph cluster together in a multidimensional morphospace defined by limb proportions, performance (running, jumping ability), and behaviour (Losos, 1990a). The existence of ecomorphs (open- versus closed-canopy habitat) has been proposed within South African dwarf chameleons (genus *Bradypodion*) stemming from climatic shifts during the Miocene and Pliocene which resulted in changes in vegetation type across the subcontinent (Tolley *et al.*, 2006; Tolley, Chase, & Forest, 2008); however, until recently empirical evidence has been lacking to support this claim.

Like all chameleons, *Bradypodion* species are highly reliant on vegetation for their survival – using crypsis and stealth to attain food and avoid predators (Tolley *et al.*, 2006;

Stuart-Fox & Moussalli, 2007). Accordingly, changes to the structure of the vegetation in which they conceal themselves likely have direct consequences for their survival and, ultimately, their evolution (Purvis, Jones, & Mace, 2000). These consequences are expected to be manifested in their prehensile tails, clamp-like feet, uniquely positioned limbs, and their sometimes ornamented heads – traits thought to be ecologically relevant in their complex arboreal habitats (Gans, 1967; Peterson, 1984; Higham & Jayne, 2004; Fischer, Krause, & Lilje, 2010; Herrel *et al.*, 2013). This hypothesis was recently tested on the Cape dwarf chameleon, *Bradypodion pumilum* (Measey, Hopkins, & Tolley, 2009; Herrel *et al.*, 2011; Hopkins & Tolley, 2011) which is comprised of two phenotypic forms or morphotypes restricted to the south-western Western Cape Province of South Africa. The open-canopy habitat form is a small, dull coloured chameleon occupying fynbos habitats, whereas the closed-canopy habitat form is a larger, conspicuously ornamented and coloured chameleon found in forest fragments, riverine thicket, and bushy, exotic vegetation in urban settings (Branch, 1998; Tolley & Burger, 2007; Tilbury, 2010). In addition to the macrohabitat differences, structural differences in the microhabitats of each form were identified with the open-canopy habitat made up of narrow vertical perches, densely clustered in isolated clumps reaching no higher than 50 cm off the ground and the closed-canopy habitat comprised of mainly horizontal, less densely packed perches of varying diameters and reaching more than 1 m off the ground (Herrel *et al.*, 2011). These ecological differences translated into functional morphological differences between forms, specifically pertaining to locomotor function and potentially signalling/fighting ability. Specifically, the open-canopy habitat *B. pumilum* possess proportionally smaller feet and tails which enable them to better grasp hold of their narrow perches, yet are less effective (weaker) on the broader perches available in the closed-canopy habitat. They also have longer limbs that may provide maximal reach to navigate across or over ground-covering

vegetation, which is abundant in this habitat (Herrel *et al.*, 2011), and wider heads but less ornamented casques (at least among males) with a correspondingly harder bite potentially for increased fighting ability. This may be because their reduced casques (believed to reduce predation risk from aerial predators: Stuart-Fox & Moussalli, 2008) make for less effective communication and there is the potential for a greater frequency of intra-sexual encounters (Measey *et al.*, 2009). Conversely, the longer tails, larger feet and shorter legs of the closed *B. pumilum* afford them stronger grip and increased stability on the wider, more elevated perches found there, thus permitting them to move faster along horizontal branches (Herrel *et al.*, 2011). Their higher casques likely allow for long-distance communication (Stuart-Fox & Moussalli, 2008; Measey *et al.*, 2009), which might reduce the frequency of harmful conspecific encounters (Stuart-Fox *et al.*, 2006a), thus explaining their proportionally weaker bites (Measey *et al.*, 2009).

In addition to the ecomorphological differences uncovered between the two *B. pumilum* forms, varying degrees of sexual dimorphism were also detected further reflecting their differential habitats. In general, males were found to be proportionally larger than females; however, closed-canopy habitat males were larger in almost all traits examined, while in the open-canopy habitat, sexual dimorphism was restricted to tail and foot size (Hopkins & Tolley, 2011). Male dwarf chameleons compete with other males for access to females to mate and use courtship displays to assess a female's willingness to mate (Burrage, 1973; Stuart-Fox & Whiting, 2005; Stuart-Fox *et al.*, 2006a; Tolley & Burger, 2007; Tilbury, 2010), with larger casqued and brightly coloured males generally found to be more successful (Stuart-Fox *et al.*, 2006a). Considering closed-canopy habitats offer increased shelter from predators, the closed-canopy habitat form of *B. pumilum* can invest in the development of these conspicuous secondary sexual characteristics resulting in increased sexual dimorphism; whereas, in the open-canopy habitat, such conspicuous

characters would increase the visibility of an individual to predators (Stuart-Fox *et al.*, 2003), likely explaining their reduced dimorphism. With this in mind, sexual dimorphism may be yet another ecomorphological trait used to test the existence of ecomorphs within this genus.

Similar open- and closed-canopy ecomorphological associations are believed to exist in a recent radiation of dwarf chameleons localized to southern KwaZulu-Natal (KZN) Province, South Africa (Tolley *et al.*, 2004), which could validate the ecomorph hypothesis. The radiation is comprised of two described species (*Bradypodion melanocephalum* and *Bradypodion thamnobates*) and three additional phenotypic forms (Raw, 1995, 2001; Tolley & Burger, 2007; Tolley *et al.*, 2008; Tilbury, 2010) herein referred to as Types A, B and C. *Bradypodion melanocephalum* (Gray, 1865) is small-bodied with a subtle casque, minute gular lobes, homogeneous scales with a few small scattered tubercles on the flanks, and is a dull brown colour. In contrast, *B. thamnobates* (Raw, 1976) has a large heavy body with conspicuous tuberculated scales, a prominent casque, large gular lobes, a bright white gular region, and a rich green colour often with reddish or orange flanks. Type A appears most similar to *B. melanocephalum* in size and colour, leading many to classify it as another population of the species (Tolley *et al.*, 2004; Tilbury, 2010); however, it can be distinguished from *B. melanocephalum* by faint green markings along its flanks and orange along its tail and dorsal crest. Type B is large in size with a prominent casque, is bright green in colour with a yellow gular region. Type C has morphological features outwardly similar to *B. thamnobates* (e.g., prominent casque and large gular lobes), although it lacks the striking coloration and heavy, tuberculated body of that species.

All five forms are allopatric in distribution (Tolley & Burger, 2007; Tilbury, 2010), but mitochondrial markers show they lack the divergence expected at the species level

(Tolley *et al.*, 2004; Tolley *et al.*, 2008). In some species, such an outcome is the result of phenotypic plasticity (e.g., Losos *et al.*, 2000; Buckley, Irschick, & Adolph, 2010); however, common garden experiments suggest this is not the case for these *Bradypodion* species (Miller & Alexander, 2009). Juveniles from both described species were raised under identical conditions and developed phenotypes similar to their original populations. As such, the lack of genetic divergence likely reflects the recent nature of the radiation, and given the short branch lengths within this clade reflected in their phylogeny (see Tolley *et al.*, 2004; Tolley *et al.*, 2008), it maybe be as recent as the late Pleistocene.

Numerous drastic climatic changes arose during the Pleistocene, especially during the Last Glacial Maximum (Mucina *et al.*, 2006), which brought upon significant changes in vegetation (Scott, 1993; Eeley, Lawes, & Piper, 1999; Tyson, 1999; Bond, Midgley, & Woodward, 2003; Mucina & Rutherford, 2006), particularly within the forest and grassland biomes (Lawes, 1990; Eeley *et al.*, 1999; Mucina & Geldenhuys, 2006; Lawes *et al.*, 2007), in which these chameleons inhabit (Tolley & Burger, 2007; Tilbury, 2010). These changes likely created distinct microhabitats (e.g., plant structure, perch size, canopy cover) within which each form had to adapt, possibly explaining the striking phenotypic diversity among them. Specifically, *B. melanocephalum* is found along the coast of southern KZN (0-150 m a.s.l.) in grasses and lowland coastal vegetation, whereas *B. thamnoba*tes is found inland in the KZN Midlands (850–1600 m a.s.l.), most often in transformed landscapes (exotic trees, bushy shrubs and urban gardens), although their primary habitat is indigenous forest which is now highly fragmented. Types A and C are also localized to the KZN Midlands; however, they are peripatric to *B. thamnobates* and each other. Type A is often found in grasslands and around transformed vegetation (including plantations), whereas Type C is restricted to Afrotemperate forest in the Karkloof area. Lastly, Type B can be found along the southern Drakensberg mountain

range up to 2000 m a.s.l., mainly in indigenous Afrotemperate forests and along river courses populated with bushes and trees; however they are occassionaly found in grasslands.

If similar open- and closed-canopy ecomorphs exist within this radiation as has been documented in *B. pumilum* then *B. melanocephalum* and Type A are expected to possess comparable ecomorphological features and functions as the open-canopy habitat *B. pumilum*, while *B. thamnobates* and Types B and C are expected to ressemble the closed *B. pumilum* in form and function. However, before any conclusions can be made, concrete evidence is required, which this thesis sets out to attain. First, morphometrics in conjunction with micro- and macrohabitat surveys are used to determine whether more tangible morphological differences exist between the five phenotypic forms and sexes apart from overall colour and size, and whether these differences are correlated to habitat structure (Chapter 2: da Silva & Tolley, 2013). Second, the functional significance of any ecomorphological differences detected is tested by comparing the performance of each form. Specifically, locomotor performance traits (running and gripping, Chapter 3: da Silva *et al.*, 2014a) and bite force (Chapter 4: da Silva *et al.*, 2014b) are investigated as they are thought to be most relevant to their survival (e.g., Losos, Walton, & Bennett, 1993; Measey *et al.*, 2009). Since an individual's phenotype will determine the limits of its performance, and limitations on performance will constrain the range of environmental resources it can exploit (Arnold, 1983; Wainwright, 1994), such performance testing is imperative to establishing and understanding the adaptive nature of this radiation (Schluter, 2000). Third, comprehensive population genetic techniques are used to test for the presence of lineage diversification in this radiation (Chapter 5). Even though previous phylogenetic studies found no significant genetic differentiation among these chameleons (Tolley *et al.*, 2004; Tolley *et al.*, 2006), sampling was extremely limited incorporating

only one or two individuals from what was assumed to be a representative locality of each form, and the genetic markers used (ND2 and 16S) were limited to the mitochondrial genome may be ineffective at detecting genetic structure in recent diversifications or below the species level. Accordingly, this study incorporates more extensive genetic sampling representing a variety of populations per form throughout southern KZN, as well as both mitochondrial DNA (mtDNA) and fast-evovling nuclear markers (microsatellites) to test for any recent genetic structuring within and between forms. Detailed spatial information is also incorporated into the population genetic analysis to help quantify the genetic effects of habitat and geographic barriers (e.g., Manel *et al.*, 2003; Spear *et al.*, 2005; Storfer *et al.*, 2007; Moore *et al.*, 2008). Using these data, patterns of ecomorphological and genetic variation will be examined in order to make inferences regarding the classification of species or taxon status at any rank (e.g., evolutionarily significant units, conservation units). Finally, ecological niche modelling (ENM) is used to examine the abiotic factors involved in shaping the ecological and evolutionary relationships within this species complex (Chapter 6). The current and past climatic niches of each of the biological units identified from the cumulative knowledge gained from Chapters 2 through 5 are projected to assess the climatic stability of southern KZN and provide insight into the demographic events that likely shaped the genetic and morphological diversity within this species complex. In cases where closely related taxa occupy divergent niches, ENM has been instrumental in delimiting species and identifying the mechanisms of speciation (e.g., Losos *et al.*, 2003; Raxworthy *et al.*, 2007; Jakob *et al.*, 2010; Hawlitschek *et al.*, 2011). This is especially so for groups exhibiting low vagility (Raxworthy *et al.*, 2007; Franklin, 2009), which is an attribute of *Bradypodion* species. As such, ENM may provide support (along with morphological and/or genetic data) for the reclassification of species in the KZN radiation.

Chapter 2

Paper I:
Ecomorphological variation and sexual dimorphism in a recent radiation of dwarf chameleons (*Bradypodion*)[*]

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ABSTRACT

Natural selection tends to favour optimal phenotypes either through directional or stabilizing selection; however, phenotypic variation in natural populations is common and arises from a combination of biotic and abiotic interactions. In these instances, rare phenotypes may possess a fitness advantage over the more common phenotypes in particular environments, which can lead to adaptation and ecological speciation. A recently radiated clade of dwarf chameleons (*Bradypodion*) restricted to southern KwaZulu-Natal Province, South Africa, is currently comprised of two species (*Bradypodion melanocephalum* and *Bradypodion thamnobates*), yet three other phenotypic forms exist, possibly indicating the clade is far more speciose. Very little genetic differentiation exists between these five phenotypic forms; however, all are allopatric in distribution, occupy different habitats and vary in overall size and coloration, which may indicate that these forms are adapting to their local environments and possibly undergoing ecological speciation. To test this, we collected morphometric and habitat data from each form and examined whether ecological relevant morphological differences exist between them that reflect their differential habitat use. Sexual dimorphism was detected in four of the five forms. Yet, the degree and number of dimorphic characters was different between them, with size-adjusted male-biased dimorphism being much more pronounced in *B. thamnobates*. Habitat differences also existed between sexes, with males occupying higher perches in more closed-canopy (forested) habitats than females. Clear morphological distinctions were detected between four of the five forms, with the head explaining the vast majority of the variation. Chameleons occupying forested habitats tended to possess proportionally larger heads and feet but shorter limbs than those in open-canopy habitats (i.e., grassland). These results show that this species complex of *Bradypodion* is morphologically variable for traits that are ecologically relevant for

chameleons, and that the variation among the five phenotypic forms is associated with habitat type, suggesting that this species complex is in the early stages of ecological speciation.

INTRODUCTION

Phenotypic variation in natural populations is intriguing from an evolutionary perspective because natural selection is assumed to favour one optimal phenotype either through directional or stabilizing selection. Consequently, a major goal of evolutionary biology is to identify processes that create and maintain phenotypic variation in natural populations. One possibility is that diversity is maintained by disruptive selection, which is driven by negative frequency dependent selection (Mather, 1955; Rueffler *et al.*, 2006) arising from biotic (e.g., competition for resources: Benkman, 1996; Swanson *et al.*, 2003) and/or abiotic interactions (e.g., temperature and climate: Davis & Shaw, 2001; Norberg *et al.*, 2001). In such instances, rare phenotypes possess a fitness advantage over the more common phenotypes in particular environments, which can lead to local adaptations, sometimes followed by ecological speciation. The most common outcome of such diversification is interspecific character displacement, in which coexisting populations diverge in resource use to mitigate the effects of competition (Grant, 1972).

Caribbean *Anolis* lizards provide one of the best examples of the rapid evolution of character displacement, where populations of *Anolis* lizards have diverged to occupy different ecological niches (e.g., crown of trees, trunk, twigs) that differ in microhabitat structure (e.g., perch diameter and height, light intensity), leading to morphological adaptations that enable them to better utilize their habitat (Losos & Sinervo, 1989; Losos, 1990b; Losos & Irschick, 1996; Losos *et al.*, 1998; Leal & Fleishman, 2002; Elstrott & Irschick, 2004). For example, shorter-limbed anoles that perch high in the canopy on thin

substrates have slower running speeds than longer-limbed anoles that utilize broader perches closer to the ground (Losos & Irschick, 1996). This has been attributed to a trade-off between stability and speed, with the shorter-limbed lizards, which rarely run, requiring greater stability in their more elevated habitats (Losos, 2001). Similarly, *Anolis* species perching high in the canopy also possess proportionally larger toe pads that confer greater clinging ability compared to species lower in the canopy (Elstrott & Irschick, 2004).

Because chameleons are highly dependent on vegetation to provide camouflage, avoid predators, and obtain food (Tolley *et al.*, 2006; Stuart-Fox & Moussalli, 2007), changes to the structure of the vegetation in which they conceal themselves likely have direct consequences for their survival and, ultimately, their evolution (Purvis *et al.*, 2000). Chameleons therefore represent ideal candidates for examining causal relationships between habitat and morphology (Losos *et al.*, 1993; Bickel & Losos, 2002; Hopkins & Tolley, 2011). Recent studies on the Cape Dwarf Chameleon (*Bradypodion pumilum*) show that chameleons from different habitats [open-canopy (e.g., fynbos) versus closed-canopy (e.g., fragments of forest, riverine thicket, and bushy, exotic vegetation in urban settings)] exhibit different body shapes (Hopkins & Tolley, 2011), enabling them to better utilize their environments (Measey *et al.*, 2009; Herrel *et al.*, 2011; Measey *et al.*, 2011). Similar associations are assumed to exist in other Bradypodion species, particularly within a recent radiation from KwaZulu-Natal (KZN) Province, South Africa (Tolley *et al.*, 2004). The KZN region has the highest alpha diversity of chameleons in southern Africa (Tolley *et al.*, 2008; Tilbury & Tolley, 2009), with seven of the 17 described *Bradypodion* species (Tilbury, 2010; Uetz, 2012); all situated within the Maputaland-Pondoland-Albany biodiversity hotspot (Mittermeier *et al.*, 2004). The majority of these species are found in Afrotemperate forest, and are separated by deep divergences dating back to the Late Miocene. However, one species complex, comprised of two described species

(*Bradypodion melanocephalum* and *Bradypodion thamnobates*) and three additional phenotypic forms (herein referred to as Types A, B and C), appears to have recently radiated (Raw, 1995, 2001; Tolley & Burger, 2007; Tolley *et al.*, 2008; Tilbury, 2010). This radiation may be so recent that it lacks the genetic divergence in mitochondrial markers expected at the species level (Tolley *et al.*, 2004; Tolley *et al.*, 2008), which is an outcome increasingly observed in species complexes as a result of insufficient time having passed for phenotypic differences to be detected in the genic regions routinely used in molecular phylogenetics (e.g., birds: Petren *et al.*, 2005; Grant & Grant, 2008; mammals: Tishkoff *et al.*, 2009; Vonholdt *et al.*, 2010; Wolf *et al.*, 2010; Rheindt *et al.*, 2011; plants: Bateman, James, & Rudall, 2012). Under some species concepts, this lack of (or limited) genetic differentiation would call into question the validity of the two described chameleon species (de Queiroz, 2007), leading some to deduce that the complex is simply comprised of phenotypically plastic forms of a single species. This hypothesis was recently disproven using a common garden experiment, where juveniles from both described species were raised under identical conditions and developed phenotypes similar to their original populations (Miller & Alexander, 2009).

The extent of phenotypic divergence within the *B. melanocephalum*-*B. thamnobates* species complex is striking (Fig. 2.1). *Bradypodion melanocephalum* (Gray, 1865) is small-bodied with a subtle casque, minute gular lobes, homogeneous scales with a few small scattered tubercles on the flanks, and is a dull brown colour. By contrast, *B. thamnobates* (Raw, 1976) has a large heavy body with conspicuous tuberculated scales, a prominent casque, large gular lobes, a bright white gular region, and a rich green colour often with reddish or orange flanks. The other three phenotypic forms have not been confidently assigned to either of these species because of ill-defined genetic and phenotypic boundaries (Tolley & Burger, 2007). Type A appears most similar to

B. melanocephalum in size and colour, leading many to classify it as another population of the species (Tolley *et al.*, 2004; Tilbury, 2010); however, it can be distinguished from *B. melanocephalum* by faint green markings along its flanks and orange along its tail and dorsal crest. Genetically, it has been found to be most similar to *B. thamnobates* (Tolley *et al.*, 2004: fig. 2, samples CT16 & CT17). Type B is large in size with a prominent casque, is bright green in colour with a yellow gular region, and also groups with B. thamnobates genetically (Tolley *et al.*, 2004). Type C has morphological features outwardly similar to *B. thamnobates* (e.g., prominent casque and large gular lobes), although it lacks the striking coloration and heavy, tuberculated body of that species.

The phenotypic diversity within this complex is likely to have arisen from the numerous drastic climatic changes during the Pleistocene, especially during the Last Glacial Maximum (Mucina *et al.*, 2006), which brought upon significant changes in vegetation (Scott, 1993; Eeley *et al.*, 1999; Tyson, 1999; Bond *et al.*, 2003; Mucina & Rutherford, 2006), especially within the forest and grassland biomes (Lawes, 1990; Eeley *et al.*, 1999; Mucina & Geldenhuys, 2006; Rebelo *et al.*, 2006; Lawes *et al.*, 2007). These changes likely created distinct microhabitats (e.g., plant structure, perch size, canopy cover) within which each form had to adapt. Indeed, all five phenotypic forms are allopatric in distribution (Fig. 1.2) and occupy different habitat types (Tolley & Burger, 2007; Tilbury, 2010). *Bradypodion melanocephalum* is found along the coast of southern KZN (0-150 m a.s.l.) in grasses and lowland coastal vegetation, whereas *B. thamnobates* is found inland in the KZN Midlands (850-1600 m a.s.l.), most often in transformed landscapes (exotic trees, bushy shrubs and urban gardens), although their primary habitat is indigenous forest. Types A and C are also localized to the KZN Midlands; however, they are peripatric to *B. thamnobates* and each other. They also occupy different habitat types. Type A is often found in grasslands and around transformed vegetation (including

plantations), whereas Type C is restricted to the Karkloof forest. Lastly, Type B can be found along the southern Drakensberg mountain range up to 2000 m a.s.l. in both indigenous forest and grasslands.

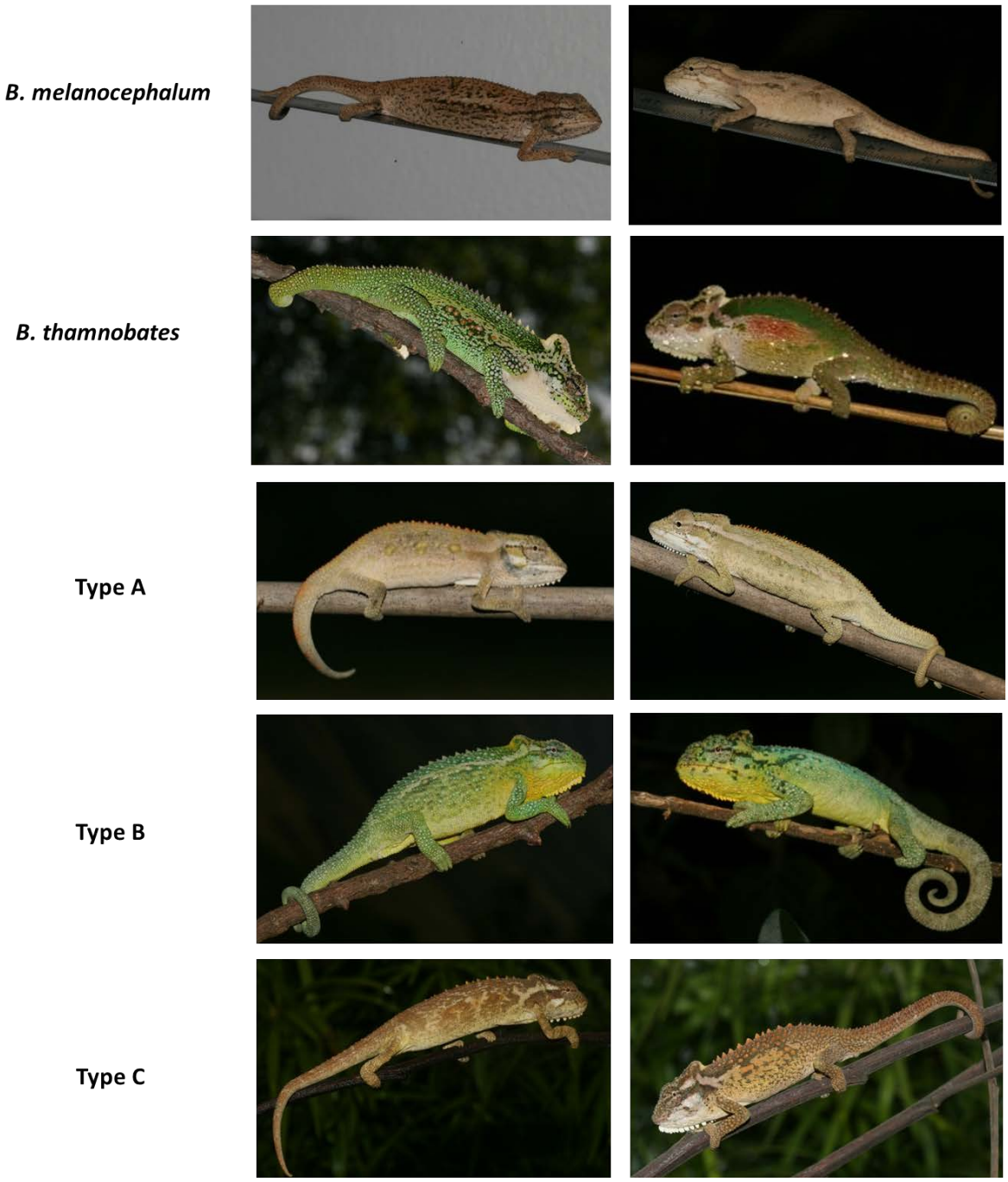

Figure 2.1 Photographs of female (left) and male (right) dwarf chameleons within the *B. melanocephalum- B. thamnobates* complex. Photos by K. A. Tolley.

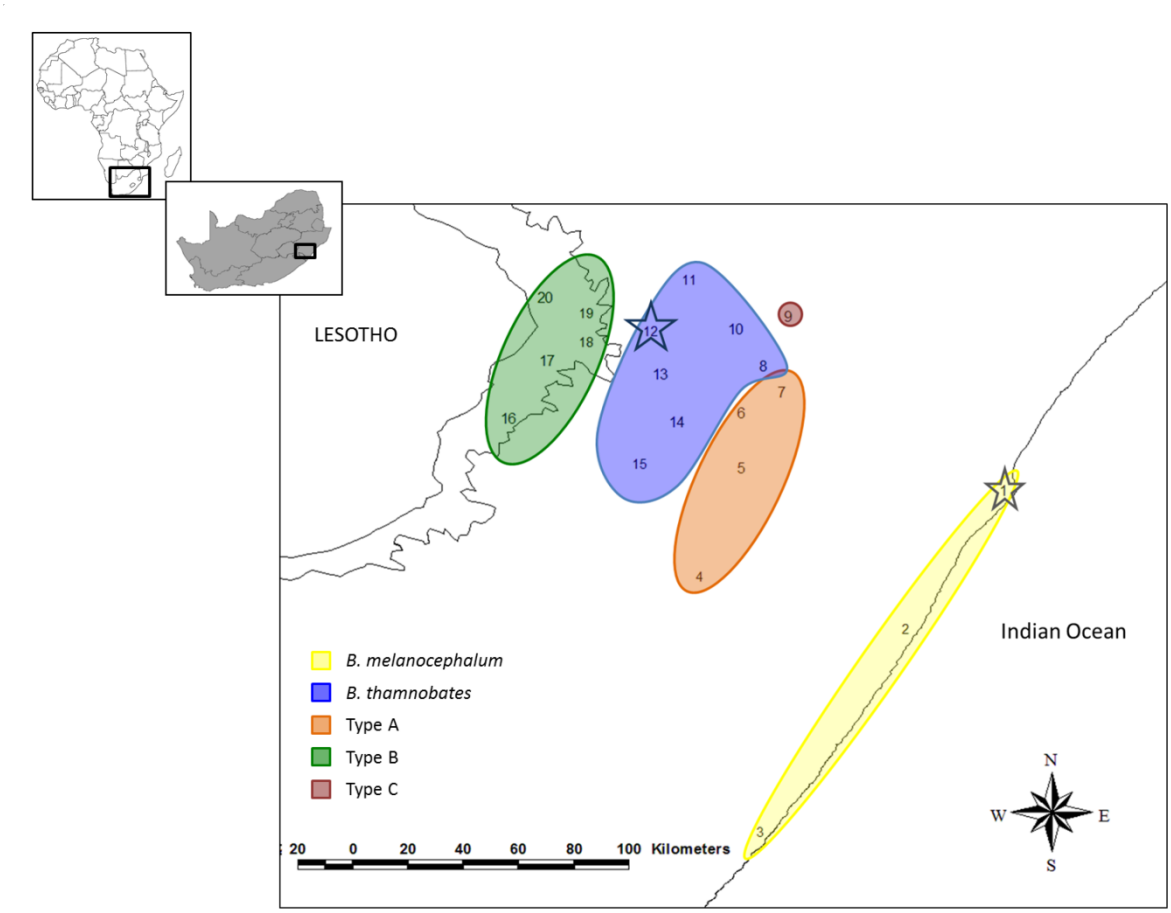

Figure 2.2 Map illustrating the general distributions of the five phenotypic forms within the *B. melanocephalum-B. thamnobates* species complex (two species, three morphotypes) and the 20 sampling sites within southern KZN, South Africa. 1-Durban; 2-Pennington; 3-Umtamvuna; 4-Ixopo; 5-Bryne Valley; 6-Stirling Farm; 7-Hilton; 8-Howick; 9-Karkloof; 10-Boschhoek; 11-Mooi River; 12-Nottingham Road; 13-Dargle; 14-Boston; 15-Bulwer;16-Sani Pass; 17-Lotheni; 18-Kamberg; 19-Giant's Castle; 20-Highmoor. Stars represent the type localities for the two described species. Contour line to the left of Lesotho delimits the Drakensberg mountain range.

To test whether habitat structure is a likely driving force of morphological variation in the *B. melanocephalum-B. thamnobates* species complex, we investigated whether tangible morphological differences exist between the five phenotypic forms (apart from overall colour and size) by comparing ecologically relevant morphological traits (i.e., limb and tail length, foot size, head shape). We also aimed to quantify and compare the microhabitat structure of each form and investigate whether the structure of vegetation reflects differences in their morphology. We hypothesize that any of the forms occupying significantly different microhabitats (i.e., perch dimensions) will show corresponding differences for traits that are ecologically relevant for chameleons (Herrel *et al.*, 2011; Herrel *et al.*, 2013).

MATERIAL AND METHODS

STUDY SITES AND SAMPLING PROCEDURES

A total of 351 dwarf chameleons within the *B. melanocephalum-B. thamnobates* complex were sampled from 20 sites throughout southern KZN (Fig. 2.2) in 2009 and 2010. Tail clips were collected as DNA samples for a separate study and served as batch marks to ensure that no individual would be sampled twice. Males were identified by the presence of hemipenal bulges or by the eversion of hemipenes. The snout–vent length (SVL) for each was recorded and the smallest SVL was noted for each phenotypic form (Table 2.1). Chameleons were identified as female if they were larger than the smallest male for that form and showed no sign of hemipenes. Individuals smaller than this with no sign of hemipenes were classified as juveniles and therefore left out of the study. Once all morphometric measurements were taken, chameleons were released at the exact point of capture.

Table 2.1 Measures of snout-vent length (SVL) for males within each phenotypic form.

	SVL (mm)		
Morph	**Minimum**	**Maximum**	**Mean**
B. melanocephalum	37.82	60.22	48.76
B. thamnobates	40.80	84.02	62.63
Type A	38.07	60.72	48.65
Type B	45.13	80.60	68.10
Type C	38.23	65.74	50.63

MORPHOMETRIC ANALYSIS

All chameleons were measured to the nearest 0.01 mm using digital callipers for 11 body and nine head measurements (Fig. 2.3): Body – SVL, interlimb length (ILL), tail length (TL), thigh length (ThL), crus length (CL), brachium length (BL), antebrachium length (AL), medial forefoot pad length (MF), lateral forefoot pad length (LF), medial hindfoot pad length (MH), and lateral hindfoot pad length (LH); Head – lower jaw length (LJL), head length (HL), casque head length (CHL), head width (HW), head height (HH), casque head height, casque height, coronoid process of mandible to snout tip (CT), and posterior surface of quadrate to snout tip (QT). Measurements were taken on the right side of the head and body for consistency. If this was not possible because of injury or disfigurement, the left side was used and noted. The mass of each chameleon was also measured using a Pesola micro-line spring scale (model 93010).

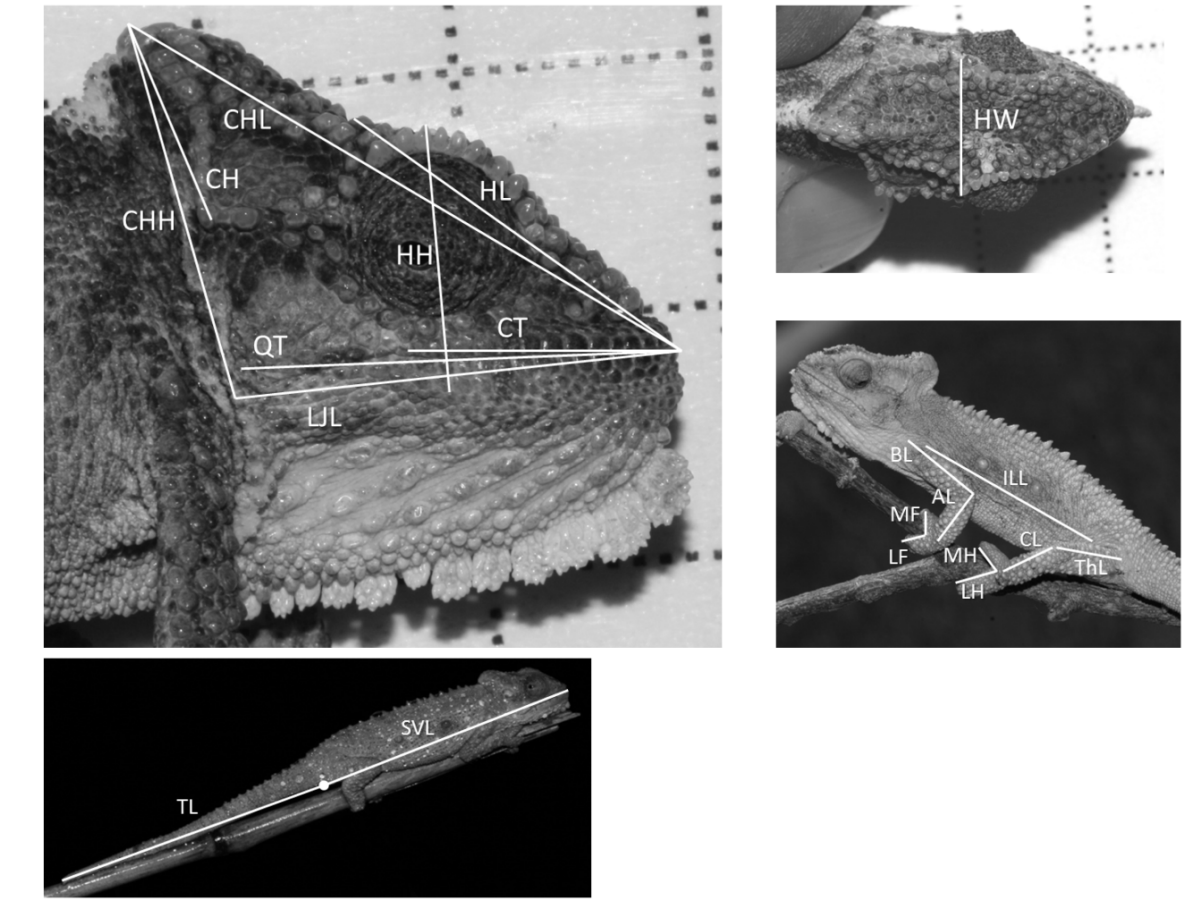

Figure 2.3 Twenty measurements recorded for each chameleon. Nine head measurements: CHL (casque head length), HL (head length), head height (HH), CHH (casque head height), CH (casque height), LJL (lower jaw length), CT (coronoid process of mandible to snout tip), QT (posterior surface of quadrate to snout tip), and HW (head width). Eleven body measurements: SVL (snout-vent length), TL (tail length), ILL (interlimb length), BL (brachium length), AL (antebrachium length), MF (medial forefoot pad length), LF (lateral forefoot pad length), ThL (thigh length), CL (crus length), MH (medial hindfoot pad length), and LH (lateral hindfoot pad length).

All analyses were carried out using SPSS, version 9.0 (SPSS Inc.). All data were log10 transformed prior to analysis to fulfil assumptions of normality and homoscedascity. To separate differences in shape from differences in body size, all data were size-corrected against $\log_{10}$SVL and the unstandardized residuals were saved for use in subsequent

analyses. The appropriateness of SVL as a common estimate of overall body size was tested using a principal component analysis (PCA) on all log_{10}-transformed data and a linear regression comparing the ratio of logILL and logLJL (both components of SVL) against logSVL. The PCA was used to examine whether variables could be accurately described using a single common measure of size (Kratochvíl *et al.*, 2003; McCoy *et al.*, 2006) and the regression was used to test whether the head and body experienced different growth trajectories between sexes (Braña, 1996). All variables fell within one principal component (PC), and the linear regression showed that head and body measurements followed similar trajectories, thereby validating the use of log_{10}SVL as a suitable covariate for all measurements.

Sexual dimorphism

A multivariate analysis of covariance (MANCOVA) using a custom general linear mode was carried out to test the equality of slopes between sexes and forms. The full model specified Sex and Form as fixed factors, Sex x Form as the interaction, log_{10}SVL as the covariate, and all other log_{10}-tranformed variables as the dependent variables (excluding LJL and ILL since they are components of SVL). A significant interaction between Sex and Form implies that slopes are intersecting (unequal) and the effect of size is sex dependent across phenotypic forms; therefore, no further analyses could be conducted to test the hypotheses as the results would not be comparable. A significant Sex effect suggests that sexes are different and should be analysed separately. For variables detected as being sexually dimorphic (see Results), a second MANCOVA based on a full factorial model was run separately by form to examine the sexually dimorphic differences between them. All *P*-values were subjected to Holm's sequential Bonferroni (Holm, 1979) correction to minimize the possibility of Type I errors (Rice, 1989).

Differences between and within phenotypic forms

To examine differences between the five phenotypic forms within the *B. melanocephalum-B. thamnobates* complex, a PCA on the unstandardized residuals for each variable was conducted. This was conducted on group linear combinations (correlated sets) of the original variables for ease of use in the subsequent analysis. The Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy and Bartlett's test of sphericity were run to determine the appropriateness of the PCA. The strength of the relationships detected in the PCA are considered strong when the KMO score is greater than 0.6 and Bartlett's test is significant, rejecting the hypothesis of an identity matrix. PC scores were saved so that the magnitude and direction of the eigenvector describing the differences between forms could be illustrated. Only PCs with eigenvalues larger than one were extracted, and the varimax rotation was used to minimize the number of variables with high loadings on each factor. Variables with communality values less than 0.5 where ignored from the analysis because low values indicate those variables are uninformative (Tabachnick & Fidell, 2007). The saved PC scores were then entered as the dependent variables in a MANOVA, with Form as the fixed factor. Bonferroni post-hoc tests were run to determine which forms differed for each PC. To ensure differences (or the lack thereof) between forms were genuine and not influenced by population level differences within them, data were split by Sex and Form and then a MANOVA, with Site (i.e., individual field sites) as the fixed factor and all size-corrected variables as the dependant variables, was carried out. For significant Site effects, a sequential Bonferroni correction was applied to all variables.

HABITAT ANALYSIS

Because the vegetation varied considerably throughout the study area, an examination of the micro- and macrohabitat structure available to chameleons was carried out. Although

all chameleon sampling was conducted at night as a result of an ease of locating them, it was assumed that night-time perches reflect day-time habitat use because this has been found in preliminary radio-tracking data on *B. pumilum* (K. Tolley & E. Katz, unpub. data). Therefore, the habitat at each sampling site was surveyed the subsequent day. Macrohabitat type and percent canopy cover were measured within a 2 m diameter circle around where each chameleon was found. Percentage canopy cover was measured at ground level using a spherical densiometer, and arranged into one of five categories: (1) 0–10%, (2) 11–25%, (3) 26–50%, (4) 51–75%, and (5) 76–100%. Category 1 is representative of grassland habitats with a very open or no canopy; whereas category 5 would be considered dense forest. From the plant on which each chameleon was found, plant type, perch height, and perch diameter were recorded in order to quantify microhabitat. Once the mean perch heights were determined for each form, field sites were re-visited to assess the density of available perches in each habitat and whether actual microhabitat use differed from microhabitat that is randomly available to the chameleons. Two 99 m transects were laid out, each made up of ten 1 m long segments separated at 10 m intervals, and the numbers and diameters of all perches that touched a 1 m long stick at the determined mean height were recorded (Herrel *et al.*, 2011). Although the two transects per sample site do not cover the entire distributional range of a given form, they are representative of the areas from which the chameleons were sampled.

Differences between forms in the categorical variables (i.e., habitat type, percent canopy cover and plant type) were explored using bar plots. Data for perch height and diameter were log10 transformed to fulfil assumptions of normality and homoscedascity. Data were then compared using two-sampled Kolmogorov–Smirnov nonparametric tests or analyses of variance (ANOVA). In the ANOVAs, Bonferroni post-hoc tests were run concurrently to highlight any pairwise differences.

RESULTS

The initial MANCOVA showed significant morphological differences between sexes (Wilks' $\lambda = 0.585$, $F_{18,72} = 12.725$, $P < 0.0001$) and forms (Wilks' $\lambda = 0.731$, $F_{18,72} = 1.464$, $P < 0.0001$). Although the interaction effect was found to be significant (Wilks' $\lambda = 0.737$, $F_{18,72} = 1.386$, $P = 0.008$), an examination of the between-subjects effects, after sequential Bonferroni correction, revealed no significant differences for any variables within the interaction. Accordingly, the assumption of equal slopes was not violated and any differences between sexes and forms could be compared in subsequent analyses.

SEXUAL DIMORPHISM

Overall, females exceeded males in mass and SVL (Table 2.2); however, when all variables were corrected for size, sexual dimorphism was detected in ten variables (Body: TL, MF, LF, MH, LH; Head: CHL, HL, HH, QT, CT) with males relatively larger than females. The degree of dimorphism differed between the five forms, with B. thamnobates being dimorphic for all ten variables and Type B exhibiting no detectable dimorphism (Table 2.3). All four dimorphic forms showed dimorphism for TL, with HH and MH also exhibiting dimorphism for B. melanocephalum and Type A, respectively. Because sexual dimorphism was detected within the *B. melanocephalum-B. thamnobates* complex, all subsequent analyses were conducted separately by sex.

Table 2.2 Mean morphological and habitat data for male (M) and female (F) dwarf chameleons used in this study, grouped by phenotypic form. Standard error shown in brackets.

	B. melanocephalum		Type A		*B. thamnobates*		Type B		Type C	
	M	F	M	F	M	F	M	F	M	F
					Ecology					
N	35	29	32	35	38	65	16	16	9	12
Perch height (m)	1.39 (0.19)	0.75 (0.09)	1.63 (0.12)	1.51 (0.06)	2.35 (0.32)	2.35 (0.21)	3.9 (0.40)	1.35 (0.22)	2.33 (0.17)	2.45 (0.15)
Perch diameter (mm)	1.68 (0.12)	2.05 (0.15)	1.59 (0.15)	1.88 (0.15)	2.21 (0.14)	1.98 (0.10)	2.39 (0.17)	2.05 (0.21)	1.55 (0.20)	1.76 (0.20)
					Morphology					
N	46	29	32	46	57	87	22	16	9	12
Mass (g)	2.15 (0.08)	4.39 (0.22)	2.54 (0.14)	3.5 (0.32)	6.73 (0.45)	8.65 (0.61)	7.82 (0.57)	11.4 (1.47)	3.27 (0.64)	4.28 (0.92)
SVL (mm)	48.76 (0.75)	56.29 (0.78)	48.65 (1.09)	50.86 (1.39)	62.63 (1.57)	66.50 (1.62)	68.10 (1.31)	71.55 (3.46)	50.63 (3.70)	51.5 (3.72)
TL (mm)	54.01 (0.96)	52.83 (0.88)	53.77 (1.29)	49.08 (1.09)	68.61 (1.90)	64.59 (1.75)	77.78 (2.02)	77.80 (4.47)	56.08 (4.43)	49.84 (3.38)
ILL (mm)	28.16 (0.73)	33.28 (0.60)	28.03 (0.93)	30.27 (1.08)	37.20 (2.20)	42.57 (2.02)	43.64 (1.00)	48.83 (3.96)	29.48 (1.01)	30.54 (3.13)
BL (mm)	9.15 (0.18)	9.94 (0.16)	9.32 (0.24)	9.57 (0.26)	11.49 (0.34)	11.69 (0.33)	13.77 (0.42)	13.84 (0.83)	8.84 (0.50)	9.54 (0.68)
AL (mm)	7.71 (0.15)	8.38 (0.12)	8.03 (0.21)	8.23 (0.20)	10.29 (0.31)	10.29 (0.26)	11.50 (0.24)	11.92 (0.63)	7.60 (0.50)	7.86 (0.66)
MF (mm)	4.29 (0.10)	4.73 (0.10)	4.56 (0.12)	4.65 (0.11)	6.30 (0.17)	6.11 (0.16)	7.03 (0.14)	7.11 (0.41)	5.31 (0.43)	4.79 (0.31)
LF (mm)	5.22 (0.11)	5.50 (0.11)	5.44 (0.10)	5.47 (0.11)	7.43 (0.22)	7.35 (0.19)	8.39 (0.29)	8.43 (0.43)	5.91 (0.45)	5.50 (0.33)
ThL (mm)	8.82 (0.15)	9.54 (0.18)	9.22 (0.24)	9.60 (0.26)	10.98 (0.32)	11.35 (0.31)	12.96 (0.38)	13.69 (0.76)	8.96 (0.80)	8.88 (0.59)
CL (mm)	7.50 (0.11)	8.35 (0.12)	7.83 (0.20)	8.16 (0.23)	10.00 (0.19)	10.14 (0.28)	11.17 (0.24)	11.52 (0.56)	7.79 (0.60)	7.76 (0.52)
MH (mm)	4.06 (0.08)	4.44 (0.09)	4.47 (0.12)	4.23 (0.11)	6.01 (0.19)	6.04 (0.16)	6.77 (0.17)	7.11 (0.48)	4.69 (0.38)	4.22 (0.33)
LH (mm)	5.48 (0.09)	5.67 (0.11)	5.48 (0.14)	5.62 (0.14)	7.58 (0.23)	7.59 (0.20)	8.74 (0.25)	8.82 (0.55)	5.85 (0.47)	5.65 (0.30)
LJL (mm)	10.87 (0.15)	11.86 (0.15)	11.06 (0.20)	11.35 (0.24)	14.26 (0.31)	14.25 (0.28)	15.01 (0.28)	15.13 (0.68)	11.69 (0.72)	11.95 (0.83)
CHL (mm)	16.14 (0.24)	17.14 (0.23)	16.30 (0.29)	16.72 (0.34)	22.18 (0.49)	21.82 (0.45)	22.49 (0.46)	22.72 (1.06)	17.35 (1.16)	17.12 (1.24)
HL (mm)	11.50 (0.17)	12.02 (0.19)	11.53 (0.19)	11.81 (0.22)	14.28 (0.30)	14.21 (0.30)	14.27 (0.28)	14.69 (0.58)	12.12 (0.56)	11.83 (0.75)
CHH (mm)	9.34 (0.15)	10.41 (0.21)	9.80 (0.20)	10.09 (0.25)	14.62 (0.41)	14.55 (0.36)	15.09 (0.40)	15.69 (0.92)	10.51 (0.77)	11.04 (0.98)
HH (mm)	6.68 (0.10)	6.93 (0.13)	7.06 (0.11)	7.09 (0.16)	9.07 (0.20)	8.90 (0.18)	9.59 (0.20)	9.79 (0.43)	7.37 (0.48)	7.19 (0.46)
HW (mm)	7.19 (0.08)	7.66 (0.12)	7.30 (0.13)	7.61 (0.16)	10.27 (0.26)	10.14 (0.22)	10.66 (0.26)	10.88 (0.52)	8.19 (0.61)	8.32 (0.55)
CH (mm)	4.31 (0.12)	4.98 (0.12)	4.80 (0.14)	4.95 (1.75)	7.97 (0.25)	7.89 (0.21)	7.78 (0.24)	8.58 (0.49)	5.40 (0.50)	5.42 (0.55)
CT (mm)	8.48 (0.11)	9.32 (0.11)	8.73 (0.15)	8.93 (0.19)	11.08 (0.24)	11.03 (0.21)	11.55 (0.19)	11.52 (0.50)	9.09 (0.49)	9.18 (0.52)
QT (mm)	9.76 (0.13)	10.58 (0.14)	9.97 (0.18)	10.07 (0.24)	12.98 (0.30)	12.92 (0.26)	13.51 (0.24)	13.54 (0.55)	10.38 (0.82)	10.52 (0.64)

SVL, snout-vent length; TL, tail length; ILL, interlimb length; BL, brachium length; AL, antebrachium length; MF, medial forefoot pad length; LF, lateral forefoot pad length; ThL, thigh length; CL, crus length; MH, medial hindfoot pad length; LH, lateral hindfoot pad length; LJL, lower jaw length; CHL, casque head length; HL, head length; CHH, casque head height; HH, head height; HW, head width; CH, casque height; CT, coronoid process of mandible to snout tip; QT, posterior surface of quadrate to snout tip

Table 2.3 *F*-values resulting from MANCOVA for sexual dimorphism in morphology for all five forms in the *B. melanocephalum-B. thamnobates* complex. Significance levels after sequential Bonferroni correction: ***$P < 0.0001$, ** $P < 0.01$, *$P < 0.05$.

		F-value				
		B. melanocephalum	*B. thamnobates*	Type A	Type B	Type C
Body	TL	73.254***	147.058***	62.463***	6.950	22.254***
	MH	0.229	4.524*	9.664**	0.997	7.707
	LH	5.370	8.401**	0.015	2.366	1.291
	MF	0.304	20.170***	0.027	2.416	5.208
	LF	5.713	10.656**	0.572	2.158	3.170
Head	CHL	5.894	36.077***	0.033	4.021	1.132
	HL	2.533	9.004**	0.001	0.005	1.816
	HH	16.523***	26.602***	3.775	1.202	1.438
	CT	0.030	19.317***	0.280	5.606	0.052
	QT	2.099	28.031***	3.040	3.251	0.110

TL, tail length; MH, medial hindfoot pad length; LH, lateral hindfoot pad length; MF, medial forefoot pad length; LF, lateral forefoot pad length; CHL, casque head length; HL, head length; HH, head height; CT, coronoid process of mandible to snout tip; QT, posterior surface of quadrate to snout tip

Multivariate Analysis of Forms

Differences between phenotypic forms

The PCA was found to be appropriate for both sexes (KMO > 0.85; Bartlett's test: $P < 0.0001$), with four PCs extracted for each sex (Table 2.4). These PCs accounted for 68% and 64% of the total variance between forms for females and males, respectively, of which the head (including casque) made up the majority (females: 41.02%; males: 51.37%).

