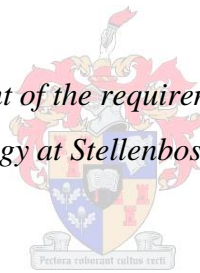


**Ecomorphological forms in Dwarf Chameleons (*Bradypodion*):
Assessment of functional morphology and gene flow across
spatially adjacent habitat types**

By

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*Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in
Zoology at Stellenbosch University*



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March 2013

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Abstract

Adaptive radiation is the process whereby clades, lineages, or species demonstrate rapid divergence into an array of phenotypic forms. Variation in ecological parameters, such as habitat use and morphology or behavioural traits related to communication; drive the evolution of ecologically relevant traits in specific habitat types. Nevertheless, such processes may be countered or enhanced by sexual selection pressures as selection acts on the phenotype to maximise reproductive output. Within the Cape Floristic region, species of dwarf chameleons (*Bradypodion*) are showing signs of such an adaptive radiation. Previous work on *B. pumilum* revealed intraspecific morphological differentiation, emphasised by functional differences in ecologically relevant traits, between those occupying the fynbos and forest/riverine thicket habitat types. Similar phenotypic divergences are hypothesised to have occurred in their allopatric, forest-dwelling neighbour, the Knysna Dwarf Chameleon (*B. damaranum*), given the presence of a closely related, morphologically divergent, undescribed species (*B. sp. 1*) found in the adjacent fynbos habitat type. With this in mind, functional morphological variation was examined between these two potential ecomorphological forms. A second unidentified fynbos species (*B. sp. 2*), which neighbours these species in its distribution, served to substantiate the proposed morphology~performance~habitat hypotheses. Given the chameleon's strong reliance of vegetation type, habitat use was explored by examining the microhabitat relevant to chameleons and ascertaining whether this habitat is used randomly. To associate variation in morphology with differences in habitat use, differences in performance capabilities were quantified, particularly those associated with grip strength (hand and tail) and sprint speed. Furthermore, twelve microsatellite markers were used in combination with the ND4 mitochondrial marker to understand the fine scale patterns of gene flow both within and between habitat types. In response to the varied pressures experienced, differences in ecologically relevant traits are found between *B. damaranum* and the two fynbos species, particularly those related to locomotion (limb length) and bite force (head width). Furthermore, analysis of microhabitat features

shows that the fynbos and forest habitat types are structurally different, facilitating differences in habitat use. Differences in performance also vary between vegetation types, with *B. damaranum* possessing stronger hand and tail grip forces as well as faster sprint speeds. Sexual dimorphism is also present; however it is more prominent in the forest-dwelling *B. damaranum*. Genetic analyses revealed high levels of geographical structure between *B. sp. 1* and *B. damaranum*, suggesting the presence of a strong barrier to gene flow. Given the congruence between morphological divergence and genetic spatial patterns, it appears that this barrier is associated with habitat type. Within each habitat type, both mtDNA and microsatellite analyses reveal congruent patterns of structuring. These patterns are, however, not governed by barriers to gene flow, but rather via isolation by distance (based on microsatellite data). Furthermore, mtDNA analysis confirmed *B. sp. 2* to be highly divergent, occupying a separate clade to *B. sp. 1* and *B. damaranum*. The adaptive differences observed between *B. damaranum* and *B. sp. 1*, coupled with its overall resemblance to those observed in *B. pumilum*; suggest the presence of true chameleon ecomorphs in the genus *Bradypodion*. This coupled with the lack of gene flow between ecomorphs is indicative of a true allopatric diversification.

Opsomming

Adaptiewe radiasie is die proses waardeur genetiese groepe, lyne, of spesies vinnige divergeer na 'n verskeidenheid van fenotipiese vorms. Variasie in ekologiese parameters, soos habitat verbruik en morfologie of gedrags eienskappe met betrekking tot kommunikasie, dryf die evolusie van ekologies relevante eienskappe in spesifieke habitat tipes. Nieteenstande, kan sulke prosesse teengewerk of versterk word deur seksuele seleksie omdat hierdie seleksie optree om die spesifieke fenotipe se reprodktiewe uitset te maksimaliseer. In die Kaapse Floristiese streek, toon spesies van die dwerg verkleurmannetjie (*Bradypodion*) tekens van 'n adaptiewe radiasie. Vorige werk op *B. pumilum* het intraspesifieke morfologiese differensiasie aan die lug gebring, beklemtoon deur funksionele verskille in ekologies relevante eienskappe, tussen diere wat uitsluitlik binne die fynbos of die woud/oewer tiepe habitat woon. Daar word gespekuleer dat soortgelyke fenotipiese verskille plaasgevind het binne hul allopatriese, woudlewende familie-lid, die Knysna dwergverkleurmannetjie (*B. damaranum*), gegewe die teenwoordigheid van 'n nou verwante, morfologies uiteenlopende, onbeskryfde spesies (*B. sp. 1*) wat in die aangrensende fynbos habitat aangetref word. Met dit in gedagte is funksionele morfologiese variasie tussen hierdie twee potensiële ekomorfologiese vorms ondersoek. 'n Tweede onbekende fynbos spesies (*B. sp. 2*), waarva dje verspreiding langsliggend is aan hierdie spesies, is gebruik om die voorgestelde morfologie ~ prestasie ~ habitat hipoteses te staaf. Omdat die verkleurmannetjie afhanklikheid is van plantegroei tipe, het hierdie studie habitat gebruik ondersoek deur die mikrohabitat te bestudeer wat deur willekeurig hierdie diere gebruik word. Om die variasie in morfologie met verskille in habitat gebruik om te assosieer, is verskille in prestasie vermoëns gekwantifiseer, veral dié wat verband hou met greep krag (hand en stert) en hardloop spoed. Verder is twaalf mikrosatelliet merkers gebruik in kombinasie met die ND4 mitochondriale merker om fyn-skaal patrone van genevloei te verstaan beide binne en tussen habitat tipes. In reaksie op die uiteenlopende evolusionêre drukke is verskille in ekologies relevante eienskappe gevind tussen *B. damaranum* en die twee fynbos spesies, veral dié met betrekking tot voortbeweging (ledemaat lengte) en byt krag (kop