For females, PC1 correlated highly with head dimensions, PC2 feet and tail, PC3 limbs, and PC4 with head length (Fig. 2.4, left). MANOVA revealed PCs 1–3 to be significantly different between forms ($F = 10.032–17.123$, $P < 0.0001$). *Bradypodion melanocephalum* was typically found to have the smallest features for all PCs, whereas *B. thamnobates* possessed a relatively larger head (including casque) and Type B,

proportionally, the longest limbs and tail, as well as the largest feet. Types A and B showed similarities in head and limb shape. The three Midlands forms (*B. thamnobates*, Types A and C) were very similar in morphology. Indeed, no differences were observed between the females of *B. thamnobates* and Type C for all PCs. However, some morphological distinctions were found with respect to Type A and the other Midlands forms, with Type A having a smaller head and longer limbs.

For males, TB and RD were excluded because they were uninformative as indicated by their communality values. PC1 possessed positive loadings for casque measurements, PC2 for the remaining head measurements, PC3 for feet, and PC4 for the remaining limb measurements and tail length (Fig. 2.4, right). All four PCs showed significant differences between forms (F = 7.358–11.941, $P < 0.0001$). Males displayed a similar pattern to the females, with B. melanocephalum turning out to have the smallest features for all but one PC (PC4), *B. thamnobates* having the largest casque and head, and Type B having the largest body (feet, limbs and tail). *Bradypodion melanocephalum* and Types A and B were found to possess similarly small casques, yet large limbs and tails. Type C proved to be fairly intermediate in head and casque shape, showing no significant differences between it and the other forms, although it did possess significantly larger feet than B. melanocephalum and had the shortest limbs and tail overall.

Site differences within forms

Type C comprised individuals from a single site; therefore, it was not included in this analysis. Of the four remaining forms, only *B. thamnobates* was found to have site-specific differences for both sexes (females: Wilks' $\lambda = 0.116$, $F_{5,85} = 2.104$, $P < 0.0001$; males: Wilks' $\lambda = 0.037$, $F_{5,85} = 1.946$, $P < 0.0001$), all involving head shape. Females were found to differ in HW and HH, and males in HL and HH. For both sexes, differences

in HH involved two sites [Boston (site 14: Fig. 2.2) and Bulwer (site 15: Fig. 2.2)], with individuals from these localities typically having shorter heads than the other *B. thamnobates* sites. Female dwarf chameleons from Boston were also found to have narrower heads, whereas Boston males possessed longer head lengths compared to the other sites.

Table 2.4 Results examining differences between forms for both sexes. PC loadings displayed according to size with the percentage of variance explained by each component. Bold values highlight variables representing a particular PC. *F*- and *P*- values calculated from MANOVA on PC scores.

	Females					Males			
	PC1	PC2	PC3	PC4		PC1	PC2	PC3	PC4
QT	**0.812**	-0.000	0.083	0.073	CHL	**0.850**	0.334	0.226	0.061
CT	**0.755**	0.049	0.195	0.033	HL	**0.779**	-0.030	0.015	0.182
CHL	**0.752**	0.219	0.041	0.504	CHH	**0.760**	0.302	0.271	0.038
CH	**0.732**	0.354	-0.162	0.049	CH	**0.755**	0.355	0.201	-0.106
HW	**0.715**	0.306	0.163	0.042	HW	0.539	**0.539**	0.254	0.019
HH	**0.707**	0.230	0.284	0.028	CT	0.295	**0.732**	0.152	0.022
CHH	**0.703**	0.435	-0.016	0.241	QT	0.351	**0.721**	0.161	0.060
LF	0.325	**0.756**	0.130	0.082	HH	0.418	**0.578**	0.316	-0.037
MF	0.113	**0.748**	0.156	0.079	MF	0.128	0.264	**0.794**	0.040
LH	0.321	**0.696**	0.263	0.031	LF	0.193	0.134	**0.727**	0.145
MH	0.402	**0.613**	0.254	0.009	MH	0.155	0.180	**0.655**	0.234
TL	-0.000	**0.545**	0.376	0.260	LH	0.263	0.071	**0.567**	0.492
CL	0.276	0.112	**0.787**	-0.133	BL	0.042	-0.125	0.236	**0.784**
ThL	-0.056	0.284	**0.749**	0.207	ThL	0.006	-0.073	0.166	**0.754**
AL	0.306	0.194	**0.739**	0.074	TL	0.100	0.210	-0.068	**0.630**
BL	-0.149	0.376	**0.566**	0.409					
HL	0.279	0.103	0.126	**0.862**					
% variance	41.02	13.79	6.87	6.04	% variance	37.10	14.27	6.55	6.19
F	50.72	33.54	49.89	1.44	*F*	37.75	33.41	36.53	25.50
P	<0.001	<0.001	<0.001	0.841	*P*	<0.001	<0.001	<0.001	<0.001

TL, tail length; BL, brachium length; AL, antebrachium length; MF, medial forefoot pad length; LF, lateral forefoot pad length; ThL, thigh length; CL, crus length; MH, medial hindfoot pad length; LH, lateral hindfoot pad length; CHL, casque head length; HL, head length; CHH, casque head height; HH, head height; HW, head width; CH, casque height; CT, coronoid process of mandible to snout tip; QT, posterior surface of quadrate to snout tip

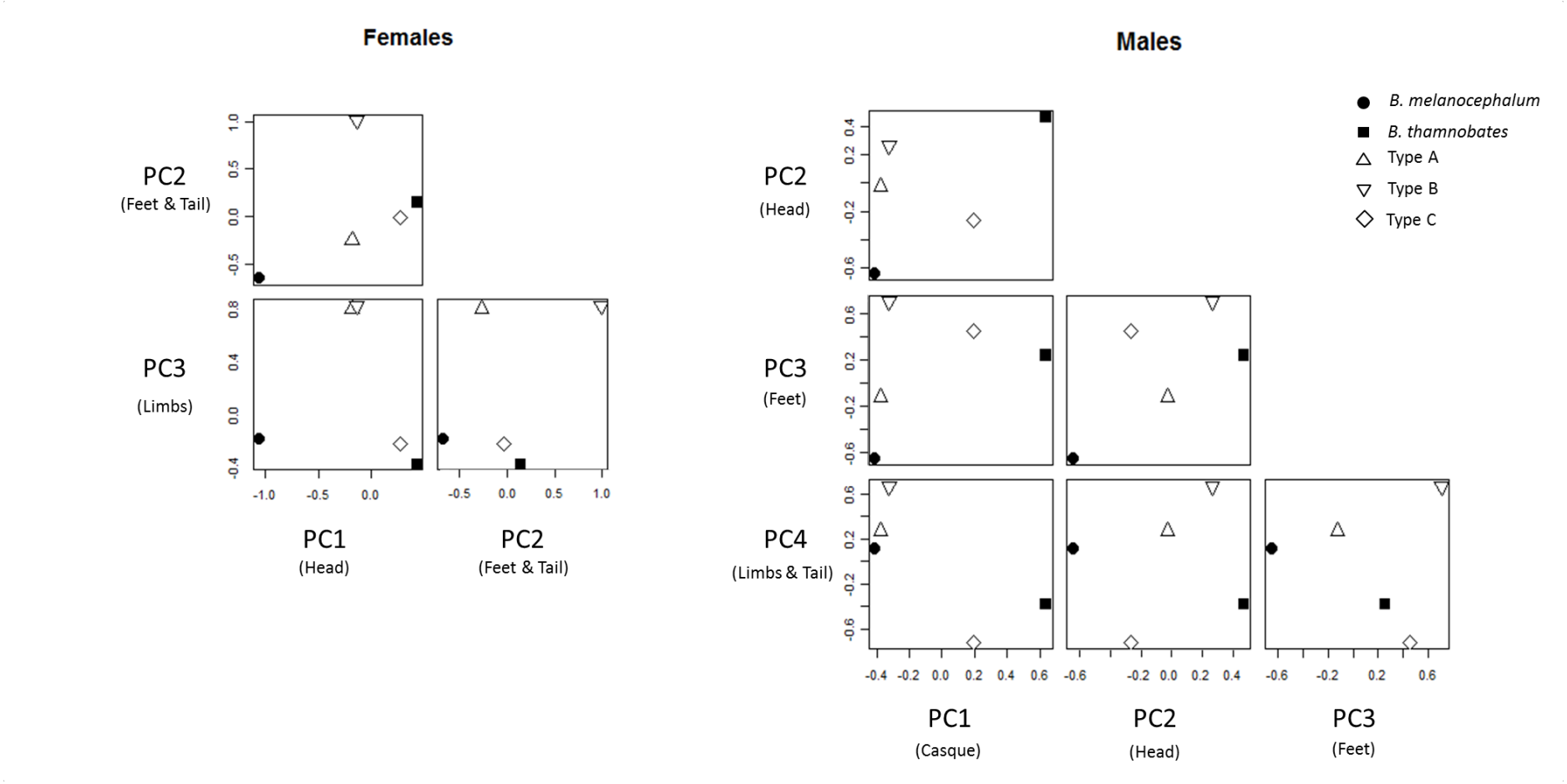

Figure 2.4 Matrix plots of average principal component (PC) scores for female and male dwarf chameleons within the *B. melanocephalum-B. thamnobates* complex. The 17 size-corrected morphometric variables were assigned to three principal components for females, and four for males. Note: PC1 for females also includes casque.

HABITAT ASSOCIATIONS

Differences between sexes and forms were observed in a variety of habitat variables. In the categorical variables percent canopy cover, plant type, and habitat type (Fig. 2.5), females tended to occupy more open-canopy habitats compared to males for all five forms, often perching on grass and herbaceous plants. Between forms, *B. melanocephalum* and Type A were more readily found in open-canopy habitats compared to the others. *Bradypodion melanocephalum* was typically found in degraded grasslands; however, some individuals were located in and around coastal forests. Type A was typically found along the edges of exotic plantations (primarily *Eucalyptus* spp.) on both trees and grasses and in degraded grasslands. Types B and C tended to occupy denser canopied habitats, often found in trees or shrubs within forests and gardens for Type B or along road verges for Type C. The presence of Type C only along road verges is likely the result of sampling bias because of difficulty accessing the forest. *Bradypodion thamnobates* was more variable in its cover and plant choice, likely as a result of the majority of individuals sampled being found in trees and shrubs in urban or semi-urban settings.

The number of random perches was found to differ between macrohabitats ($F = 16.097$, $P < 0.0001$), with the habitats of *B. melanocephalum* and Type A being far denser than the other three forms (Table 2.5). When examining microhabitat, the associated perch diameters from each habitat also differed ($F = 61.501$, $P < 0.0001$), with the perches in the *B. melanocephalum* habitat being much narrower (1.77 ± 1.96 mm) and those in the habitat of Type B being notably wider (4.04 ± 1.53 mm) than the other habitats. Within a habitat, the random perch diameters from the two transects differed only for the habitat of Type B (Transect 1: 3.57 ± 1.47 mm; Transect 2: 4.48 ± 1.47 mm; $Z = 2.149$, $P < 0.0001$). When comparing the random versus the actual perches used by each phenotypic form, *B. thamnobates* and Type C were found to use their microhabitats in

a random fashion (*B. thamnobates*: $F = 0.000$, $P = 0.986$; Type C: $F = 3.257$, $P = 0.072$), whereas *B. melanocephalum* chose wider perches than randomly available ($F = 9.055$, $P = 0.003$) and Types A and B narrower perches on average (Type A: $F = 6.199$, $P = 0.014$; Type B: $F = 3.261$, $P < 0.0001$). When comparing the actual perch diameters used by each form, no sex-specific differences were found ($F = 0.378$, $P = 0.536$); however, significant differences between forms were discovered ($F = 4.868$, $P = 0.001$), which were attributed to Type A using narrower perches than *B. thamnobates* and Type B. Differences in perch height were detected between forms ($F = 16.126$, $P < 0.0001$) and sexes ($F = 5.885$, $P = 0.017$), with *B. melanocephalum* occupying significantly lower perches compared to the other forms, and the males of each form perching higher than females; however, this was only significant within *B. melanocephalum* ($F = 4.769$, $P = 0.036$) and Type B ($F = 37.031$, $P < 0.0001$).

Table 2.5 Number of available perches in each of the two transects laid out within the habitats of each phenotypic form.

Phenotypic form	Transect		Average per meter
	1	2	
B. melanocephalum	165	141	15.45
B. thamnobates	99	105	10.20
Type A	136	147	14.15
Type B	87	92	8.95
Type C	95	119	10.7

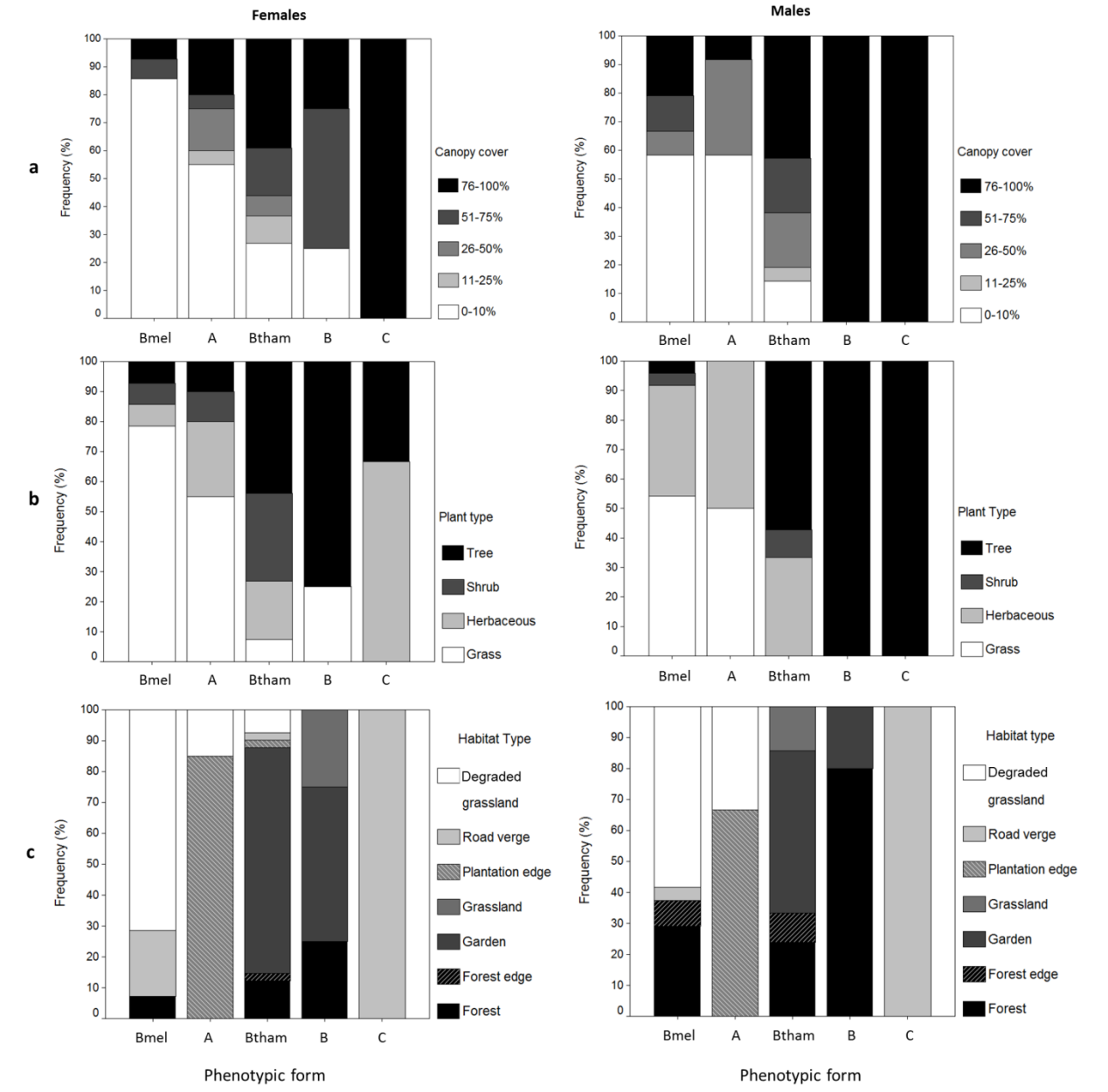

Figure 2.5 Barplots illustrating the proportion of (a) percent canopy cover, (b) plant type and (c) habitat type utilised by female and male dwarf chameleons within the *B. melanocephalum*-*B. thamnobates* complex. Bmel, *B. melanocephalum*; A, Type A; Btham, *B. thamnobates*; B, Type B; C, Type C.

DISCUSSION

Chameleons from the morphologically diverse clade of *Bradypodion* in KwaZulu-Natal Province have five distinct morphological forms, despite a lack of genetic differentiation for mitochondrial markers (Tolley *et al.*, 2004; Tolley *et al.*, 2008). The forms differ for ecologically relevant traits associated with microhabitat structure (perch dimensions), particularly with respect to hand/foot size, limbs, and tail. It is likely that differences in morphology between the forms are a result of a balancing of selection pressures (natural and sexual) closely associated with their microhabitat, suggesting that this is at least one mechanism that triggers phenotypic divergence, despite a lack of genetic divergence.

SEXUAL SIZE AND SHAPE DIMORPHISM

Overall, females were found to be larger than males in mass and body length for all five forms, which is not unexpected given that female-biased sexual size dimorphism has been documented in other chameleon species (Hebrard & Madsen, 1984; Reaney *et al.*, 2012), including *Bradypodion* (Stuart-Fox *et al.*, 2006a; Stuart-Fox, 2009; Hopkins & Tolley, 2011). In reptiles, sexual dimorphism is often attributed to sexual selection, resource partitioning and/or fecundity advantage (Shine, 1979; Fitch, 1981; Shine, 1988; Olsson *et al.*, 2002). Considering male dwarf chameleons display to females to entice mating and not vice versa (Stuart-Fox & Whiting, 2005; Tolley & Burger, 2007; Stuart-Fox & Moussalli, 2008), sexual selection in the form of female competition for males is unlikely to explain female-biased sexual size dimorphism in this species complex. However, larger female dwarf chameleons do have more offspring than smaller ones likely as a result of their larger abdominal cavity (Burrage, 1973), and the high energy demands of reproduction often require female lizards to consume more and/or different prey than males (Shine,

1989), making fecundity advantage and resource partitioning, respectively, probable explanations.

Once size was removed from all comparisons between sexes, males were relatively larger for many of the characters examined, in four of the five forms. Bradypodion thamnobates exhibited the largest relative differences, possibly indicating greater male-male competition. Tail length was found to differ in all sexually dimorphic forms, with the males having longer tails than females; typical of reptiles and of other chameleon species (Fitch, 1981; Tilbury, 2010; Hopkins & Tolley, 2011). Some possible explanations for this dimorphism include female mate choice (relative tail length may attest to a male's overall fitness), longer mating times (longer tails may allow males to better grasp their perches or females when mating, thereby enabling them to mate longer and deliver more sperm, ultimately allowing them to sire more offspring: Hofmann & Henle, 2006), and/or males simply requiring longer tails to accommodate their hemipenes (Shine *et al.*, 1999). To resolve which is the most likely explanation, a broad comparative study of the relationship between mating patterns, effective reproductive output, sexual dimorphism in tail length (and possibly width), and performance capability of the tail should be conducted.

Considering sexual selection and niche divergence are the primary explanations for sexual dimorphism in organisms (Shine, 1989), its absence may be equally associated. As such, the sexes of Type B may experience little to no sexual selection or niche divergence. However, differences in habitat structure were detected in this form, with males perching higher and occupying more closed-canopy habitats than females. If these differences are sufficiently substantial, then reduced sexual selection may be an explanation. An alternative explanation is that the number of individuals sampled is too low to allow detection of any significant differences between sexes (ß = 68%), making it difficult to draw any solid conclusions regarding the lack of sexual dimorphism in this form.

ECOMORPHOLOGICAL VARIATION

Head shape explained the vast majority of variation between forms, with casque dimensions being the predominant distinction between male forms. As with many lizards, chameleons use their heads to signal to rivals, be they conspecifics or predators, indicating that conflicts can be harmful and, on occasion, fatal. Considering *B. thamnobates* was the most ornamented for both males and females (i.e., having proportionally larger head and casque dimensions), the need to communicate with conspecifics is likely strongest in this form, followed closely by Type C. Accordingly, the diminutive head features of *B. melanocephalum* and Type A, coupled with their habitats being more visually open to predators (at least avian predators), likely indicates that the need to communicate to conspecifics is outweighed by the need to avoid predation. Surprisingly, the casque of Type B males was proportionally comparable to the open-canopy forms, yet these chameleons were found in forested environments, where detection from predators is assumed to be minimal and communication with conspecifics is key. As noted above, this result may be a consequence of limited sample size, or the reduced casque may indicate that they too utilize open-canopy environments and thus are subject to increased predation pressure. A typical forest patch in the southern Drakensberg is surrounded by vast grasslands and, although nothing is known about their movements, an individual would need to traverse the grasslands to reach the next forest patch. This association between open- and closed- canopy habitats and the balancing of selection pressures (i.e., natural: to avoid predation; sexual: to signal and acquire mates), has also been suggested in two phenotypic forms of the Cape dwarf chameleon, *B. pumilum* (Hopkins & Tolley, 2011), and lends support to the hypothesis that these chameleons are adapting to their microhabitats.

Further evidence that these chameleons are adapting to their microhabitats can be gleaned from their feet and tail. These traits were also found to be variable between forms and closely associated with microhabitat structure, with the smaller-footed *B. melanocephalum* utilizing the narrowest perches of all forms, and the larger-footed Type B utilizing the widest perches. They are considered ecologically relevant because they provide stability and support to chameleons when navigating through their arboreal habitats (Fischer *et al.*, 2010; Herrel *et al.*, 2011). Stability becomes especially important during confrontations with conspecifics, for example, which often result in fierce fighting (Stuart-Fox *et al.*, 2006a; Tolley & Burger, 2007).

Limb length is often associated with the running, and hence escape, ability of lizards, with animals that have longer limbs being able to run faster (Losos & Sinervo, 1989; Sinervo & Losos, 1991; Macrini & Irschick, 1998; Melville & Swain, 2000; Vanhooydonck & Van Damme, 2001; Calsbeek & Irschick, 2007; Herrel *et al.*, 2011). However, because chameleons run relatively infrequently and are fundamentally different from other lizards in their locomotor behaviour (Bickel & Losos, 2002; Herrel *et al.*, 2013), selection for running speed may not be high. Selection for maximal reach, on the other hand, might be high, enabling them to bridge gaps (Herrel *et al.*, 2011). The grassland chameleons (*B. melanocephalum* and Type A) were found to occupy habitats far denser than those of the other phenotypic forms and thus would not need to reach very far to grasp hold of the next available perch. Accordingly, they would be expected to have the shortest limbs of all phenotypic forms. Correspondingly, the more closed-canopy chameleons (*B. thamnobates*, Types B and C) should possess proportionally longer limbs to traverse the larger gaps found in their habitats. However, the three KZN Midlands forms (*B. thamnobates*, Types A and C) did not follow this pattern. Instead, *B. thamnobates* and Type C possessed proportionally small limbs and Type A long limbs. This discrepancy

may suggest that microhabitat openness (i.e., the density of perches) may not be the sole or primary factor influencing limb length in this dwarf chameleon species complex, or possibly that the habitat structure in the Midlands is far more variable. Although climatic changes during the Pleistocene brought about significant changes in vegetation throughout KZN (Eeley *et al.*, 1999; Mucina & Rutherford, 2006), which likely set off the phenotypic divergence within this species complex (Tolley *et al.*, 2004), more recent factors may also have contributed. Within the past 2000 years, the KZN Midlands has been almost completely transformed, largely because of anthropogenic influences such as Iron Age farming and herding (Hall, 1980; Bousman, 1998; Huffman, 2007) and, more recently (approximately 200 years), urbanization, comprising factors that are considered to have overshadowed any climatic changes during this time (Neumann *et al.*, 2010). These factors significantly changed the structure and composition of vegetation in the area, which in turn, may have forced the chameleons to rapidly adapt to their 'new' habitats with respect to limb morphology. Despite the short time scale, lizards have been shown to adapt to novel or changed environments in far shorter time periods (e.g., 36 years in Podarcis sicula: Herrel *et al.*, 2008).

Type C was the most puzzling of the five forms, possessing a blend of features from both open- and closed-canopy habitats, often resulting in it showing no significant differences between forms (e.g., dull coloration: open-canopy forms; prominent casque, large gular lobes: closed-canopy forms). This could signify that it might have been initially part of an open-canopy habitat form (such as Type A) and is in the process of adapting to a forested environment. However, considering forests are the ancestral habitat type for KZN chameleons (Tolley *et al.*, 2008: fig. 1, nodes 1-8), Type C has most probably not changed habitats but, instead, particular aspects of its habitat may have changed, requiring it to adapt. Given that the Karkloof forest, where these chameleons reside, has experienced

approximately a 75% reduction in size from 1880 to 1940 (Lawes, Macfarlane, & Eeley, 2004), the structure (e.g., canopy openness) and composition of the vegetation that they utilize has likely changed. Alternatively, the dataset for this phenotypic form may be biased. Access to the forest was so limited for this form (as a result of a dense upper canopy with no visibility for sampling) that chameleons were only found along road verges adjacent to the forest. Considering other studies have reported size dependent dispersal in chameleons, with smaller animals tolerating more marginal, sub-optimal habitat (Keren-Rotem, Bouskila, & Geffen, 2006; Tolley *et al.*, 2010), it is possible that larger Type C chameleons exist in the forest, which we were unable to sample.

Morphological differences within forms were isolated to *B. thamnobates*. Individuals from Boston, and to a lesser extent Bulwer, were found to possess different head shapes compared to the other *B. thamnobates* populations, which might be attributed to these sites being at the southern extent of the distribution where the landscape has a different geomorphology and appears more fragmented. An escarpment surrounds the Boston and Bulwer region, with a river valley dividing the two towns. In the past, forests likely covered the slopes of this escarpment down to the river and probably linked up with other forests in the KZN Midlands, such as the Dargle forest to the north (site 13; Fig. 2.2). With extensive deforestation and urbanization, only a few very small forest fragments remain. This land transformation is likely acting as a barrier to dispersal, which the escarpment is intensifying. Fragmentation has been shown to bring about morphological changes in populations by altering microclimates and potentially selection regimes (Sumner, Moritz, & Shine, 1999). Physical fluxes, such as wind, radiation, and water, change across the landscape, affecting the vegetation (Saunders, Hobbs, & Margules, 1991) and, in turn, prey availability. This potential change in prey might have brought about the observed differences in head shape, especially considering that head width,

height, and length are the traits often correlated with bite force and prey hardness, and bite force is a common performance trait used to assess the adaptive significance of differences in head morphology (Herrel *et al.*, 2001a; Verwaijen, Van Damme, & Herrel, 2002; Measey *et al.*, 2009; Measey *et al.*, 2011). To help determine whether this subset of chameleons is adapting to their environment (i.e., on their own evolutionary trajectory) and should be considered a cryptic form of *B. thamnobates* and yet another form within the *B. melanocephalum-B. thamnobates* complex, bite performance and prey type between populations should be compared. The extent of gene flow and population structure between these chameleons and the other *B. thamnobates* populations should also be determined. If no differentiation is found, the head shape of these chameleons might simply be a result of founder effects genetic drift in these small populations.

The present study has identified clear correlations between morphology and habitat in this species complex, and a variety of selection pressures have been proposed to explain how and why they arose; however, what does it all mean for the management and conservation of these chameleons? Is it suggestive of separate species or simply morphologically variable populations of the same species? These questions are valid but, before they can be answered, evidence that the differing morphological traits are indeed ecologically relevant is required to establish a fit between phenotype and environment. This is typically accomplished by testing whether traits associated with particular environments consistently enhance performance in that environment. If such a fit can be fulfilled, morphologies are said to be adaptive and ecological speciation might be taking place, in which case the observed forms should be managed as unique entities on their own evolutionary trajectory (e.g., evolutionarily significant units: Ryder, 1986; Crandall *et al.*, 2000). A fine-scale genetic assessment using nuclear microsatellites is also needed to determine how these forms relate to one another by clarifying the effects that habitat

fragmentation has had on gene flow, population structure, and genetic diversity. Additional research on the behaviour, physiology, reproduction, and historic distributions of each form should also help develop a more complete understanding of the underlying processes involved in shaping the observed patterns of morphological variation.

Chapter 3

Paper II:

Linking microhabitat structure, morphology and locomotor performance traits in a recent radiation of dwarf chameleons (*Bradypodion*)[*]

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ABSTRACT

Evidence that morphological traits associated with particular environments are functionally adapted to those environments is a key component to determining the adaptive nature of radiations. Adaptation is often measured by testing how organisms perform in diverse habitats, with performance traits associated with locomotion thought to be amongst the most ecologically relevant. We therefore explored whether there are relationships between morphology, locomotor performance traits (sprint speed, forefoot and tail grip strength on broad and narrow dowels) and microhabitat use in five phenotypic forms of a recent radiation of dwarf chameleon – the *Bradypodion melanocephalum-Bradypodion thamnobates* species complex – to determine whether morphological differences previously identified between the forms are associated with functional adaptations to their respective habitats, which can be broadly categorised as open or closed-canopy vegetation. The results showed significant differences in both absolute and relative performance values between the phenotypic forms. Absolute performance suggests there are two phenotypic groups – strong (*B. thamnobates* and Type B) and weak (*B. melanocephalum* and Types A and C). Relative performance differences highlighted the significance of forefoot grip strength among these chameleons, with the closed-canopy forms (*B. thamnobates*, Types B and C) exceeding their open-canopy counterparts (*B. melanocephalum*, Type A). Little to no differences were detected between forms with respect to sprint speed and tail strength. These results indicate that strong selection is acting upon forefoot grip strength and has resulted in morphological adaptions that enable each phenotypic form to conform with the demands of its habitat. This study provides evidence for the parallel evolution of forefoot grip strength among dwarf chameleons, consistent with the recognition of open- and closed-canopy ecomorphs within the genus *Bradypodion*.

INTRODUCTION

Trait utility – evidence that morphological traits associated with particular environments are indeed ecologically pertinent – is a key component for assessing the adaptive nature of radiations, as well as for understanding the underlying mechanisms involved in evolutionary adaptations (Schluter, 2000). Trait utility is often measured by testing how organisms perform ecologically relevant functions in diverse habitats. Because locomotion is essential for the survival (e.g., to escape predation, find food) and reproduction (to find mates, defend territories) of many animals, performance traits associated with locomotion are thought to be amongst the most ecologically relevant (Huey & Stevenson, 1979; Arnold, 1983; Aerts *et al.*, 2000).

Many animals rely on a broad repertoire of locomotor capabilities, such as running/sprinting, jumping, clinging, and climbing, to carry out functions relevant to survival; however, optimisation of one performance trait often results in a trade-off in another (e.g., Lewontin, 1978; Stearns, 1992; Irschick & Losos, 1999). This is because different performance traits may require very different organismal configurations (e.g., muscle fibre type, skeletal structure) which are beneficial in different environments (e.g., Arnold, 1983; Abu-Ghalyun *et al.*, 1988; Losos, 1990c; Aerts *et al.*, 2000). Such trade-offs have been well documented for lizards. For example, a trade-off is commonly observed between speed and stability in cases in which terrestrial and arboreal species are compared. Open-canopy, terrestrial environments, in which organisms tend to be more visible to predators, typically harbour long-legged lizards capable of running rapidly along the ground (i.e., broad substrate) to avoid predation. Conversely, lizards in closed, arboreal habitats tend to have shorter limbs, which often results in them having relatively slower running speeds, but increased stability on the narrow, sometimes vertical, substrates due to the reduced distance between their centre of mass and the surface, which minimizes

sideways torque (e.g., Pounds, 1988; Losos & Sinervo, 1989; Losos, 1990b; Sinervo & Losos, 1991; Losos *et al.*, 1993; Losos & Irschick, 1996; Arnold, 1998; Macrini & Irschick, 1998; Melville & Swain, 2000; Vanhooydonck, Herrel, & Irschick, 2006).

Chameleons, unlike most lizards, move slowly on all substrates. They are thought to be cruise foragers (Butler, 2005) that use their ballistic tongue to capture prey (Zoond, 1933; Wainwright, Kraklau, & Bennett, 1991; Wainwright & Bennett, 1992a, b; Herrel *et al.*, 2001b). To avoid predation, chameleons rely upon crypsis and, in the case of arboreal chameleons, dropping from branches (Brain, 1961; Burrage, 1973; Tolley & Burger, 2007). Although there are a number of primarily terrestrial chameleon clades that utilise low perches at night to decrease predation risk, the majority of Chamaeleonidae radiated during the Eocene into a fully arboreal niche (Tolley, Townsend, & Vences, 2013). They have specialised adaptations for such habitats, including a prehensile tail and hands/feet, which allow them to grasp perches in a fully arboreal environment (Burrage, 1973; Peterson, 1984; Tilbury, 2010; Chapter 2). These features are particularly useful for clinging and holding onto relatively narrow substrates (Peterson, 1984; Higham & Jayne, 2004). Because of their vastly different locomotor adaptations and cryptic strategies compared to other lizards (Peterson, 1984), the typical performance predictions may not apply to chameleons (i.e., Herrel *et al.*, 2011). Nevertheless, chameleon morphology has been shown to correlate with performance in particular habitats. For example, chameleons in closed-canopy habitats, such as forests and woodlands, tend to possess relatively longer tails and larger feet than do chameleons in open-canopy habitats like grasslands and heathlands (Hopkins & Tolley, 2011; Herrel *et al.*, 2013). This may enable them to grip harder on the broader perches found there (Losos *et al.*, 1993; Herrel *et al.*, 2011; Herrel *et al.*, 2013). The closed-canopy species within the genus *Bradypodion* (dwarf chameleons) also run faster than do their open-canopy counterparts, likely owing to their relatively

longer limbs (Herrel *et al.*, 2011; Herrel *et al.*, 2013). It has been suggested that closed-canopy habitats are less cluttered, and in essence more 'open' at the microhabitat level, with fewer available perches for a chameleon to grasp, compared to open-canopy habitats, which are structurally cluttered at the microhabitat level (Herrel *et al.*, 2011; Chapter 2). As such, longer limbs may be essential within closed-canopy habitats to facilitate gap bridging between perches. The associated differences in sprint speed may simply be a by-product of their limb length (e.g., longer limbs allow longer strides to be taken without necessarily increasing stride frequency: Bauwens *et al.*, 1995; Bonine & Garland, 1999; Vanhooydonck, Van Damme, & Aerts, 2002). These correlations demonstrate local adaptations to microhabitat, and thus trait utility. In the case of *Bradypodion pumilum* (the Cape dwarf chameleon), these adaptations led to the suggestion that open- and closed-canopy forms should be considered ecomorphs (Measey *et al.*, 2009; Herrel *et al.*, 2011). However, an essential component of the ecomorph concept is the parallel evolution in multiple lineages of correlations between morphology and ecology (*sensu* Williams, 1972). An assessment of trait utility in another *Bradypodion* clade – the *Bradypodion melanocephalum-Bradypodion thamnobates* species complex – may thus prove beneficial for the classification of dwarf chameleons as ecomorphs.

The *B. melanocephalum-B. thamnobates* species complex is a recent radiation of dwarf chameleons from KwaZulu-Natal (KZN) Province, South Africa (Tolley *et al.*, 2008) that is classified as being taxonomically problematic due to discordance between phylogeny and morphology (Tolley *et al.*, 2004). The complex is comprised of five recognisable phenotypic forms (Fig. 3.1), all with distinct differences in ecology and distribution (Chapter 2). Two forms are classified taxonomically – *B. melanocephalum* (Gray, 1865) and *B. thamnobates* (Raw, 1976) – and the remaining three (regarded as Types A, B and C in Chapter 2) designated as morphotypes (Tolley & Burger, 2007;

Tilbury, 2010). Type A appears most similar to *B. melanocephalum* in size and colour, leading many to classify it as another population of the species (Tolley *et al.*, 2004; Tilbury, 2010); however, it has been found to be most similar to *B. thamnobates* genetically (Tolley *et al.*, 2004: fig. 2, samples CT16 & CT17). Types B and C have morphological features outwardly similar to *B. thamnobates* (e.g., prominent casque and large gular lobes), yet differ in size and colouration. Mitochondrial DNA has grouped Type B with *B. thamnobates* (Tolley *et al.*, 2004: fig. 2, sample CT71), while Type C has been found to group with both *B. melanocephalum* and *B. thamnobates* (Tolley *et al.*, 2004: fig. 2, samples B304 & B305).

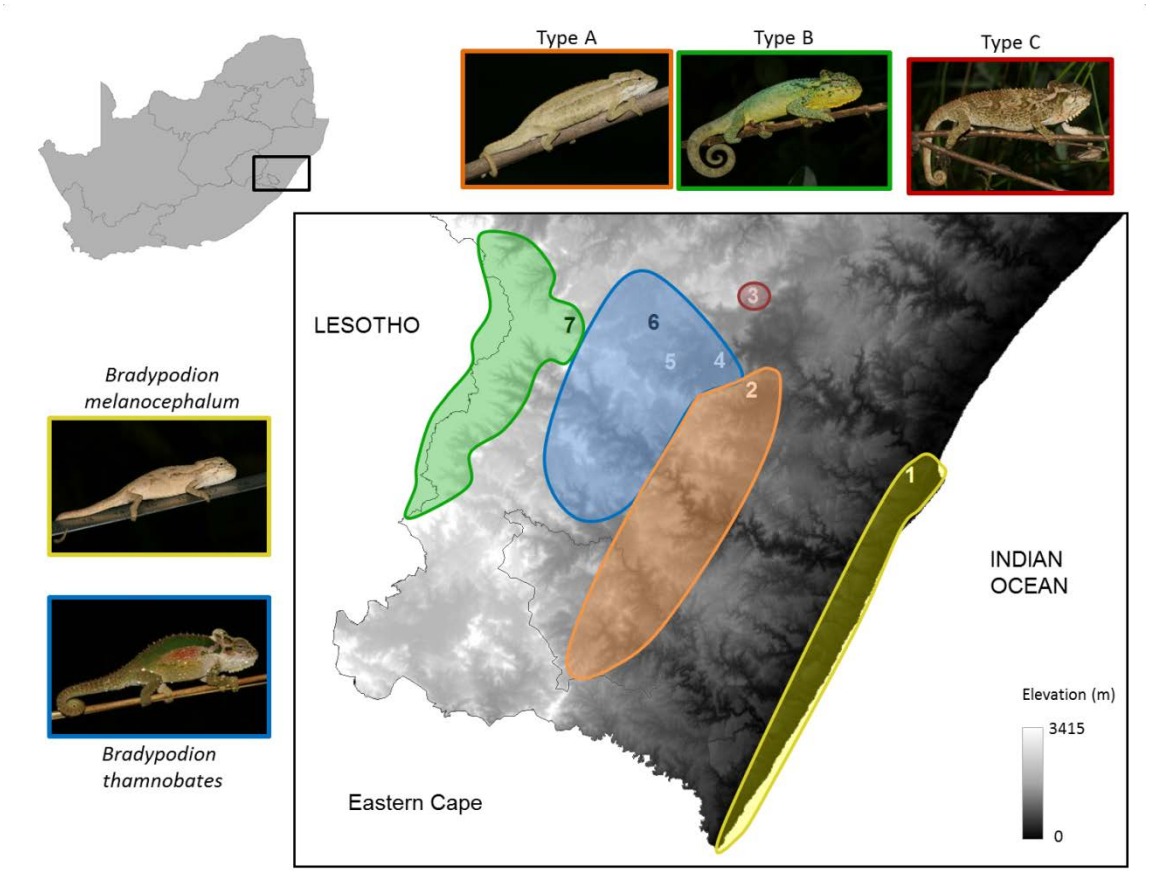

Figure 3.1 Photographs and general distributions of the five dwarf chameleon forms within the *B. melanocephalum-B. thamnobates* species complex from southern KwaZulu-Natal Province, South Africa. Only male forms are shown, although females resemble males in overall colouration (refer to fig. 2.1 in Chapter 2). Numbers indicate field sites sampled in this study: 1, Durban; 2, Hilton; 3, Karkloof; 4, Howick; 5, Dargle; 6, Nottingham Road; 7, Kamberg Nature Reserve.

Similar to their congener *B. pumilum* (Herrel *et al.*, 2011), these forms appear to fall into two broad habitat categories – either open (*B. melanocephalum* and Type A) or closed-canopy (*B. thamnobates*, Types B and C) – and have morphological features that appear to reflect adaptations to these habitats (see Chapter 2). However, some morphological features, particularly the limbs and tail, do not always correlate with these broad habitat categories (Chapter 2), potentially reflecting differences at the microhabitat level and/or the recent divergence of this radiation.

To understand whether or not the phenotypic differences in this group of chameleons are adaptive, we examined whether performance could be predicted by morphology and/or microhabitat. We expected similar patterns to be revealed as have been observed with other *Bradypodion* species (Herrel *et al.*, 2011; Herrel *et al.*, 2013). Therefore, we hypothesised that absolute differences in performance will be correlated to overall body size, but that the phenotypic forms would exhibit functional adaptations (i.e., relative differences in maximal sprint speed, forefoot and tail grip strength) associated with their microhabitats. In particular, we predicted that 1) relative sprint speed would be determined by limb length; 2) closed-canopy habitat chameleons, which possess proportionally larger feet (Chapter 2), would have a relatively stronger grip on both wide and narrow perches than do the shorter-footed open-canopy chameleons; 3) closed-canopy chameleons would possess a proportionally stronger tail grip on wide perches because their longer tails can wrap more coils around a thick substrate compared to the smaller tails of the open-canopy chameleons, increasing the contact area and creating more friction, thereby allowing for a stronger grip (Herrel *et al.*, 2013), while on narrow perches, all forms would be expected to perform comparably; and 4) morphological traits that correlate well with grip strength, will also show strong correlations to microhabitat (perch diameter). Confirmation of these predictions would corroborate the parallel evolution of open- and

closed-canopy ecomorphs within the *B. melanocephalum-B. thamnobates* species complex, as well as the genus.

MATERIALS AND METHODS

STUDY SITES AND SAMPLING PROCEDURES

A total of 171 dwarf chameleons (85 females; 86 males) representing the five phenotypic forms (see Chapter 2) within the *B. melanocephalum-B. thamnobates* species complex were sampled from seven sites within southern KZN (Fig. 3.1) in January and February 2010. To obtain an adequate sample size, *B. thamnobates* was sampled from three sites; whereas the remaining four forms were sampled from a single site each. Animals were collected at night and geo-referenced using GPS coordinates recorded at the location each chameleon was found. Marked flagging tape was placed on the perch of each chameleon to indicate the exact location at which each chameleon was found. Each chameleon, along with a section of their perch, was then placed in a separate cloth bag and brought back to the field base overnight, where they were measured and their performance tested the subsequent day. The diameter of the perch was measured to the nearest 0.01 mm using digital callipers. Once all data were collected, animals were released at their exact point of capture.

MORPHOMETRICS

All chameleons were measured to the nearest 0.1 mm using digital callipers (Fig. 3.2): snout-vent length (SVL), interlimb length (ILL), tail length (TL), thigh length (ThL), crus length (CL), medial hindfoot pad length (MH), lateral hindfoot pad length (LH), proximal hindfoot pad length (PH), brachium length (BL), antebrachium length (AL), medial forefoot pad length (MF), lateral forefoot pad length (LF), and proximal forefoot pad

length (PF). Because we worked with live animals, measurements were made externally and are, therefore, the best approximations for the actual skeletal components listed above. The limits of each component were determined by gently moving the limbs and feet at the joints and positioning each end of the callipers at either end of the bony segment. For consistency, these measurements were taken on the left side of the body. Each measurement was taken once because preliminary precision trials conducted on 10 *B. thamnobates* chameleons and based on three measurements of each variable found little error between the three recordings (± 0.77%). The mass of each chameleon was also measured using a Pesola® micro-line spring scale (model 93010: 30 g x 0.25 g ± 0.3%).

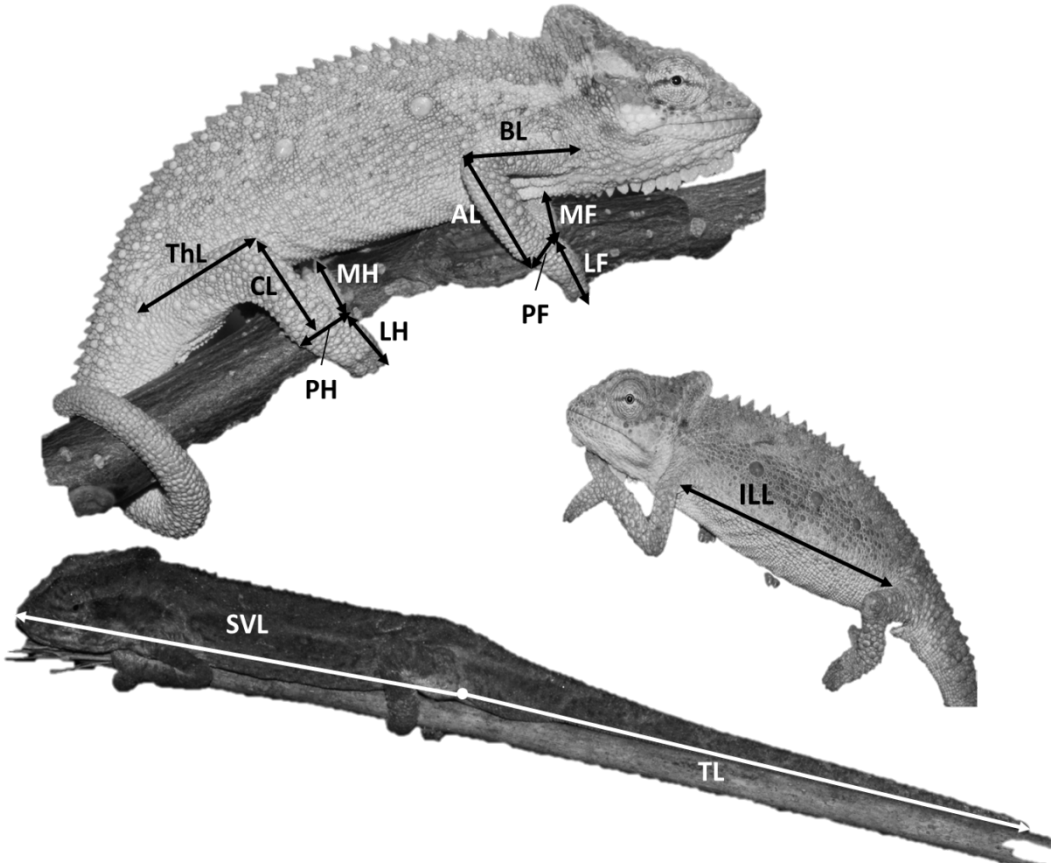

Figure 3.2 Thirteen measurements recorded for each chameleon. SVL (snout-vent length), TL (tail length), ILL (interlimb length), ThL (thigh length), CL (crus length), MH (medial hindfoot pad length), LH (lateral hindfoot pad length), PH (proximal hindfoot pad length), BL (brachium length), AL (antebrachium length), MF (medial forefoot pad length), LF (lateral forefoot pad length), and PF (proximal forefoot pad length).

PERFORMANCE

Chameleons were first allowed to thermoregulate in a sun/shade setting to attain their preferred body temperature (between 28-32˚C; see Segall *et al*., 2013). All performance trials were then performed at ambient temperature. A minimum rest period of 1hr was allowed for each chameleon during the transition between sprinting and gripping tests. Sprint speed was tested by running chameleons along a flat 1 m long track marked at 25 cm intervals. Considering chameleons move very slowly in their regular (perched) habitat and probably do not rely on running to avoid predation in their arboreal habitat (Brain, 1961; Burrage, 1973; Tolley & Burger, 2007), selection related to sprint speed might occur when animals are moving along the ground (Herrel *et al.*, 2011). Previous studies of chameleons have also shown that sprint speed is highest on a flat substrate (Abu-Ghalyun *et al.*, 1988; Losos *et al*., 1993); therefore, sprinting performance was tested by chasing chameleons along a flat track. The times at which animals crossed the 25 cm markers were recorded using a stopwatch. For dwarf chameleons, these manual recordings were found to be comparable to readings provided electronically using infrared photocells (Herrel *et al.*, 2011). The speed in centimetres per second over the fastest interval was calculated and retained for further analysis.

Grip strength was tested using two different sized horizontal dowels (broad: 9.25 mm; narrow: 4 mm) mounted separately on a piezo-electric force platform (Kistler Squirrel force plate, ± 0.1N: see Herrel *et al.*, 2012), which was connected to a Kistler charge amplifier (type 9865). The dowel sizes were chosen as they are representative of branch diameters available to these chameleons (Chapter 2), and hence, might reflect the limit of what they perch on. Moreover, they resemble the dowel sizes used in other dwarf chameleon performance studies (Herrel *et al.*, 2011; Herrel *et al.*, 2013). Forces were obtained during a 60 second recording session and recorded at 1000Hz. During the session,

chameleons voluntarily gripped the dowel with their tail and forefeet repeatedly (typically, 2-4 grips each per session), and were then pulled until they released the dowel. Animals were pulled in the vertical direction to measure tail force and in the horizontal direction to measure forefoot grip strength. Even though structural differences exist between chameleon fore- and hindfeet, which might affect their performance, such as the reverse arrangement of fused toes between the medial and lateral segments (Burrage, 1973; Peterson, 1984), we only investigated forefoot performance because it allowed for comparisons to other species in the genus (*B. pumilum*: Herrel *et al.,* 2011; *Bradypodion damaranum*: Herrel *et al.,* 2013; Potgieter, 2013; *Bradypodion occidentale*: Herrel *et al.,* 2013) and, principally, because the forefoot is much easier to measure, resulting in greater precision. Furthermore, the morphometric data show strong correlations between fore- and hind-foot sizes (Chapter 2). Accordingly, the forefoot performance results are expected to hold for the hindfoot as well. Each chameleon was tested in three separate recording sessions for each dowel, with at least 30 min rest between sessions involving the same dowel, and at least one hour of rest between sessions when changing dowels. The peak forces (*Z*, tail; *Y*, forefeet) were recorded and extracted using Bioware software (Kistler), and the highest tail and forefeet grip values per individual per dowel were retained for subsequent analysis.