breedte). Verder, het die analise van mikrohabitat kenmerke getoon dat die fynbos en woud habitat tipes struktureel verskil, en dit fasiliteer dus die verskille in habitat gebruik. Verskille in prestasie het ook gewissel ook tussen plantegroeitipes deurdat *B. damaranum* 'n sterker hand en stert greep sowel as vinniger hardloop spoed getoon het. Seksuele dimorfisme is ook teenwoordig, maar dit is meer prominent in die woud-bewoning *B. damaranum*. Genetiese analyses het hoë vlakke van geografiese struktuur tussen *B. sp. 1* en *B. damaranum* aan die lig gebring, wat dui op die teenwoordigheid van 'n formidabile grens tot genevloei. Gegewe die ooreenkomste tussen morfologiese divergensie en genetiese ruimtelike patrone, blyk dit dat hierdie grens tot genevloei verband hou met die habitat tipe. Binne elke tipe habitat, het buide mtDNA en mikrosatelliet analises dieselfde geneties patrone. Hierdie geneties patrone word nie gereeld deur grense tot genevloei beïnvloed nie, maar eerder deur isolasie met afstand (gebaseer is op die mikrosatelliet data). Verder, mtDNA analises bevestig dat *B. sp. 2* verreweg geneties verwant is en 'n afsonderlike klade vorm het relative tot die *B. sp. 1* en *B. damaranum*. Die aanpasbare verskille wat waargeneem tussen *B. damaranum* en *B. sp. 1*, tesame met die ooreenkomste aan dié waargeneem met *B. pumilum*; dui op die teenwoordigheid van ware verkleurmannetjie ekomorfs in die genus *Bradypodion*. Dit, tesame met die gebrek van genevloei tussen ekomorfs, is 'n aanduiding van 'n ware allopatriese diversifikasie.

Acknowledgements

I would like to thank the National Research Foundation (NRF) and the South African National Biodiversity Institute (SANBI) for funding as well as CapeNature and SANParks for the permits issued for sampling trips.

For assistance in chameleon hunting and/or habitat measurements thanks to Krystal Tolley, John Measey, Anthony Herrel, Jessica de Silva and Shelley Edwards. An additional thanks to Anthony Herrel and Bieke Vanhooydonck for providing the necessary apparatus needed to measure chameleon grip forces.

For assistance with the laboratory and morphometric work I would like to thank everyone at the Environmental Genomics Group laboratory at Stellenbosch University and the Leslie Hill Laboratory at SANBI for all your valuable suggestions and advice.

Finally I would like to thank my supervisors Bettine Jansen van Vuuren and Krystal Tolley for their knowledge, suggestions and patience with regards to the write-up. A very special thanks to Krystal Tolley for organising all the sampling trips, teaching me the ins and outs of morphometric analysis and for all the time and effort spent with me assessing the results.

On a personal note, I would like to thank my friends and family, with special reference to Luke Potgieter and Ann Mclachlan, for all your support over the last two years.

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Chapter 1: Introduction

The principal driving force for the evolution of species diversity resides within adaptation, historical contingency and/or chance (mutation, genetic drift) events (Darwin 1859; Travisano et al. 1995). The relative influence of each to adaptive evolution has been intensely scrutinised in the past (e.g. King & Jukes 1969; Mayr 1983; Parker & Smith 1990). Should contingency or chance facilitate species diversification, the respective degree of ancestral phenotypic integration or stochastic gene selection would direct adaptive evolution to produce divergent phenotypes in similar environments (Gould 1989). Nevertheless, studies have indicated that repeated evolution of phenotypic forms does occur in similar environments (Losos et al. 1998; Rüber et al. 1999; Blackledge & Gillespie 2003; Wiens et al. 2006), demonstrating directional selection as a principal element of adaptive radiation, with chance and contingency playing lesser roles.

Adaptive radiation and repeated evolution

Adaptive radiation is the process whereby clades, lineages, or species demonstrate rapid divergence into an array of phenotypic forms (Schluter 1996). These radiations, operating under divergent natural selection, facilitate phenotypic differentiation in response to specific ecological pressures (Schluter 1988; Schluter 1996). Under ecological theory, divergences in habitat use, trophic morphology and behaviour coincide with the allopatric or sympatric models of speciation to promote the onset of effective reproductive isolation (e.g. Schluter 1988; Schluter 1996; Streelman & Danley 2003). Well known adaptive radiations include Darwin's finches (Grant et al. 1976; Schluter 1988; Burns et al. 2002) and the African cichlids (Albertson 2003; Young et al. 2009).

The phenomenon of repeated evolution of a phenotype signifies a special case of adaptive radiation where separate lineages evolve morphologically similar phenotypes in ecologically similar environments (e.g. Rundle et al. 2000; Gillespie 2004). Among the reptilian species, *Anolis* radiations represent a prime example of repeated evolution (Losos et al. 1998; Losos 2009). Following independent colonisations on the four islands of the Greater Antilles, congruent

ecological pressures (habitat use, trophic morphology, and behaviour) on each island instigated the diversification of similar ecomorphological types (“ecomorphs”) occupying similar ecological niches (Losos 1992; Losos et al. 1998). Consequently, this produced a recently diverged, monophyletic relationship between lineages demonstrating dissimilar morphologies on each island (Losos et al. 1998; Poe 2004). Gene flow among populations of ecomorphs may occur, but generally has no effect on the morphological traits associated with trophic differentiations (Lu & Bernatchez 1999; Rundle et al. 2000; Ogden & Thorpe 2002). Ecomorphs can thus be classified based on morphological (e.g. snout-vent length, hind limb and forelimb length, tail length mass, subdigital lamellae number), ecological (e.g. perch diameter and height) or behavioural (e.g. display) characteristics (Losos & Sinervo 1989; Losos et al. 1990).

For any ecomorph hypothesis, however, underlying divergences in ecologically relevant traits are paramount to associate morphological divergences with differences in habitat use and behaviour (Losos et al. 1990). Differences in limb-length observed in anoles are directly associated with the perch diameter, inevitably affecting their locomotor abilities (Losos et al. 1998; Irschick & Losos 1998). Longer limbs permit rapid movement used for prey capture and predator evading, while shorter limbs ensure slow movement to catch immobile prey and avoid predator detection (Irschick & Losos 1998; Losos et al. 2000). Furthermore, longer tails are thought to increase balance during perching activities (Ballinger et al. 1973), while differences in head size and shape directly correlate to differences in bite force, a trait intended for resource exploitation or aggressive encounters (McBrayer 2004; Herrel et al. 2008).

In addition to natural selection, habitat structure simultaneously can drive selection of sexually dimorphic traits, resulting in conflicting demands of each on species morphology. Sexual selection can therefore counter adaptive radiation and usually follows periods of divergence in habitat use and trophic morphology (Albertson et al. 1999; Streelman et al. 2002). The conspicuousness of the sexually dimorphic traits, in the form of a signal, inevitably governs its effectiveness. These signals

can, however, increase the likelihood of predator detection in certain environments (Endler 1980; Zuk & Kolluru 1998). In various lizard species, colour is strongly correlated to habitat structure (Persons et al. 1999; Leal & Fleishman 2002) with the more ostentatious forms occurring in closed habitats (Stuart-Fox & Ord 2004; Dolman & Stuart-Fox 2010; Hopkins & Tolley 2011). Head size and shape, mentioned above as a derivative of resource exploitation, is simultaneously influenced by sexual selection pressures where it may be utilised in combat against conspecifics (Lappin & Husak 2005; Stuart-Fox et al. 2006; Measey et al. 2009) or as a mate attractant (Darwin 1859; Lailvaux et al. 2004; Hamilton & Sullivan 2005).

Taxonomic discrepancies

Classifications of recently diverged lineages, however, are not without taxonomic discrepancies, often through the lack of congruence between genetic and morphological analyses (Tolley et al. 2004; Tolley & Burger 2007), and is evident in many vertebrate species (e.g. Normark & Lanteri 1998; Heckman et al. 2006; Köhler & Deen 2010; Baldwin et al. 2011). These discrepancies should not be underestimated and can be governed by processes such as phenotypic plasticity, hybridisation and inadequate or biased sampling, among others. Examples include the variation in limb length observed when *Anolis sagrei* hatchlings were raised on both broad and narrow surfaces, respectively, suggesting plasticity (Losos et al. 2000). These developmentally plastic responses caused those reared on broad surfaces to develop longer limbs, a trait used extensively in defining anole radiations as it permits rapid movement to utilise prey and avoid predators (Irschick & Losos 1998). Consequently, such common-garden or breeding experiments are important in defining radiations as they differentiate between plastic responses and specialization to different habitats types. Introgression is another factor that can add to taxonomic discrepancies in several ways: 1) Two species may be perceived as one through shared mitochondrial DNA haplotypes (e.g. Seehausen et al. 1997); 2) genetic swamping of one species by another may reinforce reproductive isolation between partially isolated species (Dowling et al. 1997; Turelli et al. 2001); 3) adaptive evolution may be promoted via interspecific gene transfer (Grant & Grant 1992) or 4) the

generation of new species (Dowling & DeMarais 1993). Many additional factors or alternative explanations, contributing to phenotypic variation, could be available to confound taxonomy.

Study system

The genus *Bradypodion* (dwarf chameleons) comprises of medium to small sized chameleons endemic to southern Africa (Branch 1998; Tolley & Burger 2007). Their life history strategy suggests a strong reliance on vegetation type with the use of crypsis and stealth to successfully attain food and avoid predation (Tolley et al. 2006; Stuart-Fox & Moussalli 2007; Hopkins & Tolley 2011; Herrel et al. In press; Measey et al 2009). The phylogeny of *Bradypodion*, based on mitochondrial DNA, suggests the presence of several well-supported, allopatrically distributed clades (Tolley et al. 2004; Tolley et al. 2006). Those endemic to the Cape Floristic Region (CFR) show diversification patterns consistent with the palaeoclimatic events of the mid-Miocene and Plio-Pleistocene period (Tolley et al. 2006; Tolley et al. 2008). Global cooling, prompted by Antarctic ice sheet expansion, shaped the modern semi-arid environment (Udeze & Oboh-Ikuenobe 2005) ultimately restricting the Afromontane forest vegetation to refugia, sustained by orographic rainfall on the south coast, and allowing establishment of the fynbos biome (Scott et al 1997; Chase & Meadows 2007). This change in habitat type is hypothesised to have propagated *Bradypodion* diversifications in the CFR with the new lineages diversifying in the fynbos and older lineages persisting in the forest environments (Tolley et al. 2006; Tolley et al. 2008). Similar trends in environmental distributions are seen across the phylogeny where closely related lineages occupy an array of structurally different habitat types (Tolley et al. 2006; Tolley & Burger 2007; Tolley et al. 2008; Measey et al. 2009).