Statistical Analyses

All analyses were carried out using SPSS version 17.0 (2008). All data were $\log_{10}$ transformed prior to analysis to fulfil assumptions of normality and homoscedasticity. Ordinary least squares regressions were then conducted to verify that the assumptions were met. Each $\log_{10}$ transformed variable was entered as the dependent variable, separately, and a plot of the z-predicted (x-axis) against z-residual (y-axis) values was constructed. All

plots showed that the error variance (z-residual) is consistent with the varying values in the predicted variables (z-predicted), confirming homoscedasticity. To remove the effect of body size on performance, all data were size-corrected using a linear regression executed on all individuals, and the unstandardized residuals saved for use in subsequent analyses. The regression and a principal component analysis (PCA) indicated that all body and performance measurements followed similar trajectories and fit within a single principal component, with log SVL possessing the highest component score (Braña 1996; Kratochvíl *et al.*, 2003; McCoy *et al.*, 2006). Accordingly, all measurements were size-corrected using logSVL.

Although a previous study revealed significant morphometric differences between the five phenotypic forms and sexes examined in this study (Chapter 2), a multivariate analysis of variance (MANOVA) using a general linear model (GLM) was carried out to verify that the subset of data, which included only individuals used in the performance tests used here, would reproduce those results. The full model specified SEX and FORM as fixed factors, SEX x FORM as the interaction, and all size-corrected variables as the dependent variables. All *P*-values were subjected to Holm's sequential Bonferroni (Holm 1979) correction to minimize the possibility of Type I errors (Rice, 1989).

Performance

For grip strength tests, repeated-measures ANOVAs were carried out to assess whether performance was dependent on dowel size for each phenotypic form and both sexes. MANOVAs were then conducted on each of the five performance variables using both absolute ($\log_{10}$ transformed) and relative (size-corrected) values to test for differences between forms. As above, all *P*-values were subjected to Holm's sequential Bonferroni correction. To explore which morphological variables best explained the variation in sprint

speed and forefoot grip strength on both dowels for each chameleon form, separately, multiple linear regression models were carried out on size-corrected variables. The same models were run for each phenotypic form and sex. Specifically, the three performance variables were entered separately as the dependent variable in a linear regression, with all size-corrected variables used as the independent variables. Akaike's information criterion (AIC) was calculated using the residual sum of squares from each model, and the difference between the lowest AIC and all others (Δ_i) was determined. Akaike's weights (*wi*) were then calculated for each model, with the one exhibiting the highest *wi* acknowledged as the best model (Burnham & Anderson, 2002). Because tail length was the only tail variable measured in this study, a linear regression was conducted simply to assess the degree of correlation between it and tail grip strength on both dowels.

Habitat

To determine whether the perch diameter used by chameleons (i.e., microhabitat) is correlated with their morphology, linear regression analyses were run on $\log_{10}$ transformed data using perch diameter as the independent variable and variables making up the forefoot, hindfoot and tail (MF, LF, PF, MH, LH, PH, TL) as the dependent variables. Only these morphometric variables were included because they are directly involved in gripping perches. As above, AIC and *wi* were calculated for each model.

RESULTS

The initial MANOVA revealed morphological differences between the five phenotypic forms (Wilks' $\lambda = 0.267$, $F_{4,169} = 4.899$, $P < 0.001$) and sexes (Wilks' $\lambda = 0.539$, $F_{1,169} = 10.959$, $P < 0.001$), confirming previous results for this species complex (Chapter

2; refer to Table 3.1 for raw data). Given the significant sex effect, all subsequent analyses were carried out separately by sex.

Table 3.1 Summary of mean microhabitat, morphological, and performance data for male (M) and female (F) dwarf chameleons used in this study, grouped by phenotypic form. Standard deviation shown in brackets.

	B. melanocephalum		*B. thamnobates*		Type A		Type B		Type C	
	M	F	M	F	M	F	M	F	M	F
					Microhabitat					
N	*25*	*16*	*17*	*23*	*19*	*23*	*14*	*10*	*7*	*9*
Perch diameter (mm)	1.77 (0.95)	2.06 (0.94)	2.38 (0.87)	2.18 (1.47)	1.55 (0.76)	2.18 (1.00)	2.80 (1.58)	2.24 (1.00)	1.58 (0.58)	1.78 (0.73)
					Morphology					
N	*25*	*15*	*20*	*25*	*20*	*25*	*14*	*12*	*6*	*7*
Mass (g)	2.1 (0.5)	4.4 (0.9)	6.2 (3.8)	8.8 (5.9)	2.6 (0.9)	2.2 (0.7)	8.4 (1.9)	13.4 (4.5)	1.8 (0.3)	1.9 (0.5)
SVL (mm)	49.1 (4.4)	56.8 (2.9)	60.0 (14.6)	66.4 (16.6)	48.3 (7.3)	44.5 (5.4)	69.5 (4.3)	77.5 (6.8)	40.6 (3.2)	41.6 (3.9)
TL (mm)	54.7 (5.4)	51.7 (3.7)	66.7 (17.9)	65.5 (18.1)	52.5 (7.9)	44.8 (4.1)	79.4 (7.9)	85.5 (10.7)	44.8 (3.3)	41.3 (4.1)
ILL (mm)	27.6 (3.2)	33.4 (2.0)	32.9 (8.4)	37.9 (10.6)	25.5 (4.1)	24.2 (3.6)	38.0 (3.8)	44.8 (5.8)	20.8 (2.4)	21.1 (1.4)
BL (mm)	9.3 (1.1)	10.0 (0.7)	11.9 (3.1)	12.7 (3.5)	9.5 (1.6)	8.9 (1.3)	14.7 (1.1)	15.4 (1.5)	7.7 (0.3)	7.9 (0.7)
AL (mm)	7.8 (0.9)	8.4 (0.5)	9.9 (2.8)	10.5 (2.9)	8.1 (1.5)	7.35 (0.9)	11.9 (0.7)	12.9 (1.3)	6.4 (0.5)	6.2 (0.5)
MF (mm)	4.4 (0.5)	4.9 (0.4)	6.1 (1.5)	6.4 (1.6)	4.7 (0.7)	4.3 (0.5)	7.1 (0.4)	7.9 (08)	4.2 (0.4)	4.1 (0.3)
LF (mm)	5.3 (0.5)	5.7 (0.5)	7.3 (1.9)	7.8 (1.8)	5.5 (0.7)	5.1 (0.5)	8.9 (0.5)	9.2 (0.8)	4.9 (0.6)	4.7 (0.6)
PF (mm)	1.9 (0.2)	2.0 (0.2)	2.9 (0.8)	3.0 (0.9)	1.9 (0.3)	1.9 (0.3)	3.6 (0.5)	3.8 (0.4)	1.6 (0.1)	1.8 (0.3)
ThL (mm)	9.1 (0.9)	9.6 (0.6)	11.3 (3.4)	11.8 (3.3)	9.4 (1.6)	8.7 (1.1)	13.4 (1.1)	15.1 (1.50)	7.1 (0.6)	7.4 (0.7)
CL (mm)	7.7 (0.6)	8.4 (0.5)	9.5 (2.5)	10.3 (2.8)	7.8 (1.3)	7.3 (0.9)	11.3 (0.6)	12.5 (1.1)	6.3 (0.6)	6.3 (0.4)
MH (mm)	4.2 (0.3)	4.5 (0.4)	6.0 (1.8)	6.4 (1.7)	4.5 (0.8)	3.9 (0.4)	7.0 (0.7)	8.0 (1.0)	3.7 (0.5)	3.4 (0.4)
LH (mm)	5.6 (0.5)	5.8 (0.5)	7.7 (2.0)	8.0 (2.1)	5.7 (0.9)	5.1 (0.8)	9.2 (1.1)	9.8 (1.4)	4.7 (0.4)	4.9 (0.4)
PH (mm)	2.1 (0.3)	2.2 (0.3)	3.3 (1.1)	3.3 (1.2)	2.3 (0.5)	1.9 (0.4)	3.6 (0.6)	4.2 (0.7)	7.7 (0.3)	2.1 (0.3)

Table 3.1 *continued.*

	B. melanocephalum		*B. thamnobates*		Type A		Type B		Type C	
	M	F	M	F	M	F	M	F	M	F
					Performance					
N	*23*	*15*	*20*	*25*	*20*	*25*	*13*	*12*	*5*	*7*
Speed (cm s^{-1})	6.30 (1.60)	5.94 (1.31)	7.48 (3.51)	7.79 (1.85)	5.99 (1.92)	5.21 (1.29)	9.96 (2.06)	9.13 (1.84)	4.52 (1.27)	4.04 (0.84)
Max. forefoot grip force (N)										
Broad	0.08 (0.03)	0.07 (0.03)	0.18 (0.14)	0.17 (0.09)	0.05 (0.03)	0.05 (0.02)	0.19 (0.12)	0.21 (0.16)	0.06 (0.02)	0.05 (0.02)
Narrow	0.47 (0.11)	0.55 (0.13)	1.16 (0.90)	1.13 (0.68)	0.39 (0.19)	0.30 (0.12)	1.49 (0.41)	1.70 (0.53)	0.28 (0.09)	0.26 (0.06)
Max. tail grip force (N)										
Broad	0.82 (0.31)	0.68 (0.31)	1.36 (0.87)	1.52 (1.00)	0.60 (0.36)	0.62 (0.25)	2.23 (1.27)	2.17 (1.12)	0.44 (0.10)	0.50 (0.16)
Narrow	0.95 (0.42)	0.62 (0.24)	1.34 (0.99)	1.65 (1.34)	0.78 (0.39)	0.56 (0.27)	2.17 (0.94)	2.19 (1.45)	0.40 (0.11)	0.38 (0.15)

SVL, snout-vent length; TL, tail length; ILL , interlimb length; ThL , thigh length; CL , crus length; MH , medial hindfoot pad length; LH , lateral hindfoot pad length; PH , proximal hindfoot pad length; BL , brachium length; AL , antebrachium length; MF , medial forefoot pad length; LF , lateral forefoot pad length; PF , proximal forefoot pad length.

PERFORMANCE

The effect of dowel size on forefoot grip strength was significant for both sexes in all five phenotypic forms (Table 3.2), with animals exerting higher forces on the narrow dowel compared to the broad dowel (Table 3.1). In contrast, only Type A males from the KZN Midlands showed a significant difference in tail performance between the two dowels (Table 3.2), with these animals also showing a stronger grip on the narrow dowel (Narrow: 0.78 ± 0.36 N; Broad: 0.60 ± 0.36 N). Overall, chameleons exhibited stronger grip forces with their tails than with their forefeet (Table 3.1).

Table 3.2 Repeated measures ANOVA assessing the dependence of grip strength on dowel size within both sexes of each phenotypic form.

Morph	Sex	*n*	Max. Forefoot grip strength		Max. Tail grip strength	
			F	***P***	***F***	***P***
B. melanocephalum	M	23	271.81	< 0.001	0.24	0.631
	F	15	257.37	< 0.001	0.64	0.435
B. thamnobates	M	20	550.56	< 0.001	0.22	0.647
	F	25	521.00	< 0.001	0.01	0.910
Type A	M	20	396.04	< 0.001	12.15	0.003
	F	25	259.66	< 0.001	1.04	0.320
Type B	M	13	449.59	< 0.001	1.09	0.315
	F	12	230.94	< 0.001	0.18	0.682
Type C	M	5	159.31	0.001	1.918	0.225
	F	7	212.71	< 0.001	0.44	0.531

M, male; F, female; *n*, sample size; *F*, test value; *P*, significance value.

Absolute and relative performance differences were uncovered between forms for both males and females, albeit to varying degrees (Table 3.3). Both sexes showed the same pattern in terms of absolute differences, typically with the largest forms being the strongest (i.e., *B. thamnobates* and Type B). In addition, these two largest forms showed similar performance levels between them, for almost all traits (Table 3.3). Similarly, the smaller forms (*B. melanocephalum* and Types A and C) were comparable to each other for most performance traits.

Relative performance values showed fewer differences between forms and sexes. After Bonferroni correction, female forms were only found to differ from each other in forefoot grip strength on the broad dowel (Table 3.3; Fig. 3.3). This was attributed to *B. thamnobates* having a substantially stronger grip than *B. melanocephalum* and Type A. Male forms differed from each other in forefoot grip strength on both dowels, as well as tail grip strength on the broad dowel (Fig. 3.3). These differences were also attributed to the stronger gripping ability of *B. thamnobates*, particularly for forefoot strength, and *B. melanocephalum* and Type B for tail grip strength.

Table 3.3 MANOVA results investigating absolute and relative performance differences between phenotypic forms.

Performance	Males (*n*=86)				Females (*n*=85)			
	Absolute		Relative		Absolute		Relative	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Sprint speed (cm s^{-1})	10.406	< 0.001*	1.331	0.266	8.069	< 0.001*	1.139	0.344
Max. forefoot grip force (N)								
Broad	18.509	< 0.001*	4.669	0.002*	22.585	< 0.001*	3.961	0.006*
Narrow	26.643	< 0.001*	5.187	0.001*	27.066	< 0.001*	2.227	0.073
Max. tail grip force (N)								
Broad	14.910	< 0.001*	3.636	0.009*	14.207	< 0.001*	2.510	0.048
Narrow	14.932	< 0.001*	1.972	0.107	14.410	< 0.001*	1.874	0.123

F, test value; *P*, significance value; * Significant after Bonferroni correction.

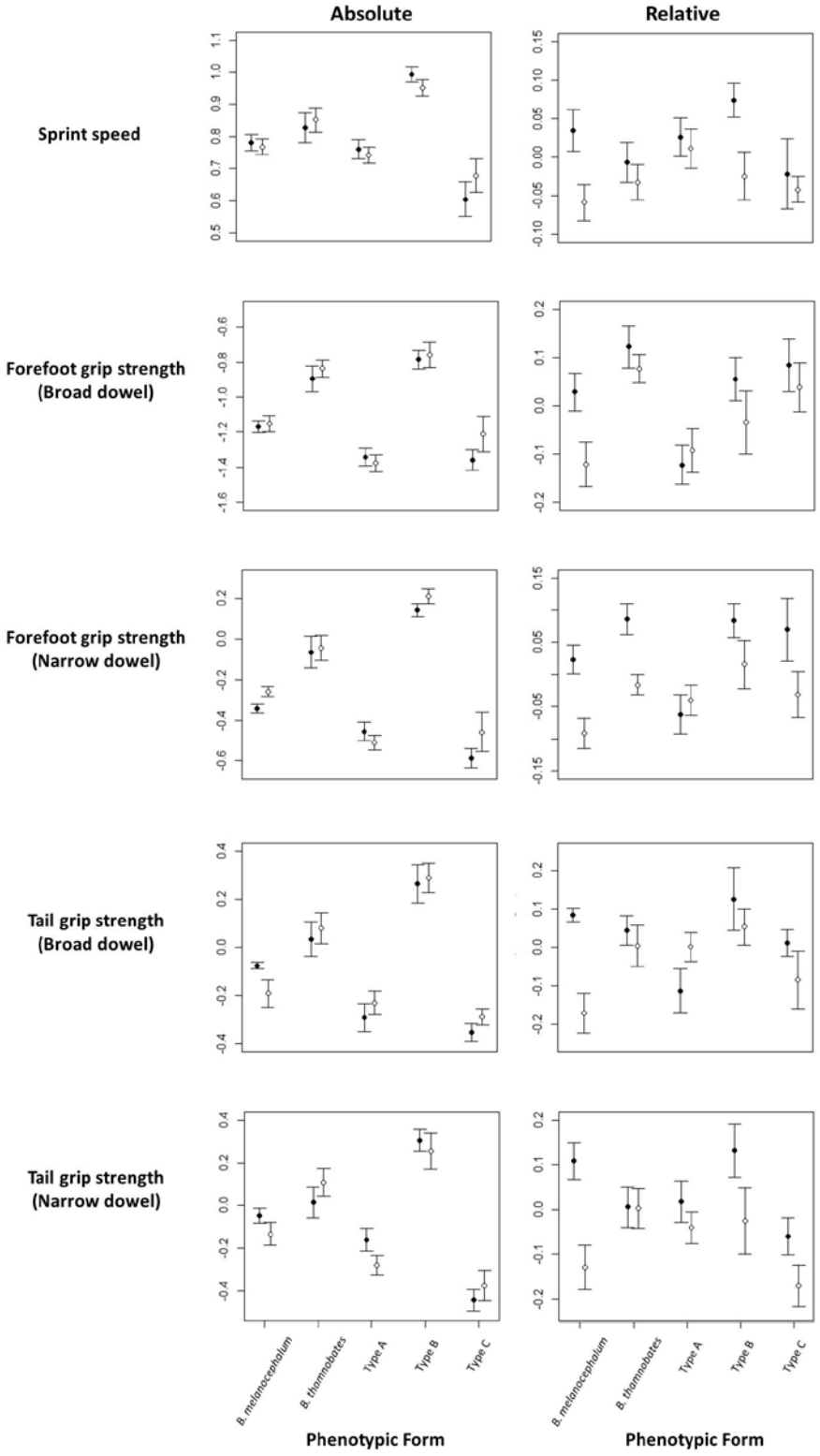

Figure 3.3 Error plots of mean absolute values (left) and mean relative values (right) for the five performance variables tested for the *B. melanocephalum-B. thamnobates* species complex. Error bars represent standard error. Absolute force equates to $\log_{10}$-transformed values, whereas relative force depicts size-corrected values. Solid circles represent males; empty circles, females.

Model selection using linear regression to find the morphological variables that best explain performance did not show a pattern that could be generalised to fit all forms (Table 3.4). In some cases, several candidate models, often involving multiple morphological variables, exhibited significant correlations with performance (Tables S3.1-S3.3); however, the best fitting models tended to include a single morphological variable (Table 3.4). Of the three performance variables that underwent model selection (sprint speed and forefoot grip strength on broad and narrow dowels), forefoot grip strength on the narrow dowel showed significant correlations for almost all forms and sexes (Table 3.4). As forefoot size increased (particularly the medial and proximal forefoot), so did the grip strength of both sexes of the open-canopy forms, *B. thamnobates* females, and Type B males. In contrast, antebrachium length, not forefoot size, was the best predictor of grip strength on the narrow dowel for *B. thamnobates* from the closed-canopy habitat. On the broad dowel, forefoot size and grip strength were not correlated for most forms; and in two cases, correlations were negative, suggesting overall that chameleons do not perform well on the broad surface and, in some cases, performance drops significantly. Sprint speed exhibited the greatest variation among forms and sexes, with the best model generally incorporating a combination of fore- and hindlimbs and feet. Only three groups showed significant correlations between tail length and grip strength (Broad dowel: *B. melanocephalum* females; Narrow dowel: Type B females and Type C males). No performance-morphology associations were uncovered for Types B and C females for any of the five performance traits, which could potentially be attributed to their low sample sizes (n = 11 and 8, respectively).

HABITAT

Model selection examining the best morphological correlates of perch diameter found significant correlations for all but Type C chameleons (Table S3.4), and was particularly strong for females of the closed-canopy habitat form, *B. thamnobates*. Of the best fitting models, proximal hindfoot pad length was correlated to perch diameter in females; although in males, no consistent pattern was observed (Table 3.5). Overall, different morphological variables were found to associate with perch diameter (Table 3.5) compared to those that associated with grip strength (Table 3.4).

Table 3.4 Results of regression analyses on the morphological variables found to best reflect the five performance variables under investigation.

Performance variable	Phenotypic form	Males				Females			
		Model	ß	R^2	*P*	Model	ß	R^2	*P*
Sprint Speed	*B. melanocephalum*	**AL**	**0.412**	**0.169**	**0.041**	**CL**	**-0.683**	**0.612**	**0.002**
						MF	**0.657**		
	B. thamnobates	**ThL**	**0.652**	**0.425**	**0.002**	PH	-0.314	0.197	0.089
						AL	0.394		
	Type A	MF	0.377	0.142	0.112	**LH**	**0.375**	**0.309**	**0.030**
						AL	**0.357**		
	Type B	**MF**	**0.627**	**0.505**	**0.015**	LH	0.570	0.325	0.053
		BL	**0.378**						
	Type C	**MH**	**0.244**	**0.001**	**0.001**	ThL	-0.371	0.138	0.326
		PH	**0.732**						
		AL	**0.436**						
Maximum Forefoot Grip Strength (Broad dowel)	*B. melanocephalum*	AL	0.378	0.143	0.135	**MH**	**-0.317**	**0.100**	**0.025**
	B. thamnobates	BL	0.395	0.156	0.084	LF	0.295	0.087	0.153
	Type A	MF	0.363	0.131	0.127	MF	0.341	0.116	0.111
	Type B	**PF**	**0.524**	**0.275**	**0.045**	AL	-0.500	0.25	0.098
	Type C	**MF**	**-0.780**	**0.608**	**0.038**	MF	0.636	0.404	0.066
Maximum Forefoot Grip Strength (Narrow dowel)	*B. melanocephalum*	**MF**	**0.411**	**0.168**	**0.042**	**PF**	**0.561**	**0.315**	**0.024**
	B. thamnobates	**AL**	**0.486**	**0.219**	**0.038**	**AL**	**0.383**	**0.304**	**0.018**
						MF	**0.307**		
	Type A	**AL**	**-0.493**	**0.351**	**0.031**	**MF**	**0.716**	**0.513**	**<0.001**
		PF	**0.489**						
	Type B	**PF**	**0.612**	**0.374**	**0.015**	**PF**	0.402	0.161	0.196
	Type C	MF	-0.509	0.259	0.244	MF	0.077	0.154	0.296

Table 3.4 *continued*.

Performance variable	Phenotypic form	Males				Females			
		Model	ß	R^2	*P*	Model	ß	R^2	*P*
Maximum Tail Grip Strength (Broad dowel)	*B. melanocephalum*	TL	0.248	0.061	0.233	TL	**0.539**	**0.291**	**0.031**
	B. thamnobates		0.342	0.117	0.140		-0.177	0.031	0.397
	Type A		0.058	0.003	0.814		0.228	0.052	0.296
	Type B		-0.069	0.005	0.807		0.233	0.050	0.486
	Type C		0.260	0.067	0.574		0.252	0.064	0.513
Maximum Tail Grip Strength (Narrow dowel)	*B. melanocephalum*	TL	-0.035	0.001	0.867	TL	0.107	0.011	0.694
	B. thamnobates		-0.018	0.000	0.939		**0.432**	**0.187**	**0.031**
	Type A		0.105	0.011	0.669		0.071	0.005	0.748
	Type B		-0.040	0.002	0.888		0.608	0.370	0.036
	Type C		**0.822**	**0.675**	**0.023**		0.254	0.065	0.509

R^2, coefficient of determination; ß, Beta coefficient depicting direction of correlation; *P*, significance value; BL, brachium length; AL, antebrachium length; ThL, thigh length; CL, crus length; MF, medial forefoot pad length; LF, lateral forefoot pad length; PF, proximal forefoot length; MH, medial hindfoot pad length; LH, lateral hindfoot pad length; PH, proximal hindfoot pad length; TL, tail length. Text in bold highlights significant morphology-performance correlations. Refer to Table 3.1 for sample sizes.

Table 3.5 Morphological variables found to best reflect perch diameter across all phenotypic forms and sexes.

Phenotypic form	Males					Females				
	n	Model	ß	R^2	*P*	*n*	Model	ß	R^2	*P*
B. melanocephalum	25	**PF**	**0.429**	**0.184**	**0.032**	15	**PH** **TL**	**0.537** **0.478**	**0.653**	**0.001**
B. thamnobates	17	**PF**	**0.513**	**0.263**	**0.035**	23	**MF** **PH**	**-1.195** **1.177**	**0.712**	**0.000**
Type A	19	LH	-0.274	0.075	0.256	23	**PH**	**-0.417**	**0.174**	**0.049**
Type B	14	**MH** **PH** **TL**	**1.022** **-0.441** **-0.511**	**0.659**	**0.011**	10	PF MF	-1.285 1.353	0.421	0.195
Type C	6	LF	0.471	0.221	0.287	7	LH	0.308	0.095	0.371

n, sample size; ß, Beta coefficient depicting direction of correlation; R^2, coefficient of determination; *P*, significance value; TL, tail length; MF, medial forefoot pad length; LF, lateral forefoot pad length; PF, proximal forefoot length; MH, medial hindfoot pad length; LH, lateral hindfoot pad length; PH, proximal hindfoot pad length. Text in bold highlights significant correlations between morphology and perch diameter.

DISCUSSION

Chameleons within the *B. melanocephalum-B. thamnobates* species complex possess functional adaptations in forefoot size and performance that correspond to their use of either open- or closed-canopy habitats. These results reflect those observed for other *Bradypodion* species (Herrel *et al.*, 2011; Herrel *et al.*, 2013; Potgieter, 2013), providing additional support for the existence of open- and closed-canopy ecomorphs within the genus. No habitat-specific correlations were uncovered between limb length and sprint speed, or between tail length and tail strength, indicating that selection is not acting upon these traits in terms of the habitat associations and measurements made.

As expected, the absolute differences detected between the five chameleon forms followed the same pattern for each performance trait investigated, demonstrating the effect of overall body size on performance. Forms that utilise closed-canopy habitats are stronger and faster than those that use open-canopy habitats. Indeed, the forms generally fit into one of two absolute performance categories – strong (Type B and *B. thamnobates*) and weak (*B. melanocephalum*, Types A and C). For many animals, body size is highly heritable (Peters, 1983) and has been shown to be influenced by habitat use (e.g., Asplund, 1974; Fleming, 1991), which might also be the case here. Moreover, each form reaches different absolute body sizes (Chapter 2), which is not a consequence of phenotypic plasticity, as demonstrated by a common garden experiment on *B. thamnobates* and *B. melanocephalum* (Miller & Alexander, 2009). Accordingly, the differences in absolute performance are likely indicative of ecological differences between them. The one exception might be with the Type C chameleons. These chameleons are the smallest (in absolute terms) of all the forms in this study, yet their primary habitat is forest. If they were to follow the other forest forms, they should be amongst the larger chameleons. Considering that the individuals sampled in this study were collected in secondary vegetation along the forest

edge, and not in the forest itself due to accessibility problems (Chapter 2), they may not be representative of mature adults, but rather sub-adults, thus biasing the data.

In addition to absolute differences, relative performance differences were detected in forefoot grip strength on both sized dowels and tail grip strength on the broad dowel, indicating that selection may be acting upon these performance traits, and their associated morphological traits, in response to habitat. As expected, forefoot grip strength produced the same pattern on either dowel, with the closed-canopy forms (including Type C) exerting greater forces for their size than the open-canopy forms. The widest perches in the open-canopy habitats do not exceed 6 mm, and average around 2 mm; whereas, the widest perches in the closed-canopy habitats can reach close to 20 mm, and average between 2.50-4.50 mm (Chapter 2). Both dowels appear to be too large for the smaller-footed open-canopy habitat chameleons to adequately grasp. Conducting similar tests using dowels that better represent perch diameters more commonly available in open-canopy habitats, and thus that are more representative of the actual perches used by those chameleons (e.g., 1.5-2 mm or narrower), may prove useful for testing the effectiveness of foot size on narrow perches.

The greater forefoot grip strength of the closed-canopy habitat chameleons likely emphasizes the importance of stability and balance within this habitat. It could be especially important during intra-specific encounters, which often result in intense fighting. These fights generally involve intense swaying, open-mouthed threat displays, chasing and biting (Burrage, 1973; Stuart-Fox *et al.*, 2006; Tolley & Burger, 2007), with both combatants grasping the branch to maintain balance and support. In open-canopy habitats, where the average plant and perch height is between 0.75 m and 1.75 m and the perches are densely clustered in a vertical orientation (Chapter 2), the risk of displacement is far less compared to closed-canopy habitats where perches are less densely arranged and perch

heights average 1.6-4.5 m (Chapter 2). This may explain why grip strength showed correlations to both limb and foot variables (Tables 3.4, S3.2 & S3.3). Grip strength is created by the flexor muscles, which extend from the limbs into the feet, and the extensor muscles in the limbs stabilize the wrist and provide leverage. As such, they cannot function in isolation.

As expected, the tails of each form were found to perform similarly on the narrow dowel, suggesting they are equally suited for grasping narrow perches. On the broad dowel, unexpected differences in tail performance were identified for males. Instead of the closed-canopy chameleons having a proportionally stronger grip owing to their relatively longer tails (see Herrel *et al.*, 2013), the tail grip of the open-canopy *B. melanocephalum* was among the strongest for males. This result is especially surprising considering that the other open-canopy form, Type A, which possesses a comparable tail length to *B. melanocephalum* (Chapter 2), was the weakest. The much weaker tail grip of Type A males is unlikely to be a consequence of microhabitat, because females from this habitat did not show the same outcome, yet they utilised the same size perches. Moreover, males have longer tails than females, so it would be expected that they would be better able to wrap their tails around the broad dowel, and hence be able to exert a proportionally stronger force; yet this was not observed. Given that tail length alone could not adequately explain tail performance for most forms, other morphological features or adaptations which were not measured here may be involved, such as the length of the distal end of the tail which is used in prehensile activities and the length of the hypaxial muscles (M. ischiocaudalis and M. inferocaudalis) which work to curl the tail (Zippel, Glor, & Bertram, 1999; Bergmann, Lessard, & Russell, 2003). As such, identification of the morphological components involved in tail performance, and whether these differ between forms, may further our understanding. However, if this result is a sampling artefact, then

the overall generalisation is that all these form are well suited for grasping onto both broad and narrow dowels with their tails. This would then mirror results found for open- and closed-canopy forms of the congeners *B. pumilum* and *B. damaranum* (Herrel *et al.*, 2011, Herrel *et al.*, 2013). Considering all forms were able to exert greater forces with their tails compared to their forefeet, the importance of the tail for stability and support in each habitat is likely to be high. Indeed, chameleons are known to pull themselves onto branches solely using their tails (Tolley & Burger, 2007). This ability allows them to move effectively both horizontally and vertically throughout their habitats (Higham & Jayne, 2004; Tolley & Burger, 2007; Tilbury, 2010; Herrel *et al.*, 2011), allowing them to reach or extend further to traverse large gaps. These abilities may be particularly important when added stability or an escape route is required, such as during aggressive confrontations with conspecifics (Herrel *et al.*, 2011) and, possibly, predators.

Sprint speed also showed no relative differences between forms, indicating that the direction and strength of selection on this performance trait may be the same within each habitat. This finding is not altogether surprising considering chameleons move extremely slowly and tend to use crypsis instead of running to avoid predation (Brain, 1961; Burrage, 1973; Tolley & Burger, 2007). These results also indicate that sprint speed is not just a by-product of limb length, as suggested as a possible explanation for performance differences between open- and closed-canopy *B. pumilum* forms (Herrel *et al.*, 2011). Indeed, the combination of limbs and feet correlated best with sprint speed for each form, but this appears to be simply a function of body size. Types A and B – the forms with, proportionally, the longest limbs – did not run faster than the other forms, again supporting the hypothesis that limb length may be more important for bridging gaps rather than increasing speed.

Hindfoot size (especially proximal hindfoot pad length) was found to correlate best with perch diameter in almost all forms and sexes; however, this feature was not tested for performance in the present study. Consequently, it is not possible to infer whether grip performance is driven by perch size for the hindfoot. However, considering the associations between the fore- and hind-foot mentioned in the Materials and Methods, and the fact that forefoot size did show strong correlations to perch diameter in two forms the forefoot performance results are expected to hold for the hindfoot as well. As such, these results indicate that microhabitat structure (i.e., the size of perches along which chameleons move) has an effect on dwarf chameleon morphology and has likely contributed to the observed differences in trait utility between forms within this species complex. Although future studies of other species and forms that have radiated into different habitats are needed to test the generality of these observations, these data provide the first evidence of the potential existence of ecomorphs in chameleons.

Supporting Information

Table S3.1 Regression models exploring the best morphological correlate of sprint speed for each of the five phenotypic forms of the *B. melanocephalum-B. thamnobates* species complex.

Phenotypic form	Males			Females		
	Model	AIC	*wi*	Model	AIC	*wi*
B. melanocephalum	ThL	-94.473	0.035	ThL	-70.05	0.002
	CL	-95.966	0.074	CL	-72.86	0.008
	MH	-94.979	0.045	MH	-69.46	0.001
	LH	-93.977	0.028	LH	-53.37	0.000
	PH	-94.640	0.038	PH	-68.01	0.001
	BrL	-93.922	0.027	BrL	-69.22	0.001
	AL	**-98.265**	**0.235***	AL	-70.18	0.002
	MF	-94.086	0.029	MF	-72.29	0.006
	LF	-94.306	0.032	LF	-70.42	0.002
	PF	-93.868	0.026	PF	-69.11	0.001
	AL+BrL	-97.745	0.149	**MF+CL**	**-81.26**	**0.384***
	AL+BrL+CL	-98.693	0.175*	CL+BrL+MF	-81.82	0.281*
	CL+AL+BrL+MF	-98.065	0.081	THL+CL+BrL+MF	-83.34	0.242*
	CL+PH+AL+BrL+MF	-96.824	0.024	THL+CL+BrL+MF+LF	-83.22	0.060*
				THL+CL+BrL+AL+MF+LF	-82.80	0.007*
B. thamnobates	**ThL**	**-89.863**	**0.513***	ThL	-101.92	0.046
	CL	-81.868	0.009	CL	-102.23	0.053
	MH	-81.060	0.006	MH	-102.85	0.073
	LH	-79.685	0.003	LH	-101.85	0.044
	PH	-80.668	0.005	PH	-103.09	0.082
	BrL	-79.982	0.004	BrL	-101.92	0.046
	AL	-79.759	0.003	AL	-104.48	0.164
	MF	-79.982	0.004	MF	-101.85	0.044
	LF	-79.466	0.003	LF	-102.15	0.051
	PF	-79.321	0.000	PF	-101.92	0.046
	ThL+LF	-89.165	0.280*	**AL+PH**	**-105.16**	**0.190**
	ThL+LH+LF	-88.418	0.130*	CL+PH+AL	-104.23	0.087
	ThL+MH+LH+LF	-87.454	0.040*	MH+PH+AL	-103.93	0.075
	ThL+MH+LH+PH+LF	-85.915	0.010*			
	ThL+MH+LH+PH+AL+LF	-84.072	0.000			
Type A	ThL	-77.528	0.051	ThL	-86.46	0.019
	CL	-77.363	0.047	CL	-85.99	0.015
	MH	-78.204	0.071	MH	-86.94	0.024
	LH	-77.528	0.051	LH	-90.30	0.128*
	PH	-77.948	0.063	PH	-88.00	0.040
	BrL	-75.125	0.015	BrL	-88.67	0.056
	AL	-77.695	0.055	AL	-90.06	0.113*
	MF	**-80.110**	**0.185**	MF	-88.30	0.047
	LF	-77.863	0.060	LF	-85.93	0.014
	PF	-77.611	0.053	PF	-86.39	0.018
	MF+LF	-80.351	0.158	**LH+AL**	**-91.92**	**0.228***
	AL+MF+LF	-81.035	0.141	LH+PH+AL	-91.21	0.110*
	AL+MF+LF+PF	-80.340	0.050	LH+PH+AL+PF	-92.18	0.104*
	PH+AL+MF+LF+PF	-81.758	0.039	MH+LH+PH+AL+PF	-92.82	0.067*
				MH+LH+PH+AL+LF+PF	-91.91	0.016

Table S3.1 *continued*.

Phenotypic form	Males			Females		
	Model	AIC	*wi*	Model	AIC	*wi*
Type B	ThL	-67.901	0.021	ThL	-50.99	0.155
	CL	-66.769	0.012	CL	-46.65	0.018
	MH	-73.000	0.274*	MH	-47.16	0.023
	LH	-69.295	0.043	LH	-47.34	0.025
	PH	-66.769	0.012	PH	-46.90	0.020
	BrL	-68.350	0.027	BrL	-46.90	0.020
	AL	-67.466	0.017	AL	-46.99	0.021
	MF	-68.199	0.025	MF	-46.65	0.018
	LF	-66.906	0.013	LF	-50.99	0.155
	PF	-66.769	0.012	PF	-41.16	0.001
	MH+BRL	**-74.610**	**0.419***	**ThL+LF**	**-54.30**	**0.478**
	CL+MH+BRL	-73.170	0.106*	LH+MF+LF+PF	-54.95	0.050
	CL+MH+BrL+MF	-71.459	0.016	LH+PH+MF+LF+PF	-57.97	0.016
Type C	ThL	-26.153	0.010	**ThL**	**-46.77**	**0.119**
	CL	-25.732	0.008	CL	-46.34	0.096
	MH	-24.134	0.003	MH	-45.93	0.078
	LH	-25.151	0.006	LH	-47.23	0.150
	PH	-29.790	0.059	PH	-45.93	0.078
	BrL	-23.757	0.003	BrL	-46.34	0.096
	AL	-27.771	0.021	AL	-45.93	0.078
	MF	-24.292	0.004	MF	-45.93	0.078
	LF	-23.980	0.003	LF	-45.93	0.078
	PF	-23.402	0.002	PF	-45.93	0.078
	BrL+MF	-27.790	0.005	THL+LH	-45.72	0.030
	MH+PH+AL	**-46.197**	**0.876***	THL+BrL	-46.23	0.038

AIC, Akaike's information criterion; *wi*, Akaike's weight; ThL, thigh length; CL, crus length; MH, medial hindfoot pad length; LH, lateral hindfoot pad length; PH, proximal hindfoot pad length; BrL, brachium length; AL, antebrachium length; MF, medial forefoot pad length; LF, lateral forefoot pad length; PF, proximal forefoot pad length; *, $P < 0.05$. Multi-trait models with *wi* less than 0.01are not shown here. Text in bold highlights the best fitting model.

Table S3.2 Regression models exploring the best morphological correlate of maximum forefoot grip strength on the broad dowel for each of the five phenotypic forms of the *B. melanocephalum-B. thamnobates* species complex.

Phenotypic form	Males			Females		
	Model	AIC	*wi*	Model	AIC	*Wi*
B. melanocephalum	BrL	-81.958	0.217*	BrL	-49.26	0.119
	AL	**-82.191**	**0.243***	AL	-48.99	0.104
	MF	-81.146	0.144*	**MF**	**-50.70**	**0.245**
	LF	-75.835	0.010	LF	-48.87	0.098
	PF	-78.458	0.038	PF	-49.95	0.168
	BrL+AL	-81.885	0.172*	MF+PF	-50.98	0.199
	BrL+AL+LF	-81.863	0.124*	MF+LF+ PF	-49.99	0.067
	BrL+AL+MF+LF	-81.018	0.052*			
B. thamnobates	**BrL**	**-63.430**	**0.380**	BrL	-90.15	0.095
	AL	-61.753	0.164	AL	-91.01	0.145
	MF	-60.265	0.078	MF	-91.79	0.215
	LF	-60.559	0.091	**LF**	**-92.19**	**0.263**
	PF	-60.613	0.093	PF	-91.05	0.149
	BrL+MF	-62.591	0.193	AL+LF	-91.23	0.133
Type A	BrL	-60.431	0.015	BrL	-65.85	0.087
	AL	-60.208	0.013	AL	-67.51	0.199
	MF	**-68.567**	**0.891**	**MF**	**-68.57**	**0.338**
	LF	-62.539	0.043	LF	-66.92	0.149
	PF	-60.367	0.014	PF	-66.34	0.111
	MF+LF	-61.926	0.024	AL+MF	-66.89	0.118
Type B	BrL	-47.092	0.041	BrL	-30.85	0.081
	AL	-47.705	0.056	**AL**	**-34.00**	**0.392**
	MF	-43.994	0.009	MF	-31.85	0.134
	LF	-48.641	0.090	LF	-31.49	0.112
	PF	**-51.525**	**0.379***	PF	-32.29	0.167
	AL+PF	-51.539	0.261	AL+PF	-32.59	0.115
	AL+MF+PF	-51.522	0.135			
	AL+MF+LF+PF	-50.466	0.029			
Type C	BrL	-21.227	0.027	BrL	-29.50	0.132
	AL	-22.160	0.043	AL	-28.27	0.071
	MF	**-26.846**	**0.445***	**MF**	**-32.29**	**0.534**
	LF	-22.874	0.061	LF	-28.44	0.078
	PF	-21.522	0.031	PF	-28.18	0.068
	BrL+PF	-29.384	0.353*	PF+MF	-30.98	0.116
	BrL+AL+PF	-33.014	0.040			

AIC, Akaike's information criterion; *wi*, Akaike's weight; BrL, brachium length; AL, antebrachium length; MF, medial forefoot pad length; LF, lateral forefoot pad length; PF, proximal forefoot pad length; *, $P < 0.05$. Multi-trait models with *wi* less than 0.01are not shown here. Text in bold highlights the best fitting model.

Table S3.3 Regression models exploring the best morphological correlate of maximum forefoot grip strength on the narrow dowel for each of the five phenotypic forms of the *B. melanocephalum*-*B. thamnobates* species complex.

Phenotypic form	Males			Females		
	Model	AIC	*wi*	Model	AIC	*wi*
B. melanocephalum	BrL	-68.66	0.070	BrL	-68.66	0.024
	AL	-70.30	0.057	AL	-70.30	0.056
	MF	**-68.66**	**0.495***	MF	-68.66	0.024
	LF	-70.05	0.083	LF	-70.05	0.049
	PF	-74.41	0.083	**PF**	**-74.41**	**0.434***
	MF+LF	-74.32	0.213	AL+PF	-74.32	0.294*
				AL+MF+PF	-73.68	0.118
B. thamnobates	BrL	-86.53	0.259	BrL	-122.51	0.021
	AL	**-87.28**	**0.377***	AL	-127.69	0.281*
	MF	-84.24	0.083	MF	-126.18	0.132*
	LF	-82.97	0.044	LF	-123.80	0.040
	PF	-82.63	0.037	PF	-122.87	0.025
	BrL+AL	-86.53	0.200	**AL+MF**	**-128.52**	**0.350***
				AL+MF+LF	-127.02	0.121*
				BRL+AL+MF+LF	-125.02	0.028
Type A	BrL	-72.31	0.075	BrL	-94.97	0.000
	AL	-73.07	0.109	AL	-96.95	0.001
	MF	-71.11	0.041	**MF**	**-110.06**	**0.516***
	LF	-70.93	0.038	LF	-94.32	0.000
	PF	-73.00	0.106	PF	-94.97	0.000
	AL+PF	**-76.38**	**0.434***	MF+LF	-109.58	0.327*
	AL+MF+PF	-75.27	0.158	MF+LF+PF	-108.29	0.121*
	BrL+AL+MF+PF	-73.83	0.039	AL+MF+LF+PF	-106.47	0.029*
Type B	BrL	-62.27	0.033	BrL	-43.36	0.131
	AL	-61.88	0.027	AL	-43.18	0.120
	MF	-61.69	0.025	MF	-44.33	0.213
	LF	-62.98	0.047	LF	-43.61	0.149
	PF	**-68.20**	**0.641***	**PF**	**-45.10**	**0.313**
	BrL+PF	-66.50	0.188	AL+PF	-43.25	0.073
	BrL+MF+PF	-64.66	0.039			
Type C	BrL	-23.265	0.157	BrL	-33.81	0.146
	AL	-24.057	0.233	AL	-33.99	0.159
	MF	**-24.213**	**0.251**	**MF**	**-35.15**	**0.285**
	LF	-22.566	0.110	LF	-34.36	0.192
	PF	-22.447	0.104	PF	-33.81	0.146
	AL+PF	-22.213	0.021	BrL+MF	-34.14	0.072
	MF+PF	-22.621	0.025			
	AL+MF+LF+PF	-25.357	0.099			

AIC, Akaike's information criterion; *wi*, Akaike's weight; BrL, brachium length; AL, antebrachium length; MF, medial forefoot pad length; LF, lateral forefoot pad length; PF, proximal forefoot pad length; *, $P < 0.05$. Multi-trait models with *wi* less than 0.01are not shown here. Text in bold highlights the best fitting model.

Table S3.4 Regression models exploring the best morphological correlate of perch diameter for each of the five phenotypic forms.

Phenotypic Form	Log_{10}Perch Diameter					
	Males			Females		
	Model	AIC	*wi*	Model	AIC	*Wi*
B. melanocephalum	TL	-72.183	0.226	TL	-51.56	0.035*
	MH	-71.942	0.051	MH	-45.94	0.002
	LH	-71.446	0.045	LH	-45.50	0.002
	PH	-71.769	0.035	PH	-53.02	0.073*
	MF	-71.468	0.041	MF	-45.20	0.001
	LF	-71.489	0.035	LF	-45.67	0.002
	PF	**-76.327**	**0.403***	PF	-45.99	0.002
	PF+MF	-75.560	0.226	**PH+TL**	**-58.15**	**0.671***
	PF+TL	-74.431	0.128	PF+PH+TL	-56.60	0.171*
				PF+TL+PH+LH	-55.26	0.035*
B. thamnobates	TL	-61.572	0.180*	TL	-57.80	0.000*
	MH	-59.204	0.055	MH	-56.68	0.000*
	LH	-59.482	0.063	LH	-56.75	0.000*
	PH	-60.743	0.119	PH	-52.31	0.000*
	MF	-59.812	0.074	MF	-61.96	0.002*
	LF	49.527	0.000	LF	-57.39	0.000*
	PF	**-61.948**	**0.217***	PF	-54.10	0.000*
	PF+TL	-52.138	0.000	PF+TL	-56.41	0.000*
	PF+MH	-61.976	0.160*	**MF+PH**	**-73.58**	**0.664***
	PF+PH+MH	-62.371	0.113	MF+PH+TL	-72.38	0.256*
	PF+PH+MF+MH	-60.443	0.020	MF+PH+TL+MH	-70.56	0.062*
				MF+PH+TL+MH+PF	-68.70	0.012*
Type A	TL	-45.332	0.154	TL	-50.91	0.007
	MH	-45.485	0.167	MH	-52.23	0.097
	LH	**-45.824**	**0.198**	LH	-50.78	0.007
	PH	-44.638	0.109	**PH**	**-54.91**	**0.054***
	MF	-44.678	0.111	MF	-51.44	0.010
	LF	-44.651	0.110	LF	-50.71	0.007
	PF	-44.572	0.106	PF	-50.94	0.007
	TL+PF	-43.415	0.045	PF+PH	-53.91	0.027
				PF+PH+MH	-52.92	0.011
Type B	TL	-36.925	0.015	TL	-29.65	0.108
	MH	-41.988	0.186*	MH	-29.71	0.111
	LH	-37.920	0.024	LH	-29.81	0.117
	PH	-36.785	0.014	PH	-29.76	0.114
	MF	-36.766	0.014	MF	-29.76	0.114
	LF	-36.925	0.015	LF	-29.76	0.114
	PF	-36.805	0.014	PF	-30.08	0.134
	TL+PF	-34.925	0.000	**PF+MF**	**-32.08**	**0.152**
	MH+TL	-44.036	0.250*	PF+TL	-28.77	0.029
	MH+PH+TL	**-46.736**	**0.633***	PF+MH+MF	-30.17	0.010
	MH+TL+PH+LF	-44.852	0.077*			
	MH+TL+PH+LF+MF	-42.969	0.010			
Type C	TL	-20.093	0.112	TL	-26.02	0.106
	MH	-20.462	0.135	MH	-26.15	0.113
	LH	-20.505	0.138	**LH**	**-26.86**	**0.161**
	PH	-19.937	0.104	PH	-26.46	0.132
	MF	-19.937	0.104	MF	-26.64	0.145
	LF	**-21.422**	**0.218**	LF	-26.64	0.145
	PF	-20.133	0.114	PF	-26.46	0.132
	PF+TL	-18.378	0.011	LF+TL	-25.84	0.041
	MH+TL	-21.980	0.064	PF+TL	-24.79	0.024

AIC, Akaike's information criterion; *wi*, Akaike's weight; TL, tail length; MH, medial hindfoot pad length; LH, lateral hindfoot pad length; PH, proximal hindfoot pad length; MF, medial forefoot pad length; LF, lateral forefoot pad length; PF, proximal forefoot pad length; *, $P < 0.05$. Multi-trait models with *wi* less than 0.01are not shown here. Text in bold highlights the best fitting model.

Chapter 4

Paper III:
Sexual dimorphism in bite performance drives morphological variation in chameleons*

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ABSTRACT

Phenotypic performance in different environments is central to understanding the evolutionary and ecological processes that drive adaptive divergence and, ultimately, speciation. Because habitat structure can affect an animal's foraging behaviour, anti-predator defences, and communication behaviour, it can influence both natural and sexual selection pressures. These selective pressures, in turn, act upon morphological traits to maximize an animal's performance. For performance traits involved in both social and ecological activities, such as bite force, natural and sexual selection often interact in complex ways, providing an opportunity to understand the adaptive significance of morphological variation with respect to habitat. Dwarf chameleons within the *Bradypodion melanocephalum-Bradypodion thamnobates* species complex have multiple phenotypic forms, each with a specific head morphology that could reflect its use of either open or closed-canopy habitats. To determine whether these morphological differences represent adaptations to their habitats, we tested for differences in both absolute and relative bite performance. Only absolute differences were found between forms, with the closed-canopy forms biting harder than their open-canopy counterparts. In contrast, sexual dimorphism was found for both absolute and relative bite force, but the relative differences were limited to the closed-canopy forms. These results indicate that both natural and sexual selection are acting within both habitat types, but to varying degrees. Sexual selection seems to be the predominant force within the closed-canopy habitats, which are more protected from aerial predators, enabling chameleons to invest more in ornamentation for communication. In contrast, natural selection is likely to be the predominant force in the open-canopy habitats, inhibiting the development of conspicuous secondary sexual characteristics and, ultimately, enforcing their overall diminutive body size and constraining performance.