Initial work on *Bradypodion pumilum* revealed prominent intraspecific morphological differentiation, emphasised by functional differences in ecologically relevant traits, between those occupying open (fynbos) and closed (forest, riverine thicket, and exotic urban vegetation) habitat types (Herrel et al. 2011; Measey et al. 2009). Structurally, the closed habitat is comprised of

numerous horizontal perches present at a multitude of angles and diameters, while the open habitat typically consists of isolated, densely clustered, vertical perches usually narrow in diameter (Herrel et al. 2011). Consequently, in an open habitat type, ecomorphs rely on smaller feet and longer limbs for both arboreal movement along and terrestrial movement between these narrow perches, respectively (Hopkins & Tolley 2011). The functional consequence of these morphological progressions sees this ecomorph possess both slower sprint speeds and weaker grip forces than the closed habitat ecomorph (Herrel et al. 2011). As sprint speed is correlated with habitat openness in non-arboreal lizards (e.g. Losos 1990b; Melville & Swain 2000; Herrel et al. 2002; Irschick et al. 2005), these results suggest that the closed habitat type may be spatially more “open” at ground level, allowing chameleons to rapidly avoid ground-dwelling predators following a fall from their perch (Herrel et al. 2011). Alternatively, it may provide a selective advantage for rapid movement along wide, horizontal perches (Herrel et al. 2011). The longer limbs of the open habitat ecomorph, on the other hand, may select for movement through the dense ground-cover indicative of fynbos habitat types, or for bridging gaps between the vertical perches. The weaker hand/foot grip strength characteristic of these open habitat ecomorphs may elucidate the ecological relevance of perch use in chameleons; given that smaller hands and a shorter tail have evolved in a habitat defined by narrow perches (Herrel et al. 2011).

Contrasts in sexual dimorphism may be indicative of the pronounced effect predator visibility has in open habitats (Measey et al. 2009; Hopkins & Tolley 2011). Enhanced ornamental characters (e.g. gular scales and casque height; Measey et al. 2009; Stuart-Fox et al. 2006), used for sexual or competitive signalling in closed habitats, would increase conspicuousness in open habitats ultimately facilitating rapid predator detection (Baird et al. 1997; Stuart-Fox et al. 2003; Stuart-Fox & Ord 2004, Dolman & Stuart-Fox 2009). Consequently, those occupying open habitats (open habitat ecomorphs) tend to be smaller in size with reduced ornamental characters and dull coloration (Measey et al. 2009). Within closed habitats, contests are generally restricted to close range displays (Stuart-Fox et al. 2006) and because open habitat ecomorphs are constrained in this

context, there may be higher investment in fighting performance. By virtue of a broader head, open habitat ecomorphs possess a stronger bite force in relation to their body size (Measey et al. 2009), suggesting that casque height, a previous indicator of bite strength in other species of chameleon (Herrel et al. 2001; Herrel & Holanova 2008), is an ornamental character in an open habitat type (Measey et al. 2009). This indicates that open habitat ecomorphs have maximised bite force by increasing head width in preference to casque height, for greater muscle mass.

Given this information, the opportunity to investigate similar patterns in other *Bradypodion* species will provide a step forward in validating both the hypothesis of repeated evolution of ecomorphs and its occurrence on a continental stage. *Bradypodion damaranum* is one of the larger members of the genus, possessing a restricted distribution to fragmented patches (closed habitat) in the Knysna Forest, located on the south-facing slopes of the Outeniqua and Tsitsikamma Mountain ranges (Tolley & Burger 2007). Naturally it boasts a high casque, colourful flanks and a long tail (Figure 1a; Tolley et al. 2006; Tolley & Burger 2007). Recently, an undescribed species (here referred to as *B. sp. 1*) was located in the adjacent fynbos (open habitat) region on the north-facing slopes of the Tsitsikamma and Kouga mountains. In contrast to *B. damaranum*, *B. sp. 1* is characterised by a reduced casque, plain coloration and a reduced tail (Figure 1b; Tolley et al. 2006; Tolley & Burger 2007). Mitochondrial DNA studies indicate *B. sp. 1* to be closely related to *B. damaranum*, suggesting a recent divergence between the two lineages (Tolley et al. 2004; Tolley et al. 2006). Furthermore, a single *B. damaranum* individual was shown to have a haplotype more closely related to *B. sp. 1*, suggesting either introgression events, the presence of current gene flow, or lack of lineage sorting (Tolley et al. 2004; Tolley et al. 2006).

To provide insights into the evolution of ecomorphs in *Bradypodion*, fast evolving nuclear markers (i.e. microsatellite loci) were used in combination with mitochondrial markers to test fine-scale patterns of gene flow both within and between habitat types, while morphometric measurements allowed for correlations of morphological variation with variation in habitat. In this study, I

hypothesise that the microhabitat structure relevant to chameleons (e.g. perch diameter and height) of fynbos (open habitat) and forest (closed habitat) differs significantly, facilitating morphological differentiation between *B. sp. 1* and *B. damaranum* for ecologically relevant traits (i.e. limb length, fore and hindfoot size, tail length, head size, casque height). Furthermore, I hypothesise that this ecological/morphological separation would create a strong barrier to gene flow across macrohabitats (fynbos vs. forest), with isolation by distance predominating genetic patterns within each habitat.

Aims and questions

- 1) To quantify differences in microhabitat between the fynbos and forest habitat types.
 - a. Are there differences in microhabitat (fynbos vs. forest)?
 - b. Are there differences in microhabitats utilised by chameleons (perch size in fynbos vs. forest)?
 - c. Is there a difference between perches chosen by chameleons and perches available within a habitat type?
- 2) To correlate these microhabitat differences with differences in ecologically relevant traits.
 - a. Does morphology correlate with habitat use (perch size) in each habitat type?
 - b. Do ecologically relevant traits differ between vegetation types?
- 3) To quantify the functionality of these morphological differences observed.
 - a. Do performance traits (sprint speed, hand and tail grip strength) correlate to morphology in each habitat type?
 - b. Do differences in performance traits occur between habitat types?
- 4) To test for fine scale patterns of gene flow within and between habitat types.
 - a. Does an ecological barrier exist between ecomorphs or microhabitats which restrict gene flow?
 - b. Is gene flow governed by isolation by distance within a single habitat type?