INTRODUCTION

Evolutionary and ecological processes that drive adaptive divergence and, ultimately, speciation can be influenced by phenotypic performance in different environments. As new environmental niches become available for populations to exploit, morphological and physiological adaptations arise, often resulting in enhanced performance in the novel habitat (Schluter, 2000). Evidence for these adaptations can be found in the improved performance of animals in their new environment (Schluter, 2000). For example, habitat structure or complexity is known to influence a range of lizard behaviours, including communication and anti-predator defences. Densely vegetated, structurally complex habitats may afford lizards greater cover from predators, thereby enabling them to invest more in conspicuous features, such as ornamentation and bright colouration, for increased detectability to conspecifics in those habitats; whereas, the converse is true in less vegetated habitats, where visibility to predators is high, thereby increasing the need for crypsis (e.g., Leal & Fleishman, 2004; Stuart-Fox & Moussalli, 2008). Because the head is involved in many ecologically and socially relevant activities, such as feeding, mating and aggressive interactions, its morphology and association to bite performance and habitat have been widely investigated to better understand the adaptive significance and the underlying processes shaping phenotypic variation within and between species (e.g., Herrel *et al.*, 1999; Herrel *et al.*, 2001a; Husak *et al.*, 2006; Huyghe *et al.*, 2006; Lappin, Hamilton, & Sullivan, 2006; Herrel, McBrayer, & Larson, 2007; Lailvaux & Irschick, 2007; Measey *et al.*, 2009; Herrel *et al.*, 2010; Vanhooydonck *et al.*, 2010; Kaliontzopoulou *et al.*, 2012). Many of these studies have shown that bite force is influenced by both natural and sexual selection, yet the relative contribution of these selective pressures remains difficult to unravel as they often interact in complex ways. Moreover, sexual and natural selection can act in opposition, with sexual selection

favouring conspicuous coloration or ornamentation for effective communication and natural selection favouring cryptic coloration and reduced ornamentation to avoid injury from predation or intraspecific encounters (Andersson, 1982; Endler, 1983). This results in a trade-off between the two selective pressures, with the relative strength of natural and sexual selection on particular head traits being partly dependent on the environment (e.g., Herrel, Vanhooydonck, & Van Damme, 2004; Measey *et al.*, 2011; Vanhooydonck *et al.*, 2011). This complex interaction often results in interspecific variation; however, it can also lead to intraspecific variation in the form of varying degrees of sexual dimorphism (e.g., Butler & Losos, 2002; Butler, Sawyer, & Losos, 2007; Stuart-Fox & Moussalli, 2007; Kaliontzopoulou, Carretero, & Llorente, 2010), both of which have been shown to contribute significantly to adaptive radiations (Schluter, 2000; Butler *et al.*, 2007).

Chameleons have radiated into multiple habitats, including forests, grasslands, heathlands, savannah, and desert; and their colonisation of these different niches corresponds with the emergence of these biomes on the landscape (Tolley *et al.*, 2013). Indeed, chameleon morphology may be under rapid directional selection in instances where novel habitats are colonised (Tolley *et al.*, 2004; Tolley *et al.*, 2006; Tolley *et al.*, 2008), and this process may be well illustrated by a radiation of dwarf chameleons (*Bradypodion*) from KwaZulu-Natal (KZN) Province, South Africa. The species complex is comprised of five phenotypic forms, two of which are described species (*Bradypodion melanocephalum*, *Bradypodion thamnobates*) and the remaining three (Types A, B and C) designated as morphotypes (Gray, 1865; Raw, 1976; Tolley & Burger, 2007; Tilbury, 2010; Chapter 2) (Fig. 4.1). All forms are allopatric in distribution, but mitochondrial markers show they lack the divergence expected at the species level, which reflects the recent nature of the radiation (Tolley *et al.*, 2004; Tolley *et al.*, 2008). However, it is likely that, at present, no gene flow takes place between forms given that the habitats in which

they occur are fragmented and isolated. There are also ecological differences between their macro- and micro-habitats, with *B. melanocephalum* and Type A occupying more open-canopy habitats (e.g., grasslands), which contain densely clustered, vertically-oriented vegetation for chameleons to perch upon; while *B. thamnobates* and Types B and C occupy closed-canopy habitats (e.g., forests, transformed landscapes) that contain broader perching substrates arranged both vertically and horizontally (Chapter 2). These ecological differences were found to correlate to functional differences in forefoot grip strength, suggesting that the forms are adapted morphologically to their different environments (Chapter 3). However, variation in head size and shape was found to be the most important component in differentiating between phenotypic forms in this radiation, accounting for approximately half of the total variation in both sexes (Chapter 2). Moreover, the degree of sexual dimorphism varied between forms, with little to no dimorphism in head size and shape detected among open-canopy habitat chameleons, yet extensive dimorphism among the closed-canopy *B. thamnobates* (Chapter 2). As such, it is expected that considerable sexual and interspecific (interform) variation will be uncovered in bite performance, lending further support for the designation of this radiation as adaptive.

Like most lizards, dwarf chameleons use their heads in intraspecific communication signalling to rivals that confrontations can be harmful, and displaying to females to assess their willingness to mate (Rand, 1961; Burrage, 1973; Bickel & Losos, 2002; Stuart-Fox & Whiting, 2005; Stuart-Fox *et al.*, 2006a; Tolley & Burger, 2007; Tilbury, 2010). Considering that different structural habitats can select for different types of communication behaviour (Waser & Brown, 1984; Fleishman, 1992; Leal & Fleishman, 2004; Stuart-Fox & Moussalli, 2008), the effectiveness of a particular head design may depend upon the environment. Given that closed-canopy habitats can constrain the effectiveness of aerial predators, sexual selection may be the predominant force within

these habitats, enabling chameleons, especially males, to invest more in ornamentation, such as the casque, for communication; while, in the open-canopy habitats, natural selection may outweigh sexual selection to increase crypsis (Stuart-Fox *et al.*, 2003; Stuart-Fox *et al.*, 2006a; Stuart-Fox & Moussalli, 2007). This likely explains why chameleons with large heads and ornaments (*B. thamnobates* and Types B and C) occupy closed-canopy habitats, while those with proportionally smaller heads and ornaments (*B. melanocephalum* and Type A) typically occupy more open-canopy habitats (Tolley & Burger, 2007; Chapter 2). If differential degrees of natural and sexual selection are, in fact, influencing chameleon head morphology between and within these habitats, this should be reflected in their bite performance and in the morphological features used to produce it. Accordingly, if ornaments are honest signals, in closed-canopy habitats, bite force should correlate best to ornamentation, especially in males. The result would be high levels of sexual dimorphism, with males generating a greater force to assist them during intrasexual competitions. In contrast, in open-canopy habitats, bite force is expected to correlate with non-ornamented, functional characters, with the proportionally larger headed chameleons producing a greater force, irrespective of sex. Regardless of the degree of natural selection within each habitat, its influence between habitats is expected to be strong enough to ensure that larger headed chameleons possess a harder bite, which in this radiation would be the closed-canopy forms.

To test these predictions and gain insight into the adaptive nature of the chameleon head within this recent radiation, we use a combination of morphometric and bite force data for multiple phenotypic forms. Specifically, we investigate whether the heads of the phenotypic forms and sexes are morphologically and functionally differentiated with respect to habitat structure, and which morphological variables are most closely associated

with bite force within each form. The latter allows for inferences to be made regarding ornamental features and behaviour.

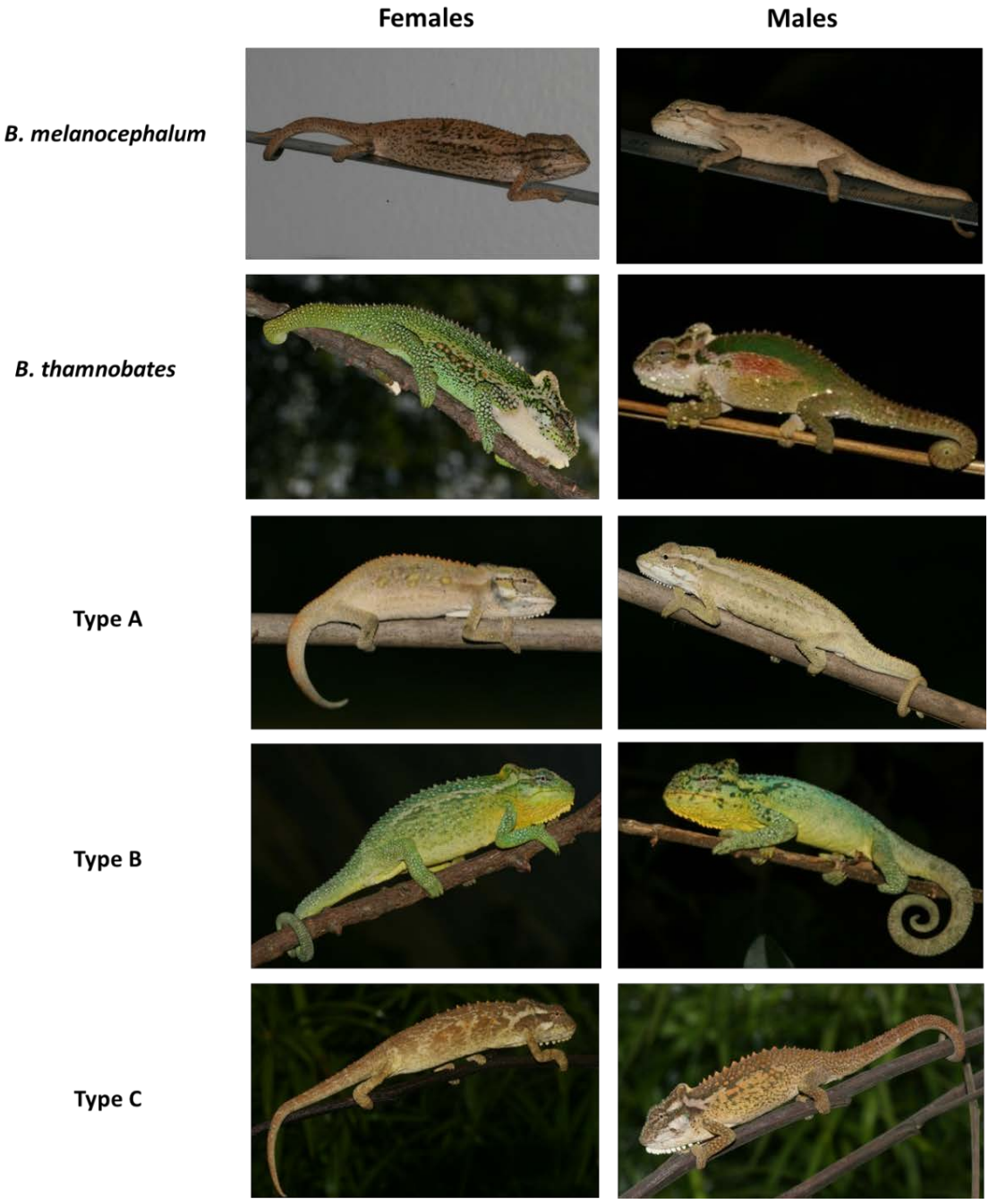

Figure 4.1 Photographs of the five dwarf chameleon forms within the *B. melanocephalum*-*B. thamnobates* species complex from southern KwaZulu-Natal Province, South Africa. Figure taken from Chapter 2 (fig. 2.1).

MATERIALS AND METHODS

ETHICS STATEMENT

Ethics clearance was obtained from Stellenbosch University (Clearance No. 2009B01007) and the South African National Biodiversity Research (Clearance no. 0010/08), and permits for scientific research and collections were obtained from Ezemvelo KZN Wildlife (OP 3538/2009; OP 4351/2009; OP 4596/2010), permitting the collection and handling of the lizards.

STUDY SITES AND SAMPLING PROCEDURE

Dwarf chameleons representing four of the five phenotypic forms of the *B. melanocephalum-B. thamnobates* species complex were sampled from six field sites within southern KZN (Fig. 4.2) between January and February 2010. Although sampled, Type C was not included due to insufficient sample sizes. Animals were collected at night and geo-referenced at the exact location each chameleon was found. They were placed in separate cloth bags then brought back to the field base overnight, where they were measured and their bite force tested the subsequent day. Once all data were collected, animals were released at the exact site of capture.

MORPHOMETRICS

For all chameleons, snout-vent length (SVL) and nine head measurements (ornamented or non-ornamented) were measured to the nearest 0.01 mm using digital callipers (Table 4.1, Fig. 4.3). The non-ornamented measurements included lower jaw length (LJL), head length (HL), head width (HW), head height (HH), the distance from the coronoid process of the mandible to snout tip (i.e., snout length, CT), and posterior surface of quadrate to snout tip (QT); and the ornamented measurements include casque head length (CHL), casque head

height (CHH), and casque height (CH). The mass of each chameleon was also measured using a Pesola® micro-line spring scale (model 93010).

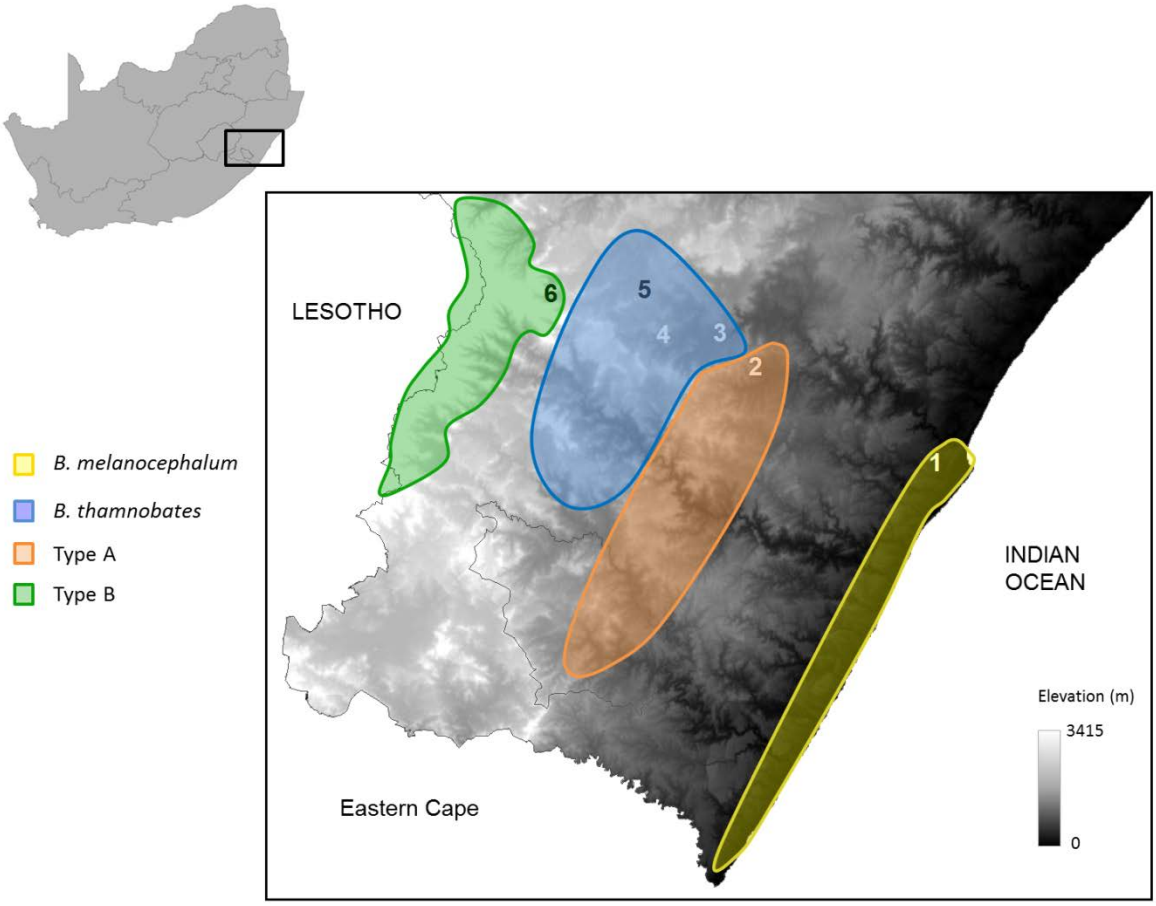

Figure 4.2 Distributions of four of the five phenotypic forms of the *B. melanocephalum*-*B. thamnobates* species complex. Numbers indicate field sites sampled in this study: 1, Durban; 2, Hilton; 3, Karkloof; 4, Howick; 5, Dargle; 6, Nottingham Road; 7, Kamberg Nature Reserve.

Table 4.1 Summary of morphological and bite performance data for male (M) and female (F) dwarf chameleons used in this study, grouped by phenotypic form. Standard deviation shown in brackets.

	B. melanocephalum		Type A		*B. thamnobates*		Type B	
	M	F	M	F	M	F	M	F
				Morphology				
n	*25*	*16*	*19*	*23*	*20*	*25*	*15*	*12*
SVL (mm)	49.14 (0.88)	57.47 (0.95)	48.23 (1.68)	45.34 (1.37)	60.00 (3.27)	66.42 (3.32)	68.97 (1.20)	77.49 (1.98)
Non-ornamented								
LJL (mm)	11.55 (0.84)	11.05 (0.86)	13.27 (3.15)	14.29 (2.29)	14.39 (3.19)	13.56 (3.06)	11.06 (2.27)	11.65 (2.56)
HL (mm)	11.68 (0.72)	11.23 (0.85)	13.08 (2.41)	13.96 (2.18)	14.47 (2.56)	14.17 (2.76)	11.12 (1.89)	12.72 (2.60)
HH (mm)	6.98 (0.67)	6.89 (0.47)	8.44 (2.24)	9.08 (1.65)	9.00 (1.96)	8.73 (2.10)	6.81 (1.41)	7.42 (1.45)
HW (mm)	7.54 (0.48)	7.33 (0.59)	9.28 (2.59)	9.87 (2.10)	10.41 (2.50)	9.84 (2.60)	7.51 (1.19)	7.87 (1.71)
CT (mm)	9.05 (0.67)	8.82 (0.80)	10.22 (2.29)	10.94 (1.71)	10.84 (2.32)	10.57 (2.41)	8.48 (1.44)	9.07 (1.64)
QT (mm)	10.29 (0.68)	9.79 (0.87)	11.82 (3.00)	12.50 (2.05)	13.08 (2.93)	12.18 (3.00)	9.51 (1.86)	10.36 (2.15)
Ornamented								
CH (mm)	4.66 (0.84)	4.44 (0.70)	7.04 (2.86)	7.23 (1.84)	7.85 (2.42)	7.49 (2.42)	5.12 (1.41)	5.48 (2.05)
CHL (mm)	16.75 (1.12)	16.18 (1.20)	19.90 (4.79)	21.46 (3.90)	22.28 (4.83)	21.25 (5.12)	15.75 (3.17)	17.44 (3.94)
CHH (mm)	9.85 (1.29)	9.57 (1.04)	13.29 (4.68)	14.41 (3.03)	14.69 (4.09)	14.53 (4.23)	10.11 (2.45)	11.25 (3.29)
				Performance				
n	*23*	*15*	*19*	*20*	*20*	*25*	*13*	*12*
Bite force (N)	10.37 (3.11)	13.88 (4.11)	11.77 (5.55)	9.05 (5.23)	23.57 (17.89)	24.74 (16.35)	30.40 (7.58)	34.25 (13.04)

LJL, lower jaw length; HL, head length; HH, head height; HW, head width; CT, coronoid process of mandible to snout tip; QT, posterior surface of quadrate to snout tip; CH, casque height; CHL, casque head length; CHH, casque head height.

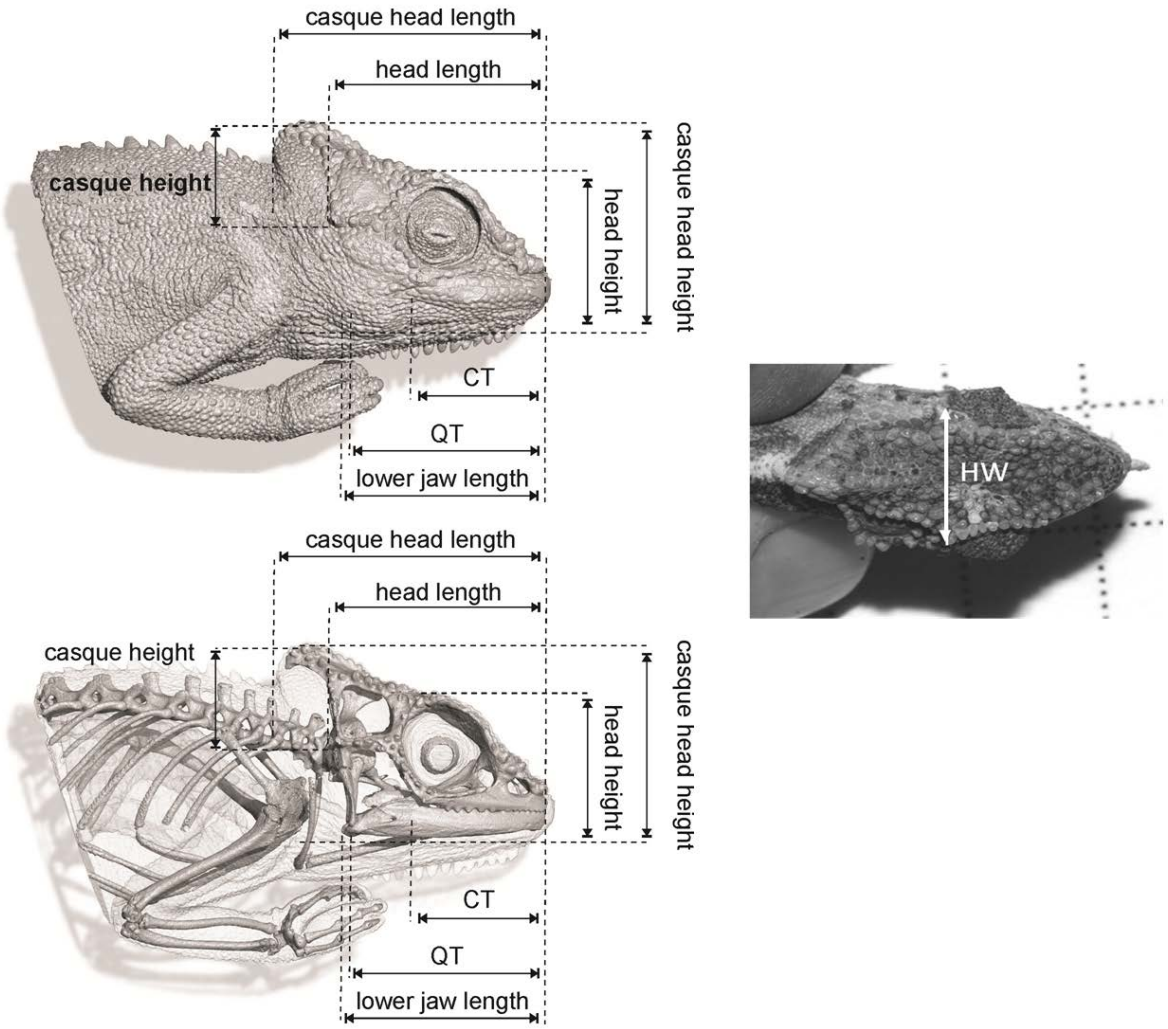

Figure 4.3 Nine head measurements recorded for each chameleon. Images on the left are based on a µCT-scan, courtesy of R. Boistel, Université de Poitiers. CT, coronoid process of mandible to snout tip; QT, posterior surface of quadrate to snout tip; HW, head width.

BITE FORCE

Chameleons were allowed to thermoregulate in a sun/shade setting to obtain their preferred body temperature (between 28-32˚C: Segall *et al.*, 2013). *In vivo* bite force was then measured in Newtons (N) at ambient temperature using an isometric force transducer (Kistler type 9203, ±500 N) connected to a bite plate and a Kistler charge amplifier (type

5995A, Kistler Inc., Winterthur, Switzerland) (Herrel *et al.*, 1999; Stuart-Fox & Moussalli, 2008). The bite plate was then placed between the jaws of the chameleon, which typically resulted in the chameleon biting down on the plate repeatedly. When necessary, chameleons were induced to bite by gently tapping the sides of their jaws. Five independent measures were recorded per chameleon and the highest value retained for analysis.

STATISTICAL ANALYSES

All analyses were carried out using SPSS version 17.0 (2008). All data were $\log_{10}$ transformed prior to analysis to fulfil assumptions of normality and homoscedascity. To separate differences in shape and performance from differences in body size, all data were size-corrected against $\log_{10}$SVL and the unstandardized residuals saved for use in subsequent analyses. Although studies have shown that the head can develop at a different rate than overall body size (e.g., Braña, 1996; Kratochvíl *et al.*, 2003), this was not found to be the case for these chameleons. After applying the methods of Braña (1996) and McCoy and colleagues (2006) across all phenotypic forms and sexes, all morphometric variables were found to share a common growth axis and follow similar trajectories, and SVL was recognized as having the highest principal component loading validating its use as a suitable covariate for all measurements.

Although a previous study showed significant differences in head morphology between the four phenotypic forms and sexes in this study (Chapter 2), a multivariate analysis of covariance (MANCOVA) using a general linear model (GLM), and a principal component analysis (PCA) were conducted to verify those results on this dataset, which is a subset from Chapter 2. The full GLM model specified SEX and FORM as fixed factors, SEX x FORM as the interaction, $\log_{10}$SVLas the covariate, and all $\log_{10}$-tranformed head

variables as the dependent variables. The unstandardized residuals for the nine head variables were then entered into a PCA and the principal component (PC) scores were saved so that the magnitude and direction of the eigenvector describing the differences between forms could be illustrated. Only PCs with eigenvalues larger than one were extracted, and the varimax rotation was used to minimize the number of variables with high loadings on each factor. Variables with communality values less than 0.5 were omitted from the analysis, as low values indicate those variables are uninformative (Tabachnick & Fidell, 2007). The saved PC scores were then entered as the dependent variables in analyses if variance (ANOVAs), with FORM as the fixed factor to assess more fine-scale differences in head morphology between forms. Bonferroni post-hoc tests were run to determine which forms differed for each principal component. Next, additional ANOVAs were conducted on both absolute (log_{10}-transformed) and relative (size-corrected) bite force to test for differences in performance between forms. All *P*-values were subjected to Holm's sequential Bonferroni correction.

Because the morphological variables found to be most relevant to bite performance differ between species (e.g., Herrel, De Grauw, & Lemos-Espinal, 2001c; Lappin *et al.*, 2006; Measey *et al.*, 2009; Vanhooydonck *et al.*, 2011), multiple regression models were carried out on size-corrected variables to explore which ones best explained the variation in bite force within each form. Akaike's information criterion (AIC) was calculated using the residual sum of squares from each model, and the difference between the lowest AIC and all others (Δ_i) was determined. Akaike's weights (*wi*) were then calculated for each model, with the one exhibiting the highest *wi* acknowledged as the best fitting model (Burnham & Anderson, 2002).

RESULTS

Morphological and performance data were gathered from 155 dwarf chameleons within the *B. melanocephalum-B. thamnobates* species complex (Table 4.1). A MANCOVA revealed differences in head morphology between the four phenotypic forms (Wilks' $\lambda = 0.363$, $F_{3,36} = 4.890$, $P < 0.001$) and sexes (Wilks' $\lambda = 0.859$, $F_{1,9} = 2.745$, $P = 0.005$), with the PCA and subsequent ANOVA indicating that *B. melanocephalum* had proportionally the smallest head in both sexes, *B. thamnobates* the biggest, and Types A and B being intermediate in head size, confirming that this subset of data shows the same pattern as the previous study (Chapter 2).

Bite force was found to correlate positively with body size (SVL) in all phenotypic forms and sexes (Fig. 4.4). A comparison of bite performance between the sexes revealed different patterns in absolute and relative forces (Table 4.2). Females tended to have a stronger absolute bite force than males (Table 4.1), with the most pronounced difference detected in *B. melanocephalum* ($F = 8.283$, $P = 0.006$; see Fig. 4.5). However, once bite force was corrected for body size, *B. thamnobates* and Type B males were found to bite proportionally harder than females (*B. thamnobates*: $F = 9.437$, $P = 0.004$; Type B: $F = 10.770$, $P = 0.003$; see Fig. 4.6). The open-canopy habitat forms showed no sexual variation in bite performance (*B. melanocephalum*: $F = 2.660$, $P = 0.111$; Type A: $F = 0.870$, $P = 0.357$).

When examining bite force between forms, differences were only found for absolute (Males: $F_{3,78} = 19.431$, $P < 0.0001$; Females: $F_{3,75} = 13.716$, $P < 0.0001$) and not relative bite forces (Males: $F_{3,78} = 1.437$, $P = 0.229$; Females: $F_{3,75} = 1.575$, $P = 0.189$). Similar patterns were detected for both sexes, with the four phenotypic forms fitting into one of two strength categories: weak (*B. melanocephalum*, Type A) or strong

(*B. thamnobates* and Type B) (Fig. 4.5). In males, Type B further differentiated from *B. thamnobates* by possessing a significantly stronger bite.

Model selection using linear regression to find the morphological variables that best explain bite force found different correlations between the four phenotypic forms and sexes (Table 4.3). *Bradypodion thamnobates* was the sole form whose performance could only be explained by a single model (Table S4.1), and this model was the same for both sexes (HH+CT). For the other forms, several candidate models displayed significant correlations to bite performance (Table S4.1). Apart from *B. thamnobates*, different parts of the casque were identified as contributing to bite force in males; however, the contribution was only significant for *B. melanocephalum* (CHL) and Type B (CH, CHH). In comparison, non-ornamented features (HH, HL, CT, QT) explained bite force in females (Table 4.3).

Table 4.3 Regression models (i.e., morphological variables) found to best reflect bite force within each phenotypic form and sex. All variables were size-corrected prior to analysis. Bold values represent significant correlations.

Phenotypic Form	*Males*					*Females*				
	Model	AIC	*wi*	R^2	*P*	Model	AIC	*wi*	R^2	*P*
B. melanocephalum	CHL	-117.81	0.399	0.391	**0.001**	QT	-68.14	0.269	0.345	**0.017**
B. thamnobates	CT HH	-85.95	0.562	0.334	**0.031**	CT HH	-113.13	0.378	0.247	**0.044**
Type A	CH LJL	-88.85	0.189	0.245	0.105	HH HL QT	-83.89	0.370	0.362	**0.033**
Type B	CH CHH HH LJL CT	-76.49	0.195	0.735	**0.031**	CT QT	-60.59	0.507	0.594	**0.017**

ß, Beta coefficient; AIC, Akaike's information criterion; *wi*, Akaike's weight; R^2, coefficient of determination; *ß*, Beta coefficient; *P*, significance value; CHL, casque head length, CHH, casque head height; CH, casque height; HL, head length; HW, head width; HH, head height; LJL, lower jaw length; CT, coronoid process of mandible to snout tip; QT, posterior surface of quadrate to snout tip.

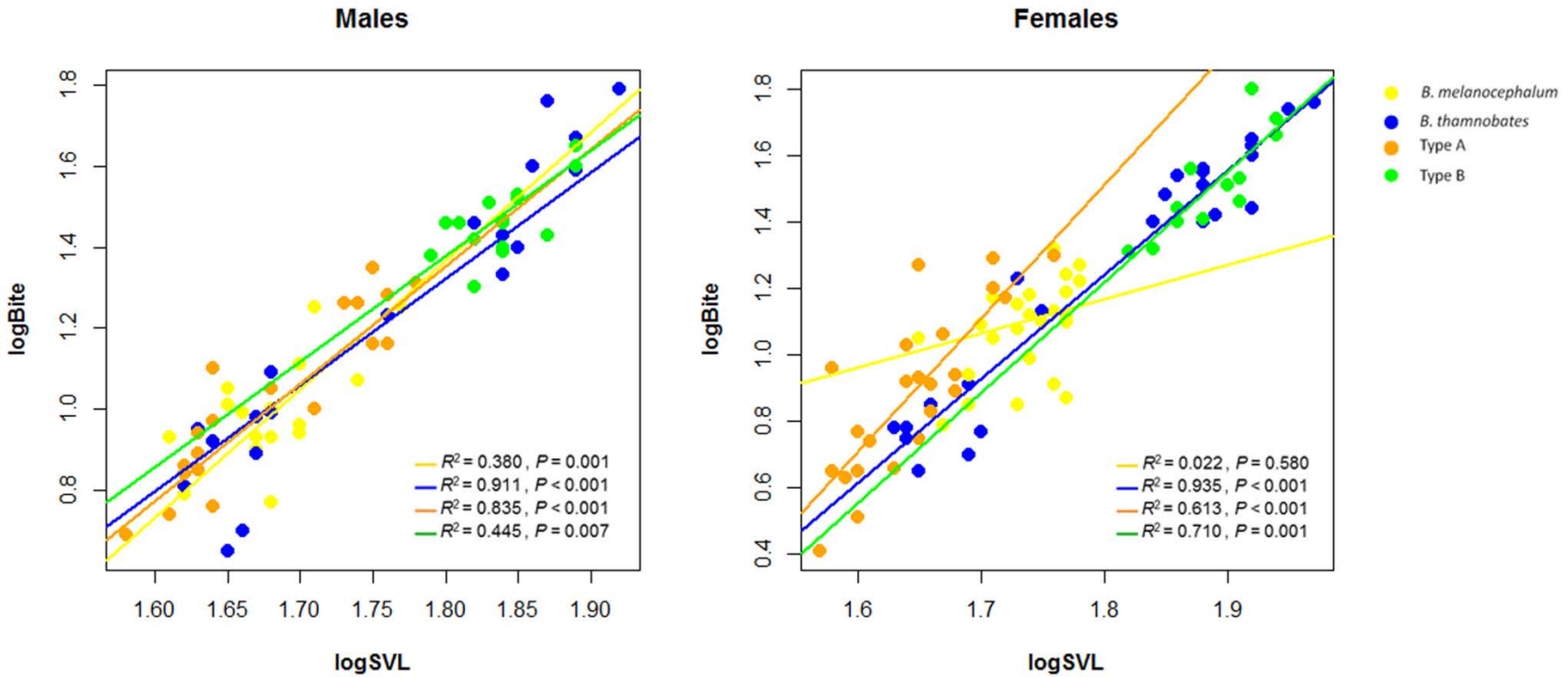

Figure 4.4 Regression plots illustrating the correlation between SVL and bite force within the *B. melanocephalum-B. thamnobates* species complex.

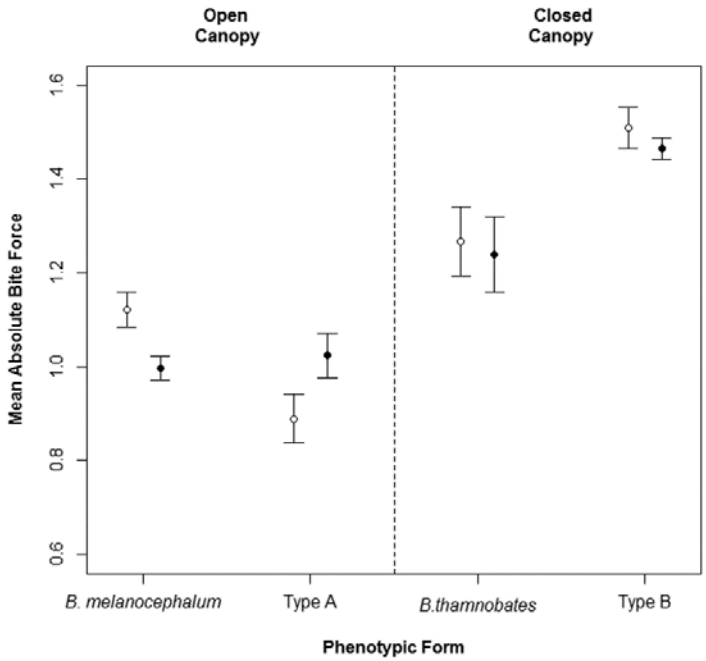

Figure 4.5 Error plots depicting mean absolute bite force for the five phenotypic forms. Absolute force equates to $\log_{10}$-transformed bite force. Solid circles represent males; empty circles, females.

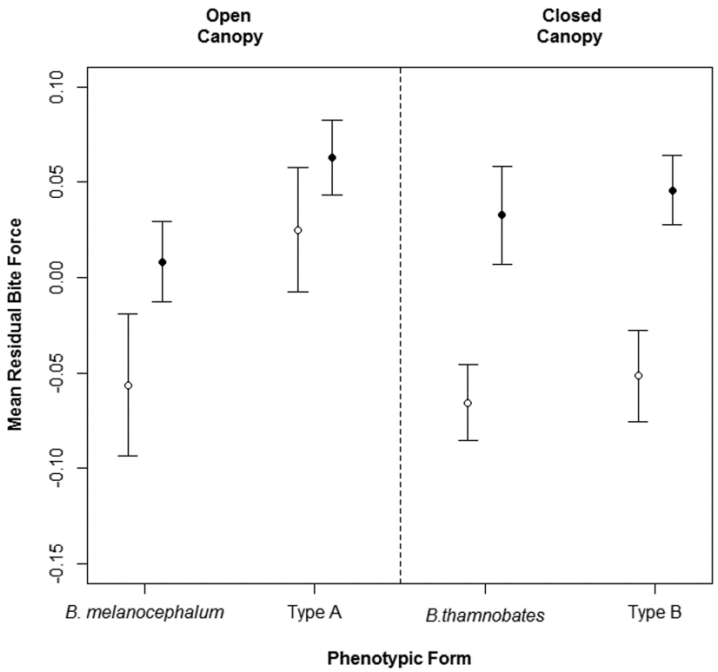

Figure 4.6 Error plots depicting mean relative bite force for the five phenotypic forms. Relative forces represent the residual values from regressing $\log_{10}$Bite Force against $\log_{10}$SVL. Solid circles represent males; empty circles, females.

DISCUSSION

Head morphology and bite performance within the *B. melanocephalum-B. thamnobates* species complex is influenced by varying degrees of natural and sexual selection, and the intensity of each appears to depend, at least partly, on the structure of the habitat. For all forms, bite force was found to correlate to overall body size, with the larger, closed-canopy forms possessing a stronger bite, as predicted under natural selection. Moreover, the degree of sexual dimorphism in head shape resulted in comparable levels of dimorphism in bite performance, with closed-canopy males biting proportionally harder than females, as predicted under sexual selection, and no dimorphism in bite performance within the open-canopy forms, possibly due to natural selection curbing sexual dimorphism for increased crypsis.

The influence of selective forces on performance is typically assessed through an examination of the proportional (size-corrected) differences between groups because morphological traits, and their associated performance, typically scale with an organism's overall body size. Consequently, differences in trait values among individuals within populations, and between populations and species, will often arise simply because individuals or populations differ in body size. With this in mind, the lack of proportional differences in bite force between phenotypic forms might suggest that natural selection is weak or not acting upon this performance measure, possibly indicating that their differential head morphologies may be a consequence of some other factor, such as founder effects. However, the absolute differences detected between open- and closed-canopy forms may be of significance considering, for many animals, body size is highly heritable (Peters, 1983) and has been shown to be influenced by habitat use (e.g., Asplund, 1974; Fleming, 1991). Each form approaches different body sizes (Chapter 2), so the detected differences in absolute bite force are likely indicative of ecological differences

between them, such as differences in diet (e.g., Verwaijen *et al.*, 2002; Herrel & Holanova, 2008) or how they conduct their social interactions.

The snout length (CT) was the common variable found to explain bite force amongst both sexes of *B. thamnobates* and Type B – in absolute terms, the two strongest forms. The muscles attaching to the coronoid (see Rieppel, 1981 for details) aid in bite force generation. Bite force has been associated with prey size and hardness in lizards, with animals possessing greater bite forces capable of consuming larger and/or harder prey (e.g., Verwaijen *et al.*, 2002; Herrel & O'Reilly, 2006; Measey *et al.*, 2011). If similar correlations exist here, then these results suggest that *B. thamnobates* and Type B are likely to consume larger and/or harder prey items than *B. melanocephalum* and Type A.

Absolute bite force might also reveal something about the social system in place within each habitat. In closed-canopy habitats, larger body sizes are advantageous because they provide an honest signal of bite force, enabling chameleons to display their potential threat from farther distances through the use of their ornamentation and, if necessary, engage in combat (see Cuadrado, 2001; Stuart-Fox & Whiting, 2005). Chameleons in the open-canopy habitat, however, have experienced a reduction in their secondary sexual characteristics, suggesting they might be better at communicating in close proximity (Measey *et al.*, 2009). The casque of *B. melanocephalum* and Type A males was found to contribute to bite performance; therefore, despite its reduced size, it may be effective enough to ward off unwanted encounters at close range.

Much like between forms, absolute differences in bite performance were also detected between the sexes. The general trend showed that females bite harder than males, because they are on average, larger in body size. Even though this relationship was only significant for B. melanocephalum, it is possibly present within other forms, yet could not be detected due to the reduced power (ß < 0.2) brought on by limited sample sizes.

Accordingly, the greater absolute bite forces of females may reduce niche overlap (Schoener, 1967) as has been suggested for other lizards (Herrel *et al.*, 1999; Herrel *et al.*, 2001a; Verwaijen *et al.*, 2002; Herrel *et al.*, 2006). For these chameleons, the bite of females was dictated by non-ornamented features, namely QT which, along with CT represents the out-lever for jaw closing. Due to the high energy demands of reproduction, females often need to consume more and/or different prey items than males (Shine, 1989). Considering that insect abundance and diversity can vary in vertical (canopy versus understory) and horizontal (between habitats) stratification (Rivers-Moore & Samways, 1996; Samways, Caldwell, & Osborn, 1996; Clark & Samways, 1997; DeVries, Murray, & Lande, 1997; DeVries, Walla, & Greeney, 1999; Kotze & Samways, 1999; Lawrence & Samways, 2002; Pryke & Samways, 2003; Grimbacher & Stork, 2007), and females within the *B. melanocephalum-B. thamnobates* species complex have been found to perch lower and occupy more open-canopy habitats than males for all forms (Chapter 2), the observed differences in bite performance between the sexes may allow for differences in dietary exploitation. However, a thorough dietary analysis needs to be undertaken to test this hypothesis.

The stronger bite of females may also provide them with an advantage during female-male interactions. In female dwarf chameleons, the need to mate after each litter is reduced because they have relatively long gestation periods for their body size (~ three months) and are able to store sperm, which enables them to have asynchronous reproduction (Burrage, 1973; Tolley & Burger, 2007; Tilbury, 2010). Consequently, 40-80% of females are gravid at a given time (Burrage, 1973). Moreover, female dwarf chameleons do not change colour to illustrate their receptive or gravid state (Burrage, 1973); therefore, the chances of males encountering a receptive female are rare. Consequently, males use courtship displays to assess a female's willingness to mate, with

females often responding with aggressive rejection behaviours (Burrage, 1973; Stuart-Fox & Whiting, 2005; Tolley & Burger, 2007; Tilbury, 2010), including biting (Stuart-Fox & Whiting, 2005). As a result, males tend to court smaller females, which are less able to dominate or inflict injury (Stuart-Fox & Whiting, 2005). Considering that our study has shown that large females possess a stronger bite than small females, the aggressive behaviour of females is potentially an honest signal of their ability to ward off unwanted encounters.

In addition to sexual dimorphism in absolute bite force, relative differences were also detected with closed-canopy males biting harder than females of the same size. A likely explanation is that closed-canopy habitats allow for increased competition between males for access to females, (as was found with increased colour change within these habitats: Stuart-Fox & Moussalli, 2008), resulting in a greater investment in the jaw muscle in males, which is also reflected in their proportionally higher and longer heads. Indeed, snout length and head height were found to best explain male bite performance, possibly by increasing the available space for jaw adductor muscles, resulting in a higher physiological cross-section and hence bite force (Herrel *et al.*, 1999; Herrel *et al.*, 2001c; Huyghe *et al.*, 2009).This is particularly relevant because altercations between males can be aggressive and often involve biting (Stuart-Fox *et al.*, 2006a; Tolley & Burger, 2007). Within Type B males, the casque (CH, CHH) was also found to contribute to bite performance, and is almost certainly used as an honest visual signal, notifying other males of the potential cost of fighting. Even though the casque did not explain bite force in *B. thamnobates*, it still appears to be an honest signal of bite performance, as larger bodied males possess larger casques and have a correspondingly harder bite.

Within the open-canopy forms (B. melanocephalum and Type A), little to no sexual dimorphism in head morphology was uncovered, which resulted in a lack of dimorphism in

bite performance. The comparable bite forces between the sexes suggests that within more open-canopy habitats there is either reduced direct competition between males for access to females or the need for increased crypsis is so strong it outweighs intrasexual selection. While there is no evidence to support the former, the trade-off between crypsis and communication/signalling ability in dwarf chameleons has been studied extensively (Stuart-Fox, Whiting, & Moussalli, 2006b; Stuart-Fox, Moussalli, & Whiting, 2007; Stuart-Fox & Moussalli, 2008; Measey *et al.*, 2009). For example, the spectral properties of chameleon signals varies predictably with habitat structure, with the display colours of open-canopy chameleons having lower UV reflectance than that of closed-canopy chameleons (Stuart-Fox *et al.*, 2007). High UV reflectance has been found to increase an animal's detectability (Fleishman, 2000); and, although, the low reflectance of open-canopy chameleons decreases their detectability to conspecifics, it is also thought to protect them from UV-sensitive avian predators (Stuart-Fox & Moussalli, 2008). Accordingly, natural selection is likely to be the predominant force in open-canopy habitats, inhibiting the development of conspicuous secondary sexual characteristics and, ultimately, enforcing their overall diminutive body size and constraining performance. However, the casque was found to contribute to bite force in the open-canopy habitat forms (B. melanocephalum: CHL; Type A: CH) and likely acts as an honest signal of performance, indicating that sexual selection might also be influencing performance in these chameleons. In fact, both selective forces are certainly operating simultaneously, but to varying degrees in each habitat.

Similar habitat-specific sexual differences have helped explain the ecomorphological diversity produced by the adaptive radiations of West Indian *Anolis* lizards (Schoener, 1967; Butler, Schoener, & Losos, 2000; Butler & Losos, 2002; Butler *et al.*, 2007). In general, anoles in low-visibility microhabitats, such as the tree crown which

has dense branches and leaves, tend to have low dimorphism; whereas those in high-visibility microhabitats, such as the tree trunk or open ground, have high dimorphism (Butler *et al.*, 2000; Butler & Losos, 2002). This relationship is similar to that found with the KZN dwarf chameleons given that the microhabitats of the open-canopy forms were actually found to have a higher density of perches and, hence, are more likely to have low-visibility, and vice versa in the closed-canopy habitats (Chapter 2). The overall extent of sexual variation in anoles can be so great, in fact, that it can exceed interspecific variation (Butler *et al.*, 2007). Consequently, overlooking sexual dimorphism could underestimate the adaptive component of an evolutionary radiation (Butler *et al.*, 2007). In light of this, sexual dimorphism should be deemed yet another ecomorphological trait used to assess divergence within a radiation or species complex. Accordingly, this study, coupled with the functional differences in forefoot grip strength already detected between open- and closed-canopy forms in this species complex (Chapter 3), proves that these five phenotypic forms have adapted morphologically to their different environments.

Supporting Information

Table S4.1 Regression models exploring the best morphological correlate of bite force for each of the five phenotypic forms of the *B. melanocephalum-B. thamnobates* species complex.

Phenotypic form	Males			Females		
	Model	AIC	*wi*	Model	AIC	*wi*
B. melanocephalum	HL	-110.395	0.010	HL	-56.730	0.022
	HW	-116.242	0.182*	HW	-60.216	0.123*
	HH	-111.388	0.016*	HH	-52.630	0.003
	LJL	-113.507	0.046*	LJL	-56.882	0.023
	CT	-110.287	0.009*	CT	-59.118	0.071
	QT	-115.697	0.139*	QT	-61.539	0.239*
	CH	-109.546	0.006	CH	-53.987	0.005
	CHH	-107.544	0.002	CHH	-56.188	0.016
	CHL	**-117.809**	**0.399***	CHL	-55.480	0.012
	HW+CT+QT	-114.557	0.047*	**HL+HW+QT+CH+ CHL**	**-68.136**	**0.269***
	HW+QT	-115.084	0.084*	HW+CT+QT	-59.932	0.042
	HW+CH	-114.380	0.059*	HW+QT	-61.611	0.175*
B. thamnobates	HL	-80.437	0.046	HL	-109.546	0.058
	HW	-80.437	0.046	HW	-110.18	0.079
	HH	-80.981	0.061	HH	-109.967	0.071
	LJL	-80.208	0.041	LJL	-109.755	0.064
	CT	-80.208	0.041	CT	-109.132	0.047
	QT	-80.437	0.046	QT	-109.441	0.055
	CH	-80.981	0.061	CH	-109.234	0.049
	CHH	-80.513	0.048	CHH	-109.755	0.064
	CHL	-80.513	0.048	CHL	-109.546	0.058
	HH+CT	**-85.950**	**0.562***	**HH+CT**	**-113.697**	**0.378***
				HW+CT+QT	-111.163	0.078

Table S4.1 *continued.*

Phenotypic form	Males			Females		
	Model	AIC	*wi*	Model	AIC	*wi*
Type A	HL	-86.751	0.058	HL	-80.012	0.048
	HW	-86.751	0.058	HW	-79.805	0.043
	HH	-86.613	0.054	HH	-80.990	0.078
	LJL	-86.751	0.058	LJL	-79.518	0.038
	CT	-86.890	0.062	CT	-80.053	0.049
	QT	-86.751	0.058	QT	-79.518	0.038
	CH	-86.751	0.058	CH	-81.121	0.084
	CHH	-86.613	0.054	CHH	-80.903	0.075
	CHL	-86.751	0.058	CHL	-80.860	0.074
	LJL+CH	**-89.654**	**0.189**	**HL+HH+QT**	**-85.225**	**0.370***
	HH+LJL+ CH	-89.171	0.094	HW+HH+CT+QT+CHH	-85.085	0.103*
	LJL+QT+CH	-89.774	0.127			
	LJL +CT+QT+CH	-89.946	0.070			
Type B	HL	-74.335	0.060	HL	-52.580	0.016
	HW	-73.431	0.039	HW	-52.580	0.016
	HH	-73.651	0.043	HH	-52.580	0.016
	LJL	-72.582	0.025	LJL	-52.580	0.016
	CT	-72.378	0.023	CT	-52.724	0.017
	QT	-73.214	0.035	QT	-52.724	0.017
	CH	-74.103	0.054	CH	-52.724	0.017
	CHH	-72.582	0.025	CHH	-53.017	0.020
	CHL	-72.378	0.023	CHL	-52.580	0.016
	HH+HW	-76.677	0.134	**CT+QT**	**-60.588**	**0.508**
	LJL+CT+CH	-70.335	0.003	HW+CT+QT	-58.588	0.071
	HH+HL+ QT	-76.011	0.050	CT+QT+CH	-60.955	0.233*
	HL+HW+CHH	-76.011	0.059	HL+QT+CT+CH+CHL	-65.985	0.040*
	HH+HW+LJL+CT+CH+CHH	-88.085	0.171*			
	HH+LJL+ CT+CH+CHH	**-83.990**	**0.210***			
	HL+HW+HH +LJL+CT+CH+CHH+CHL	-102.275	0.046*			

Table S4.1 *continued.*

Phenotypic form	Males			Females		
	Model	AIC	*wi*	Model	AIC	*wi*
Type C	HL	-17.263	0.104	HL	-21.113	0.002
	HW	-17.414	0.112	HW	-22.026	0.003
	HH	-17.214	0.101	HH	-21.167	0.002
	LJL	-17.288	0.105	LJL	-22.943	0.005
	CT	-17.288	0.105	CT	-22.026	0.003
	QT	-17.239	0.102	QT	-21.946	0.003
	CH	**-17.517**	**0.118**	CH	-27.591	0.051*
	CHH	-17.239	0.102	**CHH**	**-33.300**	**0.879***
	CHL	-17.214	0.101	CHL	-23.196	0.006
	CH+CHL	-18.765	0.049	HH+CH	-25.632	0.008
				CT+CH	-26.764	0.014*
				HW+CH	-25.838	0.009
				CH+CHL	-26.954	0.015*

AIC, Akaike's information criterion; *wi*, Akaike's weight; CHL, casque head length, CHH, casque head height; CH, casque height; HL, head length; HW, head width; HH, head height; LJL, lower jaw length; CT, coronoid process of mandible to snout tip; QT, posterior surface of quadrate to snout tip; *, $P < 0.05$. Text in bold highlights the best fitting model.

Chapter 5

Paper IV:

Isolation of novel microsatellite loci in dwarf chameleons from KwaZulu-Natal province, South Africa and their cross-amplification in other *Bradypodion* species[*]

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ABSTRACT

A recently radiated clade of dwarf chameleon (genus *Bradypodion*) localised to central-southern KwaZulu-Natal province, South Africa is considered taxonomically problematic due to the observed discordance between morphology and genetics within and between its species. The clade is made up of two described species (*Bradypodion melanocephalum-Bradypodion thamnobates*) and possibly others—all of which are experiencing significant reductions in the quality and quantity of available habitat due to natural and anthropogenic factors. To better understand the effects past and present habitat fragmentation has had on gene flow, population structure, and genetic diversity within this clade, we developed seven new microsatellite markers for the *B. melanocephalum-B. thamnobates* complex, plus two markers for *B. pumilum* using an enrichment protocol. We tested these nine markers, along with eight markers previously designed for *B. pumilum*, for cross-species transferability across five species within the genus *Bradypodion* (*B. melanocephalum, B. thamnobates, Bradypodion dracomontanum, Bradypodion* sp. and *Bradypodion pumilum*). The number of alleles ranged from 1 to 29 with observed heterozygosities ranging from 0.00 to 1.00. Several loci did not meet HW expectations, but this may be a result of extreme demographic fluctuations that have been noted for these species. Ten loci were found to be polymorphic across all species examined, making them ideal for studies examining the population genetics of dwarf chameleons.

INTRODUCTION

Dwarf chameleons (genus Bradypodion) distributed in central-to-southern KwaZulu-Natal (KZN) province, South Africa, are considered taxonomically problematic given discordance between morphology and genetics (Alexander, 2006; Tolley & Burger, 2007; Tolley *et al.*, 2008). The clade encompasses two species, *Bradypodion melanocephalum*

(the KwaZulu-Natal dwarf chameleon) and *Bradypodion thamnobates* (the Natal Midlands dwarf chameleon), which show substantial morphological distinctness (in size, colour, and skull shape) and habitat partitioning (Branch, 1998; Tolley & Burger, 2007), yet they are not reciprocally monophyletic for mitochondrial markers – ND2 and 16S (Tolley *et al.*, 2004; Tolley *et al.*, 2006). Explanations for this range from shared ancestral polymorphism as a result of recent radiation, selective sweeps on mitochondrial genes, strong selection on the phenotype as a result of environmental pressure, and phenotypic plasticity. The latter explanation can be ruled out, as common garden experiments have shown this is unlikely (Miller & Alexander, 2009). Comprehensive field surveys within the distribution of *B. thamnobates-B. melanocephalum* have uncovered other dwarf chameleon populations that appear to vary in appearance and/or habitat utilisation compared to the two described species, leading some to believe that this clade may be a more species rich than currently accepted.

The conservation status of *B. thamnobates* is Near Threatened (IUCN, 2010) primarily due to a small area of occupancy, increasing habitat fragmentation, and loss of habitat quality. It is found in fragmented forests in both rural and urban areas, as well as transformed areas (road verges and urban gardens). The status of *B. melanocephalum* has not been assessed; however, it is distributed in a highly fragmented, critically endangered grassland ecosystem. Some populations are found in small fragments of exotic vegetation along roads in highly transformed areas. Given the threatened status of their forest and grassland habitats (Driver *et al.*, 2005; Mucina & Rutherford, 2006) and the increasing pressures from anthropogenically induced habitat change (Houniet, 2007; Armstrong, 2008), there is a high likelihood these chameleons will continue to experience significant threats, as has been shown in a range of studies across both terrestrial and marine environments (Pimm *et al.*, 1995; Vitousek *et al.*, 1997; Kotze & O'Hara, 2003; Munday,

2004; Thomas *et al.*, 2004). Determining the underlying processes of speciation and morphological variation within this clade is essential before adequate conservation action can be considered and implemented. To do this, an understanding of each species population structure and genetic diversity is required. Nuclear microsatellite markers are currently considered one of the most popular types of genetic markers for such studies (Barbará *et al.*, 2007). Here, we describe nine newly developed markers developed for *B. thamnobates, B. melanocephalum,* and their congener *Bradypodion pumilum* and examine whether cross-species amplification of these new and several existing microsatellite markers (Feldheim *et al.*, 2010) is possible within the genus *Bradypodion*. Cross-amplification success is useful information, as conflict between morphology and molecules exists in other *Bradypodion* clades (Tolley *et al.*, 2006). Cross-species transferability of microsatellite loci can facilitate comparisons among closely related taxa for addressing the processes involved in population divergence and speciation (Noor & Feder, 2006), while under strict time and financial constraints, in a cost-effective manner. However, cross-species amplification is only effective if primer sequences are conserved between species. Even though there are cases showing extreme conservation of loci between species (e.g., Schlötterer, Amos, & Tautz, 1991; FitzSimmons, Moritz, & Moore, 1995; Rico, Rico, & Hewitt, 1996), the number of polymorphic loci successfully amplified tends to decrease with increasing divergence between species (e.g., Primmer, Møller, & Ellegren, 1996; Peakall *et al.*, 1998; Primmer *et al.*, 2005). With this in mind, we examined cross-amplification success between diverse Bradypodion species using the existing (Feldheim *et al.*, 2010) and new markers developed. In addition to *B. thamnobates* and *B. melanocephalum,* we included *B. pumilum, Bradypodion dracomontanum,* and *Bradypodion* sp., all of which diverged at different times from the *B. melanocephalum*-*B. thamnobates* clade; 10, 5, and < 1 Myr, respectively (Tolley *et al.*, 2008).

MATERIALS AND METHODS

Chameleons were sampled from diverse regions within their distributions in KZN and Western Cape provinces (Fig. 1), namely KZN Midlands (*B. thamnobates*), southern coast of KZN (*B. melanocephalum*), Drakensberg Mountains (*B. dracomontanum*), central KZN (*B.* sp.), and Cape Town (*B. pumilum*). Two millimetre tail clips were taken from each chameleon and stored in 99% ethanol until subsequent DNA extraction.

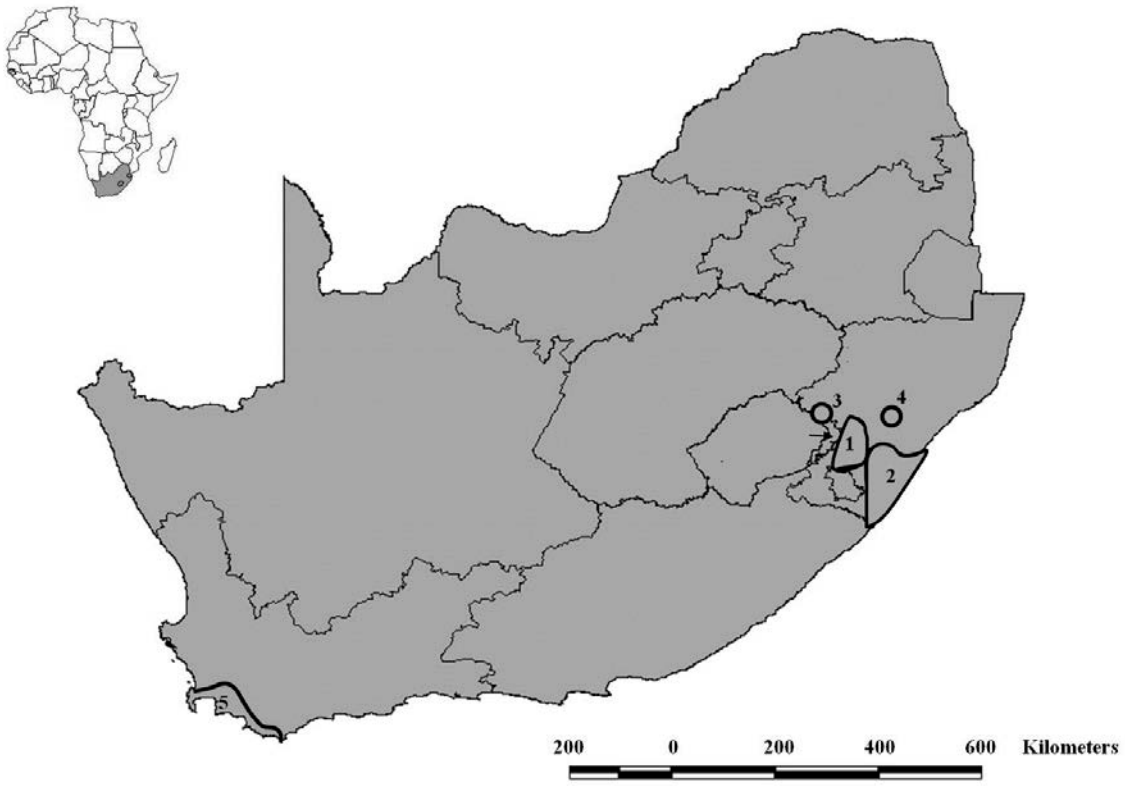

Figure 5.1 Map showing sampling sites/species distributions for each of the five Bradypodion species screened. Overall sampling regions for each species are defined as: 1, *B. thamnobates* (KZN Midlands); 2, *B. melanocephalum* (southern KZN coast); 3, *B. dracomontanum* (Drakensberg); 4, *B.* sp. (central KZN); 5, *B. pumilum* (Cape Town, Western Cape).

We developed seven new microsatellite markers for the *B. melanocephalum* (Bme)-*B. thamnobates* (Bth) complex, plus two new markers for *B. pumilum* (Bpu) (Table 5.1), using an enrichment protocol (Glenn & Schable, 2005). Genomic DNA (gDNA) from one

individual was digested with RsaI and XmnI, and SuperSNX24 linkers were ligated onto the ends of gDNA fragments. Linkers act as priming sites for polymerase chain reactions (PCR) in subsequent steps. Five tetranucleotide [$(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$] and biotinylated probes were hybridized to gDNA. Probe-gDNA complexes were added to streptavidin-coated magnetic beads (Dynabeads® M-280 Invitrogen, Carlsbad, California). This mixture was washed twice with 29 SSC, 0.1% SDS and four times with 29 SSC, 0.1% SDS at 52˚C. Between washes, a magnetic particle collecting unit was used to capture the magnetic beads which are bound to the biotin-gDNA complex. This allows us to capture gDNA containing repeats while other fragments (i.e. those not containing repeats) are washed away. Enriched fragments were removed from the biotinylated probes by denaturing at 95˚C and precipitated with 3M sodium acetate and 95% ethanol. To increase the amount of fragments, a ‘‘recovery’’ PCR was performed in a 25 µl reaction containing 1 x PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM MgCl2, 0.16 mM of each dNTP, 0.52 µM of the SuperSNX24 forward primer, 10 x BSA, 1U Taq DNA polymerase, and approximately 25 ng enriched gDNA fragments. Thermal cycling, performed in an MJ Research DYAD, was as follows: 95˚C for 2 min followed by 25 cycles of 95˚C for 20 s, 60˚C for 20 s, and 72˚C for 90 s, and a final elongation step of 72˚C for 30 min. Subsequent PCR fragments were cloned using the TOPO-TA Cloning® kit following the manufacturer’s protocol (Invitrogen). Bacterial colonies containing a vector with gDNA (i.e. white colonies) were used as a template for subsequent PCR. PCR products were then cleaned using Exonuclease I and Shrimp Alkaline Phosphatase according to the manufacturer’s protocol (Affymetrix, Santa Clara, California). DNA sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California). Sequencing reactions were precipitated with 125 mM EDTA and ethanol and run on an ABI 3730 DNA Analyzer.

Primer3 (http:// frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used to develop microsatellite PCR primers.

Optimisation of the nine markers was carried out in a 10 µl reaction volume containing approximately 5–50 ng of DNA template, 0.6259 Colourless GoTaq Flexi Buffer, 80 µM of dNTPs, 0.2 µM of each primer, and 0.5 U of Promega GoTaq® DNA Polymerase (Promega: Madison, Wisconsin, USA). MgCl2 concentrations and annealing temperatures (T_a) varied between loci and species (Table 5.1). Thermal cycling parameters were as follows: 95˚C for 4 min, followed by 40 cycles at 95˚C for 30 s, T_a for 30 s, 72˚C for 45 s and a final extension at 72˚C for 5–10 min. PCR products were combined in a multiplex format of no more than two primers. Samples were run on a 50 cm capillary on the ABI 3130XL Genetic Analyzer (16 capillaries) using Rox 500 as the internal size standard and POP-7 as the polymer, as per manufacturer's recommendations.

RESULTS AND DISCUSSION

Seven loci were found to be polymorphic across all species with the number of alleles ranging from 2 to 29 (Tables 5.2, 5.3). Bth76 was found to be polymorphic for all but one species (*B. dracomontanum*), but this could be a result of the small sample size (n = 4). Bpu535 was not polymorphic for any species except the one that was used to develop this locus (i.e. *B. pumilum*: Table 5.3).

Of the eight primers previously developed for *B. pumilum* (Feldheim *et al.*, 2010), only two (Bpu94 and Bpu557) cross-amplified consistently for all species (Tables 5.2 & 5.3). Bpu238 did not amplify for *B. dracomontanum*, yet clear products were achieved for all other species; however, only *B.* sp. and *B. pumilum* were polymorphic. Bpu557 was polymorphic for all, except *B. dracomontanum*, likely due to the small sample size. Bpu94 was the only polymorphic locus for all species.

Table 5.1 Characteristics of microsatellite loci and primers developed for *B. melanocephalum* (Bme), *B. thamnobates* (Bth) and *B. pumilum* (Bpu).

Locus	Repeat Motif	Primer Sequence (5' – 3')	Label	Genebank Access. No.	*B. melanocephalum* & *B. thamnobates*		*B. pumilum*	
					T_a (^{0}C)	$MgCl_2$ (mM)	T_a (^{0}C)	$MgCl_2$ (mM)
Bme45	$(ATAC)_{20}$	F: GAT TGG GCG GAA TAC AAG TC R: TCC CTG CCA GTT ATT GTT GC	6Fam	JN086456	59	1.0-1.5	59	1.0
Bme58	$(AG)_{32}$	F: TTG AAG CAA TGC ACA CAC AC R: GCA CCG GTT CTT TAG CTT TG	Hex	JN086457	59	0.75-1.25	59	0.75
Bme128	$(AC)_{28}$	F: TCT GTT CTG TTG CTT TTC CTC R: CCC CAA TGA TCT CTC AAT GT	6Fam	JN086458	59	1.0-1.25	59	1.25
Bth10	$(TC)_3(AC)_5(TC)_{14}(AC)_{26}$	F: TGG AGT AGA GAC TGC GCT TG R: TGT GGA TAC CCA TTT CAC CA	Hex	JN086459	59-62	0.75-1.0	59	0.75
Bth76	$(ATAG)_{37}$	F: TTG TGG TTA GAG GGG CAT TG R: CCC CAA TCT CGT TGT TCT GT	Hex	JN086460	56	1.0-1.5	56	1.0
Bth93	$(ATAG)_{24}$	F: AAG GGC ACA TCA CTG AAT CC R: CGC CAG AGA TGA TGG AAT TT	6Fam	JN086461	59	1.0-1.5	59	1.0
Bth161	$(ATCT)_{31}$	F: CCC CAA TCT CGT TGT TCT GT R: TCC AAT GCA CAC ACG TTA GC	Hex	JN086462	59	1.0-1.5	59	1.0
Bpu26*	$(TTAC)_{26}$	F: TGA AAT CTC GCT ATC CTT GT R: CTT TCG AGT AAG GGA GAC CT	Hex	GU066308	—	—	63	6.0
Bpu28*	$(TATC)_{30}$	F: CTGGAAACCTCCCTGCCTAT R: TGGACTTATAGTCCGCCTTCC	Hex	GU066310	?		58	1.0
Bpu94*	$(GTT)_{17}$	F: CAG CTT TGG CGT CTT ACA CA R: GCC TTA AAG GAA GGA AAG TGG	Hex	GU066305	48-58	1.0-2.0	48	1.25
Bpu115*	$(TAGA)_{14}$	F: GCT GTG ATA TGT AAA TTC AGG G R: CAC TTT GTT TTG GTC TCC CAC T	Hex	GU066306	?		55	1.0
Bpu132*	$(TATG)_{27}$	F: CGC TAT TTC CCC TCA AAA TC R: TGG CTC CAT ATA GCA ACA CG	6Fam	GU066309	?		48	0.75
Bpu238*	$(TATC)_{26}$	F: CCC CAA TCT CGT TGT TCT GT R: CTC ATT TCC TCC TCC CCA TT	6Fam	GU066307	58	1.0	58	1.0

Table 5.1 *continued*.

Locus	Repeat Motif	Primer Sequence (5' – 3')	Label	Genebank Access. No.	*B. melanocephalum* & *B. thamnobates*		*B. pumilum*	
					T_a (^{0}C)	$MgCl_2$ (mM)	T_a (^{0}C)	$MgCl_2$ (mM)
Bpu507	$(TG)_{23}$	F: AAT CCC TCA CCT TCA CAT GC R: CCA GGT TCA AAA TCC CAT CA	6Fam	JN086463	53	1.0-1.25	53	1.0
Bpu535	$(AG)_{17}$	F: ACC AGC TCC TTT GCA TGC TC R: GTC CAG AAC AAA CTG GAC TGC	Hex	JN086464	53	1.5	53	1.5
Bpu557*	$(GT)_8$	F: GGC ACT GGC ATC CCT AAA TA R: GAC TTG CTG AGG GAT ATT AC	6Fam	GU066303	48	1.0	48	1.25
Bpu571*	$(GA)_{11}$	F: CAA TAT GCC ACC TAA CCA TC R:CCA TGA CAA ATT ACA CAA ACC TC	6Fam	GU066304	—	—	57	6.0

* = primers previously published (Feldheim *et al.*, 2010); — = amplification failed; ? = equivocal output.

Table 5.2 Testing the cross-amplification of *Bradypodion* microsatellite loci.

Loci	Species				
	B. melanocephalum	*B. thamnobates*	*B. pumilum*	*B. dracomontanum*	*B. sp.*
Bth10	p	p	p	p	p
Bth76	p	p	p	p	P
Bth93	p	p	p	p	P
Bth161	p	p	p	p	p
Bme45	p	p	p	p	P
Bme58	p	p	p	p	p
Bme128	p	p	p	p	p
Bpu26	—	—	p	—	—
Bpu28	±	±	p	—	—
Bpu94	p	p	p	m	p
Bpu115	—	—	p	—	—
Bpu132	±	±	p	—	—
Bpu238	m	m	p	—	p
Bpu507	p	p	p	p	p
Bpu535	m	m	m	m	m
Bpu557	p	p	p	m	p
Bpu571	—	—	p	—	—

—: no PCR product; ±: equivocal output - unclear, weak or inconsistent PCR product; m: monomorphic locus in the tested set of isolates; p: polymorphic locus in the tested set of isolates

Table 5.3 Descriptive statistics of genetic variability for 12 microsatellite loci across five *Bradypodion* species.

Locus	*B. melanocephalum* (n = 50)					*B. thamnobates* (n = 38)					*B. pumilum* (n = 29)				
	N_A	S	H_O	H_E	HWe	N_A	S	H_O	H_E	HWE	N_A	S	H_O	H_E	HWE
Bth10	29	141-229	0.61	0.95	0.0000	21	167-223	0.71	0.95	0.0000	8	149-177	0.62	0.72	0.0864
Bth76	19	100-228	0.61	0.72	0.0634	18	100-236	0.79	0.92	0.0014	15	104-244	0.76	0.92	0.0227
Bth93	17	64-192	0.60	0.93	0.0000	15	128-188	0.86	0.91	0.0863	8	136-204	0.14	0.81	0.0000
Bth161	21	157-265	0.51	0.93	0.0000	23	173-285	0.81	0.93	0.0183	13	209-265	0.66	0.83	0.0032
Bme45	25	86-186	0.68	0.94	0.0000	19	90-198	0.86	0.93	0.1545	11	142-206	0.83	0.88	0.2182
Bme58	16	131-231	0.39	0.90	0.0000	11	131-163	0.55	0.80	0.0000	14	131-181	0.46	0.90	0.0000
Bme128	27	137-217	0.69	0.94	0.0000	12	135-205	0.55	0.72	0.0016	3	135-143	0.00	0.14	0.0005
Bpu94	10	109-199	0.52	0.76	0.0000	7	115-205	0.19	0.90	0.0000	7	160-193	0.50	0.75	0.0024
Bpu238	1	106	-	-	-	1	106	-	-	-	18	155-239	0.82	0.91	0.4560
Bpu507	10	182-204	0.70	0.87	0.0004	9	182-262	0.53	0.83	0.0000	16	202-262	0.90	0.92	0. 0208
Bpu535	1	254	-	-	-	1	254	-	-	-	1	256	-	-	-
Bpu557	3	104-108	0.40	0.51	0.1354	3	90-106	0.09	0.09	1.0000	5	104-116	0.24	0.49	0.0000

Locus	*B. dracomontanum* (n = 4)					*B. sp.* Greytown (n = 19)				
	N_A	S	H_O	H_E	HWE	N_A	S	H_O	H_E	HWE
Bth10	3	153-159	0.75	0.82	0.7709	13	157-233	0.67	0.86	0.0129
Bth76	1	100	-	-		2	100-204	0.11	0.10	1.0000
Bth93	5	100-176	1.00	0.93	1.0000	11	108-160	1.00	0.88	0.9355
Bth161	6	177-209	1.00	0.96	1.0000	8	185-233	0.63	0.82	0.0553
Bme45	3	134-150	0.75	0.64	1.0000	10	110-190	0.89	0.90	0.7753
Bme58	4	163-187	0.75	0.86	0.6541	7	133-147	0.78	0.82	0.0000
Bme128	5	151-193	0.50	0.86	0.0831	14	141-205	0.84	0.90	0.1809
Bpu94	3	121-160	0.50	0.50	1.0000	7	121-217	0.44	0.80	0.0025
Bpu238	0	-	-	-	-	3	102-110	1.00	0.67	1.0000
Bpu507	2	196-200	0.50	0.57	1.0000	7	184-202	0.63	0.81	0.0185
Bpu535	1	254	-	-	-	1	254	-	-	-
Bpu557	1	104	-	-	-	2	104-108	0.00	0.34	0.0001

N_A, Number of observed alleles; S, allele size range; H_O, observed heterozygosity; H_E, expected heterozygosity; HWE, Chi-square tests for Hardy-Weinberg equilibrium after Bonferroni correction.

Levels of expected and observed heterozygosities were estimated, and tests for deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) at each locus were performed using ARLEQUIN v.3.11 (Excoffier, Laval, & Schneider, 2005). Tests for linkage and Hardy-Weinberg disequilibria were corrected for multiple comparisons by applying sequential Bonferroni corrections (Rice, 1989). No significant linkage disequilibrium was detected for any of the loci. There were a number of significant deviations from HWE depending on the species examined, except for *B. dracomontanum* which showed all loci to be in HWE (Table 5.3).

The resulting deviations from HWE are all due to homozygote excess, which can be a consequence of null alleles or of biological factors such as the Wahlund effect or inbreeding (Chakraborty *et al.*, 1992). Multilocus analyses can normally distinguish these causes because such factors should register more or less concordantly across loci, whereas the effects of null alleles are locus-specific. The presence of null alleles for each locus was examined in Micro-checker version 2.2.3 (Van Oosterhout *et al.*, 2004), and three were found to shown signs of null alleles: Bpu94, Bpu557, and Bth10. The homozygote excess at the remaining loci could be due to the Wahlund effect (e.g., Johnson & Black, 1984). Given the highly fragmented nature of Bradypodion habitat coupled to an expectation of low vagility (Tolley *et al.*, 2010), non-random mating across larger distribution is a potential explanation, despite random mating within a small locale. In this case, small populations could become fixed for diverse alleles, reducing the observed heterozygosity across the entire sample, as globally ‘‘expected’’ heterozygotes will not occur in the population. Indeed, *Bradypodion* could also be subject to large demographic fluctuations, with repeated founder events in small populations (Tolley *et al.*, 2010), which would have an effect on HWE.

In conclusion, seven new polymorphic microsatellite markers are optimised for the *B. melanocephalum-B. thamnobates* complex and two additional polymorphic markers optimised for *B. pumilum*. These markers cross-amplify in other species in the same genus without a drastic reduction in allelic diversity in non-target species for most loci. These markers likely have conserved primer sequences for the genus as a whole, thus proving useful for the evaluation of population genetic diversity, construction of genetic linkage maps, and mapping of quantitative trait loci in the *B. melanocephalum-B. thamnobates* complex, as well as other related species.

Paper V:

Population genetic structure informs species delimitation within a recent radiation of dwarf chameleons*

* To be submitted for publication as: da Silva, J. M. & Tolley, K.A. Population genetic structure informs species delimitation within a recent radiation of dwarf chameleons. *Molecular Ecology*.

ABSTRACT

Species are the fundamental units of biodiversity, yet how to delimit them remains one of the most contentious debates in the fields of systematics, evolution, ecology and conservation; thus explaining the numerous species concepts currently used by biologists. The main difference between concepts is how far evolutionary population differentiation needs to proceed before populations should be considered distinct species. A lineage might lack support from one or more lines of evidence even if it is evolving separately because it is in the early stages of divergence. This is commonly observed in adaptive radiations with species identified based on distinct ecomorphological evidence, yet show little to no genetic divergence. Such discordance between morphology and genetics has been reported in a phenotypically diverse group of dwarf chameleons restricted to southern KwaZulu-Natal Province, South Africa – the *Bradypodion melanocephalum-Bradypodion thamnobates* species complex. However, the lack of genetic differentiation may be attributed to too few samples and a limited number of genetic markers used in the initial phylogenetic studies. Accordingly, in this study we incorporated extensive genetic sampling and utilised both mtDNA and fast-evolving nuclear microsatellite markers to identify genetic structure and assess levels of genetic diversity and gene flow between chameleons. We also incorporated detailed spatial information into the analyses to quantify the effects of landscape and geographic barriers on their genetic structure. The mitochondrial and microsatellite data revealed three and seven genetic clusters, respectively, likely reflecting the evolution of southern KZN dwarf chameleons in response to forest extent. Based on these results, we are able to recognise four distinct species and, at least, five adaptively distinct conservation units within the *B. melanocephalum-B. thamnobates* species complex.

INTRODUCTION

Species are the fundamental units of biodiversity and central to our understanding of many evolutionary processes. Consequently, over- or under-resolving species boundaries will confound studies aimed at understanding these processes (Sites & Marshall, 2003, 2004), yet how to delimit them remains one of the most contentious debates in the fields of systematics, evolution, ecology and conservation, which is evident in the numerous species concepts currently used by biologists (see Wilkins, 2009; Hausdorf, 2011). These debates typically involve the philosophy over what constitutes a species, temporal scale considered (years, decades, centuries, etc.), perspective followed (i.e., looking at possible future divergences versus looking to past divergences), reliability of methods used, and relevance of the data (de Queiroz, 2007). Traditionally, taxonomists used a variety of morphological characters and measurements to identify and classify species. Systematists expanded upon this work by investigating the evolutionary histories of species and their environmental adaptations, which, in the last decades, has been greatly influenced by molecular data. Today, DNA sequencing, both nuclear (nDNA) and mitochondrial (mtDNA), are integral tools in delimiting species (e.g., Avise *et al.*, 1987; Moritz, Dowling, & Brown, 1987; Doyle, 1992; Pitra *et al.*, 2006; Thomé *et al.*, 2012; Krück *et al.*, 2013), with species often identified as the terminal branches of a phylogenetic tree (following the genealogical basis of the phylogenetic species concept [PSC]: see Avise & Ball, 1990; Baum & Shaw, 1995). However, complications arise when described species previously classified by other means (e.g., morphology, ecology) are not accurately reflected in these gene trees (Pamilo & Nei, 1988; Doyle, 1992; Maddison, 1997; Maddison & Knowles, 2006). This is a fairly common occurrence often attributed to hybridization, incomplete lineage sorting (shared polymorphisms inherited from a common ancestor) or gene duplication (Nei, 1987; Doyle, 1992; Maddison, 1997). Even discrepancies between nDNA and mtDNA are common due

to their different modes of inheritance and rates of mutation (e.g., Shaw, 2002; Wake, 2006; Egger *et al.*, 2007; Leaché & Cole, 2007; Yang & Kenagy, 2009). Such discordance may call into question the validity of the proposed species under some species concepts; however, the absence of conformity should not constitute the rejection of possible lineage separation (de Queiroz, 2007). Many species concepts are actually similar in that they classify a species as a cohesive group of individuals that have at least partially different evolutionary paths representing different lineages (de Queiroz, 2005, 2007; Hausdorf, 2011). The main difference between them is how far evolutionary population differentiation needs to proceed before populations should be considered distinct species (de Queiroz, 2005, 2007). Accordingly, a lineage might lack support from one or more lines of evidence even if it is evolving separately because it is in the early stages of divergence. This has been well documented in adaptive radiations, such as Darwin's finches (Freeland & Boag, 1999; Grant *et al.*, 2005; Petren *et al.*, 2005), African cichlids (e.g., Verheyen *et al.*, 2003; Salzburger & Meyer, 2004; Seehausen, 2004), threespine sticklebacks (e.g., Taylor & McPhail, 1999; Kristjánsson, 2005) and *Anolis* lizards (e.g., Losos *et al.*, 1998). Corroboration from multiple lines of evidence should therefore be sought after, which an increasing number of studies are adopting by incorporating morphological, behavioural, ecological and genetic data (e.g., Rees *et al.*, 2001; Leaché *et al.*, 2010; Evin, Horacek, & Hulva, 2011; Taylor *et al.*, 2011; Carrasco *et al.*, 2012).

Discordance between morphology and genetics has been reported in a phenotypically diverse group of dwarf chameleons (*Bradypodion*) restricted to southern KwaZulu-Natal Province (KZN), South Africa (Tolley *et al.*, 2004). The group is comprised of five phenotypic forms, two of which are classified taxonomically – *Bradypodion melanocephalum* (Gray, 1865) and *Bradypodion thamnobates* (Raw, 1976) – and the remaining three (regarded as Types A, B and C in Chapter 2) designated as

morphotypes (Tolley & Burger, 2007; Tilbury, 2010). All are allopatric in distribution and occupy different macro- and micro-habitats, which can be broadly classified as either open- or closed-canopy (Chapter 2). Yet, despite these morphological and ecological differences, initial phylogenetic and phylogeographic studies using mitochondrial markers (ND2 and 16S) found very little, if any, genetic differentiation between them (Tolley *et al.*, 2004; Tolley *et al.*, 2008). Thus, under the genealogical PSC, this lack of genetic resolution would result in their collapse into a single species. However, this was not a satisfactory solution given that common garden experiments ruled out the possibility of phenotypic plasticity within this group, at least for *B. melanocephalum* and *B. thamnobates* (Miller & Alexander, 2009), and detailed ecomorphological studies showed that each form possessed functional adaptations to their specific habitats (Chapters 2-4). Consequently, the morphological variation between forms represents true ecological or evolutionary differences between them, lending support for their classification as separate lineages and the designation of the group as an adaptive radiation.

In some cases, phylogenetic studies fail to recognise divergence between species, when in fact it existed (see Shaffer & Thomson, 2007 for examples). This has been attributed to the incorporation of too few samples from what was assumed to be representative localities of each lineage, the reliance on particular genomic regions which might show sufficient variation, the use of a limited number of genetic markers, and the paucity of appropriate methods used to detect differences. To eliminate these as possible explanations for the discordance observed between genetics and morphology within this species complex, we incorporated extensive genetic sampling and utilised both mtDNA and fast-evolving nuclear microsatellite markers to identify genetic structure and assess levels of genetic diversity and gene flow between chameleons. We also incorporated detailed spatial information into the analyses to quantify the genetic effects of habitat and

geographic barriers (e.g., Manel *et al.*, 2003; Spear *et al.*, 2005; Selkoe & Toonen, 2006; Storfer *et al.*, 2007; Moore *et al.*, 2008). This approach allows for the examination of the hypothesis that the phenotypic forms previously identified in the *B. melanocephalum-B. thamnobates* species complex are reflected in their genetics. Based on the currently available data, there are four species concepts most applicable to this chameleon complex: the morphological species concept (MSC: Mayr, 1982), the phylogenetic species concept (PSC: Eldredge & Cracraft, 1980; Cracraft, 1997), the genotypic cluster species concept (GSC: Mallet, 1995), and the ecological species concept (ESC: Van Valen, 1976). The MSC was widely used historically; whereas, today, the PSC is one of the most widely used concept, and the GSC and ESC are often adopted for the classification of species undergoing adaptive radiations. If discordance between the data persists, then depending on the line of evidence one deems most important for delimiting species, the number of dwarf chameleons will vary drastically. This has significant implications for their conservation and management, affecting factors, such as area of occupancy, which are necessary in assessing a species' risk of extinction.

MATERIALS AND METHODS

SAMPLE COLLECTION

Chameleons within the *B. melanocephalum-B. thamnobates* species complex were sampled from multiple localities throughout southern KZN, focussing on three zones where the five forms occur: southern Drakensberg (Type B), KZN Midlands (*B. thamnobates*, Types A and C), and the south coast (*B. melanocephalum*) (Fig. 5.2). All individuals were geo-referenced (+/- 5 m) at the exact location each chameleon was found. Two to three millimetre tail clips were taken from each chameleon and stored in 99% ethanol. Although chameleons use their tails as a prehensile organ to navigate through their environments

(Tolley & Burger, 2007; Tolley *et al.*, 2010), tail clipping has been shown to have no significant effect on their performance ability (Herrel *et al.*, 2012).

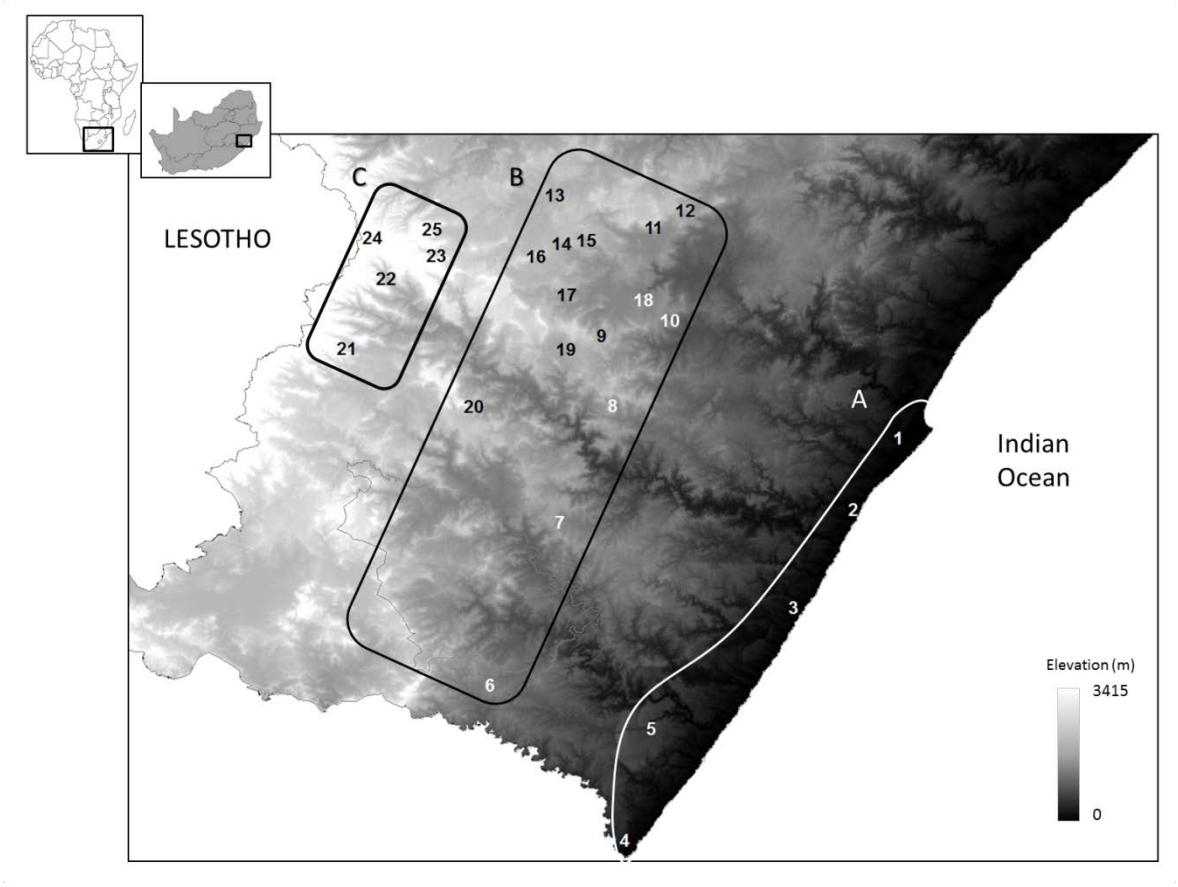

Figure 5.2 Map depicting the 25 sampling sites within southern KZN, South Africa. **A- south coast**: 1-Durban; 2-Illovo; 3-Pennington; 4-Umtamvuna; 5-Oribi Gorge. **B- KZN Midlands**: 6-Weza; 7-Ixopo; 8-Bryne Valley; 9-Stirling Farm; 10-Hilton; 11-Karkloof; 12-Gilboa Plantation; 13-Mooi River; 14-Gowrie Village; 15-Boschhoek Golf Course; 16-Notthingham Road; 17-Dargle; 18-Howick; 19-Boston; 20-Bulwer. **C- southern Drakensberg**: 21-Sani Pass; 22-Lotheni; 23-Kamberg; 24-Giant's Castle; 25-Highmoor.

DNA Extraction, Microsatellite Genotyping and mtDNA Sequencing

DNA extractions were performed using a standard salt extraction protocol (Aljanabi & Martinez, 1997). Seven microsatellite markers developed for the *B. melanocephalum-B. thamnobates* species complex, plus three additional markers developed for the congener *Bradypodion pumilum* were used to amplify microsatellites

(*Paper IV*: Chapter 5). Optimisation of the ten markers was carried out in a 10 µl reaction volume according to protocols in *Paper IV*. PCR products were combined in a multiplex format of no more than two primer pairs. Samples were run on a 50 cm capillary on the ABI 3130XL Genetic Analyzer (16 capillaries) using Rox 500 as the internal size standard and POP-7 as the polymer, as per manufacturer's recommendations. PCR profiles were analysed using Peak Scanner™ Software, Version 1.0. We used the program Micro-Checker version 2.2.3 (Van Oosterhout *et al.*, 2004) to test for the presence of genotyping errors, null alleles, and heterozygote deficiencies.

One mtDNA marker – NADH dehydrogenase 4 (ND4) – was used to generate sequences for a subsample of individuals from each sampling site. For amplification, 20-50 ng genomic DNA were added to a 25µL reaction containing a thermophilic buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT), 1.5 mM MgCl2, 0.08 µM of each primer, 0.2 µM dNTPs, and 0.3 U/µl SuperTherm *Taq* DNA polymerase. Sufficient PCR product for direct sequencing was generated after 35 cycles (94 °C for 30 s, 57 °C for 30 s, 72 °C for 45 s). These products were run on an ABI 3730XL DNA Analyzer, and the sequences aligned and edited using SEQUENCHER v. 4.1.

MICROSATELLITE STATISTICAL ANALYSES

For each locus, the number of alleles, allelic size range, observed (H_o) and expected (H_e) heterozygosities (Nei, 1987), and deviations from Hardy-Weinberg equilibrium (HWE) were calculated for all sampling locations using the program ARLEQUIN version 3.5.1.2 (Excoffier & Lischer, 2010). HWE was calculated based on a procedure described by Guo and Thompson (1992) using a test analogous to Fisher's exact test, but it employs a triangular contingency table based on a modified version of the Markov-chain random

walk algorithm. The test was run using 1.0 x10^6 Markov chains and 100 000 dememorization steps.

GENEPOP on the web (Raymond & Rousset, 1995; Rousset, 2008) and the program LinkDos (Garnier-Gere & Dillmann, 1992) were used to test for linkage disequilibrium (LD) between pairs of microsatellite loci within and between sampling sites. Standard pairwise measures of LD between loci were calculated according Cockerham and Weir's (1977) definition based on genotypic data. A MCMC dememorization of 1 000 were used for 100 batch runs of 1 000 iterations. LinkDos was used to compute three variance components of LD – D_{IS}, D_{ST}, D_{IT} – devised by Ohta (1982), which are similar to Wright's (1940) partitioning of deviations from HWE frequencies. D_{IS} is the average disequilibrium within sampling sites, D_{ST} is the contribution to the overall disequilibrium caused by differences in allele frequencies among sampling sites, and D_{IT} is classified as the total variance of disequilibrium. Ohta (1982) further classified two other components: D'_{IS} is the variance of disequilibrium of in a subpopulation relative to the total population; and, D'_{ST} is the variance of disequilibrium of the total population. This partitioning of LD is important in investigating the possible factors responsible for differences in LD, such as natural selection, genetic drift, bottlenecks, inbreeding, inversions and gene conversions (Slatkin, 2008). For example, Ohta (1982) found that when $D'_{IS} > D'_{ST}$ and $D_{ST} > D_{IS}$, limited migration is likely the main reason for the observed LD; whereas, when the reverse relationships arise, epistatic natural selection is likely to be the underlying factor since loci with favourable combinations of alleles would increase in every sampling site.

Two Bayesian model-based clustering algorithms were used to infer population structure using the microsatellite data and to probabilistically assign individuals to clusters based on multi-locus genetic data without *a priori* knowledge of population units and

limits. For all algorithms, subdivision of the data into clusters was done by maximising HWE and minimising LD. The first algorithm, implemented in STRUCTURE version 2.3.3 (Pritchard, Stephens, & Donnelly, 2000; Falush, Stephens, & Pritchard, 2003), bases its inference on genetic data alone; whereas, the second algorithm used in GENELAND version 3.3.0 (Guillot, 2005; Guillot, Mortier, & Estoup, 2005a; Guedj & Guillot, 2011; Guillot, Santos, & Estoup, 2011) incorporates spatial information. For both programs, no prior assumptions regarding the model that would best fit the data were made. Instead, simulations were conducted under each model available (e.g., no admixture/admixture; correlated/uncorrelated) with long Markov chain Monte Carlo (MCMC) runs to ensure convergence of the chain. For each program, K_{max} was set to 30 as it is larger than the actual number of field sites sampled; hence the true K will fall within this range. K_{min} was set to 1.

For STRUCTURE, 10 independent runs were carried out for each fixed K between K_{min} and K_{max}, with a MCMC of 500 000 iterations following a burn-in of 50 000 for each model combination (four in total). STRUCTURE HARVESTER Web v0.6.92 (Earl & vonHoldt, 2012) was then used to apply the ad hoc statistic developed by Evanno *et al.* (2005) to calculate ΔK – the rate of change in the estimated log probability of data between successive K values. The modal value of ΔK is considered to be the 'true' K or the uppermost hierarchical level of population structure that best describes the data (Evanno *et al.*, 2005). The10 runs associated with the modal ΔK were further processed in CLUMPP version 1.1.2 (Jakobssen & Rosenberg, 2007) using the *Greedy* algorithm (M = 2). This was done to correct for possible label switching or genuine multimodality issues (Pritchard *et al.*, 2000). Label switching refers to a scenario in which different runs obtain the same membership coefficient estimates, except with a different permutation of the cluster (Stephens, 2000). Genuine multimodality refers to different runs producing substantially

different answers, which increasing the run length cannot fix (Pritchard *et al.*, 2000; Jakobssen & Rosenberg, 2007). CLUMPP outputs cluster membership coefficient matrices, transformed so that the cluster labels across the different runs align and all replicates have as close a match as possible. DISTRUCT version 1.1 (Rosenberg, 2004) was then used to visualise the results as barplots of individual cluster membership. We assigned each individual to the cluster with the greatest proportion of membership, and each site to the cluster to which the majority of individuals at that site were assigned.

For GENELAND, data were analysed under both correlated and uncorrelated allele frequency models using spatial parameters. Estimates of posterior probabilities of each K (between $K_{\min}$ and K_{max}) occur via a reversible jump algorithm within a single run (Guillot *et al.*, 2005b). For each simulation, parameters were set to 10 independent runs with 500 000 MCMC iterations, thinning of 50, no filtering of null alleles, and the delta coordinate (representing the potential error for spatial coordinates) set at 0. This delta coordinate was considered appropriate given that each sample possessed its own geographic coordinates and these chameleons are believed to have low very vagility; therefore, the location of capture is expected to be representative of their home range. All other parameters were set to default values. The 10 runs were post-processed with a burn-in of 100 iterations in order to obtain posterior probabilities of population membership for each individual and each pixel of the spatial domain (200 pixels along the X and Y axes). The consistency of the results across the 10 runs was checked visually. Because post-processing in GENELAND already corrects for label-switching, CLUMPP was not required. However, DISTRUCT was used to create barplots of cluster membership.

Although comparing outputs of different models and different programs is a difficult statistical exercise, a method by Guillot et al. (2011) was employed to help select the model that best represented the data. The patterns obtained from the different models in

each program were checked for consistency over several runs and to make sure that the inferred clusters complied with model assumptions. If these preliminary checks were held, ARLEQUIN and GENEPOP on the web were used to examine whether the inferred clusters were in HWE and if there was LD between loci, respectively. If an inferred pattern passed these checks, there was a good chance it was not an artefact of the model algorithm.

Once the number of clusters was identified, a comprehensive examination of each cluster's genetic diversity and structure was undertaken in ARLEQUIN. To better understand the connectivity and gene flow among clusters and sites, hierarchical AMOVAs were conducted using 10 000 permutations and pairwise genetic distance matrices calculated using Slatkin's (1995) R_{ST} for microsatellite data. This statistic was used instead of F_{ST} because it relies on a stepwise mutation model which is better suited for the high mutation rates and memory dependent allele mutations found within microsatellite loci (Di Rienzo *et al.*, 1994; Slatkin, 1995). In contrast, F_{ST} relies upon the infinite allele model, which assumes low mutation rates and a mutation process independent of the prior allelic state (Weber & Wong, 1993; Slatkin, 1995).

To gain perspective on the extent of genetic differentiation between clusters, microsatellite data from *B. pumilum*, *Bradypodion dracomontanum*¸ and *Bradypodion nemorale* (Greytown population) from *Paper IV* were included in the analysis. These species were chosen as they vary in the degree of genetic divergence and geographic proximity to chameleons in the *B. melanocephalum-B. thamnobates* species complex. *Bradypodion pumilum* is the most distant, both genetically and geographically; whereas, *B. dracomontanum* and *B. nemorale* are sister taxa to the complex and occur in central KZN (Tolley & Burger, 2007; Tolley *et al.*, 2008; Tilbury, 2010). The function hclust in R (R Development Core Team, 2011) was then used to illustrate the hierarchical structuring of the microsatellite clusters identified.

We tested for signatures of genetic bottlenecks in all seven clusters using two methods. First, heterozygosity excess was tested using the program BOTTLENECK 1.2.02 (Piry, Luikart, & Cornuet, 1999). BOTTLENECK incorporates the method developed by Cornuet and Luikart (1996), which uses a single population sample to test whether there has been a recent reduction in allelic variation. Specifically, heterozygosity excess is defined as H_e (Hardy-Weinberg heterozygosity) minus H_{eq} (equilibrium heterozygosity at mutation-drift equilibrium) (see Piry *et al.*, 1999). Studies have found this method to be most effective at accurately detecting recent, low-magnitude declines in effective population size (N_e) (Cornuet & Luikart, 1996; Beebee & Rowe, 2001; Williamson-Natesan, 2005; Goossens *et al.*, 2006; Spear *et al.*, 2006; Funk *et al.*, 2010). The stepwise mutation model (SMM) and the two-phase mutation model (TPM) were used to generate null distributions under mutation-drift equilibrium, as these are considered the most appropriate models for microsatellites (Di Rienzo *et al.*, 1994; Garza & Williamson, 2001). A wide range of values was used for the percent multi-step mutations (2, 5, 10, 20, and 30%), which represent values most commonly tested in the literature (e.g., Piry *et al.*, 1999; Busch, Waser, & DeWoody, 2007; Funk *et al.*, 2010; Peery *et al.*, 2012). The variance among multiple steps was set to 12, as recommended by Piry *et al.* (1999). The significance of heterozygosity excess across all loci was determined with a one-tailed Wilcoxon sign rank test. Second, we used ARLEQUIN to estimate the *M*-ratio according to the equation $M = k / R+1$, where k represents the number of alleles at a locus and R is the associated allelic range (Garza & Williamson, 2001). Populations that have experienced a reduction in their effective population size exhibit a larger reduction in allele numbers than range (Excoffier & Lischer, 2010). Accordingly, an *M*-ratio less than 0.68 (value derived from stable wild populations) would indicate that the population has been through a bottleneck at the locus under examination, whereas a value closer to one is indicative of

stationary/stable populations (Garza & Williamson, 2001; Peery *et al.*, 2012). Studies have found that the *M*-ratio is most effective at detecting older, more severe declines (Spear *et al.*, 2006; Funk *et al.*, 2010) compared to BOTTLENECK, which is better at uncovering more recent reductions in allelic variation.