Chapter 2: The effects of habitat structure and use on the functional morphology of Dwarf chameleons (*Bradypodion*) in adjacent habitat types

Introduction

In its purist form, an adaptively radiated lineage, operating under divergent natural selection, occurs when phenotypic differentiation is driven by divergence in habitat use, morphology and communication (e.g. Schluter 1988; Schluter 1996; Streelman & Danly 2003). Repeated evolution, a special case of adaptive radiation involving morphological convergence in different species occupying ecologically similar habitats, demonstrates that natural selection can lead to predictable evolutionary diversification (Losos et al. 1998; Rüber, Verheyen & Meyer 1999; Rundle et al. 2000; Schluter 2000; Nosil, Crespi & Sandoval 2002). Ecologically-induced sexual selection pressures can, however, disrupt or enhance the natural selection process, leading to variable contributions of each to a species' morphology and, in some cases, can counter the adaptive radiation process. Interactions between morphological divergence and habitat use are often used in evolutionary biology to demonstrate the power of natural selection, with many examples across a broad range of species (Endler 1983; Schluter & Grant 1984; Garland & Losos 1994; Wiens, Brandley & Reeder 2006).

Among the most well-known examples of repeated evolution in body form are the Caribbean *Anolis* radiations where similar ecological pressures drove the repeated and independent diversification of different morphological groups, termed “ecomorphs”, each possessing a similar morphology and occupying similar ecological niches (Williams 1983; Losos et al. 1998). For ecomorphs to evolve, however, the abiotic environment (e.g. habitat structure) must impose selection pressures on traits that are ecologically relevant in that specific habitat. For example, anoles with longer limbs tend to occupy microhabitats with wider perches (Williams 1983; Losos 1990a). Limb length correlates directly to sprint speed on broad substrates, providing longer-limbed anoles with the selective advantage (prey capture, predator avoidance) in this specific microhabitat (Irschick & Losos 1998;

Losos et al. 2000; Vanhooydonck, Herrel & Irschick 2006). Differences in these ecologically relevant traits can therefore drive differences in morphology, ultimately facilitating divergence in both habitat use and behaviour (Streelman & Danley 2003). Ecomorphs can thus be defined based on similarities in ecological (perch diameter and height), morphological (e.g. snout-vent length; tail length; limb length), performance and behavioural (e.g. display) characteristics (Williams 1983; Losos 2009).

Ecological pressures can, however, also drive selection of sexually dimorphic traits, creating a balance between natural and sexual selection (Arnold 1983; Thorpe & Malhotra, 1996; Vanhooydonck et al. 2007; Vanhooydonck et al. 2009; Hopkins & Tolley 2011). Any variation in either of these selective pressures, or in their interaction, can effectively shape phenotypic divergence and mediate speciation (Endler 1983; Price 1998; van Doorn, Noest & Hogweg 1998; Boughman 2002). In some cases, sexual selection may be seen as countering the adaptive radiation process. This occurs when organisms invest energy into traits, normally influenced by natural selection, to maximise their reproductive output (McLain 1993). For example, in many lizard species, habitat density is directly correlated to colour intensities, ornamentation and large body size (Persons et al. 1999; Leal & Fleishman 2002), traits that would serve as a visual cue to predators in a less dense environment (Endler 1980; Zuk & Kolluru 1998). Similarly, both head size and shape, traits regulating bite force, are influenced by natural selection through diet (McBrayer 2004; Herrel et al. 2008; Herrel et al. 2011, Measey et al 2009). These, however, may be reinforced or offset by sexual selection as head size and bite force are traits known to influence the outcome of aggressive encounters (Lappin & Husak 2005; Stuart-Fox, Whiting, & Moussalli 2006; Measey et al. 2009) or may act as a mate attractant (Darwin 1859; Lailvaux et al. 2004; Hamilton & Sullivan 2005).

The existence of repeated evolution of habitat-specific phenotypes is becoming more prevalent in the southern African endemic Dwarf Chameleon, genus *Bradypodion*. A general trend across the phylogeny sees closely related, monophyletic lineages distributed into an array of structurally

different microhabitat types (Tolley et al. 2006; Tolley & Burger 2007; Tolley, Chase & Forest 2008; Measey et al. 2009). The allopatric nature of *Bradypodion* distributions means that these microhabitat types are non-overlapping and adjacently distributed. Given the chameleon's strong reliance on vegetation (Tolley et al. 2006; Stuart-Fox & Moussalli 2007; Tolley & Burger 2007; Measey et al. 2009), it is suggested that their distributions are typically confined to a single structural microhabitat (Tolley et al. 2006; Tolley & Burger 2007). Morphologically, however, species are divergent from their sister lineage, suggesting that each microhabitat type imposes a different ecological selection regime. Indeed, work on the Cape Dwarf Chameleon, *Bradypodion pumilum*, revealed intraspecific morphological variation, associated with variation in ecologically and functionally relevant traits in each microhabitat type. In the open habitat, chameleons have proportionally smaller feet, longer limbs and a shorter tail (Hopkins & Tolley 2011; Herrel et al. 2011). Sprint speed, a relevant indicator of performance in lizards (e.g. Losos & Sinervo 1989; Losos 1990b; Bauwens et al. 1995), also differed between populations from different habitats, with those from the closed habitat type sprinting faster (Herrel et al. 2011). Although generally associated with open habitat species (e.g. Losos 1990b; Melville & Swain 2000; Herrel et al. 2002; Irschick et al. 2005), these results suggests that the forest vegetation may be spatially more "open", potentially selecting for rapid escape from predators following a fall to the ground, or faster sprint speeds may be associated with rapid movements across wider horizontal perches (Williams 1983; Losos 1990a; Herrel et al. 2011). The fynbos habitat, alternatively, possesses a dense ground-cover, made up of low grasses, brush and leaf litter where longer limbs (but not sprint speed) may provide a selective advantage while negotiating the dense vegetation, or for bridging gaps between the vertical perches (Herrel et al. 2011). Hand and tail grip strengths also differed between the ecomorphs, with chameleons from the closed habitat type being stronger (Herrel et al. 2011). Chameleons living in a habitat characterized by narrow perches have smaller hands and a shorter tail (Herrel et al. 2011) suggesting that perch use imposes selection on morphology through differences in performance.