To test whether populations form a genetic cline or whether their gene flow is limited by environmental barriers, we analysed the pattern of isolation by distance (IBD) using two approaches: an individual-based approach carried out in ALLELES IN SPACE (AIS: Miller, 2005) and a cluster-based approach implemented in MANTEL for Windows, version 1.15 (Cavalcanti, 2002). The genetic distance implemented in AIS is an analogue of Nei's distance (Nei, Tajima, & Tateno, 1983) applied to pairs of individuals; whereas, MANTEL incorporated Slatkin's (1995) R_{ST} pairwise genetic distances calculated in ARLEQUIN. Mantel tests used 10 000 permutations to evaluate the significance between genetic and $\log_{10}$-transformed geographic distances. A spatial autocorrelation analysis was also conducted in AIS as an alternative measure of spatial genetic patterns. It has the advantage over Mantel testing by providing results on the shape of the spatial relationship (Stow *et al.*, 2001). The analysis was run using 10 distance classes and 10 000 permutations. The shape of the genetic landscape was subsequently interpolated using AIS to identify genetic discontinuities or landscape regions where relatively high or low genetic distances (or diversity) exist. The procedure was performed by initially generating a Delaunay triangulation-based connectivity network of sampling locations. Given that there was variation in the geographical distances between sampling locations (see Fig. 5.2), residual genetic distances were used. These distances were derived from the linear regression of pairwise genetic distances versus the natural logarithm of geographical distances in analyses. This approach accounted for correlations between genetic and geographical distances that may be present and ensured that large interpolation peaks were

not resolved solely due to the fact that one or a few sampling areas were geographically isolated (Manni, Guérard, & Heyer, 2004). The plot surface was calculated based on the midpoints of pairwise distances of all observations throughout the remainder of the interpolation procedure, and the final interpolated surface was produced using a 50 × 50 grid and a distance weighting value of 0.25. Areas of high genetic diversity are represented by high peaks.

mtDNA Analyses

A spatial analysis of molecular variance (SAMOVA) was conducted to define the population structure found within the mtDNA sequences. Populations that are geographically homogeneous and maximally differentiated from each other were clustered using SAMOVA 1.0 (Dupanloup, Schneider, & Excoffier, 2002). Mitochondrial DNA data were analysed according to field site and the central geographic coordinates of each site were used for the spatial layer. The number of proposed clusters (K) ranged from 2 to 30 with an annealing time of 100 simulations. Once all simulations were complete for all values of K, the output files were run in ARLEQUIN. AMOVAs (10,000 permutations) employing Wright's (1951) F_{ST} were then conducted to partition the genetic variation among the different hierarchical levels of structure. The resulting F_{CT} values (variance explained among clusters) were recorded and the difference in F_{CT} between subsequent K's (ΔF_{CT}) was plotted. The K displaying the largest ΔF_{CT} was considered to be the optimal K. Standard genetic diversity estimates, including haplotype (h) and nucleotide (π) diversities were subsequently calculated for each cluster in ARLEQUIN, and the relationships among haplotypes examined with a median-joining network using NETWORK version 4.6.1.1 (Bandelt, Forster, & Röhl, 1999). A Mantel test, spatial autocorrelation and interpolated

genetic landscape were analysed using the same parameters and conditions as the microsatellite genotypes.

Estimates of net evolutionary divergence between inferred populations were determined using the *p*-distance method in MEGA5 (Tamura *et al.*, 2011) to assess the level of sequence divergence in comparison to distinct evolutionary lineages (i.e., species). Three grouping scenarios were considered: 1) the SAMOVA clusters identified, 2) the two currently recognized species, inclusive of the other forms; and 3) the five phenotypic forms. For comparison, four other *Bradypodion* species (obtained from Tolley *et al.*, 2013) were included in the analysis: *Bradypodion gutturale*, *Bradypodion occidentale*, *B. dracomontanum*, and *B. nemorale*. *Bradypodion gutturale* and *B. occidentale* are within the sister clade to all KZN dwarf chameleons (Tolley *et al.*, 2004; Tolley & Burger, 2007; Tolley *et al.*, 2008) and divergence between them dates to 4 and 10 Ma, respectively (Tolley *et al.*, 2008). Using the node age estimates from a dated *Bradypodion* phylogeny that included these four species (obtained from Tolley *et al.*, 2008), we were able to obtain rough estimates of ND4 mutation rates and to approximate divergence times within this species complex.

RESULTS

MICROSATELLITE GENOTYPE ANALYSIS

Genotypes were generated for 279 individuals from 25 sampling sites based on 10 microsatellite loci. No null alleles or genotyping errors were detected for these loci. Heterozygote deficiencies were found for most sites and loci, with the observed levels up to four times lower than expected (Table S5.1). Nine of the 25 sites showed deviations from HWE (after Bonferroni correction) at one to six loci; whereas locus Bme58 deviated from HWE for all nine sites (Table S5.1). Only five pairwise comparisons of loci showed

significant linkage disequilibrium (Bonferroni corrected: $P < 0.005$); however, none of these pairs showed significant non-random associations at more than one locality, and no loci showed evidence of LD across all sites. An examination of the variance components of LD found D_{ST} to be greater than D_{IS} in 78% of the pairwise comparisons of loci, and D'_{ST} to be greater than D'_{IS} in approximately 50% of cases, suggesting that the observed LD has been influenced by limited migration between sampling sites.

The two clustering programs, STRUCTURE and GENELAND, identified two and seven genetic clusters, respectively (Fig.5.3). Regardless of the model chosen in STRUCTURE, the same two clusters were found to best fit the data with the two described species placed in separate clusters. Sites 1-12, comprising all south coast sites and seven KZN Midland sites, represented the '*B. melanocephalum* cluster', while sites 13-25, including all Drakensberg sites and the remaining KZN Midland sites, made up the '*B. thamnobates* cluster' (refer to Fig. 5.2). Both clusters were found to deviate from HWE at nine and 10 loci, respectively; and four loci were found to be significant for LD (Table 5.4). In GENELAND, the spatial uncorrelated model delimited seven clusters (1: northern south coast & north-eastern Midlands – herein classified as the *B. melanocephalum* cluster due to the inclusion of its type locality; 2: central south coast & central eastern Midlands; 3: southern south coast & southern Midlands; 4: Karkloof; 5: Boston – site 19; 6: KZN Midlands – herein classified as the *B. thamnobates* cluster due to the inclusion of its type locality; 7: Drakensberg. These seven clusters showed no significant LD for any locus and the fewest deviations from HWE (Table 5.4). Using the method recommended by Guillot et al. (2011) of comparing the HWE and LD results of each model, we find that the model produced by GENELAND to be more representative, highlighting the importance of spatial data in assessing the population structure of these low vagility animals.

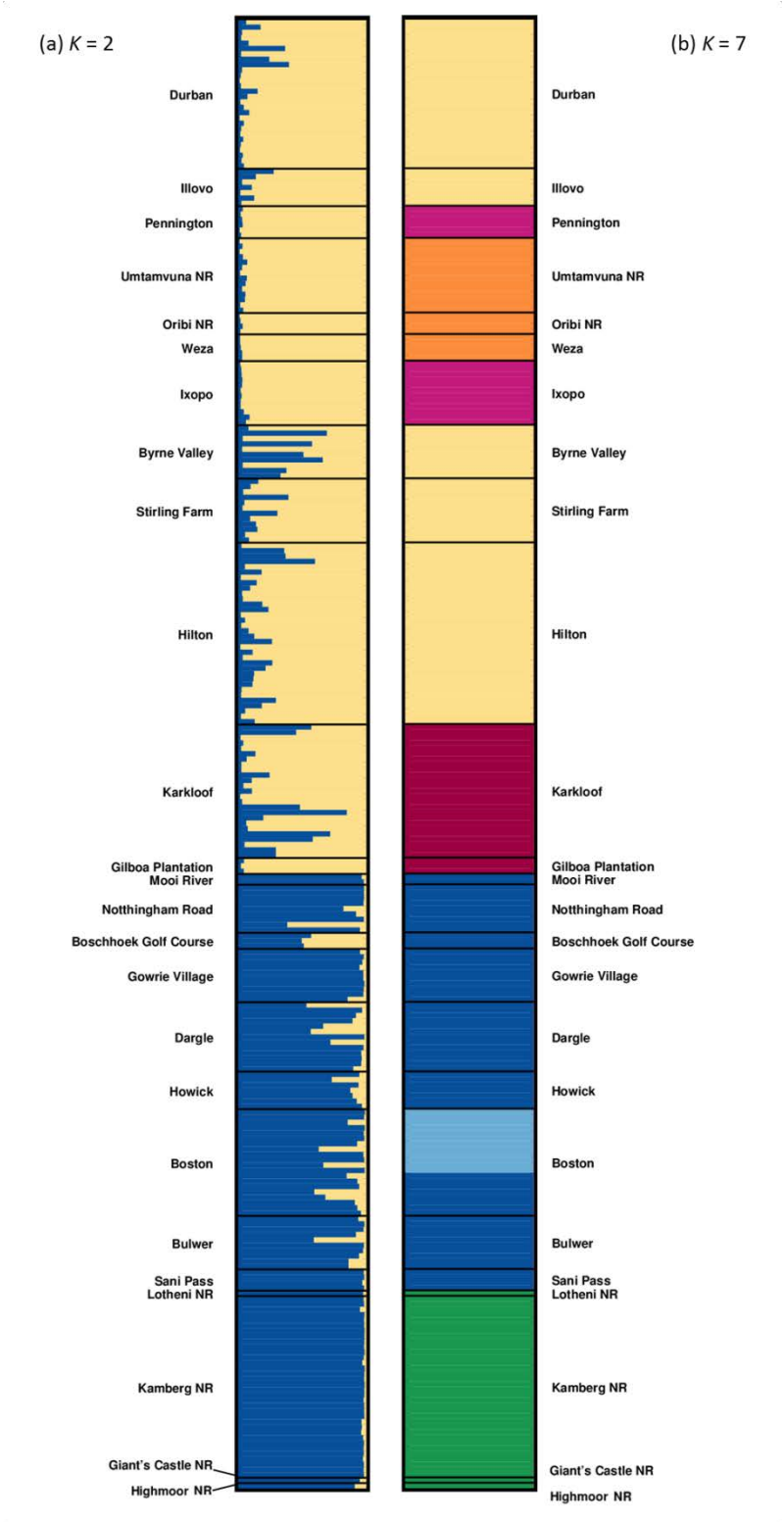

Figure 5.3 Barplots of estimated population structure from (a) STRUCTURE and (b) GENELAND analyses. Each individual is represented by a thin horizontal line divided into *K* coloured segments that represent the individual's estimated membership. Black lines separate individuals from different field sites arranged from the east (top) to west (bottom) (refer to Fig. 5.2). NR = Nature Reserve.

Table 5.4 Descriptive statistics for the clusters identified by STRUCTURE and GENELAND. Bold values indicate deviations from HWE based on a Bonferroni significance value of 0.005 ($P < 0.05/10$).

STRUCTURE clusters										
Site	*B. melanocephalum* cluster (n = 162)					*B. thamnobates* cluster (n = 117)				
Locus	N_A	S	H_O	H_E	HWE	N_A	S	H_O	H_E	HWE
Bpu94	13	109-205	0.45	0.68	**0.0000**	9	115-205	0.21	0.64	**0.0000**
Bpu557	5	90-108	0.41	0.48	0.0423	5	90-106	0.14	0.20	**0.0008**
Bpu507	16	164-254	0.70	0.87	**0.0000**	22	164-266	0.53	0.88	**0.0000**
Bth10	35	141-243	0.65	0.91	**0.0000**	30	167-227	0.72	0.95	**0.0000**
Bth76	27	96-228	0.52	0.62	**0.0000**	27	100-256	0.78	0.93	**0.0000**
Bth93	23	68-192	0.63	0.92	**0.0000**	20	72-220	0.84	0.93	**0.0006**
Bth161	27	129-265	0.68	0.94	**0.0000**	28	173-285	0.80	0.95	**0.0000**
Bme45	33	86-270	0.81	0.95	**0.0000**	26	90-198	0.82	0.94	**0.0000**
Bme58	25	129-245	0.37	0.93	**0.0000**	15	129-168	0.31	0.82	**0.0000**
Bme128	36	133-217	0.66	0.93	**0.0000**	15	131-171	0.56	0.74	**0.0000**
GENELAND clusters										
Site	Cluster 1. *B. melanocephalum* (n = 91)					Cluster 2 (n = 19)				
Locus	N_A	S	H_O	H_E	HWE	N_A	S	H_O	H_E	HWE
Bpu94	11	109-205	0.52	0.71	**0.0000**	4	121-199	0.13	0.62	**0.0000**
Bpu557	4	90-108	0.39	0.44	0.3989	3	104-108	0.61	0.67	0.6734
Bpu507	12	164-242	0.69	0.83	**0.0000**	8	184-210	0.82	0.82	0.8568
Bth10	32	149-229	0.69	0.92	**0.0000**	11	141-227	0.31	0.89	**0.0000**
Bth76	19	100-228	0.44	0.54	0.0380	8	96-192	0.32	0.46	0.0101
Bth93	19	72-192	0.70	0.91	**0.0000**	11	64-160	0.68	0.92	0.0125
Bth161	26	157-273	0.66	0.94	**0.0000**	15	129-257	0.63	0.92	**0.0003**
Bme45	25	86-194	0.81	0.95	**0.0023**	13	86-170	0.84	0.94	0.4707
Bme58	19	129-245	0.38	0.88	**0.0000**	8	129-181	0.37	0.89	**0.0000**
Bme128	27	137-211	0.70	0.90	**0.0000**	12	141-217	0.56	0.91	**0.0000**
Site	Cluster 3 (n = 24)					Cluster 4 (n = 28)				
Locus	N_A	S	H_O	H_E	HWE	N_A	S	H_O	H_E	HWE
Bpu94	4	121-190	0.60	0.65	0.0572	2	154-157	0.33	0.28	1.0000
Bpu557	4	98-108	0.50	0.51	0.6123	2	104-106	0.27	0.29	1.0000
Bpu507	8	184-254	0.67	0.80	0.1347	7	184-202	0.68	0.79	0.0696
Bth10	15	165-243	0.70	0.93	**0.0000**	9	175-209	0.68	0.69	0.6025
Bth76	12	100-196	0.71	0.80	0.0436	8	100-220	0.77	0.75	0.3742
Bth93	4	68-144	0.08	0.41	**0.0000**	11	84-172	0.82	0.86	0.9279
Bth161	12	189-253	0.71	0.89	**0.0018**	15	197-265	0.73	0.89	**0.0073**
Bme45	14	118-178	0.73	0.90	**0.0000**	19	110-270	0.85	0.94	0.0544
Bme58	11	145-171	0.50	0.87	**0.0000**	7	133-161	0.22	0.83	**0.0000**
Bme128	14	137-201	0.70	0.83	**0.0032**	18	141-219	0.56	0.93	**0.0000**

Table 5.4 *continued.*

GENELAND clusters										
Site	Cluster 5 (n = 12)					Cluster 6. *B. thamnobates* (n = 66)				
Locus	N_A	S	H_O	H_E	HWE	N_A	S	H_O	H_E	HWE
Bpu94	4	121-190	0.33	0.40	0.3380	8	115-205	0.25	0.71	**0.0000**
Bpu557	2	104-106	0.08	0.08	1.0000	4	90-106	0.19	0.29	**0.0007**
Bpu507	4	182-260	0.42	0.43	0.5659	15	164-262	0.55	0.82	**0.0000**
Bth10	3	173-221	0.33	0.62	0.0693	26	167-227	0.74	0.94	**0.0000**
Bth76	5	100-228	0.83	0.68	0.0168	24	100-256	0.74	0.94	**0.0000**
Bth93	9	72-172	0.75	0.84	0.6307	16	128-192	0.83	0.92	**0.0050**
Bth161	4	189-273	0.58	0.66	0.4493	25	173-285	0.81	0.95	0.0057
Bme45	5	110-186	0.75	0.69	0.6924	26	90-198	0.84	0.95	**0.0001**
Bme58	6	139-163	0.25	0.83	**0.0000**	14	131-163	0.38	0.78	**0.0000**
Bme128	6	141-181	0.45	0.59	0.3428	14	135-205	0.55	0.69	**0.0000**

Site	Cluster 7 (n = 39)				
Locus	N_A	S	H_O	H_E	HWE
Bpu94	5	154-172	0.05	0.40	**0.0000**
Bpu557	2	98-104	0.06	0.06	1.0000
Bpu507	16	164-266	0.51	0.84	**0.0000**
Bth10	22	167-227	0.78	0.93	0.0077
Bth76	15	164-244	0.85	0.90	0.8365
Bth93	16	128-220	0.89	0.91	0.0533
Bth161	19	185-281	0.85	0.93	0.1109
Bme45	12	110-190	0.82	0.89	0.1155
Bme58	9	129-163	0.21	0.78	**0.0000**
Bme128	4	141-171	0.61	0.54	0.0945

A hierarchical AMOVA of the GENELAND clusters revealed most of the genetic variation to be partitioned among individuals within sites (56.30%, $F_{ST} = 0.44$, $P < 0.001$) and among clusters (37.15%, $F_{CT} = 0.37$, $P < 0.001$). Very little variation could be explained among sites within clusters (6.54%, $F_{SC} = 0.10$, $P < 0.001$). Pairwise R_{ST} values were found to be significant across all the seven clusters (Table 5.5), with Cluster 3 being the most genetically distant (Fig. 5.4). When comparing R_{ST} values against the three other *Bradypodion* species, significant differences were detected between all pairings, except between Cluster 2 (the central *B. melanocephalum* group) and the KZN species, *B. dracomontanum* and *B. nemorale*, despite reasonably large R_{ST} values. This low statistical power is likely brought on by the limited sample sizes for the comparison species (Table 5.5).

Table 5.5. Pairwise genetic distances (R_{ST}) between each of the seven GENELAND clusters and three *Bradypodion* species.

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6	Cluster 7	Bpu	Bdr	Bnem
Cluster 1	—									
Cluster 2	0.030*	—								
Cluster 3	0.360**	0.291**	—							
Cluster 4	0.117**	0.138**	0.327**	—						
Cluster 5	0.477**	0.548**	0.664**	0.403**	—					
Cluster 6	0.403**	0.491**	0.586**	0.338**	0.065*	—				
Cluster 7	0.503**	0.599**	0.712**	0.457**	0.110*	0.074**	—			
Bpu	0.547**	0.644**	0.711**	0.518**	0.505**	0.395**	0.376**	—		
Bdr	0.211*	0.118	0.485**	0.336*	0.734*	0.640**	0.739**	0.772**	—	
Bnem	0.063*	0.040	0.379**	0.120**	0.605**	0.507**	0.614**	0.654**	0.218*	—

Bpu, *B. pumilum*; Bdr, *B. dracomontanum*; Bnem, *B. nemorale*; *, $P < 0.05$; **, $P < 0.001$

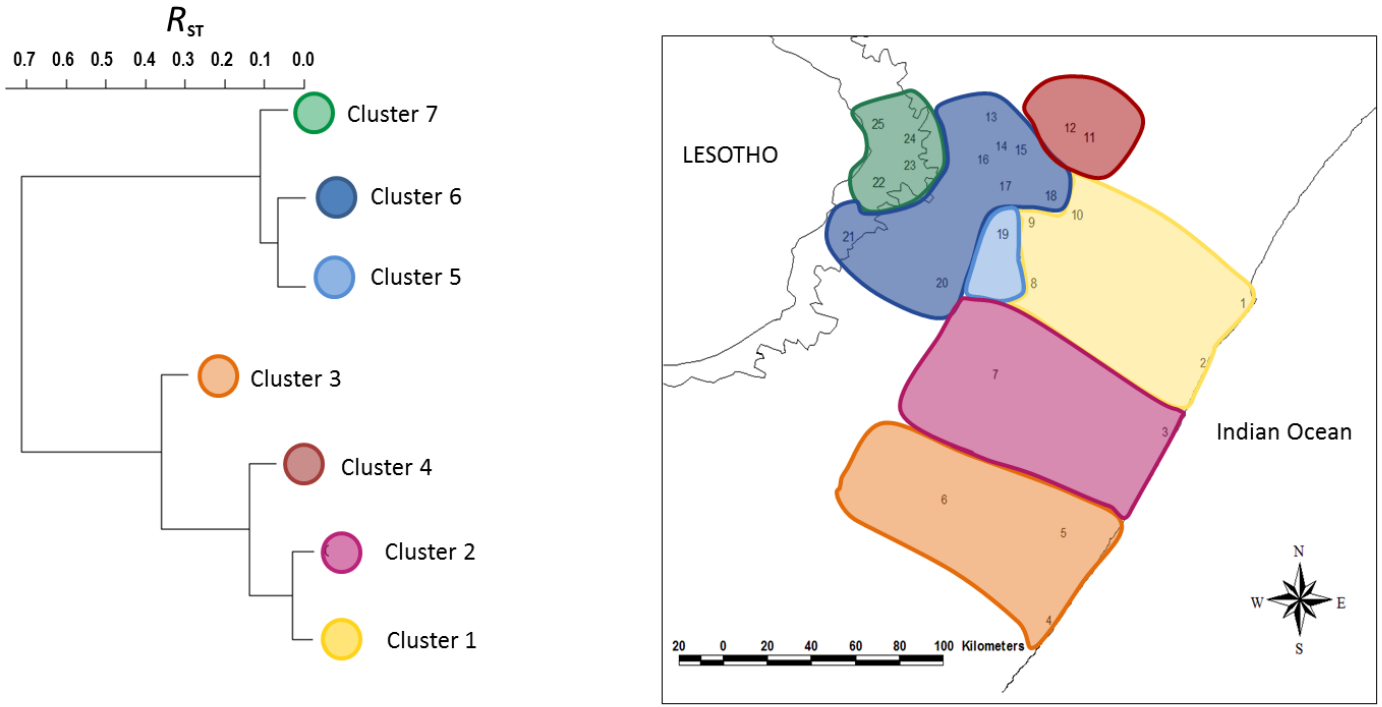

Figure 5.4 Hierarchical dendrogram and map depicting the seven microsatellite clusters identified by GENELAND. Dendrogram is based on R_{ST} values calculated in ARLEQUIN. Numbers 1-25 on map refer to field sites (see Fig. 5.2).

Bottlenecks were not detected using the Cornuet and Luikart (1996) method for any of the GENELAND clusters regardless of mutation model or parameters tested; however, significant signatures were detected for all clusters using Garza and Williamson's *M*-ratio test (Table 5.6). Mean *M* values ranged from 0.19 to 0.28, falling well below the critical *M*-value of 0.68, indicating that each cluster is likely to have experienced a population bottleneck with a prolonged recovery time.

Mantel tests revealed modest, but significant IBD between individuals (AIS: r = 0.323, $P_{(\text{random correlation} \geq \text{observed correlation})} < 0.001$) and clusters (MANTEL: r = 0.566, $P_{(\text{random} \geq \text{observed})} = 0.010$). Likewise, spatial autocorrelations illustrated that pairwise genetic distances were significantly smaller than average over shorter distances ($P < 0.003$ for two distance classes) and larger than average over greater distances ($P < 0.009$ for seven distance classes), suggesting a strong genetic cline. Visualizations of the genetic landscape revealed higher peaks in the west, which reflect higher genetic diversity areas across the landscape in that area (Fig. 4). The highest peaks were observed in the western Midlands, and the lowest within the eastern Midlands and along the coast.

Table 5.6 Results from the two bottleneck tests for the seven GENELAND clusters. For the heterozygosity excess tests, values listed are *P*-values from the one-tailed Wilcoxon sign rank tests.

	M-ratio tests[*]		Heterozygosity excess tests					
			SMM	TPM				
	θ	*M*		2%[†]	5%[†‡]	10%[†Ψ]	20%[†]	30%[†]
Cluster 1	2.65	**0.22**	0.99	0.99	0.99	0.99	0.98	0.93
Cluster 2	2.67	**0.19**	0.28	0.22	0.12	0.10	0.10	0.05
Cluster 3	2.25	**0.19**	0.78	0.65	0.62	0.54	0.50	0.50
Cluster 4	2.02	**0.28**	0.86	0.88	0.78	0.68	0.62	0.46
Cluster 5	1.56	**0.19**	0.99	0.99	0.99	0.99	0.93	0.88
Cluster 6	2.60	**0.24**	0.92	0.92	0.90	0.84	0.72	0.69
Cluster 7	1.97	**0.22**	0.99	0.99	0.95	0.88	0.65	0.62

SMM, stepwise mutation model; TPM, two-phase mutation model; θ, $4N_e\mu$. [*]Critical *M* is 0.68, below which *M* is significant at $\alpha = 0.05$. Bold values indicate significant relationships. [†]Percent multi-step mutations under the two-phased mutation model. [‡]Parameters recommended by Di Rienzo *et al.* (1994). [Ψ]Parameters recommended by Piry *et al.* (1999).

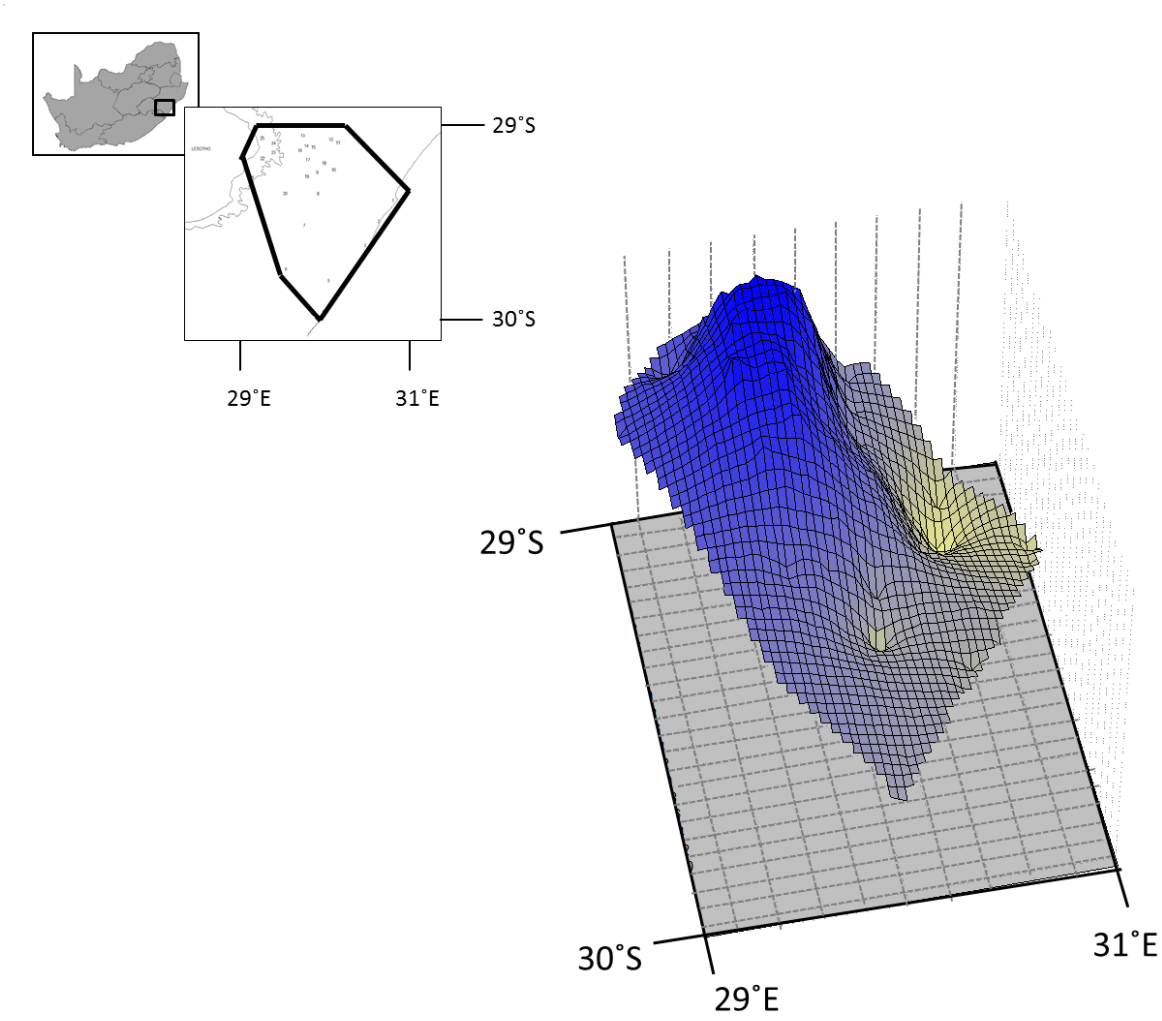

Figure 5.5 Genetic landscape for the *B. melanocephalum-B. thamnobates* species complex based on microsatellite genotypes. Landscapes were standardised to a grid of 50 x 50 cells across a geographic block encompassing all samples in the southern KZN. Dark blue shading and higher peaks show areas of greater genetic diversity.

MTDNA SEQUENCE ANALYSIS

A 477 base pair region of ND4 showed 15 haplotypes from 130 individuals from the 25 sampling sites. Of the 19 possible cluster arrangements identified by SAMOVA (K = 2-20), K = 3 was found to best fit the data (i.e., largest ΔF_{CT}), with Cluster A made up of all coastal populations and three Midlands populations, Cluster B comprised of six eastern Midlands populations, and Cluster C the remaining Midlands populations and all southern

Drakensberg populations (Fig. 5.6, top). Pairwise F_{ST} values show that the three clusters differ significantly from each other (Table 5.7), and it is these differences that explain the majority of the mtDNA variation in this species complex (AMOVA: 61.26%, $F_{CT} = 0.61$, $P < 0.001$). This is clearly illustrated by the median-joining network, which shows no shared haplotypes between clusters (Fig. 5.6, bottom). To a lesser degree, genetic variation was also detected among sites within clusters (27.49%, $F_{SC} = 0.71$, $P < 0.001$). This is most likely attributed to Cluster A because it has very high levels of genetic and nucleotide diversity, as well as the greatest number of haplotypes, which show strong geographic structure (Table 5.8; Fig. 5.6). The differential levels of genetic diversity are especially evident when looking at the mtDNA genetic distance landscape (Fig. 5.7). Given that more than three clusters are required to conduct a cluster-based IBD analysis, only the individual-based approach was performed using AIS. The greatest distances were observed among individuals in Cluster A (Fig. 5.7, blue area), while individuals in the KZN Midlands show very little variation (Fig. 5.7, yellow area), contradictory to what was found with the microsatellite data (Fig. 5.5). Similar to the microsatellite data, however, moderate but significant IBD was detected between individuals (AIS: r = 0.493; $P_{(\text{random correlation} \geq \text{observed correlation})} < 0.001$) with pairwise genetic distances being significantly smaller than average over shorter distances ($P < 0.001$ for two distance classes) and larger than average over greater distances ($P < 0.03$ for eight distance classes), suggesting a strong genetic cline.

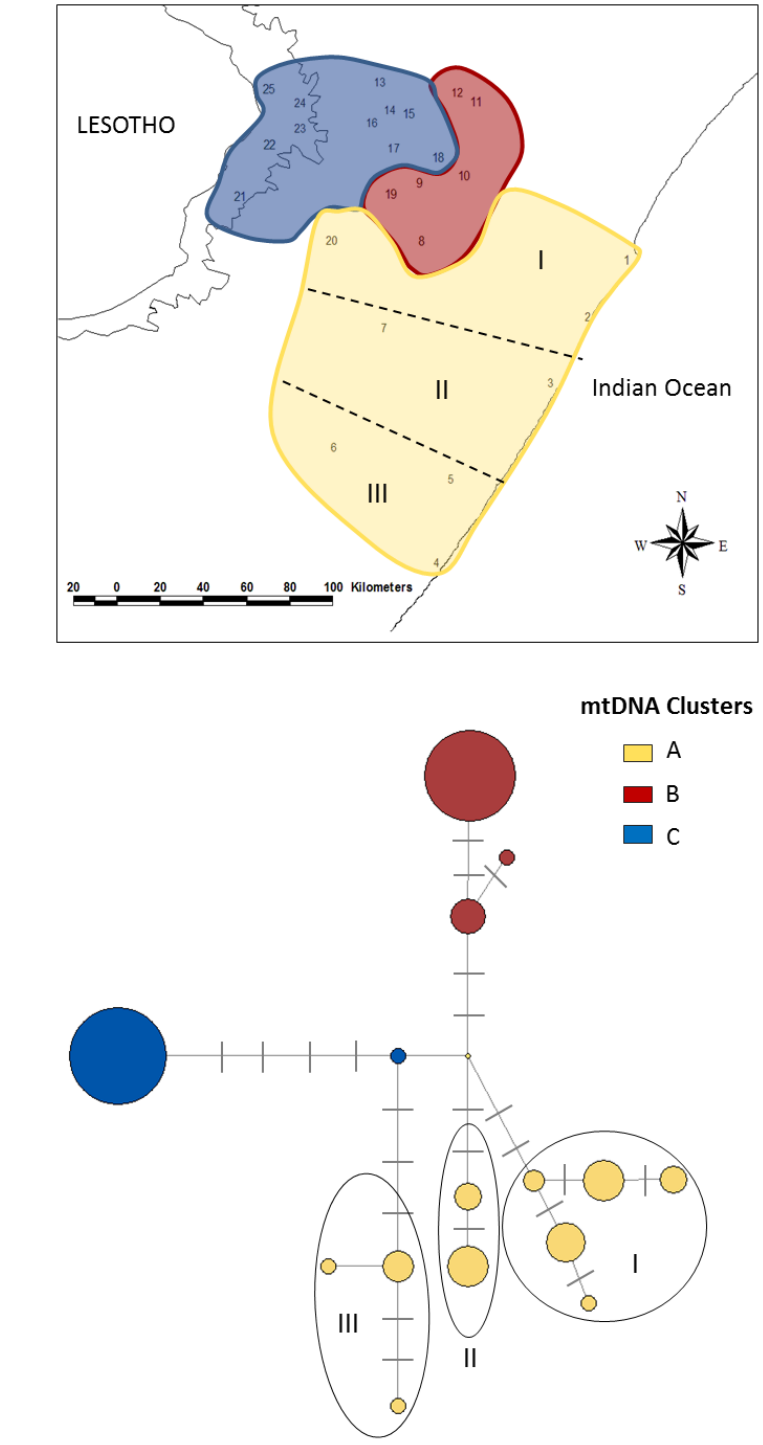

Figure 5.6 Map and median-joining haplotype network colour-coded to represent the three mtDNA clusters. The haplotypes of Cluster A can be further subdivided into three geographic segments (I, II and III). Numbers 1-25 on map refer to field sites (see Fig. 5.2).The sizes of the circles in the network indicate the frequency of the haplotypes and the branch lengths indicate genetic distance between haplotypes as determined by the number of basepair mutations (designated by dashes).

Table 5.7 Pairwise genetic distances (F_{ST}) between the three SAMOVA clusters.

	Cluster A	Cluster B	Cluster C
Cluster A	—		
Cluster B	0.4736*	—	
Cluster C	0.4835*	0.7761*	—

* $P < 0.001$

Table 5.8 Genetic diversity measures for the three mtDNA clusters of the *B. melanocephalum-B. thamnobates* species complex identified by SAMOVA.

	n (a)	H	*h*	π	Tajima's D
Cluster					
A	49 (8)	10	0.79 (0.75 – 0.84)	0.0079 (0.0034 – 0.0123)	0.3483
B	44 (6)	3	0.25 (0.17 – 0.33)	0.0011 (0.0001 – 0.0022)	-0.5007
C	37 (11)	2	0.20 (0.12 – 0.28)	0.0017 (0.0003 – 0.0030)	-0.4199

The number of individuals (*n*), number of sites sampled (*a*), number of haplotypes (H), haplotype diversity (*h*), and nucleotide diversity (π) with 95% confidence intervals in parentheses.

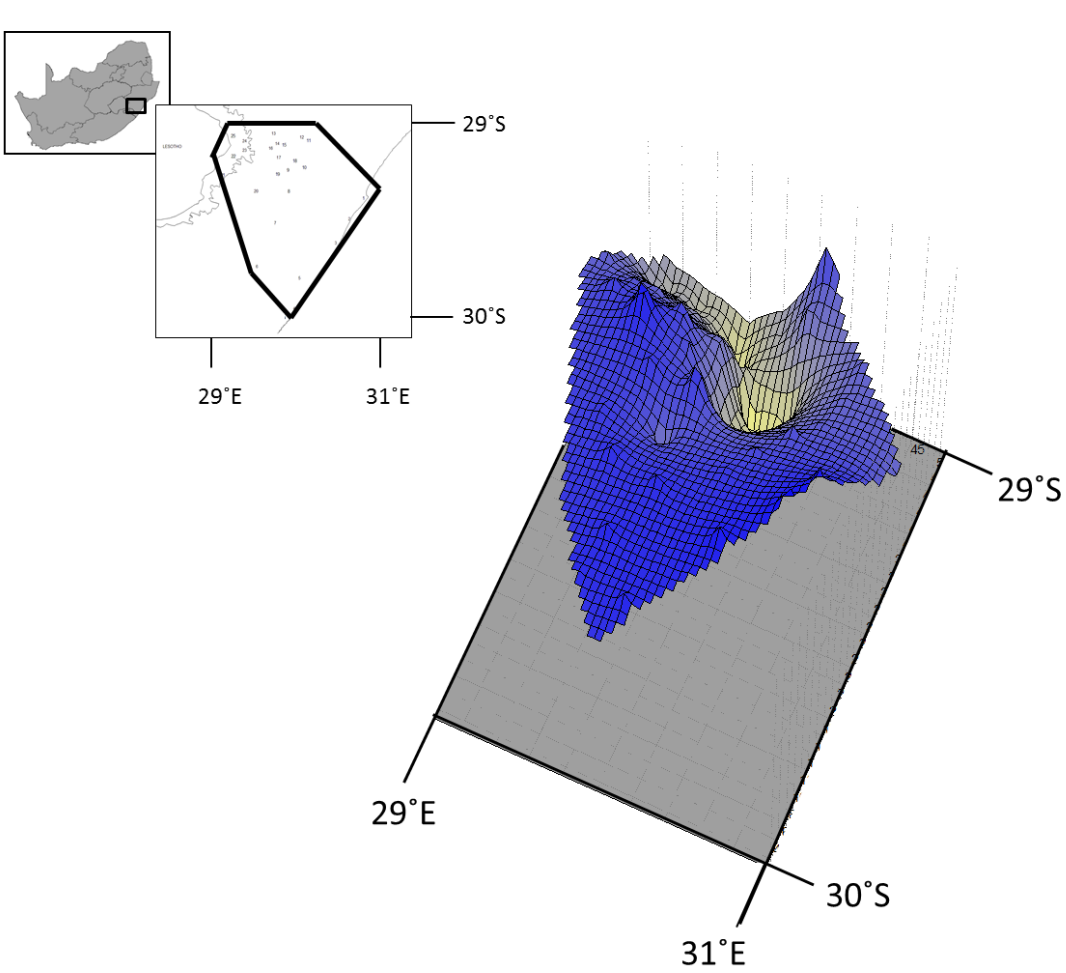

Figure 5.7 Genetic landscape for the *B. melanocephalum-B. thamnobates* species complex based on mtDNA haplotypes. Landscapes were standardised to a grid of 50 x 50 cells across a geographic block encompassing all samples in the southern KZN. Dark blue shading and higher peaks show areas of greater genetic diversity.

Table 5.9 Estimates of net evolutionary divergence (*p*-distances) between groups of sequences. Data is partitioned according to three scenarios: 1, the SAMOVA clusters identified in this study; 2, the two currently recognized species; and 3, the five phenotypic forms that show functional adaptations to their respective habitats.

Scenarios						1			2		3				
		Bgu	Bocc	Bdr	Bnem	Cluster A	Cluster B	Cluster C	Bmel	Btham	Bmel†	Type A	Btham†	Type B	Type C
	Bgu	0.027													
	Bocc	0.063	0.015												
	Bdr	0.048	0.054	0.031											
	Bnem	0.047	0.061	0.034	0.007										
	Cluster A	0.069	0.084	0.051	0.060	0.009									
1	Cluster B	0.071	0.091	0.058	0.067	0.014	0.001								
	Cluster C	0.071	0.092	0.060	0.069	0.011	0.009	0.001							
2	Bmel	0.067	0.085	0.053	0.061				0.008						
	Btham	0.068	0.088	0.055	0.065				0.006	0.009					
	Bmel	0.068	0.085	0.051	0.061						0.009				
	Type A	0.069	0.087	0.056	0.064						0.006	0.007			
3	Btham	0.068	0.088	0.054	0.065						0.006	0.007	0.008		
	Type B	0.070	0.089	0.058	0.067						0.010	0.009	0.000	0.003	
	Type C	0.070	0.087	0.056	0.062						0.004	0.003	0.007	0.011	0.003

Bgu, *Bradypodion gutturale;* Bocc, *B. occidentale*; Bdr, *B. dracomontanum*; Bnem, *B. nemorale*; Bmel, *B. melanocephalum;* Btham, *B. thamnobates.* Bmel† *and* Btham† depict the *B. melanocephalum* and *B. thamnobates* phenotypic forms, respectively, as identified in Chapter 2. Grey cells contain within group divergence.

Table 5.10 Node age estimates (million years ago, Ma) for the three SAMOVA clusters based on estimates of ND4 net sequence divergence (*p*-distances). The ages were calculated using an average mutation rate and corresponding 95% credibility intervals (lower, upper) of 0.55% (0.63, 0.32)[†]. Grey cells contain within group divergence.

		Node age estimates[‡] (million years ago, Ma)		
		Cluster A	Cluster B	Cluster C
ND4 *p-distance*	Cluster A	1.6 (1.4,2.8) 0.009	2.5 (2.2,4.4)	2.0 (1.7,3.4)
	Cluster B	0.014	0.18 (0.16,0.31) 0.001	1.6 (1.4,2.8)
	Cluster C	0.011	0.009	0.18 (0.16,0.31) 0.001

[†] Calibrated from the *B. nemorale-B. dracomontanum* ND2 node age of 5.4-10.5 Ma (as determined by Tolley *et al.*, 2008). [‡] Node age = ND4 *p*-distance / (Mutation Rate percentages[†]/100%).

A comparison of net divergence (uncorrected *p*-distances) revealed low values between populations in the *B. melanocephalum-B. thamnobates* species complex, regardless of the population structure scenario considered (Table 5.9). Of the three scenarios, the greatest divergence between populations was found with scenario 1 (i.e., the SAMOVA clusters), with between 1.1-1.4% divergence. Nevertheless, these differences were substantially less than those typically found between species, as illustrated by the 3.4-6.3% sequence divergence among the comparison *Bradypodion* species. If we take the pair among these with the lowest *p*-distance (*B. dracomontanum* and *B. nemorale*: 0.034; Table 5.9), as the lower limit for recognizing species, then species are estimated to diverge between 5.4 and 10.5 Ma (refer to table S3 in Tolley *et al.*, 2008); which corresponds to a

ND4 mutation rate between 0.63% and 0.32%, respectively. If we apply these rough estimates of mutation rates to the scenario with the greatest among group divergence (scenario 1: the three SAMOVA clusters), clusters are estimated to have diverged between 1.4 and 4.4 million years ago (Ma) (Table 5.10).

DISCUSSION

Recent phylogenetic analyses have shown that described species within this southern KZN species complex are not reciprocally monophyletic for mitochondrial markers (Tolley *et al.*, 2004; Tolley *et al.*, 2008), but the use of more sensitive nuclear markers and fine-scale sampling has uncovered geographic structure, revealing seven different evolutionary units. Depending on the molecular marker considered, varying degrees of genetic structure were observed. However, the different genetic patterns between microsatellites and mtDNA are not surprising considering microsatellites have relatively high mutation rates compared to mtDNA, making them suitable for the examination of species complexes that are not well-resolved through conventional phylogenetic techniques. Nevertheless, commonalities do exist between the results provided by the two markers and, taken together, these data provide valuable insights into the evolution of southern KZN dwarf chameleons.

The seven clusters have low sequence divergence among them, as compared to typical species-level mtDNA sequence divergence usually found for chameleons. This is especially apparent when looking at scenario 2 (the two currently recognised species), which has greater within-group sequence divergence compared to that between groups (Table 5.9), which is in agreement with phylogenetic analyses (Tolley *et al.*, 2004; Tolley *et al.*, 2008). Thus, the application of the genealogical PSC would collapse the two species into a single species. However, there is evidence to suggest that the southern KZN dwarf chameleons may be in the early stages of divergence, and on different evolutionary

trajectories despite the low genetic divergence values. We have dated the divergence of clusters within Scenario 1, which has the greatest among group divergence, to be 1.4 to 4.4 Ma, which is much more recent than most other *Bradypodion* species. Yet, these clusters have no shared haplotypes and fairly high fixation indices (R_{ST} & F_{ST} > 0.15: Frankham, Ballou, & Briscoe, 2012), suggesting that they are indeed isolated from each other and undergoing allopatric speciation. The low dispersal ability of these chameleons and their naturally fragmented habitats make it unlikely that any migration currently occurs between clusters. As such, any shared genetic signatures are most likely due to shared ancestral polymorphisms, resulting from relatively recent isolation.

The divergence estimates for the three clusters fall within the Plio-Pleistocene, which was a period of gradual, but prolonged long-term atmospheric cooling (Lisiecki & Raymo, 2005). These climatic conditions allowed for the expansion of grasslands, which transitioned from C_3 to C_4 grasses (Poaceae) in the summer rainfall regions of South Africa (Vogel, Fuls, & Ellis, 1978; Hopley *et al.*, 2007). The associated regression of forests during this period, may have led to the fragmentation of Scarp and southern Mistbelt forests (Mucina & Geldenhuys, 2006), isolating Cluster A from Clusters B and C; and, subsequently, further fragmentation within each forest type, separating Cluster B from C, and dividing Cluster A into three segments that appear to follow major water catchments. The fragmented forests surviving this period would have provided suitable habitats for chameleons to persist; however, their extent would have been considerably reduced, isolating chameleon populations.

To a large extent, the three subsets of Cluster A are represented in the microsatellite data as Clusters 1-3 (Fig. 5.4). The main discrepancies between the mtDNA and microsatellite data occur within the KZN Midlands, involving sites 8-10. The merging of Cluster A with the lower part of Cluster B may have been precipitated by the climatic

conditions during the Mid-Pleistocene, especially during marine isotope stage (MIS) 7, which is characterized by considerable climatic variability (Desprat *et al.*, 2006). During the warm phases of MIS 7 (i.e., MIS 7a, c & e), grasslands were at their maximum, while *Podocarpus* (yellowwood) forests regressed (Dupont *et al.*, 2011), likely isolating sites 8-10 from sites 11 and 12 (see Figs 5.4 & 5.6). The timing of this internal split can be validated by Cluster B's within group divergence estimated between approximately 160 and 310 thousand years (ka) ago (Table 5.10, grey cells). Following these warm periods, were cooler conditions (i.e., MIS 7b & d), which allowed forests to re-expand and eventually reach their maximum (Dupont *et al.*, 2011). This may have precipitated the mixing of Mistbelt and Coastal forests along a Scarp forest belt; thereby providing a corridor for chameleons from these different habitats to move and interact, resulting in introgressive hybridization forming Cluster 1.

The evolution of chameleon populations in response to forest extent can also be illustrated in the discrepancies between the mtDNA and microsatellite patterns involving Cluster C. This cluster's within group divergence is also estimated between 160 and 310 ka, suggesting a Mid-Pleistocene split between Afrotemperate and Mistbelt forests influenced the separation of Cluster C into Clusters 6 and 7 (see Figs 5.4 & 5.6). However, the placement of chameleons from site 21 (Sani Pass) into Cluster 6 was unexpected given their overall appearance and morphological similarities to the other southern Drakensberg chameleons in Cluster 7 (Chapter 2). These results may also indicate a period of hybridization following the warmer phases of MIS 7 when Afrotemperate and Mistbelt forests may have overlapped. This overlap might have been more extensive at the southern extent of the Drakensberg, permitting gene flow between the Afrotemperate and western Mistbelt chameleons.

Another discrepancy between microsatellite and mtDNA patterns involves the origins of chameleons from sites 19 (Boston) and 20 (Bulwer). The mtDNA groups them with *B. melanocephalum*, but their microsatellite genotypes show a closer relationship with *B. thamnobates*. Moreover, chameleons from these sites have the same overall body design (size, shape and colouration) as *B. thamnobates* (Chapter 2). As above, these results likely indicate the introgressive hybridization of Boston and Bulwer chameleons into the *B. thamnobates* cluster. The subsequent separation of Boston chameleons may represent another period of forest regression, possibly during the Last Glacial Maximum (~18 ka: Eeley *et al.*, 1999)

All of the main discrepancies between mtDNA and microsatellite patterns indicate a cycling of population bottlenecks in association with forest regression and expansion, which are reflected in the low *M*-ratio values uncovered for each cluster, and substantial amounts of gene flow through hybridization as forests re-expanded. Gene flow will typically diminish divergence; however, it can also facilitate it by introducing novel genetic variation, especially after a bottleneck, which natural or sexual selection can then act upon, resulting in populations becoming isolated (Grant & Grant, 1992; Seehausen, 2004; Barrett & Schluter, 2008; Keller *et al.*, 2013). This has been suggested as the most probable explanation for the numerous East African cichlid radiations (e.g., Keller *et al.*, 2013) and could help explain the considerable levels of phenotypic diversity observed within this complex.

Overall, current ecomorphological evidence supports the structure defined by microsatellite data and provides evidence of more recent structuring (Chapters 2-4; Fig. 5.8). In particular, three of the microsatellite clusters (4, 6, and 7) coincide directly with the three closed-canopy habitat phenotypic forms (Type C, *B. thamnobates* and Type B, respectively). Moreover, the Boston chameleons (Cluster 5) were identified as a distinct

morphological population within *B. thamnobates* (Fig. 5.8A; Chapter 2). At first glance, some inconsistencies stand out between the microsatellite Clusters 1-3 compared to the open-canopy habitat phenotypic forms, *B. melanocephalum* and Type A, even though the same individuals were included in both datasets. The microsatellite pattern groups these chameleons on an east-west axis, from the coast into the Midlands, with major water catchments acting as a barrier between clusters; whereas, the distributions of *B. melanocephalum* and Type A are arranged on a north-south axis, with a clear disjunction between them, which corresponds well with the distribution of Scarp forests (see fig. 12.3 in Mucina & Geldenhuys, 2006). However, functional traits differentiating these two phenotypic forms have not been investigated (Chapters 3 and 4). Only chameleons from sites 1 and 10 (Fig. 5.2) were tested; hence, any functional distinctions made between *B. melanocephalum* and Type A can only really hold for the northern extent of these forms, which corresponds to Cluster 1 (see Fig. 5.8 A,B). If the same ecomorphological patterns exist along the full extent of these two phenotypic forms (i.e., within Clusters 2 and 3), then there might actually be 10 distinct chameleon groups within the *B. melanocephalum*-*B. thamnobates* species complex (Fig. 5.8C). To confirm this, however, additional functional and ecomorphological work is needed on chameleons from sites 3-7.

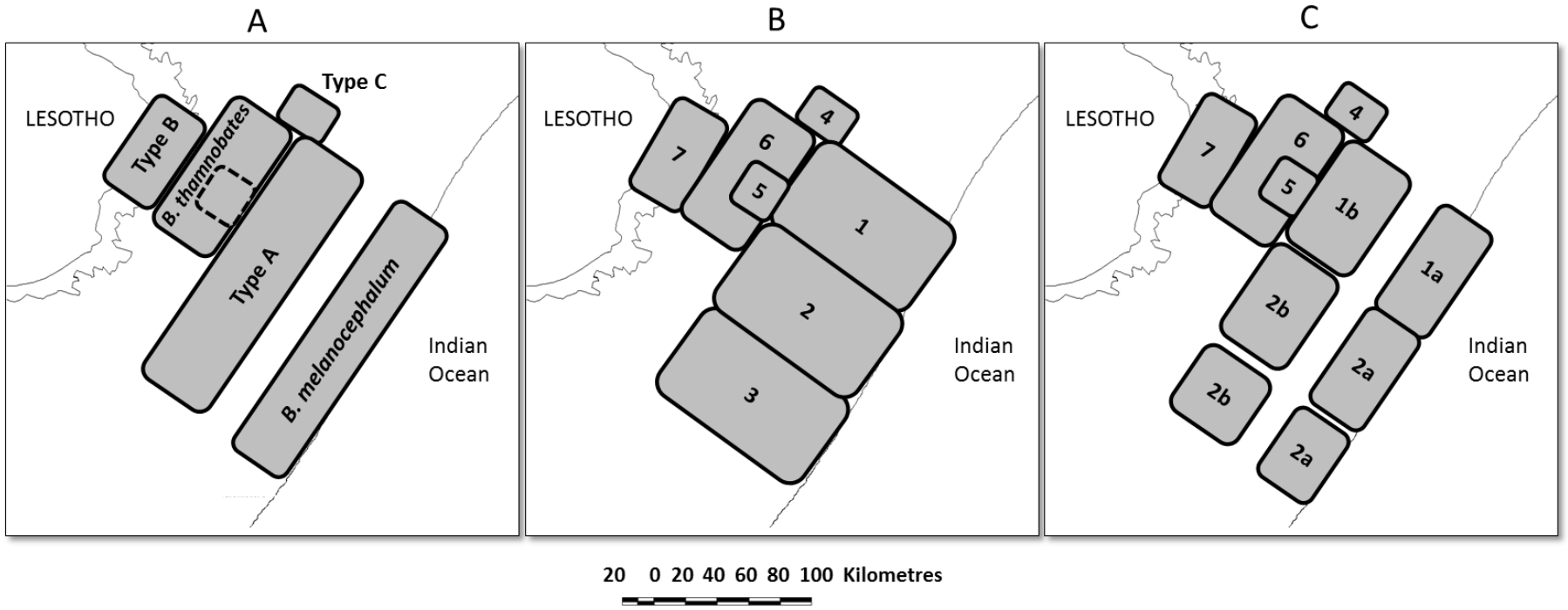

Figure 5.8 Population clustering scenarios for chameleons within the *B. melanocephalum-B. thamnobates* species complex based on morphological data (A: Chapter 2), microsatellite genotyping (B), and a combination of the two (C). Closed-canopy habitat phenotypic forms (*B. thamnobates* and Types B and C) are represented by microsatellite clusters 4-7. The dashed box in scenario A depicts the morphologically distinct *B. thamnobates* chameleons from Boston, which are referred to as cluster 5 in scenarios B and C. Open-canopy habitat forms (*B. melanocephalum* and Type A) are represented as microsatellite clusters 1-3. In scenario C, 'a' denotes coastal populations of open-canopy forms, while 'b' denotes the Midlands populations.

SPECIES CONCEPTS AND DELIMITATION

Of the four species concepts initially identified as being the most applicable to this species concept, the genealogical PSC is the one that requires the greatest genetic differentiation. Based on the results presented here, there is insufficient genetic divergence between groups of chameleons, despite more intensive sampling, confirming the findings by Tolley et al. (2004; 2008). As such, the genealogical PSC would warrant the collapse of the two currently recognized species and all the morphological forms into synonymy. However, applying this species concept would ignore all morphological, adaptive, and ecological evidence that these are independently evolving lineages (Chapters 2-4). As such, we should not apply a strict molecular-based PSC to this species complex.

Under the three remaining species concepts, multiple species can be recognized. Applying the genotypic cluster species concept (GSC), seven species would be recognised, corresponding to the seven genetic clusters indicated by the spatial genetic analysis. Three of these (Clusters 4, 6 and 7) are also supported by morphological and ecological data, and would also be recognised within the morphological and ecological species concepts framework. The four remaining 'GSC species' (Clusters 1, 2, 3 and 5) show discrepancies between genetic and ecomorphological data. Cluster 1 might be comprised of one or two species and a conservative approach would be to retain this as a single species (*B. melanocephalum*). However, because of the adaptive morphological distinctiveness between the coastal and Midland chameleons (Chapters 3 & 4), the Midlands cluster (i.e., Cluster 1b: Fig. 5.8C) should be classified as a separate ecologically distinct population conservation unit; thereby allowing management efforts to be aimed at preserving the adaptive diversity and evolutionary processes within it. This is particularly important considering the already narrow distributions of these chameleons are being threatened by extensive deforestation, agriculture, and urbanization (van Wyk, 1998;

Driver *et al.*, 2005; Mucina & Rutherford, 2006; Driver *et al.*, 2012), as well as increased pressures from natural climatic fluctuations (Houniet, 2007; Armstrong, 2008). Such instances of niche narrowness, accompanied by the low dispersal ability of these chameleons, can greatly increase their risk of extinction (Lawton *et al.*, 1994; McKinney, 1997). Although Clusters 2, 3 and 5 would be considered separate species under the GSC, there is insufficient ecomorphological evidence to confidently assign each as species according to the MSC and ESC. Each of these clusters is based on a very limited sampling area, which may not be representative of their full distribution, potentially biasing the results. Moreover, the functional performance of their morphology was not examined. Consequently, the adaptive nature of any morphological differences between and within each of these clusters compared to all others is unknown. Until this has been established, we recommend Clusters 2 and 3 be designated as separate genetic conservation units of *B. melanocephalum,* and Cluster 5 a genetic conservation unit of *B. thamnobates*, in an attempt to preserve the genetic integrity of these populations. In total, this study recognizes four species (*B. melanocephalum, B. thamnobates, B.* 'southern Drakensberg' *sp.*, and *B.* 'Karkloof' *sp.*) and four conservation units within the *B. melanocephalum*-*B. thamnobates* species complex.

Supporting Information

Table S5.1 Standard genetic diversity measures for each microsatellite locus at each field site.

Site	*Nottingham Road* (n = 9)					*Mooi River* (n = 2)				
Locus	N_A	S	H_O	H_E	HWE	N_A	S	H_O	H_E	HWE
Bpu94	4	154-205	0.14	0.49	0.0063	1	154	—	—	—
Bpu557	1	104	—	—	—	1	104	—	—	—
Bpu507	5	184-202	0.44	0.81	0.0071	3	186-252	1.00	0.83	1.0000
Bth10	13	167-223	0.89	0.97	0.3291	2	177-203	1.00	1.00	1.0000
Bth76	12	104-228	0.75	0.94	0.0116	3	172-208	0.50	0.83	0.3333
Bth93	8	132-176	0.89	0.88	0.2438	3	132-160	0.50	0.83	0.3322
Bth161	9	189-269	0.75	0.92	0.2608	4	201-261	1.00	1.00	1.0000
Bme45	10	98-170	0.89	0.93	0.2299	2	130-150	1.00	1.00	1.0000
Bme58	5	131-139	0.44	0.67	0.0343	2	133-139	0.50	0.50	1.0000
Bme128	4	135-165	0.56	0.63	0.5184	1	167	—	—	—
Site	*Gowrie* (n = 10)					*Dargle* (n = 13)				
Locus	N_A	S	H_O	H_E	HWE	N_A	S	H_O	H_E	HWE
Bpu94	3	154-190	0.22	0.45	0.0596	6	115-190	0.20	0.80	**0.0000**
Bpu557	2	90-104	0.10	0.10	1.0000	3	90-106	0.38	0.53	0.0433
Bpu507	4	186-202	0.60	0.68	0.7403	6	164-202	0.23	052	**0.0029**
Bth10	8	167-219	0.60	0.90	0.0728	11	169-215	0.77	0.88	0.1227
Bth76	7	100-256	0.63	0.87	0.0725	11	100-236	0.58	0.90	0.0091
Bth93	8	132-180	0.80	0.88	0.7770	9	128-184	0.91	0.89	0.4339
Bth161	9	189-265	0.50	0.87	0.0087	14	181-277	1.00	0.94	0.7720
Bme45	8	90-194	0.75	0.84	0.3006	15	102-190	0.92	0.94	0.7486
Bme58	3	133-147	0.40	0.61	0.0851	8	131-157	0.33	0.84	**0.0006**
Bme128	6	135-171	0.50	0.64	0.3361	4	141-179	0.25	0.59	0.0117
Site	*Boschhoek* (n = 3)					*Howick* (n = 7)				
Locus	N_A	S	H_O	H_E	HWE	N_A	S	H_O	H_E	HWE
Bpu94	4	121-160	0.67	0.80	0.6000	2	121-154	0.20	0.20	1.0000
Bpu557	3	90-106	1.00	0.73	1.0000	2	90-104	0.14	0.14	1.0000
Bpu507	3	186-200	1.00	0.83	1.0000	7	182-262	1.00	0.88	0.8791
Bth10	4	199-219	1.00	0.87	0.4666	9	167-213	0.83	0.94	0.3876
Bth76	3	104-192	0.33	0.73	0.1993	6	100-228	0.83	0.80	0.6192
Bth93	4	136-188	0.67	0.87	0.4682	7	140-188	0.86	0.82	0.1155
Bth161	4	209-249	1.00	1.00	1.0000	8	173-265	0.86	0.91	0.2149
Bme45	5	114-186	1.00	0.93	1.0000	7	110-174	0.71	0.87	0.4921
Bme58	4	135-153	1.00	0.80	1.0000	3	135-139	0.71	0.65	0.7199
Bme128	4	135-169	1.00	0.87	0.4670	4	141-205	0.71	0.63	0.7797
Site	*Bulwer* (n = 10)					Boston (n = 20)				
Locus	N_A	S	H_O	H_E	HWE	N_A	S	H_O	H_E	HWE
Bpu94	3	121-157	0.38	0.64	0.1440	5	121-190	0.25	0.61	**0.0006**
Bpu557	2	104-106	0.20	0.44	0.1328	2	104-106	0.05	0.05	1.0000
Bpu507	5	184-208	0.50	0.51	0.5208	7	182-260	0.45	0.75	**0.0005**
Bth10	12	173-227	0.90	0.95	0.5577	10	173-221	0.44	0.84	**0.0001**
Bth76	8	180-248	0.90	0.89	0.1037	10	100-228	0.85	0.86	**0.0001**
Bth93	10	148-192	1.00	0.93	0.0929	11	72-172	0.65	0.89	0.0066
Bth161	12	201-285	1.00	0.95	1.0000	10	189-273	0.65	0.84	**0.0014**
Bme45	10	110-198	1.00	0.92	1.0000	12	110-186	0.79	0.86	0.0500
Bme58	3	139-143	0.30	0.58	**0.0018**	8	139-163	0.2	0.85	**0.0000**
Bme128	6	135-165	0.70	0.78	0.6609	9	141-181	0.67	0.74	0.0290

Table S5.1 *continued*.

Site	*Kamberg Nature Reserve* (n = 34)					*Sani Pass* (n = 4)				
Locus	N_A	S	H_O	H_E	HWE	N_A	S	H_O	H_E	HWE
Bpu94	4	154-163	0.06	0.37	**0.0001**	2	157-163	0.33	0.60	1.0000
Bpu557	2	98-104	0.07	0.07	1.0000	1	104	—	—	—
Bpu507	12	164-266	0.50	0.81	**0.0000**	5	182-254	0.75	0.86	0.6636
Bth10	20	165-227	0.78	0.92	0.0111	3	195-205	0.25	0.68	0.0844
Bth76	14	168-244	0.83	0.89	0.8699	6	200-240	1.00	0.93	1.0000
Bth93	13	128-220	0.94	0.90	0.1671	4	140-160	1.00	0.80	1.0000
Bth161	17	185-281	0.85	0.92	0.1676	2	209-229	0.33	0.60	1.0000
Bme45	12	110-190	0.85	0.87	0.3561	2	110-154	0.25	0.25	1.0000
Bme58	6	129-147	0.21	0.76	**0.0000**	1	133	—	—	—
Bme128	4	141-169	0.62	0.50	0.0120	1	141	—	—	—
Site	*Lotheni Nature Reserve* (n = 2)					*Highmoor Nature Reserve* (n = 2)				
Locus	N_A	S	H_O	H_E	HWE	N_A	S	H_O	H_E	HWE
Bpu94	1	157	—	—	—	1	172	—	—	—
Bpu557	1	104	—	—	—	1	104	—	—	—
Bpu507	4	182-246	0.50	0.83	0.3313	3	240-256	1.00	0.83	1.0000
Bth10	2	197-203	1.00	1.00	1.0000	4	177-209	1.00	1.00	1.0000
Bth76	2	208-220	1.00	1.00	1.0000	4	164-220	1.00	1.00	1.0000
Bth93	4	144-172	0.50	0.83	0.3364	4	128-180	1.00	1.00	1.0000
Bth161	4	185-241	0.50	0.83	0.3349	4	193-281	1.00	1.00	1.0000
Bme45	1	150	—	—	—	3	146-158	1.00	0.83	1.0000
Bme58	4	133-163	0.50	0.83	0.3348	2	133-139	0.00	0.67	0.3325
Bme128	1	141	—	—	—	2	141-167	1.00	1.00	1.0000
Site	*Giant's Castle* (n = 1)					*Karkloof* (n = 28)				
Locus	N_A	S	H_O	H_E	HWE	N_A	S	H_O	H_E	HWE
Bpu94	—	—	—	—	—	2	154-157	0.37	0.31	1.0000
Bpu557	1	104	—	—	—	2	104-106	0.22	0.20	1.0000
Bpu507	1	200	—	—	—	5	184-200	0.68	0.77	0.1770
Bth10	1	191	—	—	—	8	175-209	0.64	0.67	0.3872
Bth76	2	192-212	1.00	1.00	1.0000	7	100-220	0.78	0.75	0.6115
Bth93	1	148	—	—	—	10	84-156	0.80	0.86	0.8976
Bth161	2	225-249	1.00	1.00	1.0000	15	197-265	0.75	0.89	0.0289
Bme45	2	134-146	1.00	1.00	1.0000	18	110-270	0.92	0.94	0.2263
Bme58	1	139	—	—	—	7	133-161	0.23	0.83	**0.0000**
Bme128	2	141-171	1.00	1.00	1.0000	17	141-219	0.56	0.93	**0.0000**
Site	*Gilboa* (n = 3)					*Stirling* (n = 12)				
Locus	N_A	S	H_O	H_E	HWE	N_A	S	H_O	H_E	HWE
Bpu94	1	157	—	—	—	5	121-166	0.40	0.74	0.0059
Bpu557	2	104-106	0.67	0.53	1.0000	4	90-108	0.50	0.51	1.0000
Bpu507	3	186-202	0.67	0.73	1.0000	8	164-242	0.50	0.80	**0.0025**
Bth10	3	177-197	1.00	0.73	1.0000	7	159-197	0.64	0.81	0.2545
Bth76	3	100-208	0.67	0.73	1.0000	6	100-208	0.64	0.69	0.3926
Bth93	4	112-172	1.00	0.87	1.0000	11	72-156	0.82	0.90	0.6377
Bth161	3	213-245	0.50	0.83	0.3323	12	181-253	0.73	0.94	0.0630
Bme45	4	110-142	0.33	0.87	0.0644	11	90-174	0.82	0.93	0.4097
Bme58	1	157	—	—	—	6	135-213	0.25	0.85	**0.0003**
Bme128	3	157-177	0.50	0.83	0.3309	11	137-181	0.75	0.87	0.3261

Table S5.1 *continued*.

Site	*Hilton* (n = 34)					*Byrne Valley* (n = 10)				
Locus	N_A	S	H_O	H_E	HWE	N_A	S	H_O	H_E	HWE
Bpu94	6	109-184	0.50	0.65	**0.0000**	4	154-163	0.33	0.63	0.0360
Bpu557	3	104-108	0.36	0.42	0.0794	3	104-108	0.20	0.35	0.3078
Bpu507	9	164-202	0.70	0.79	**0.0009**	5	182-200	0.80	0.71	0.7634
Bth10	16	159-207	0.72	0.85	0.3117	11	157-229	0.60	0.93	0.0109
Bth76	10	100-204	0.48	0.53	0.3866	7	100-216	0.50	0.58	0.2797
Bth93	14	72-180	0.63	0.86	**0.0017**	8	72-180	0.80	0.87	0.3886
Bth161	20	157-273	0.73	0.92	**0.0000**	7	173-245	0.40	0.85	**0.0011**
Bme45	22	94-186	0.88	0.96	0.3487	10	90-170	0.80	0.91	0.4733
Bme58	11	129-245	0.42	0.87	**0.0000**	7	137-163	0.50	0.88	**0.0024**
Bme128	16	141-203	0.62	0.83	**0.0000**	9	139-191	1.00	0.91	0.8905
Site	*Durban* (n = 28)					*Illovo* (n = 7)				
Locus	N_A	S	H_O	H_E	HWE	N_A	S	H_O	H_E	HWE
Bpu94	5	137-205	0.62	0.73	0.0540	4	121-205	1.00	0.73	0.0344
Bpu557	3	104-108	0.44	0.51	0.7575	2	104-106	0.43	0.36	1.0000
Bpu507	8	182-218	0.73	0.79	0.1134	5	182-200	0.67	0.83	0.5599
Bth10	18	149-227	0.76	0.94	**0.0016**	6	155-197	0.50	0.68	0.1486
Bth76	8	100-228	0.35	0.44	0.1851	4	100-212	0.14	0.58	0.0068
Bth93	14	112-176	0.70	0.84	0.0531	5	128-192	0.71	0.74	0.4043
Bth161	13	177-265	0.67	0.86	**0.0012**	7	193-245	0.57	0.85	0.0484
Bme45	17	102-158	0.82	0.94	0.1044	4	86-174	0.43	0.71	0.1212
Bme58	10	137-177	0.29	0.83	**0.0000**	6	139-163	0.50	0.86	0.0873
Bme128	11	121-163	0.68	0.86	0.0055	6	141-201	0.71	0.77	1.0000
Site	*Umdoni Park, Pennington* (n = 7)					*Ixopo* (n = 12)				
Locus	N_A	S	H_O	H_E	HWE	N_A	S	H_O	H_E	HWE
Bpu94	1	121	—	—	—	4	121-199	0.20	0.68	**0.0021**
Bpu557	3	104-108	0.71	0.66	1.0000	4	98-108	0.58	0.71	0.4530
Bpu507	5	184-198	1.00	0.79	1.0000	7	184-210	0.64	0.82	0.1104
Bth10	6	141-229	0.57	0.77	0.2777	9	163-215	0.20	0.88	**0.0000**
Bth76	4	100-116	0.57	0.65	0.6287	7	96-192	0.23	0.42	**0.0016**
Bth93	6	72-160	0.86	0.85	0.6463	10	64-160	0.54	0.90	0.0061
Bth161	5	157-225	0.57	0.80	0.2026	14	129-257	0.62	0.92	**0.0010**
Bme45	8	114-170	0.86	0.91	0.6666	11	86-170	0.77	0.93	0.0747
Bme58	3	157-181	0.29	0.65	0.0540	6	129-155	0.38	0.85	**0.0000**
Bme128	5	141-217	0.71	0.73	0.3472	9	143-181	0.50	0.89	**0.0048**
Site	*Weza* (n = 5)					*Umtamvuna Nature Reserve* (n = 15)				
Locus	N_A	S	H_O	H_E	HWE	N_A	S	H_O	H_E	HWE
Bpu94	3	121-190	0.33	0.73	0.2020	3	121-160	0.71	0.54	0.2670
Bpu557	2	104-106	0.25	0.25	1.0000	3	104-108	0.60	0.48	0.7416
Bpu507	4	186-254	0.50	0.75	0.0877	6	184-200	0.73	0.84	0.4527
Bth10	3	183-211	0.75	0.75	0.0568	12	165-243	0.60	0.91	0.0101
Bth76	4	100-124	1.00	0.86	0.2384	8	100-196	0.64	0.75	0.2644
Bth93	2	68-144	0.00	0.43	0.1425	3	72-144	0.13	0.13	1.0000
Bth161	2	205-221	0.25	0.25	1.0000	10	189-253	0.80	0.89	0.2338
Bme45	2	142-174	0.25	0.25	1.0000	13	122-178	0.87	0.89	0.0367
Bme58	2	155-157	0.00	0.43	0.1427	9	145-167	0.67	0.88	0.1912
Bme128	4	143-167	1.00	0.82	0.7720	11	137-201	0.64	0.80	0.0348

Table S5.1 *continued.*

Site	*Oribi Gorge Nature Reserve* (n = 4)				
Locus	N_A	S	H_O	H_E	HWE
Bpu94	3	121-190	0.33	0.73	0.2009
Bpu557	2	104-108	0.25	0.54	0.4284
Bpu507	5	184-202	0.75	0.79	0.7680
Bth10	5	165-215	1.00	0.93	1.0000
Bth76	3	100-164	0.50	0.83	0.3361
Bth93	1	72	—	—	—
Bth161	7	205-253	1.00	0.96	1.0000
Bme45	3	118-134	1.00	0.83	1.0000
Bme58	2	149-171	0.50	0.43	1.0000
Bme128	2	143-147	0.50	0.43	1.0000

N_A, number of alleles; S, allelic size range; H_O, observed heterozygosity; H_E, expected heterozygosity. Bold values indicate deviations from HWE based on a Bonferroni significance value of 0.005 ($P < 0.05/10$).

Table S5.2 ND4 mutation rates calibrated from the node age estimates (million years ago, Ma) of five *Bradypodion* species based on ND2 net sequence divergence (Tolley et al. 2008). The Ages were obtained using a Bayesian relaxed clock and corresponding Bayesian 95% credibility intervals (lower, upper).

		Node age estimates (million years ago, Ma)				
		Bgu	Bocc	Bdr	Bnem	Btham
ND4 Mutation rate[†] (%)	Bgu		10.1 (5.5,16.7)	12.2 (7.0,19.4)	12.2 (7.0,19.4)	12.2 (7.0,19.4)
	Bocc	0.62 (0.38,1.15)		12.2 (7.0,19.4)	12.2 (7.0,19.4)	12.2 (7.0,19.4)
	Bdr	0.39 (0.25,0.69)	0.44 (0.28,0.77)		6.2 (5.4,10.5)	9.0 (5.1,14.7)
	Bnem	0.39 (0.24,0.67)	0.50 (0.31,0.87)	0.55 (0.32,0.63)		9.0 (5.1,14.7)
	Btham	0.56 (0.35,0.97)	0.72 (0.45,1.26)	0.61 (0.37,1.08)	0.72 (0.44,1.27)	

Bgu, *Bradypodion gutturale;* Bocc, *B. occidentale*; Bdr, *B. dracomontanum*; Bnem, *B. nemorale*; Btham, *B. thamnobates*. [†]Mutation rate = ((ND4 p-distance[‡])/node age)*100%. [‡]Refer to Table 5.9 for ND4 *p*-distances.

Chapter 6

Paper VI:
Reconstructing the Pleistocene geography of the *Bradypodion melanocephalum-Bradypodion thamnobates* species complex[*]

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ABSTRACT

Morphological, ecological and genetic data have been used to investigate the ecology and evolution of chameleons within the *Bradypodion melanocephalum-Bradypodion thamnobates* species complex. These data have greatly improved our understanding of the biotic factors involved in shaping the present-day chameleon diversity. However, given that these chameleons are greatly affected by their environment, an examination of abiotic factors is needed to obtain a complete understanding of their evolution. In particular, an investigation of their past climatic niches would greatly improve our understanding of their biogeographic history and the speciation mechanisms involved in their diversity and distribution. We hypothesize that the distribution of chameleons within this species complex is closely linked with that of their respective forests, each of which has its own evolutionary history. Accordingly, we expect corresponding patterns of paleoclimatic change. Afrotemperate and Mistbelt forests are more ancient and typically support highly resilient species due to their experience with paleoclimatic extinction filters. As such, chameleons within these environments are expected to show high climatic stability. In contrast, Coastal forests have been more recently established and typically have less resilient species; and, therefore, chameleons in these habitats are expected to show climatic niche. To test this, we used ecological niche models to project the past climatic niches of these chameleons during the Last Interglacial and the Last Glacial Maximum. Their climatic niches were found to correlate to different climatic variables and a climatic niche stability gradient was uncovered across southern KZN, with the highest climatic stability associated with Afrotemperate forests in the west and the lowest stability with Coastal forests to the east, as predicted. These results help explain the observed patterns of morphological and genetic variation within this species complex, with chameleons in areas of high climatic niche stability tending to show accordance between morphological and

genetic data, while chameleons in climatically labile areas show discordance between these data.

INTRODUCTION

Predicting the past distributions of species' climatic niches has greatly improved our understanding of a variety of evolutionary questions, such as speciation mechanisms (e.g., Peterson & Nyári, 2008), ecological niche conservatism (e.g., Martínez-Meyer, Townsend Peterson, & Hargrove, 2004; Peterson & Nyári, 2008), species extinctions (e.g., Martínez-Meyer *et al.*, 2004; Nogués-Bravo *et al.*, 2008), and historical migration pathways (e.g., Ruegg, Hijmans, & Moritz, 2006; Carstens & Richards, 2007). Ecological niche modelling, coupled with molecular and phylogeographic data, have also provided great insight into the biogeographic history of species and communities (e.g., Knowles, Carstens, & Keat, 2007; Waltari *et al.*, 2007; Araújo *et al.*, 2008; Peterson & Ammann, 2013). Many of these studies have focused on the role Pleistocene climatic fluctuations have had in the generation of modern biodiversity (Hewitt, 2000). This is because the Pleistocene is either the period in which much of the present diversity was generated, or in which biodiversity generated earlier had to respond to dramatically changing conditions involving severe glacial-interglacial cycles (e.g., Klicka & Zink, 1997; Avise & Walker, 1998; Dynesius & Jansson, 2000; Weir & Schluter, 2004; Tolley *et al.*, 2008).

Despite the lack of glaciation on the African continent, the glacial-interglacial fluctuations of the Pleistocene greatly affected the distribution of biomes (Deacon, 1983; Hamilton & Taylor, 1991; Scott, Homgren, & Partridge, 2008; Potts *et al.*, 2013). The forest biome, for example, predominated during the warmer, humid interglacials; while during the glacials, forests became fragmented and open vegetation, such as grasslands, expanded (Scott, Anderson, & Anderson, 1997; Eeley *et al.*, 1999; Dupont *et al.*, 2001).

This is referred to as the expansion-contraction model of Pleistocene biogeography (e.g., Provan & Bennett, 2008; Bagley *et al.*, 2013; Potts *et al.*, 2013). During the glacials, the fragmented forests could either act as refugia for forest specialists, enabling them to persist during these unfavourable climatic periods or isolating populations further, providing opportunities for speciation (Haffer, 1969). This hypothesis has been proposed for the diversity and distribution of dwarf chameleons (*Bradypodion*) within KwaZulu-Natal (KZN) Province, South Africa (Tolley *et al.*, 2008).

Dwarf chameleons, like the majority of Chamaeleonidae, are fully arboreal species (Tolley, Townsend & Vences 2013) and, thus, are highly reliant on vegetation for their survival (Tolley *et al.*, 2006; Stuart-Fox & Moussalli, 2007). Most *Bradypodion* within KZN reside predominantly in forests (Tolley *et al.*, 2008); consequently, their histories are expected to be intrinsically linked. Several studies have suggested that most northern KZN forests are refugia (Eeley *et al.*, 1999; Mazus, 2000; Lawes *et al.*, 2007) and their contraction during glacial periods produced localized faunal extinctions (Lawes *et al.*, 2007). This has been validated by several dwarf chameleon lineages separated by deep genetic divergences and known only from a single forest each (Tolley *et al.*, 2008; Tilbury & Tolley, 2009). However, a recent radiation of dwarf chameleons from southern KZN appears to have been affected by different historical processes during these climatic cycles, with environmental changes likely eliciting rapid morphological adaptation to novel habitats, without significant genetic evolution (Chapters 2-5).

The *Bradypodion melanocephalum-Bradypodion thamnobates* species complex is a phenotypically diverse group of dwarf chameleons currently comprised of two taxonomically classified species – *B. melanocephalum* (Gray, 1865) and *B. thamnobates* (Raw, 1976); however, at least two other species and up to six conservation units have been proposed based on a combination of ecomorphological and population genetic

information (Chapters 2-5; see Fig. 6.1). In this chapter, these proposed species and conservation units will be referred to as evolutionarily significant units (ESUs) following the adaptive evolutionary conservation framework of Fraser and Bernatchez (2001). This framework defines an ESU as a species, group, or population that maintains and sustains itself over time in a definable area with highly restricted gene flow. The 10 distinct ESUs in this species complex are allopatric in distribution and occupy different macro- and micro-habitats (Chapters 2), yet their genetics suggests they have only recently diverged, possibly starting with the arrival and expansion of C_4 grasses, and the corresponding regression of *Podocarpus* forests during the Plio-Pleistocene transition (Chapter 5). Since then, the numerous glacial phases of the Pleistocene are thought to have further isolated chameleons, while the subsequent interglacials may have provided multiple contact events between forests, enabling them to radiate into new habitats (Chapter 5). Today, each ESU is known from one of four forest types: Afrotemperate, Mistbelt, Scarp, and Coastal forests. The montane Afrotemperate and Scarp forests each contain a single ESU (the southern Drakensberg and southern Coastal *B. melanocephalum* ESUs, respectively [Clusters 7 & 3a: Fig. 6.1]). In contrast, six ESUs are known from the Midlands' Mistbelt forests (both *B. thamnobates* ESUs [Cluster 5 & 6: Fig. 6.1], the Karkloof ESU [Cluster 4: Fig. 1], the three Midlands *B. melanocephalum* ESUs [Clusters 1b, 2b, 3b: Fig. 6.1]) and two from the Coastal forests (the northern and central Coastal *B. melanocephalum* ESUs [Clusters 1a & 2a: Fig. 6.1). Each forest type has its own evolutionary history, with Afrotemperate and Mistbelt forests being more ancient and typically supporting highly resilient species due to their experience with paleoclimatic extinction filters; whereas, Coastal forests being more recently established (within the past 8,000 years), have less resilient species (Eeley *et al.*, 1999; Lawes, Eeley, & Piper, 2000; Mucina & Geldenhuys, 2006; Lawes *et al.*, 2007). If the distribution of chameleons within this complex is closely

linked with that of their respective forests, we expect corresponding patterns of paleoclimatic change. Accordingly, we hypothesize that Afrotemperate and Mistbelt ESUs were highly resilient through the glacial-interglacial cycles of the Pleistocene, while the Scarp and Coastal ESUs were less stable, showing drastic changes in distribution in response to relict Scarp populations taking advantage of the new Coastal forest.

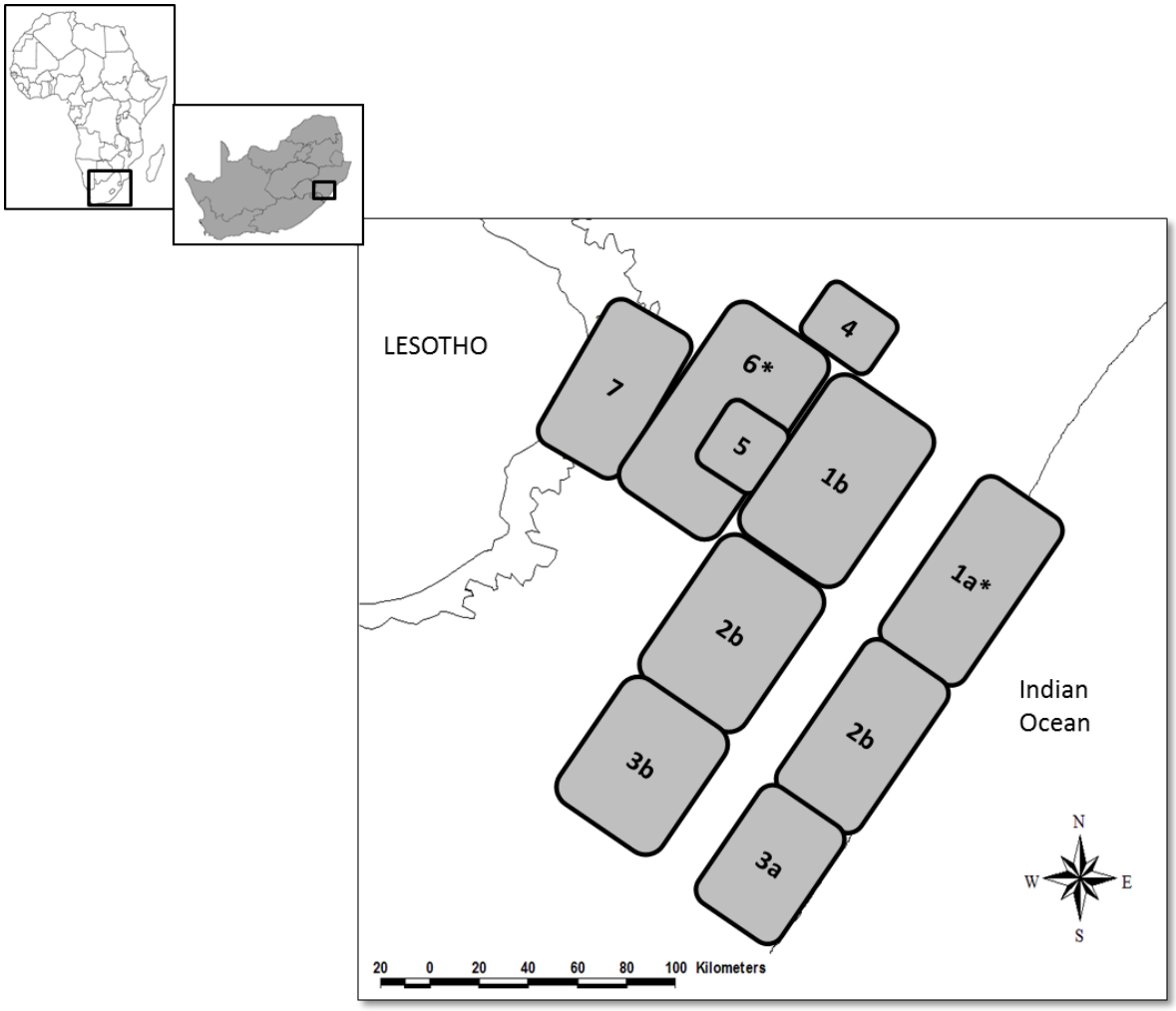

Figure 6.1 Schematic of the approximate distributions of the 10 proposed chameleon ESUs within the *B. melanocephalum-B. thamnobates* species complex. Cluster 1 = *B. melanocephalum* north; Cluster 2 = *B. melanocephalum* central; Cluster 3 = *B. melanocephalum* south; Cluster 4 = Karkloof; Cluster 5 = *B. thamnobates* (Boston population); Cluster 6 = *B. thamnobates*; Cluster 7 = southern Drakensberg. For the *B. melanocephalum* ESUs (Clusters 1-3), 'a' denotes Coastal populations, while 'b' denotes Midland populations. * = type localities of described species.

To test these hypotheses, we used bioclimatic analyses and niche modelling to project the past climatic niches of each ESU during the Last Glacial Maximum (LGM) and the Last Interglacial (LIG: ~120,000-140,000 years BP). These climatic periods were selected because they represent the most extreme climatic conditions during the Pleistocene (Deacon, 1983; Tyson, 1986); thereby, enabling us to gain insight into the climatic niche of these ESUs during the other glacial-interglacial cycles within this geological epoch. Because niche models utilize associations between environmental variables and known species or population occurrence data to define the abiotic conditions within which populations can be maintained (Guisan & Thuiller, 2005), the modeled climatic niche is thought to approximate a set of physical variables of Hutchinson's (1957) fundamental niche (Soberón & Peterson, 2005). This, of course, does not necessarily equate to a population's actual geographic distribution or realized niche, which is influenced by a variety of biotic interactions, such as competition and predator/prey interactions (Hutchinson, 1957; Austin, Nicholls, & Margules, 1990; Malanson, Westman, & Yan, 1992; Guisan & Zimmermann, 2000). However, considering chameleons are highly reliant on crypsis, and hence vegetation, for their survival, they are vulnerable to climatic shifts that lead to shifts in vegetation structure and composition (Tolley *et al.*, 2006; Tolley *et al.*, 2008). In fact, habitat shifts in connection with climatic fluctuations have been shown to impact the phylogenetic patterns in this genus (Tolley *et al.*, 2008). Accordingly, the results presented here are expected to correspond closely with the actual distributions of chameleons within the *B. melanocephalum-B. thamnobates* species complex through time. These analyses should, therefore, help us identify the location of past refugia and the ranges of chameleon ESUs within this species complex.

MATERIALS AND METHODS

STUDY AREA AND DATA COLLECTION

A total of 464 occurrence records for chameleons within the *B. melanocephalum-B. thamnobates* complex were obtained from southern KZN. These records are from the same individuals used in the genetic analysis, and therefore represent the 10 proposed genetic clusters (Chapter 5). All individuals were geo-referenced using GPS coordinates recorded at the precise moment and location of capture (+/- 5 m). Given that other *Bradypodion* species have small home ranges (i.e., a few hundred metres: Katz, 2012), these GPS readings are considered representative of the geographic extent of each chameleon.

NICHE MODELLING

Current climatic niche

Niche modelling was carried out in the program MAXENT version 3.3.3 (Phillips, Anderson, & Schapire, 2006; Phillips & Dudik, 2008). Input files consisted of all records available, plus 24 environmental variables from the 2009 Idrisis predictive variable modelling suite (Table 6.1: Ezemvelo KZN Wildlife, Biodiversity Conservation Planning Division, unpublished data) scaled at a resolution of approximately 200m (~6-arc seconds). This modelling suite was chosen because it represents the most accurate and current biological information for KZN and covers a finer scale and greater geographic extent of the province compared to the 19 bioclimatic (Bioclim) variables provided by WorldClim (Hijmans *et al.*, 2005), which are commonly used in niche modelling. Bioclim variables are averaged over a 50-year time period (1950-2000), at a resolution of approximately 1 km (30-arc seconds) and are limited to temperature and precipitation in defining the climatic niche of organisms.

Table 6.1 2009 Idrisis predictive modelling suite variables included in MAXENT models.

Variable
Total Annual Rain Days ≥ 2mm
Total Annual Rain Days ≥ 10mm
Aspect (˚)
Clay
Elevation (m)
Geology
Mean Annual Precipitation (mm)
Mean Evaporation: A-Pan Equivalent (mm)
Median Rainfall for February (mm)
Median Rainfall for July (mm)
Monthly Mean Daily Minimum Relative Humidity for February (%)
February Means of Daily Minimum Temperature (˚C)
Monthly Mean Daily Maximum Relative Humidity for February (%)
February Means of Maximum Daily Temperature (˚C)
Monthly Mean Daily Minimum Relative Humidity for July (%)
July Means of Minimum Daily Temperature (˚C)
Monthly Mean Daily Maximum Relative Humidity for July (%)
July Means of Maximum Daily Temperature (˚C)
Total Profile Plant Available Water (mm)
Slope (˚)
Consolidated Land Types
Soil Potential
Solar Radiation for January (mJ/m^2)
Solar Radiation for July (mJ/m^2)

Preliminary models were run to assess which variables most influenced the climatic niche of each of the 10 chameleon ESUs. Default settings in MAXENT were used except that the maximum iterations were increased to 2,000 to allow the models to converge. Ten replicates of each were run; each replicate with a random subsample of 25% of the data to

determine the model dependence on the locations used. The averaged results from the 10 runs was examined noting the area under the curve (AUC) statistic of the receiver operating characteristic (ROC) plots, the table of variable contributions, and the three jackknife tests (regularized training gain, test gain, and AUC). Variables that contributed < 1% to the model and permutation importance and/or whose presence resulted in no gain or a decline in the predictive performance of the model according to at least one of the jackknife tests were discarded. The model was then re-run on the reduced variable-set following the same procedure. This continued until all remaining variables improved the predictive power of the model according to all three jackknife tests. A final model of 10 replicates was then run for each ESU using all coordinate data (i.e., 0% test data).

Next, ArcGIS Desktop 10.1 was used to identify suitable and non-suitable habitat of each ESU. This was done by using a 10% training presence logistic threshold provided by MAXENT. This threshold considers 10% of the presence locations used to develop the model (training data) to be misclassified. Although this metric is less conservative than the minimum training presence threshold where all training locations would be correctly classified, the 10% threshold is less sensitive to outliers (i.e., over-predictions). The 2008 KZN Province land cover dataset (Ezemvelo KZN Wildlife, 2010) was then overlaid on each of the final models to get a better indication of their distributions. Although chameleons may be able to withstand some level of disturbance or transformation (e.g., along roadsides, in gardens, surrounding plantations: Tolley & Burger, 2007; Tilbury, 2010; Chapter 2), ground-truthing has revealed that they do not occupy completely transformed areas (such as sugarcane or other crops, and dense invasions by alien plants) and areas that are intensively grazed and frequently burnt (Tolley & Measey, 2007; Armstrong, 2009); therefore, these areas were excluded from the final MAXENT models.

Past climatic niche

Paleoclimatic data for the LIG and LGM were obtained from Worldclim (LIG - Otto-Bliesner *et al.*, 2006; LGM - Paleoclimate Modelling Intercomparison Project Phase II [PMIP2]: Braconnot *et al.*, 2007). Because the same climatic variables are required for each time series in order to hindcast the climatic niches of organisms, and because no paleoclimatic data is available for the Idrisis dataset, the current climatic niche of each ESU was reassessed using the 19 Bioclim variables (Table S6.1), following the same procedure as above (Hijmans *et al.*, 2005). The suitable climatic niches for each ESU during the LIG and LGM were then predicted by projecting the chosen Bioclim variables. LIG variables were available at a resolution of 30-arc seconds (~1 km) (Otto-Bliesner *et al.*, 2006), while the LGM variables were at a lower resolution of 2.5-arc minutes (~25 km) (Braconnot *et al.*, 2007). As with the present distributions, a 10% training presence logistic threshold was incorporated to identify suitable and non-suitable habitat.

NICHE OVERLAP AND CLIMATIC STABILITY

For each climatic period, the extent of climatic niche overlap between ESUs was calculated by adding all suitable and non-suitable habitat classifications together in ArcGIS. ArcGIS was also used to determine climatic stability of each ESU by comparing the persistence of a climatic niche between the three time slices (LIG, LGM, present). It is hypothesized that climate stability allows the persistence of populations (Hugall *et al.*, 2002; Carnaval *et al.*, 2009) or, in this case, ESUs. If suitable climate is 'present' in a grid cell across multiple time slices, then that grid cell has high climatic stability. Conversely, if suitable climate is not present across multiple time slices, then that grid cell has low stability.

RESULTS

The central and southern Midlands *B. melanocephalum* ESUs (see Fig. 6.1, clusters 2b and 3b) were excluded from all analyses because there was insufficient locality data (n = 4 and 1, respectively) to obtain any measure of climatic influence (all variables showed 0% contribution). MAXENT has produced good models using limited samples (for example, *n* = 5: Hernandez *et al.*, 2006; Pearson *et al.*, 2007); however, some researchers have found that models using less than 30 points are inconsistent (Wisz *et al.*, 2008) and that robust models tend to be achieved with *n* > 50 (Stockwell & Peterson, 2002). Accordingly, caution will be taken in the interpretation of the central and southern Coastal *B. melanocephalum* ESUs (Fig. 6.1, clusters 2a and 3a; n = 7 and 17, respectively).

NICHE MODELLING

Current climatic niche

Of the 24 Idrisis variables considered, 14 were found to correlate highly with the present distributions of chameleons within the *B. melanocephalum-B. thamnobates* species complex (Table 6.2). Two variables, in particular, were found to correlate with the climatic niches of four of the eight ESUs examined: February's (summer) mean daily maximum relative humidity and July's (winter) mean minimum daily temperature. Each ESU appears to have a geographically restricted climatic niche, with considerable fragmentation (Fig. 6.2), and a clear divide in suitable climatic niche is apparent between the interior (i.e., Midlands & Drakensberg) and Coastal ESUs, with no climatic niche overlap between them geographically (Fig. 6.3). Within each of the two regions, however, extensive geographic overlap is detected. Along the coast, the predicted climatic niche of the central *B. melanocephalum* ESU is completely overlapped by the northern and southern

B. melanocephalum ESUs (i.e., 84% overlapped with northern; 16% overlapped with southern); and, approximately 20% of the predicted niches the northern and southern Coastal *B. melanocephalum* ESUs are shared. In terms of area, the most extensive overlap was found within the KZN Midlands, involving *B. thamnobates*, the Karkloof ESU and the northern Midlands *B. melanocephalum* ESU. Approximately 30% and 80% of the potential ESU ranges are overlapped by the other, respectively. *Bradypodion thamnobates* and the southern Drakensberg ESU also show considerable overlap, with 24% of the climatic niche of the southern Drakensberg ESU shared with *B. thamnobates*, and 13% of *B. thamnobates* with the southern Drakensberg ESU. At present, the only ESU not showing geographic overlap of climate niches is Karkloof.

Table 6.2 Variables found to best represent each ESU's climatic niche.

ESU	Variable	% Contribution*
Northern Coastal *B. melanocephalum*	**July Means of Minimum Daily Temperature**	31.5 (43.9)
	Solar Radiation for January	11.5 (12.9)
	Elevation	57.0 (43.2) +/++
Northern Midlands *B. melanocephalum*	February's Mean Daily Minimum Relative Humidity	48.6 (46.9) ++
	July Means of Minimum Daily Temperature	35.4 (42.7)
	February's Mean Daily Maximum Relative Humidity	16.0 (10.4) +
Central Coastal *B. melanocephalum*	Total Annual Rain Days ≥ 2mm	23.9 (27.3)
	February's Mean Daily Maximum Relative Humidity	6.6 (2.2)
	February's Mean Daily Minimum Temperature	30.2 (60.3) ++
	July's Mean Daily Maximum Relative Humidity	39.3 (10.2) +
Southern Coastal *B. melanocephalum*	Total Annual Rain Days ≥ 2mm	29.2 (19.6)
	February's Mean Daily Maximum Relative Humidity	11.9 (3.7) ++
	July Means of Minimum Daily Temperature	33.3 (76.0) +
	Solar Radiation for July	25.6 (0.6)

Table 6.2 *continued*.

ESU	Variable	% Contribution*
Karkloof	Median Rainfall for February	50.4 (3.3) +
	Median Rainfall for July	14.8 (10.0) ++
	February's Mean Daily Maximum Relative Humidity	12.3 (71.7)
	Consolidated Land Types	22.4 (15.0)
B. thamnobates (Boston)	Geology	65.9 (80.1) +/++
	February's Mean Daily Minimum Relative Humidity	18.9 (16.7)
	Solar Radiation for July	15.2 (0.1)
B. thamnobates	February's Mean Daily Minimum Relative Humidity	54.1 (46.5) +/++
	July Means of Minimum Daily Temperature	45.9 (53.5)
Southern Drakensberg	Geology	28.9 (53.8) ++
	February Means of Maximum Daily Temperature	14.8 (37.4)
	July's Mean Daily Maximum Relative Humidity	56.2 (8.8) +

* Percent contribution of each variable depends on the path used to obtain the optimal solution. Numbers in parentheses indicate the variable's permutation importance to the final model, not the path used to obtain it. + Highest gain in model's predictive power when used in isolation. ++ Greatest decline when omitted from model. Bold highlights most common variable.

Figure 6.2 Current climatic niches predicted for eight ESUs within the *B. melanocephalum-B. thamnobates* species complex according to the Idrisis dataset.

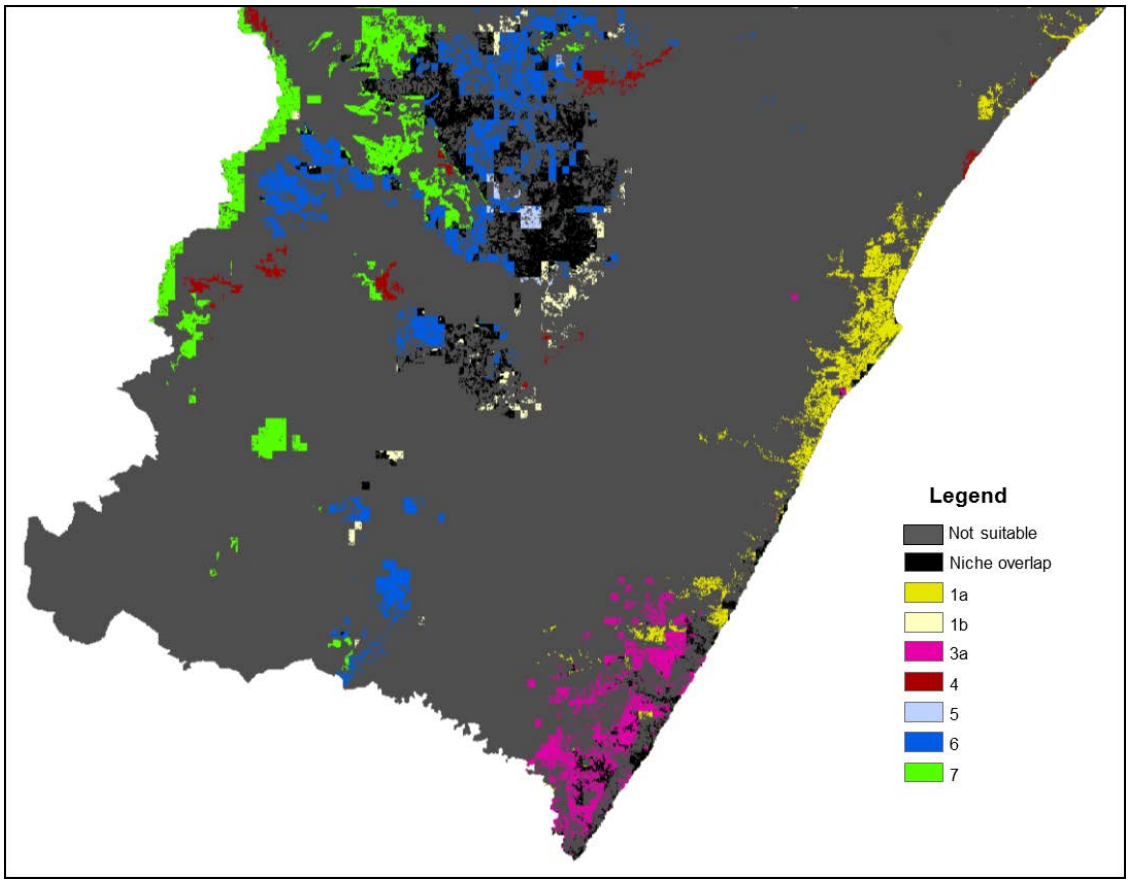

Figure 6.3 Map depicting the current climatic niches of eight ESUs within the *B. melanocephalum-B. thamnobates* species complex from southern KZN Province, South Africa, as well as their predicted overlap. Legend number codes correspond to ESUs as depicted in Fig. 1: Cluster 1b = northern Midlands *B. melanocephalum* ESU; Cluster 2a = central Coastal *B. melanocephalum* ESU; Cluster 3a = southern Coastal *B. melanocephalum* ESU; Cluster 4 = Karkloof ESU; Cluster 6 = *B. thamnobates* ESU; Cluster 7 = southern Drakensberg ESU. Area of niche overlap (black) predominantly involves Clusters 1b and 6.