Differences in sexual selection pressures between the two habitats are congruent with known relationships between sexual dimorphism and habitat density. The larger body size, more vivid coloration and larger ornaments (e.g. casque height and gular scales, Measey et al. 2009; Stuart-Fox, Whiting, & Moussalli 2006) of the closed habitat chameleons are used in a context of sexual signalling (Darwin 1859; Lappin & Husak 2005; Stuart-Fox, Whiting, & Moussalli 2006; Measey et al. 2009). However, such traits would increase the likelihood of detection by predators in an open environment (Endler 1980; Zuk & Kolluru 1998; Stuart-Fox & Ord 2004, Dolman & Stuart-Fox 2009). Consequently, the open habitat ecomorphs are smaller in size with reduced ornaments and a duller coloration (Measey et al. 2009; Hopkins & Tolley 2011). This means that contests via close range displays, as seen in the closed habitat ecomorphs (Stuart-Fox, Whiting, & Moussalli 2006), are selected against in an open environment, potentially facilitating a greater demand for fighting performance. By virtue of a wider head, open habitat ecomorphs possess a stronger bite force in relation to their body size (Measey et al. 2009).

The generality of these findings remains to be tested in other *Bradypodion* species. If, however, comparable to the scenario observed for *B. pumilum*, such data could provide an important step forward in confirming the existence of repeated evolution of habitat-specific morphology within this Dwarf Chameleon lineage. *Bradypodion damaranum*, the allopatric neighbour of *B. pumilum*, possesses a restricted distribution to fragmented forest patches on the south-facing slopes of the Outeniqua and Tsitsikamma Mountains, South Africa (Tolley & Burger 2007). Naturally it boasts a high casque, colourful flanks and a long tail (Figure 1a; Tolley et al. 2004; Tolley et al. 2006). Mitochondrial DNA studies have, however, confirmed the existence of an unidentified closely related lineage (*Bradypodion* species 1, or *B. sp. 1*; Tolley et al. 2004; Tolley et al. 2006) residing in the adjacent fynbos vegetation (Figure 1b). This species seems to exhibit similar morphological features (reduced casque, dull coloration, short tail) to those observed in fynbos *B. pumilum*. Divergence between *B. damaranum* and *B. sp. 1* was fairly recent (± 3.6 Ma) with their diversification patterns consistent with the palaeoclimatic events experienced during the Plio-

Pleistocene period (Tolley et al. 2006; Tolley et al. 2008). A second unidentified fynbos species (*Bradypodion* species 2, or *B. sp. 2*; Tolley et al. 2004; Tolley et al. 2006), shown to be closely related to *Bradypodion ventrale* (Tolley et al. 2004; Tolley et al. 2006), neighbours *B. sp. 1* in its distribution and is found in similar fynbos habitat (Figure 1c). Both undescribed species are similar in appearance and possess a reduced casque, plain coloration and a short tail (Figure 1b, c; Tolley et al. 2006; Tolley & Burger 2007). They are, however, highly divergent with an ancestral split dating back to approximately 14.1 million years ago (Tolley et al. 2006; Tolley et al. 2008).

In this study, morphological variation is examined between chameleons from the two habitat types (forest and fynbos) to quantify differences in ecologically relevant traits (e.g. limb length, fore and hind foot size, tail length, head size, casque height). The functionality of these differences will then be tested by measuring and comparing performance traits (sprint speed, hand and tail grip strength) thought to be relevant to chameleons (Herrel et al. 2011). Finally, both the structural microhabitat and its use (perch diameter, perch height) by chameleons in different macrohabitats will be quantified to explore correlations with morphology. We hypothesise that if microhabitat structure does indeed drive morphology in *Bradypodion*, as previously observed in *Bradypodion pumilum*, then morphological differentiation between *B. damaranum* and the two fynbos species (*B. sp. 1* and *B. sp. 2*) should occur.

Methods

Data collection

Data were obtained from multiple localities representing both *B. damaranum* and *B. sp. 1* distributions (Figure 2). For *B. damaranum*, this included the forested areas of the Outeniqua and Tsitsikamma Mountains (Bloukrans pass, Garden of Eden, Tsitsikamma and Keurbooms Nature Reserve). For *B. sp. 1*, samples from the Kouga Mountains (within the Baviaanskloof Mega Reserve) and Tsitsikamma Mountains (Geelhoutbos, Joubertina, Grootnek and Louterwater farms) were

obtained. *B. sp. 2* individuals were sampled on the nearby Baviaanskloof Mountains (Bosrug) only. All individuals were caught by hand during night-time surveys. Upon discovery, both perch height and diameter were measured and recorded. As perch diameters recorded are indicative of the branch size on which chameleons sleep, they may not reflect daytime perch use. Preliminary data, however, based on radio tracking in *Bradypodion pumilum* suggest that night time perch use is indicative of daytime use (K. A. Tolley and E. Katz, unpub. data.). Chameleons were brought back to the field station (at each site) where morphometric and performance measurements were recorded. Males were identified by the presence of a hemipenal bulge or by everting the hemipenes, while females were identified as individuals larger than the smallest male without hemipenes (*e.g.* snout-vent length ≥ 45 mm, Jackson 2007). The latitude/longitude of all captures was recorded and chameleons were returned to their initial perches after data collection was completed (typically within 24-32 hours).

To examine differences in morphology between *B. damaranum*, *B. sp. 1* and *B. sp. 2*, 21 morphological characters presumed to be ecologically relevant in chameleons (Herrel et al. 2011) and other lizards (Losos et al. 1990a; Losos et al. 1992) were measured using a digital calliper (accuracy 0.01mm). These characters included: Snout-vent length (SVL); tail length (TL); head width (HW1): width of skull behind the eyes; (HW2): widest points of skull; head length (KHL): tip of snout to tip of casque, (AHL): tip of snout to the back of the squamosal; lower jaw length (LJL): tip of snout to the back mandible; head height (KHH): bottom of maxillary to the top of the eye, (AHH): bottom of mandible to the top of the eye; from the tip of the snout to the back of the quadrate (QT); from the tip of the snout to the back of the jugal (CT); casque height (KCH); from the posterior edge of the mandible to the tip of the casque, (ACH): from the posterior ventral edge of the superior temporal fossa to the tip of the casque; femur length (FM): from the body wall to the insertion of the femur at the knee; tibia length (TB): from the base of the tibia at the knee to the insertion of the tibia above the carpal; medial and lateral hindfoot pad length (MH and LH); humerus length (HM): from the body wall to the insertion of the humerus at the elbow; radius

length (RD): from the base of the radius at the elbow to the insertion of the radius above the tarsal; medial and lateral forefoot pad length (MF and LF).

Sexual Dimorphism

To investigate sexual dimorphism within *B. damaranum*, *B. sp. 1* and *B. sp. 2*, the data set was separated by species and sexes were compared using an ANCOVA for each variable. In some lizards, the body and head demonstrate disparate scaling between sexes, therefore head measurements were compared using LJL as the covariate (Braña 1996; Kratochvil et al. 2003) while tail and limb measurements were evaluated using SVL as the covariate. To ensure SVL and LJL as appropriate covariates and that the assumptions of ANCOVA are met, equality of slopes for dependent variables was assessed using a General Linear Model. Consequently, equality of slopes was violated for tail length and head width measurements in both *B. sp. 1* (TL: $F = 9.13$, $P < 0.01$; HW: $F = 7.27$, $P = 0.009$; HW2: $F = 8.29$, $P < 0.01$) and *B. sp. 2* (HW2: $F = 8.30$, $P = 0.006$; TL: $F = 5.92$, $P < 0.05$). For *B. damaranum*, equality of slopes was validated for all characters. Following the ANCOVA, all subsequent probability values were subjected to Bonferroni corrections to minimize the effects of Type I errors (Rice 1989). All analyses were carried out using SPSS software (SPSS Inc).

Multivariate Analysis of Species

To assess morphological variation between *B. damaranum*, *B. sp. 1* and *B. sp. 2* the dataset was separated by sex with juveniles excluded. Each variable was examined for outliers using scatterplots (SVL and dependent variable) and assessed for normality using the Kolmogorov-Smirnov test. Given the disparate scaling between the head and body in lizards, head measurements were compared using LJL as the covariate (Braña 1996; Kratochvil et al. 2003) while tail and limb measurements were evaluated using SVL as the covariate. Equality of slopes was then evaluated using a General Linear Model (separated by sex) to ensure both SVL and LJL as appropriate covariates. All equal sloped, log-transformed variables were then regressed against SVL and LJL,

saving the resulting unstandardized residuals for use in the principal components analysis (see below). To reduce the likelihood of Type I errors, all consequent probability values were subjected to Bonferroni corrections (Rice 1989).

Principal components analyses were carried out, using the saved unstandardized residual scores, to explore the morphometric relations between the different species. If differences in the microhabitat relevant to chameleons do facilitate morphological divergence, then differences in these ecologically relevant traits should be evident between species in the two habitat types. Given the disparate scaling in head and body measurements, two independent PCAs (PCA1: Head; PCA2: Body) were carried out. To justify character inclusion into the PCA analyses, sampling adequacy was investigated using a Kaiser-Meyer-Olkin (KMO) test, while communalities measured the relevance of each character to the analysis (Tabachnick & Fidell, 1996). Following varimax rotation of the component matrix, all ensuing principal components (PCs) with an eigenvalue greater than 1.0 were extracted.

Habitat use

To investigate whether the three species utilise different microhabitats, several analyses were conducted. Firstly, the variation in microhabitat structure available to chameleons was quantified in both vegetation types. Subsequently, the perch height and size utilised by chameleons were compared both within and between vegetation types, respectively. Finally, to assess whether chameleons prefer specific perch sizes, the habitat available was compared against perches chosen.

To investigate differences in the microhabitat structure, random perch diameters (RPD's) were measured for both habitat types. A 100 metre transect was run in at least two sites per habitat where chameleons were sampled. At 10m intervals, a digital calliper (accuracy 0.01mm) was used to record diameter of every perch crossing a 1 metre radius. To account for variation in available perch size within the forest canopy, RPDs were taken at three levels: 1.5 m; 2.5 m and 3.5 m. RPDs were recorded at two localities within the forest habitat type, namely at the Garden of Eden and Plaatbos

(Figure 2a). As fynbos vegetation is structurally low, measurements were taken 10 cm below the top of the average vegetation height (± 1 m), which is where chameleons are typically found roosting at night. For *B. sp. 1*, RPDs were measured at Geelhoutbos, Joubertina, Grootnek farm and Louterwater farm, while for *B. sp. 2* measurements were recorded at Bosrug (Figure 2). To determine if the available microhabitat for chameleons differs between vegetation types, both perch diameter and perch height were compared between habitats using a Student's t-test (two-tailed, $\alpha = 0.05$). In addition, to examine variability within each habitat, mean perch diameters were compared 1) between height levels within forest; 2) between transects within each habitat type.