Past climatic niche and climatic stability

Eleven of the 19 Bioclim variables were found to correlate to the present distributions of chameleons within the *B. melanocephalum-B. thamnobates* species complex (Table S6.2). Compared to the Idrisis models, these models were found to over-predict the present climatic niches of five ESUs by approximately 77% (ranging from 55-92% per ESU); while, the northern and southern Coastal *B. melanocephalum* ESUs were under-predicted by approximately 11% and 52%, respectively (Fig. S6.1). Consequently,

the extent of suitable paleoclimatic niches may also be over- and under-predicted, respectively. Inconclusive results were obtained for the present Bioclim distribution of the Boston ESU (Cluster 5: Fig. 6.1); hence, it was omitted from these analyses.

The paleoclimatic models of each ESU revealed considerable differences in their potential distributions (Figs 6.4 & 6.5) compared to present (Fig. 6.2). The projected climate during the LIG suggests that suitable niches were only available for *B. thamnobates*, the southern Drakensberg ESU, and the central and southern Coastal *B. melanocephalum* ESUs, with no overlap between them (Fig. 6.4a). During the LGM, *B. thamnobates* and the southern Drakensberg ESU persist, while the climatic niches of the Coastal *B. melanocephalum* ESUs are not predicted by the model (Figs 6.4b & 6.5). Moreover, the climatic niches of the northern Midlands *B. melanocephalum* and Karkloof ESUs emerge, overlapping extensively with each other and that of *B. thamnobates*, resulting in approximately 50% of the projected niches of the former two clusters being shared, and 36% of *B. thamnobates* shared (Fig. 6.4b). The absence of suitable climatic niches for the northern Coastal *B. melanocephalum* ESU from the LIG and LGM (Fig. 6.5) suggests its climatic niche has only recently become available (within the past 20,000 years). Since the LGM, the climatic niches of the other Coastal ESUs have also re-emerged, while that of the northern Midlands *B. melanocephalum* ESU has experienced a considerable decline, and that of *B. thamnobates*, a westward shift. The niches of the Karkloof and southern Drakensberg ESUs appear to have remained stable within the past 20,000 years (Fig. 6.5b).

Overall, the climate stability across southern KZN since the LIG has been low. However, some areas of high stability have been detected, predominantly in the southern Drakensberg and, to a lesser extent, within the climatic niche of *B. thamnobates* (Fig. 6.5c).

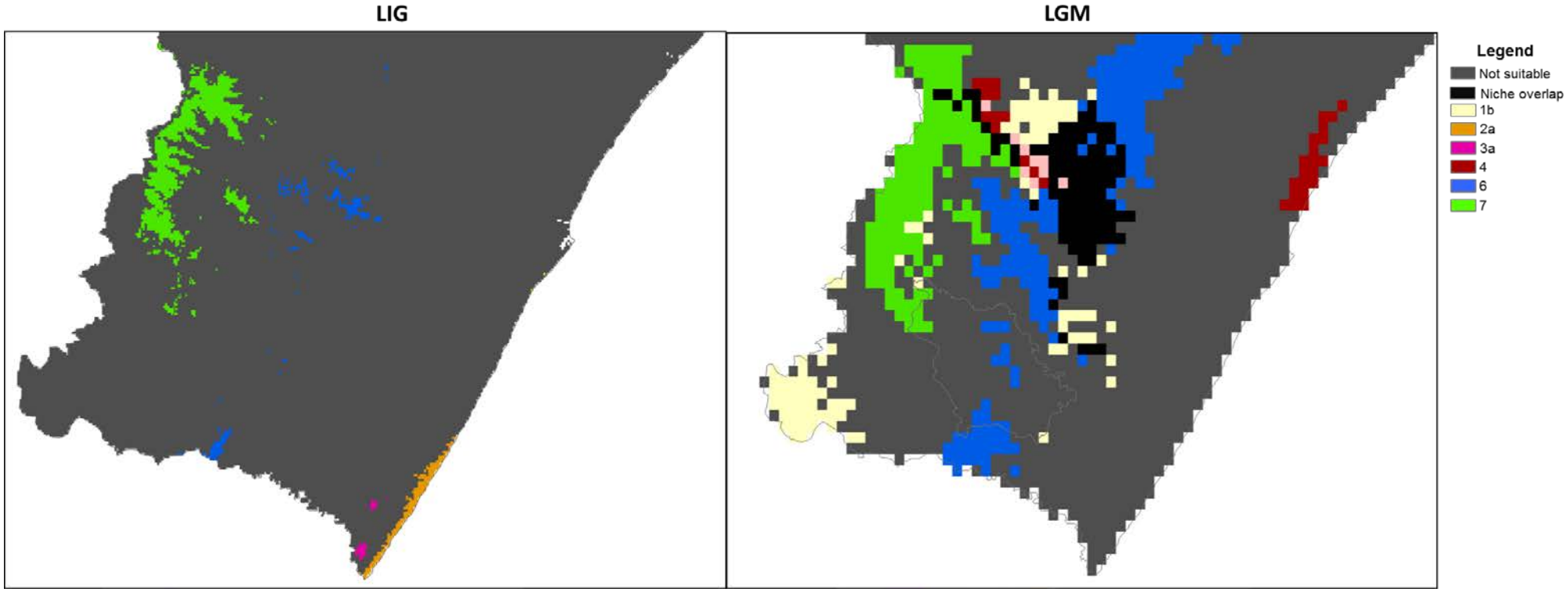

Figure 6.4 Projected paleoclimatic niches and niche overlap for ESUs within the *B. melanocephalum-B. thamnobates* species complex dating back to the LGM and LIG. Resolution: LGM = 2.5-arc minutes; LIG = 30-arc seconds. Legend number codes correspond to ESUs as depicted in Fig. 1: Cluster 1b = northern Midlands *B. melanocephalum* ESU; Cluster 2a = central Coastal *B. melanocephalum* ESU; Cluster 3a = southern Coastal *B. melanocephalum* ESU; Cluster 4 = Karkloof ESU; Cluster 6 = *B. thamnobates* ESU; Cluster 7 = southern Drakensberg ESU. Area of niche overlap (black) predominantly involves Clusters 1b and 6.

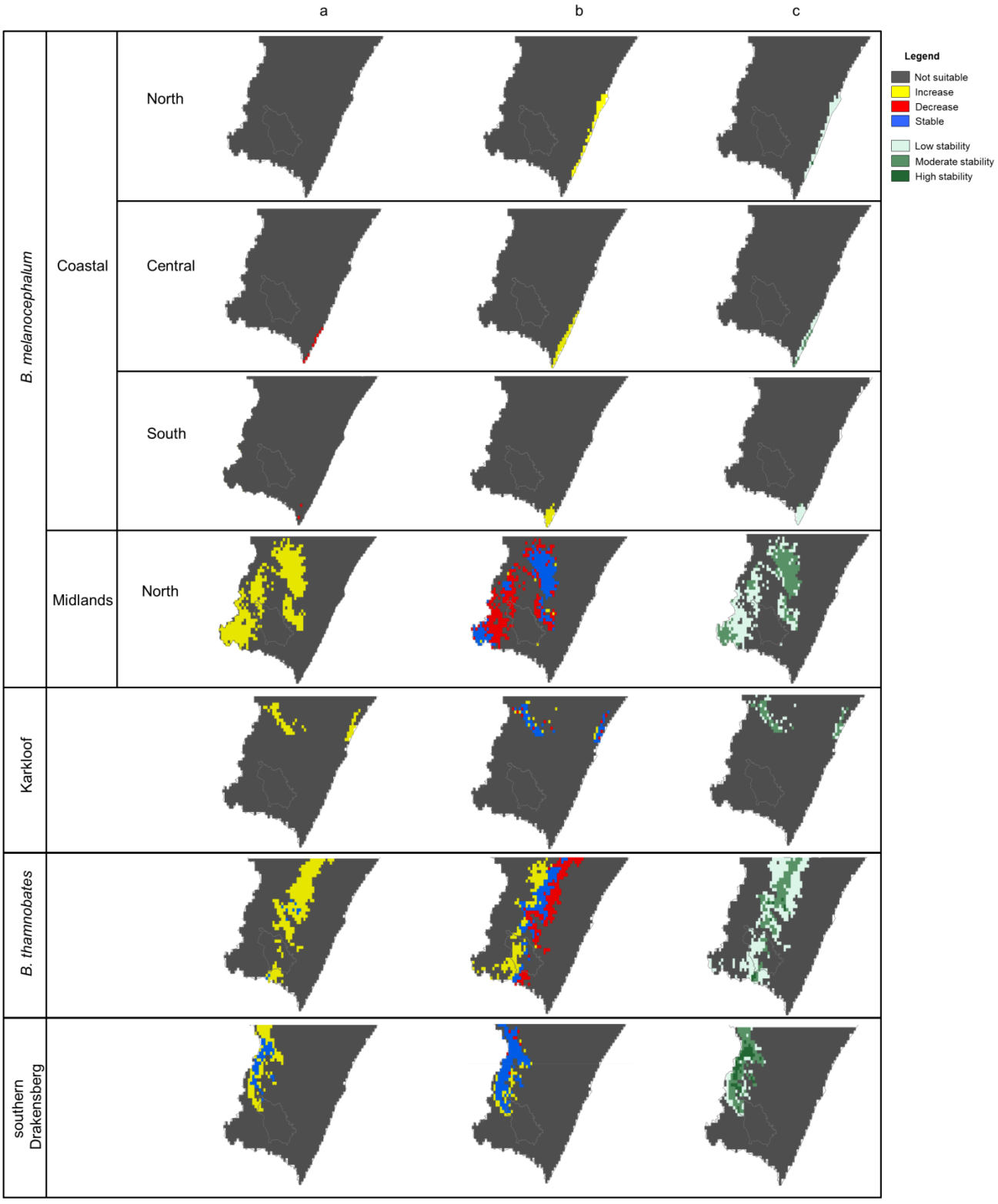

Figure 6.5 Change in climatic niche from (a) LIG to LGM and (b) LGM to present and (c) the degree of climatic stability through time for seven ESUs within the *B. melanocephalum*-*B. thamnobates* species complex. High stability denotes climatic niches that have remained stable across the three time periods; moderate stability are areas that have remained stable for two periods; and low stability areas showed suitable climate niches in only one time period.

DISCUSSION

Chameleons within the *B. melanocephalum-B. thamnobates* species complex are estimated to have diverged during the Early and Middle Pleistocene in response to the contraction and subsequent re-expansion of forests during glacial and interglacial cycles (Chapter 5) – much earlier than the Last Interglacial (LIG) and Last Glacial Maximum (LGM) periods modelled in this study. However, given that these periods represent examples of the most extreme climatic conditions during the Pleistocene (Deacon, 1983; Tyson, 1986), we consider the paleo-projections presented here to be conservative estimates of the cycling of climatic niches for these chameleons throughout the Pleistocene. Accordingly, the data provided here offer valuable insights into the potential distributions of these chameleons in response to changing climate, which may have affected their evolutionary history.

The climatic niches of the seven ESUs examined were found to correlate to different climatic variables, which is not surprising considering species will likely manifest distinct, individualistic responses to climate change (Ackerly *et al.*, 2010; Serra-Diaz *et al.*, 2013). A climatic niche stability gradient was uncovered across southern KZN, with the highest climatic stability associated with the southern Drakensberg mountain range in the west and the lowest stability along the coast to the east (Fig. 6.5c). These results indicate that this species complex is affected by both climatic niche conservatism (the tendency of an organism to retain their ancestral ecological traits and environmental distributions: Wiens & Graham, 2005) and lability (the tendency of a niche to change over time: Losos *et al.*, 2003), which could explain the observed patterns of morphological and genetic diversity between ESUs (refer to Chapter 2-5).

Since the LIG, the geographic extent of the climatic niche of the southern Drakensberg ESU has changed very little, indicating that it likely acted as a refugium for

these chameleons. These results are in accordance with our hypothesis that the southern Drakensberg chameleons were highly resilient through the glacial-interglacial cycles of the Pleistocene, and that they reflect the evolutionary history of Afrotemperate forests in KZN (Eeley *et al.*, 1999; Lawes *et al.*, 2007). These high levels of climatic stability are reflected in the strong accordance observed between morphological and genetic lines of evidence, with the southern Drakensberg ESU having a very distinctive morphology compared to the other ESUs, both in overall size, colouration and in proportional anatomy (Chapters 2-4), and by being genetically well differentiated from them (Chapter 5).

The Midlands' Mistbelt ESUs, however, were also expected to be resilient and show high stability, yet only a few small fragments show stability since the LIG. These fragments are associated with the climatic niche of *B. thamnobates* (refer to Fig. 6.5c). For the most part, the KZN Midlands show moderate stability, with the major extent of the climatic niches of *B. thamnobates*, the Karkloof and northern Midlands *B. melanocephalum* ESUs arising during the LGM, and remaining fairly consistent through to present. The climatic niches of these Midlands chameleons are predicted to have overlapped extensively during the LGM (Fig. 6.4) and possibly during other glacial periods throughout the Pleistocene. However, the genetic data indicate that only the Karkloof and northern Midlands *B. melanocephalum* ESUs exchanged genes prior to the Mid-Pleistocene (Chapter 5: fig. 5.6, table 5.10). The genetic and morphological distinctiveness of *B. thamnobates* in the presence of climatic niche overlap with the two other Midlands ESUs suggests that resource partitioning may have isolated *B. thamnobates* from the others as far back as the Plio-Pleistocene transition, driving divergence in phenotypic traits and making them incompatible, thereby facilitating ecological speciation (Schluter, 2000, 2001; Rundle & Nosil, 2005). More recently, the Karkloof and northern Midlands *B. melanocephalum* ESUs show both genetic and morphological differentiation (Chapters

2, 3 & 5), which might have been facilitated by the potential climatic niche lability of the Midlands *B. melanocephalum* ESUs and the present separation of their climatic niches (Fig. 6.3).

Regarding the *B. melanocephalum* chameleons, climatic niche lability could explain why no climatic niche overlap is predicted to have existed between the Coastal and Midlands *B. melanocephalum* ESUs, while the genetic data indicate the presence of gene flow between them (Chapter 5). The Coastal ESUs may be more recent in origin, dating to the emergence of Scarp and Coastal forests approximately 8 ka (Eeley *et al.*, 1999; Lawes *et al.*, 2000; Mucina & Geldenhuys, 2006; Lawes *et al.*, 2007), and resulting from one or more founder events from the Midlands *B. melanocephalum* ESUs. The Midlands chameleons may have gradually moved eastward, utilising the more widespread grasslands during the glacials and forests during the interglacials. This could explain their intermediately sized head and feet (Chapter 2) – features found to correlate closely with habitat (Chapters 3 & 4). Chameleons occupying closed-canopy habitats typically possess larger heads and feet; whereas, in open-canopy habitats, chameleons are proportionally smaller (Chapters 2-4). These morphologies allow them to better utilize their respective habitats (Chapters 3 & 4). The intermediate features of the Midlands *B. melanocephalum* ESUs may be a consequence of their cyclical adaptation to open- and closed-canopy vegetation. Subsequently, within the past 8,000 years, the emergence of Scarp and Indian Ocean Coastal Belt forests created new habitats (Coastal forests and grasslands), as well as barriers (Scarp forests). The Midlands ESUs could have expanded their climatic and vegetative niches to utilise the Coastal vegetation and overall climatic conditions and, with time, directional selection may have acted to favour individuals able to tolerate these new conditions (Pearman *et al.*, 2008). The Scarp forests eventually became a barrier, further isolating the Coastal chameleons from the founding Midlands populations. This would

explain the genetic similarity, as well as the morphological distinctiveness between the Coastal and Midlands ESUs (Chapters 2 &5), as well as the low levels of climatic niche stability predicted for the Coastal ESUs. Such instances of niche lability are often detected in adaptive radiations (Schluter, 2000), such as *Anolis* lizards (Losos *et al.*, 2003; Knouft *et al.*, 2006) and in species with limited dispersal ability (Ackerly, 2003; Franklin, 2009), which is believed to be an attribute of *Bradypodion* species.

CONCLUSION

Utilizing predictions from three bioclimatic time slices, we have made inferences about the potential geographic distributions and movements of chameleons within the *B. melanocephalum-B. thamnobates* species complex throughout the Pleistocene that could have produced the observed patterns of morphological and genetic variation we see today. Overall, chameleons in areas of high climatic niche stability (or climatic niche conservatism) tend to show accordance between their genetics and morphology, while chameleons in areas that are climatically labile tend to show discordance between these two lines of evidence. Although we consider the predictions made here to be valid, we recognize that more accurate assessments of the climatic stability within southern KZN would greatly improve our understanding of the evolution of these chameleons. Higher resolution (30-arc seconds for all time periods) datasets that incorporate additional biologically-relevant paleo-environmental layers, such as land cover (including vegetation type), solar radiation and humidity (variables found to correlate highly with chameleon distributions: Table 6.2) should be included in the paleoclimatic models to increase the quality of the predictions. Moreover, these datasets should extend beyond the LIG and LGM, in order to span the history of these chameleons. Accordingly, time slices covering the numerous Pleistocene glaciation events (both glacial and interglacial phases), and

going back into the Pliocene, as well as into the Holocene should be included, if possible. This would allow for a detailed examination of the changes in the paleo-distributions of these ESUs, and thus be greatly useful in understanding their historical biogeography.

Supporting Information

Table S1 Bioclim variables included in the final MAXENT models.

Variable Code	Description
BIO2	Mean Diurnal Range [Mean of monthly (max temp - min temp)]
BIO3	Isothermality (BIO2/BIO7) (* 100)
BIO4	Temperature Seasonality (standard deviation *100)
BIO5	Max Temperature of Warmest Month
BIO6	Min Temperature of Coldest Month
BIO8	Mean Temperature of Wettest Quarter
BIO12	Annual Precipitation
BIO13	Precipitation of Wettest Month
BIO14	Precipitation of Driest Month
BIO15	Precipitation Seasonality (Coefficient of Variation)
BIO16	Precipitation of Wettest Quarter
BIO17	Precipitation of Driest Quarter
BIO18	Precipitation of Warmest Quarter
BIO19	Precipitation of Coldest Quarter

Table S2 Bioclim variables found to best represent each ESU's current climatic niche.

ESU	Variable Code	% Contribution*
Northern Coastal *B. melanocephalum*	BIO2	73.5 (78.8) +/++
	BIO8	14.3 (18.1)
	BIO14	12.2 (3.1)
Northern Midlands *B. melanocephalum*	BIO13	13.2 (9.6)
	BIO14	57.1 (47.3) +/++
	BIO15	29.7 (43.0)
Central Coastal *B. melanocephalum*	BIO2	100 (100) +/++
Southern Coastal *B. melanocephalum*	BIO4	51.4 (26.4) +/++
	BIO12	48.6 (73.6)
Karkloof	BIO13	59.6 (34.0)
	BIO18	40.4 (66.0) +/++
B. thamnobates (Boston)	----------	
B. thamnobates	BIO6	62.2 (77.7) +/++
	BIO12	21.4 (11.2)
	BIO15	16.4 (11.2)
Southern Drakensberg	BIO5	32.7 (0.3) +
	BIO12	37.3 (35.7) ++
	BIO19	30.0 (64.0)

* Percent contribution of each variable depends on the path used to obtain the optimal solution. Numbers in parentheses indicate the variable's permutation importance to the final model, not the path used to obtain it. + Highest gain in model's predictive power when used in isolation. ++ Greatest decline when omitted from model.

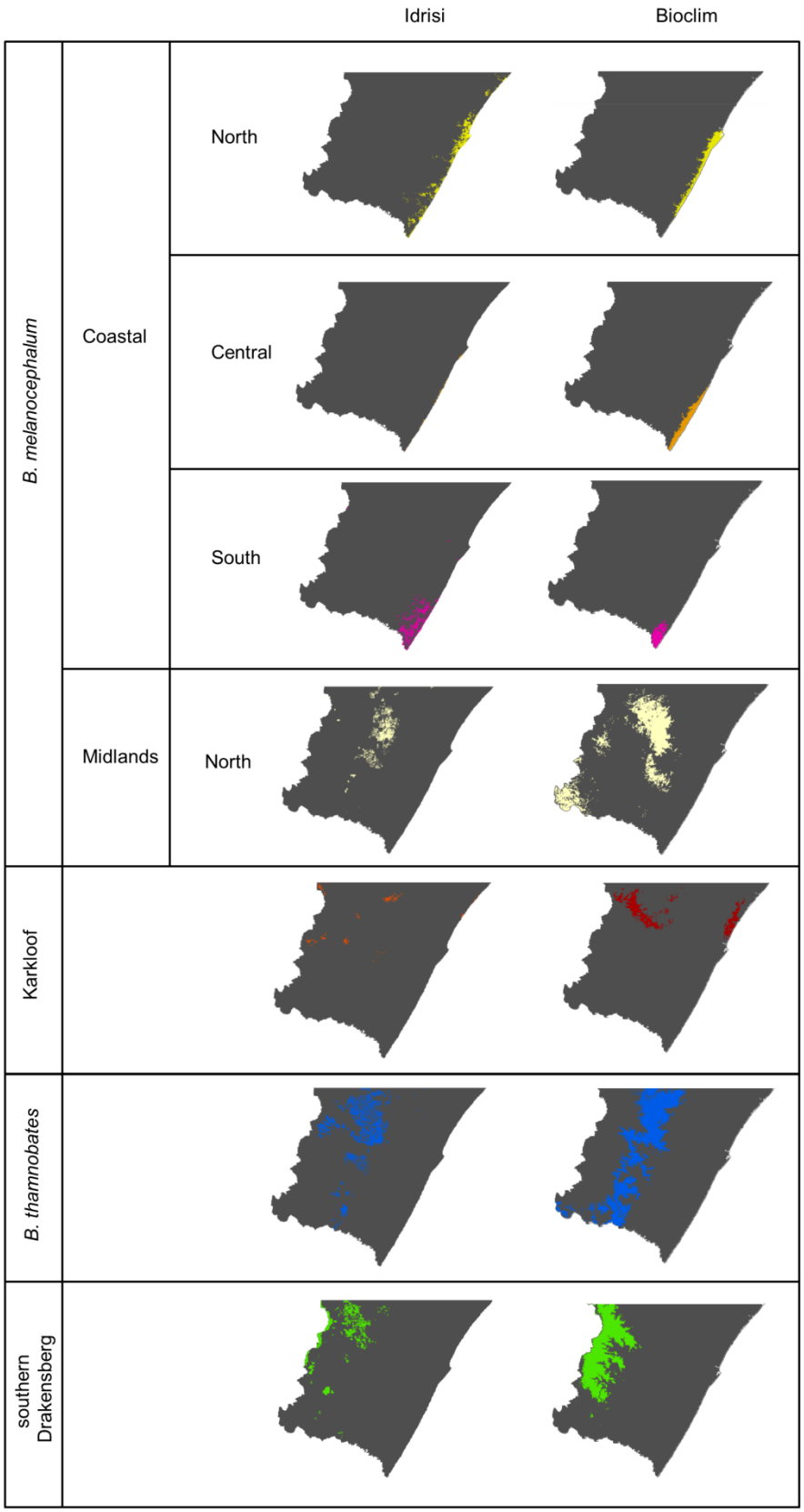

Figure S6.1 Comparing the current climatic niches predicted for each chameleon ESU using the Idrisis (left) and Bioclim (right) datasets.

Chapter 7

Conclusion

My primary aim in this dissertation was to investigate the underlying processes of speciation and morphological variation that have led to the observed discordance between morphology and genetics within the *Bradypodion melanocephalum-Bradypodion thamnobates* species complex – a recent radiation of dwarf chameleons from southern KwaZulu-Natal (KZN) Province, South Africa. Early studies recognised at least five phenotypic forms within this complex that differed in overall size, colouration and distribution (Raw, 2001; Tolley & Burger, 2007), yet showed little to no genetic differentiation (Tolley *et al.*, 2004; Tolley *et al.*, 2008). This could suggest that the different phenotypic forms are polymorphisms of a single species or that this species complex represents an adaptive radiation, with the different forms showing adaptations to their specific habitats and the lack of genetic divergence indicating that these chameleons are in the early stages of ecological speciation. However, because the adaptive significance of the different phenotypic forms had not been tested and because very few samples were included in both the morphological survey and genetic studies (i.e., 1-2 individuals per form), it was impossible to determine the actual explanation. Accordingly, my aim was to determine whether the previously recognised discordance was genuine or an artefact of inadequate sampling.

I achieved this by first extensively sampling dwarf chameleons throughout southern KZN and analysing morphometric and habitat data for every individual (Chapter 2). Clear distinctions in head, foot, limb and tail morphology were found between the five phenotypic forms, which appeared to correlate to their differential habitats, which can be broadly classified as either open or closed-canopy vegetation. Specifically, chameleons in open-canopy habitats have proportionally smaller features than their closed-canopy counterparts. Moreover, varying degrees of sexual dimorphism associated with these habitats were detected, with the closed-canopy forms being more sexually dimorphic than

the open-canopy forms. This indicated that sexual selection is likely to be a more predominant force within the closed-canopy habitats, which are more protected from aerial predators, thereby enabling them to invest more in communication; while, in open-canopy habitats, natural selection is likely to be the more predominant force, ultimately enforcing their overall diminutive body size.

To determine whether the morphological differences identified were adaptive, and hence whether chameleons in this species complex constitute an adaptive radiation, I tested several locomotor performance traits (running speed, foot and tail grip strength: Chapter 3) and bite force (Chapter 4) – traits thought to be most relevant for their survival. The phenotypic forms were found to differ in both absolute and relative forefoot grip strength and absolute bite force, with the proportionally smaller open-canopy forms possessing correspondingly weaker forefoot strengths and bite forces than the closed-canopy forms. Furthermore, sexual differences in both absolute and relative bite forces were also detected within the closed-canopy forms, with males generating a greater force than females. The lack of differentiation between forms for the other performance traits either indicates that these chameleons are equally well suited for grasping perches with their tails and running or that the tests were not representative of how they would perform in their environments. To clarify this, additional performance tests should be conducted that incorporate multiple perch sizes and orientations (horizontal and vertical) that are more representative of the actual perches used by chameleons. Irrespective of the outcomes, the performance results presented in this dissertation indicate that both natural and sexual selection are acting within both habitat types, but to varying degrees, as suggested in Chapter 2.

In closed-canopy habitats, larger body sizes are advantageous because they provide an honest signal of performance, which is especially useful during intraspecific encounters, which can result in intense fighting. The proportionally larger heads of closed-canopy

chameleons enable them to display their potential threat from farther distances through the use of their ornamentation (prominent casques) and, if necessary, engage in combat, which generally involves chasing and biting (Burrage, 1973; Stuart-Fox *et al.*, 2006a; Tolley & Burger, 2007). In such instances, their greater forefoot grip strength provides them increased stability as they grasp hold of branches to maintain balance and support. In open-canopy habitats, where the average plant and perch height is far lower and the perches are more densely clustered than in closed-canopy habitats (Chapter 2), the risk of injury from displacement is less, potentially explaining their proportionally weaker grip strength.

The observed patterns of forefoot grip strength between open and closed-canopy dwarf chameleons are shared with another *Bradypodion* species, *Bradypodion pumilum* (Herrel *et al.*, 2011). Accordingly, this dissertation provides initial evidence for the parallel evolution of this performance trait among dwarf chameleons in response to microhabitat structure, lending support for the existence of open and closed-canopy ecomorphs within the genus.

To determine whether the ecomorphological patterns within this species complex were reflected in their genetics or whether this complex truly lacked genetic differentiation, I conducted a comprehensive population genetic study using a combination of mitochondrial and nuclear microsatellite DNA markers and incorporated detailed spatial information into the analyses to quantify the genetic effects of landscape and geographic barriers (Chapter 5). Compared to typical levels of genetic divergence used to discriminate between chameleon species, very little genetic differentiation was detected within this species complex, confirming the findings of previous phylogenetic studies (Tolley *et al.*, 2004; Tolley *et al.*, 2008). However, there was evidence to suggest that the southern KZN dwarf chameleons are in in the early stages of divergence, and on different evolutionary trajectories despite the low levels of genetic differentiation.

Both mitochondrial and microsatellite markers were able to detect geographic structure, with three mitochondrial clusters and seven microsatellite clusters identified (Fig. 7.1: Maps A & B). The differing genetic patterns between the two markers reflect their different mutation rates and modes of inheritance, with the microsatellite data depicting the more recent structuring and the mitochondrial structure showing shared ancestral polymorphisms. Taken together, these molecular data inform the timing and mode of evolution within this species complex. The mitochondrial data estimate these chameleons to have first diverged between 1.4 and 4.4 million years ago during the Plio-Pleistocene transition, which was characterized by the emergence and expansion of C_4 grasslands and the corresponding regression of the remaining forests patches in the region (Vogel *et al.*, 1978; Mucina & Geldenhuys, 2006; Hopley *et al.*, 2007). The molecular data also suggest repeated periods of fragmentation and gene flow between clusters during the Middle and Late Pleistocene, which helped shape the microsatellite structure. This cycling of genetic connectivity is also likely to have occurred in response to forest extent, which changed considerably throughout the glacial-interglacial cycles during this time (Eeley *et al.*, 1999; Dupont *et al.*, 2001; Dupont *et al.*, 2011).

The intimate link proposed between the genetics of this species complex and their habitat is supported by the ecomorphological evidence presented in this dissertation (Chapters 2-4), which closely resembles the structure defined by the microsatellite data. This is most evident for the three closed-canopy forms, which show strong accordance between their morphologies and genetics (Fig. 7.1: Maps B & C). Thus, under the morphological, ecological, and genotypic cluster species concepts (Van Valen, 1976; Mayr, 1982; Mallet, 1995), each of these closed-canopy forms should be recognized as separate species (i.e., *B. thamnobates*, *Bradypodion* 'southern Drakensberg' *sp.*, and *Bradypodion* 'Karkloof' *sp.*: Chapter 5). The open-canopy forms, however, continue to

show discordance between their morphologies and genetics. Where the ecomorphological evidence identified two phenotypic forms along a north-south axis, the microsatellite data grouped these same chameleons into three clusters along an east-west axis (Fig. 7.1: Maps B & C). This suggests that similar ecological factors affect each of these microsatellite clusters. The failure of this to be recognized in their genetics likely reflects the recentness of these morphological distinctions. In fact, the two ecomorphological forms within each cluster correspond to two forest types within the Midlands and Coastal regions of southern KZN, specifically the Mistbelt and Indian Ocean Coastal Belt forests, respectively (refer to Mucina & Geldenhuys, 2006); the latter of which emerged only 8,000 years ago (Eeley *et al.*, 1999; Lawes *et al.*, 2000; Mucina & Geldenhuys, 2006; Lawes *et al.*, 2007). Given the low dispersal ability of these chameleons and the present divide between Mistbelt and Coastal forests (Mucina & Geldenhuys, 2006), migration within any of the open-canopy microsatellite clusters is unlikely. Accordingly, these chameleons are likely in the early stages of ecological and/or allopatric speciation, and there may actually be six open-canopy clusters (Fig. 7.1: Map D). However, ample data was only obtained for the northern open-canopy clusters (1a & 1b). For the central and southern open-canopy clusters, only a few individuals from limited localities were sampled for morphometric and genetic data, and no performance testing was conducted on these chameleons. As such, these clusters have simply been inferred. To validate their distinctiveness, increased sampling for morphometric and performance data should be conducted at multiple field sites, if possible. Until such time, these chameleons should be grouped according to their microsatellite clusters and managed as genetically distinct conservation units, while the two northern open-canopy clusters (1a & 1b) should, at the very least, be recognized as ecologically distinct populations or “adaptive evolutionary” conservation units (*sensu* Fraser & Bernatchez, 2001) of *B. melanocephalum* (Chapter 5).

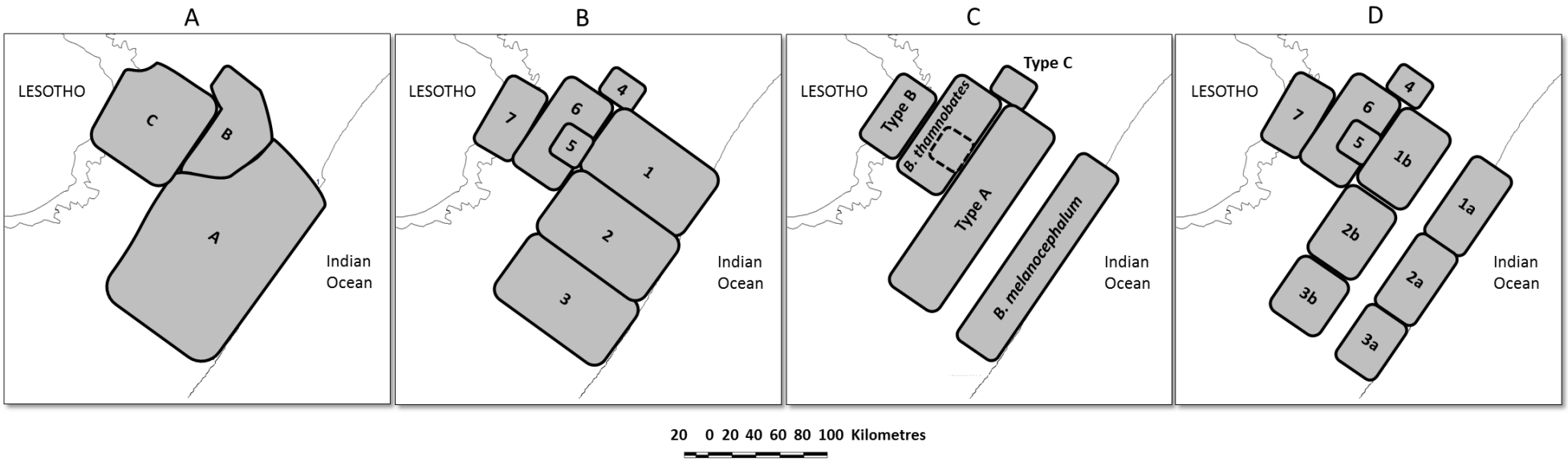

Figure 7.1 Maps depicting the approximate geographical distributions of chameleons within the *Bradypodion melanocephalum-Bradypodion thamnobates* species complex through time. Map A illustrates the ancestral structure recognised by mitochondrial DNA sequences dated approximately 1.4-4.4 million years ago (Chapter 5). Map B portrays the microsatellite structure estimated at approximately 160,000-310,000 years ago (Chapter 5). Map C depicts the structure based on morphological data (Chapter 2) and Map D represents the likely distribution of distinct chameleon clusters at present. Closed-canopy habitat phenotypic forms (*B. thamnobates*, Type B [southern Drakensberg] and Type C [Karkloof]) are represented by mtDNA clusters B and C, and microsatellite clusters 4-7. Open-canopy habitat forms (*B. melanocephalum* and Type A) are represented as mtDNA clusters A and B, and microsatellite clusters 1-3. In Map D, 'a' denotes coastal populations of open-canopy forms, while 'b' denotes the Midlands populations.

The data discussed thus far support a close association between the evolution of chameleons within this species complex and their habitat. To further explore this relationship, I used ecological niche modelling to identify the abiotic variables shaping the present distributions of chameleons and projected these variables backward in time to the Last Interglacial (LIG) and Last Glacial Maximum (LGM) (Chapter 6). These periods were chosen as they represent the most extreme climatic conditions during the Pleistocene (Deacon, 1983; Tyson, 1986); thereby, enabling me to gain insight into the potential climatic niches of these chameleon clusters throughout this epoch. If the distributions of chameleons within this species complex are truly linked with that of their habitats, I expected corresponding patterns of paleoclimatic change. The climatic niches of the three proposed closed-canopy species showed moderate to high levels of stability since the LIG, mimicking that of their closed-canopy forested habitats (Eeley *et al.*, 1999). The Afrotemperate and Mistbelt forests that these chameleons inhabit are more ancient and, consequently, have experience with paleoclimatic extinction filters (Eeley *et al.*, 1999; Lawes *et al.*, 2000; Mucina & Geldenhuys, 2006; Lawes *et al.*, 2007), which has enabled them to act as refugia for these chameleons throughout the numerous glacial-interglacial cycles throughout the Pleistocene. These results suggest that these chameleons have very conservative climatic niches.

The climatic niches of the open-canopy chameleons, however, were predicted to be far less stable and not fully reflective of the distributions of forests during the Pleistocene. In fact, the apparent gene flow between the open-canopy chameleons in the Midlands and the Coast as revealed by the microsatellite data (Fig. 7.1: Map B) suggest that these chameleons experienced climatic niche lability. The Midlands chameleons may have gradually moved eastward, utilising the more widespread grasslands during the glacials and forests during the interglacials, which could explain their proportionally smaller, open-

canopy habitat features. With the emergence of Scarp and Indian Ocean coastal belt forests approximately 8,000 years ago (Eeley *et al.*, 1999; Lawes *et al.*, 2000; Mucina & Geldenhuys, 2006; Lawes *et al.*, 2007), the Midlands chameleons could have expanded their climatic and vegetative niches to utilise the coastal climate and vegetation. Directional selection may have then acted to favour individuals able to tolerate these new conditions (Pearman *et al.*, 2008). The Scarp forests eventually became a barrier between them, further isolating the Coastal chameleons from the founding Midlands chameleons. This would explain the apparent gene flow, yet morphological distinctiveness between the Coastal and Midlands chameleons (Chapters 2 &5), as well as the low levels of climatic niche stability predicted for these chameleons.

Overall, this dissertation has shown that a combination of biotic and abiotic factors have influenced the evolution of dwarf chameleons within this adaptive radiation. The variable climate throughout the Pleistocene created unique, often isolated habitats in which these chameleons could persist and, through intraspecific competition, resource partitioning and predation, these chameleons became locally adapted to these habitats. These processes allowed for both ecological and allopatric speciation to occur, creating the observed patterns of morphological and genetic diversity. The different lines of evidence used in this thesis provide a timeline of this evolution, with the most recent structure believed to be represented by Map D of Figure 7.1. Even though particular clusters require further research, this thesis will contribute significantly to refining the taxonomic status of this species complex.

The evolutionary and ecological knowledge gained in this dissertation will also prove valuable in informing conservation management decisions. The forest and grassland habitats in which these chameleons inhabit are amongst the most threatened ecosystems in South Africa (Driver *et al.*, 2005; Driver *et al.*, 2012) and, as such, there is a high

likelihood these chameleons are experiencing and will continue to experience significant threats to their survival and persistence. Thus, management efforts should be aimed at preserving the adaptive diversity and evolutionary processes across the geographic range of this species complex.

Moreover, while this dissertation has focused specifically on the *B. melanocephalum-B. thamnobates* species complex, it has advanced our understanding of the evolutionary processes shaping the *Bradypodion* genus as a whole. It might also benefit similar studies looking at problematic taxa, especially those involving low mobility, specialty groups.

Reference List†

†This list comprises (as per SUN regulations) a complete list of references from all the papers comprising this thesis, including the Introduction, Papers I to VII which comprise the body of the thesis, and the Conclusion.

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Appendix

Published Chapters
(First page only)

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Biological Journal of the Linnean Society, 2013, **109**, 113–130. With 5 figures

Ecomorphological variation and sexual dimorphism in a recent radiation of dwarf chameleons (*Bradypodion*)

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Natural selection tends to favour optimal phenotypes either through directional or stabilizing selection; however, phenotypic variation in natural populations is common and arises from a combination of biotic and abiotic interactions. In these instances, rare phenotypes may possess a fitness advantage over the more common phenotypes in particular environments, which can lead to adaptation and ecological speciation. A recently radiated clade of dwarf chameleons (*Bradypodion*) restricted to southern KwaZulu-Natal Province, South Africa, is currently comprised of two species (*Bradypodion melanocephalum* and *Bradypodion thamnobates*), yet three other phenotypic forms exist, possibly indicating the clade is far more speciose. Very little genetic differentiation exists between these five phenotypic forms; however, all are allopatric in distribution, occupy different habitats and vary in overall size and coloration, which may indicate that these forms are adapting to their local environments and possibly undergoing ecological speciation. To test this, we collected morphometric and habitat data from each form and examined whether ecological relevant morphological differences exist between them that reflect their differential habitat use. Sexual dimorphism was detected in four of the five forms. Yet, the degree and number of dimorphic characters was different between them, with size-adjusted male-biased dimorphism being much more pronounced in *B. thamnobates*. Habitat differences also existed between sexes, with males occupying higher perches in more closed canopy (forested) habitats than females. Clear morphological distinctions were detected between four of the five forms, with the head explaining the vast majority of the variation. Chameleons occupying forested habitats tended to possess proportionally larger heads and feet but shorter limbs than those in open canopy habitats (i.e. grassland). These results show that this species complex of *Bradypodion* is morphologically variable for traits that are ecologically relevant for chameleons, and that the variation among the five phenotypic forms is associated with habitat type, suggesting that this species complex is in the early stages of ecological speciation. *Biological Journal of the Linnean Society*, 2013, **109**, 113–130.

ADDITIONAL KEYWORDS: adaptation – habitat – lizards – morphology – South Africa – Squamata.

INTRODUCTION

Phenotypic variation in natural populations is intriguing from an evolutionary perspective because natural selection is assumed to favour one optimal phenotype either through directional or stabilizing selection. Consequently, a major goal of evolutionary biology is to identify processes that create and maintain phenotypic variation in natural populations. One possibility is that diversity is maintained by disruptive selection, which is driven by negative frequency-dependent selection (Mather, 1955; Rueffler *et al*., 2006) arising from biotic (e.g. competition for resources: Benkman, 1996; Swanson *et al*., 2003) and/or abiotic interactions (e.g. temperature and climate: Davis & Shaw, 2001; Norberg *et al*., 2001). In such instances, rare phenotypes possess a fitness

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Linking microhabitat structure, morphology and locomotor performance traits in a recent radiation of dwarf chameleons

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Summary

1. Evidence that morphological traits associated with particular environments are functionally adapted to those environments is a key component to determining the adaptive nature of radiations. Adaptation is often measured by testing how organisms perform in diverse habitats, with performance traits associated with locomotion thought to be among the most ecologically relevant.

2. We therefore explored whether there are relationships between morphology, locomotor performance traits (sprint speed, forefoot and tail grip strength on broad and narrow dowels) and microhabitat use in five phenotypic forms of a recent radiation of dwarf chameleon – the *Bradypodion melanocephalum–Bradypodion thamnobates* species complex – to determine whether morphological differences previously identified between the forms are associated with functional adaptations to their respective habitats, which can be broadly categorized as open or closed-canopy vegetation.

3. The results showed significant differences in both absolute and relative performance values between the phenotypic forms. Absolute performance suggests there are two phenotypic groups – strong (*B. thamnobates* and Type B) and weak (*B. melanocephalum* and Types A and C). Relative performance differences highlighted the significance of forefoot grip strength among these chameleons, with the closed-canopy forms (*B. thamnobates*, Types B and C) exceeding their open-canopy counterparts (*B. melanocephalum*, Type A). Little to no differences were detected between forms with respect to sprint speed and tail strength. These results indicate that strong selection is acting upon forefoot grip strength and has resulted in morphological adaptations that enable each phenotypic form to conform with the demands of its habitat.

4. This study provides evidence for the parallel evolution of forefoot grip strength among dwarf chameleons, consistent with the recognition of open and closed-canopy ecomorphs within the genus *Bradypodion*.

Key-words: Chamaeleonidae, lizards, morphometrics, perch diameter, South Africa

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Sexual Dimorphism in Bite Performance Drives Morphological Variation in Chameleons

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Abstract

Phenotypic performance in different environments is central to understanding the evolutionary and ecological processes that drive adaptive divergence and, ultimately, speciation. Because habitat structure can affect an animal's foraging behaviour, anti-predator defences, and communication behaviour, it can influence both natural and sexual selection pressures. These selective pressures, in turn, act upon morphological traits to maximize an animal's performance. For performance traits involved in both social and ecological activities, such as bite force, natural and sexual selection often interact in complex ways, providing an opportunity to understand the adaptive significance of morphological variation with respect to habitat. Dwarf chameleons within the *Bradypodion melanocephalum-Bradypodion thamnobates* species complex have multiple phenotypic forms, each with a specific head morphology that could reflect its use of either open- or closed-canopy habitats. To determine whether these morphological differences represent adaptations to their habitats, we tested for differences in both absolute and relative bite performance. Only absolute differences were found between forms, with the closed-canopy forms biting harder than their open-canopy counterparts. In contrast, sexual dimorphism was found for both absolute and relative bite force, but the relative differences were limited to the closed-canopy forms. These results indicate that both natural and sexual selection are acting within both habitat types, but to varying degrees. Sexual selection seems to be the predominant force within the closed-canopy habitats, which are more protected from aerial predators, enabling chameleons to invest more in ornamentation for communication. In contrast, natural selection is likely to be the predominant force in the open-canopy habitats, inhibiting the development of conspicuous secondary sexual characteristics and, ultimately, enforcing their overall diminutive body size and constraining performance.

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Introduction

Evolutionary and ecological processes that drive adaptive divergence and, ultimately, speciation can be influenced by phenotypic performance in different environments. As new environmental niches become available for populations to exploit, morphological and physiological adaptations arise, often resulting in enhanced performance in the novel habitat [1]. Evidence for these adaptations can be found in the improved performance of animals in their new environment [1]. For example, habitat structure or complexity is known to influence a range of lizard behaviours, including communication and anti-predator defences. Densely vegetated, structurally complex habitats may afford lizards greater cover from avian predators. If indeed predation pressure is released in dense vegetation, chameleons may invest more in conspicuous features, such as ornamentation and bright colouration, for increased detectability to conspecifics. However, in less vegetated habitats, where visibility to predators is high, rather than being visible chameleons may need to be cryptic to avoid detection (e.g., [2,3]). Because the head is involved in many ecologically and socially relevant activities, such as feeding, mating and aggressive interactions, its morphology and association to bite performance and habitat have been widely investigated to better understand the adaptive significance and the underlying processes shaping phenotypic variation within and between species (e.g., [4–15]). Many of these studies have shown that bite force is influenced by both natural and sexual selection, yet the relative contribution of these selective pressures remains difficult to unravel as they often interact in complex ways. Moreover, sexual and natural selection can act in opposite ways, with sexual selection favouring conspicuous coloration or ornamentation for effective communication and conflict avoidance, and natural selection favouring

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APPLICATION ESSAYS

Isolation of novel microsatellite loci in dwarf chameleons from KwaZulu-Natal province, South Africa and their cross-amplification in other *Bradypodion* species

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Abstract A recently radiated clade of dwarf chameleon (genus *Bradypodion*) localised to central-southern KwaZulu-Natal province, South Africa is considered taxonomically problematic due to the observed discordance between morphology and genetics within and between its species. The clade is made up of two described species (*B. melanocephalum–B. thamnobates*) and possibly others—all of which are experiencing significant reductions in the quality and quantity of available habitat due to natural and anthropogenic factors. To better understand the effects past and present habitat fragmentation has had on gene flow, population structure, and genetic diversity within this clade, we developed seven new microsatellite markers for the *B. melanocephalum–B. thamnobates* complex, plus two markers for *B. pumilum* using an enrichment protocol. We tested these nine markers, along with eight markers previously designed for *B. pumilum*, for cross-species transferability across five species within the genus *Bradypodion* (*B. melanocephalum, B. thamnobates, B. dracomonatum, B. sp. and B. pumilum*). The number of alleles ranged from 1 to 29 with observed heterozygosities ranging from 0.00 to 1.00. Several loci did not meet HW expectations, but this may be a result of extreme demographic fluctuations that have been noted for these species. Ten loci were found to be polymorphic across all species examined, making them ideal for studies examining the population genetics of dwarf chameleons.

Keywords Reptiles · Chamaeleonidae · Africa · Microsatellites

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Dwarf chameleons (genus *Bradypodion*) distributed in central-to-southern KwaZulu-Natal (KZN) province, South Africa, are considered taxonomically problematic given discordance between morphology and genetics (Alexander 2006; Tolley and Burger 2007; Tolley et al. 2008). The clade encompasses two species, *B. melanocephalum* (the KwaZulu-Natal dwarf chameleon) and *B. thamnobates* (the Natal Midlands dwarf chameleon), which show substantial morphological distinctness (in size, colour, and skull shape) and habitat partitioning (Branch 1998; Tolley and Burger 2007), yet they are not reciprocally monophyletic for mitochondrial markers—ND2 and 16S (Tolley et al. 2004; Tolley et al. 2006). Explanations for this range from shared ancestral polymorphism as a result of recent radiation, selective sweeps on mitochondrial genes, strong selection on the phenotype as a result of environmental pressure, and phenotypic plasticity. The latter explanation can be ruled out, as common garden experiments have shown this is unlikely (Miller and Alexander 2009). Comprehensive field surveys within the distribution of *B. thamnobates–B. melanocephalum* have uncovered other

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