Performance of grip strength and sprint speed

In order to quantify the grip strength of both the hand and tail between all species, one of two dowels (broad: 10 mm diameter; narrow: 5 mm diameter) were mounted on a piezo-electric force plate attached to a metal base and connected to an amplifier. Grip forces for each chameleon, on each dowel, were measured at 1000 Hz during three separate 60s intervals (Herrel et al. 2012). During each interval, chameleons were allowed to repeatedly grip each dowel with their hands and tail. Each chameleon was given approximately 30 min rest between each interval and an hours rest between sessions using different sized dowels. Maximum tail grip strength was measured by allowing each chameleon to voluntarily wrap their tails around each dowel before drawing them away in a vertical path, allowing extraction of peak forces in a Z-direction. Maximum hand grip strength was measured by allowing each chameleon to voluntarily grip each dowel before drawing them away in a horizontal path, allowing extraction of peak forces in a Y-direction. Each interval typically involved two to three tail and hand measurements. All forces were extracted using Bioware software, retaining the three highest measurements for subsequent analyses.

Differences in sprint speed between *B. damaranum*, *B. sp. 1* and *B. sp. 2* were quantified by chasing chameleons down a 2 metre long flat track marked at 25 cm intervals. Using a stopwatch, the time

taken for each chameleon to cross each 25 cm interval was recorded. The fastest interval times were converted into centimetres per second and retained for further analyses.

All performance data were log₁₀-transformed and assessed for normality using a Kolmogorov-Smirnov test. Correlation analyses were then run to explore which morphological variable best explained the variation in performance. Differences in performance (grip strength, tail strength and sprint speed) between species were then assessed using an ANCOVA, incorporating the morphological trait best correlated with the respective performance as the covariate. Finally, the effects of grip strength on dowel size for each species were assessed using repeated-measures ANOVA. All analyses were performed using SPSS software (SPSS Inc).

Results

Sexual Dimorphism

Female body size (SVL) exceeded that of males for both *B. sp. 1* ($F = 26.80$, $P < 0.001$; females: 59.22 ± 6.20 mm; males: 51.95 ± 4.60 mm) and *B. sp. 2* ($F = 19.25$, $P < 0.01$; females: 63.6 ± 4.32 mm; males: 53.42 ± 5.18 mm), while *B. damaranum* males possessed a larger body size than females ($F = 13.87$; $P < 0.001$; females: 57.41 ± 12.75 mm; males: 67.02 ± 8.17 mm).

Following both head (LJL) and body (SVL) size adjustments, sexual dimorphism was not evident for any characters, other than head width and tail length, in both *B. sp. 1* and *B. sp. 2*. Once scaled to SVL, both tail ($F = 15.33$; $P < 0.001$) and radius ($F = 13.18$; $P < 0.01$) measurements showed dimorphism in *B. damaranum*, with tail length larger in males and radius length larger in females. For the head measurements, *B. damaranum* males possess a higher casque (KCH: $F = 4.19$, $P < 0.05$; ACH: $F = 5.53$, $P < 0.05$), but not a wider head in comparison to females.

Multivariate Analysis of Species

Equality of slopes between species was validated for all variables, leading to the inclusion of their residual values into either PCA1 (head measurements) or PCA2 (tail and body measurements). The KMO test indicated adequate sampling for both sexes in both analyses (PCA 1: Females: KMO = 0.674; Males: KMO = 0.745; PCA 2: Females: KMO = 0.714, Males: KMO = 0.741) and most communalities were high, confirming each variable as a positive contributor to the analysis (Tabachnick & Fidell 1996). For PCA 1, communalities were low for two variables in females (AHL and QT) and one (HW2) in males, leading to their removal from subsequent analyses.

For PCA 1: All variables loaded strongly on the first three axes of the PCA for females and the first four axes for males, accounting for approximately 66.7% and 76.7% of the variation, respectively (Table 1). The MANOVA revealed significant differences for PC1 (Females: positive loading for casque height; Males: positive loading for casque height and head length) in both sexes and PC2 (positive loading QT/CT) in males (Table 3). In both females and males, *post hoc* (LSD) analysis revealed PC1 to differ significantly between chameleons in different habitat types (Table 3; Figure 3a, c). In males, PC2 (positive loading for QT/CT) was significantly different for *B. sp. 2* (Table 3; Figure 3c).

For PCA 2: All variables loaded strongly on the first two axes of the PCA for both females and males, accounting for approximately 56.7% and 53.0% of the total variation, respectively (Table 2). The MANOVA for PC1 (positive loading for hands, feet and tail) indicated significant differences for both females and males (Table 3). *Post hoc* comparison (LSD) for PC1 revealed significant differences between chameleons from the two vegetation types for both sexes, with no difference between *B. sp. 1* and *B. sp. 2* which are both from fynbos vegetation (Table 3; Figure 3b, d).

Habitat Use

Within the fynbos habitat type for *B. sp. 1* (open habitat), the distribution of RPDs did not vary between most sites i.e. Louterwater, Grootnek and Joubertina (Table 4). Geelhoutbos, however,

differed significantly from both Grootnek and Joubertina dam, possessing smaller perches on average (Geelhoutbos: 1.68 ± 1.71 mm; Grootnek: 1.66 ± 1.17 mm; Joubertina: 1.87 ± 1.76 mm). Bosrug (fynbos habitat, locality of *B. sp2*) differed significantly from all other fynbos localities (Table 4), possessing narrower perches on average (1.57 ± 0.98 mm). Within the forest habitat type, no variations in RDPs were observed at different heights within sites (Garden of Eden and Plaatbos). In addition, there was no difference in perch diameter between Garden of Eden and Plaatbos sites ($Z = -1.898$; $P > 0.05$). RPDs did, however, differ between the open and closed sites ($Z = -18.462$; $P < 0.001$), with the available perches in the open habitat type being considerably narrower on average than the forest (Fynbos: 1.31 ± 0.77 mm; Forest: 2.69 ± 1.38 mm). Assessment between RPDs and actual perch diameters showed that chameleons in the open habitat type routinely utilise perches that are, on average, wider than those randomly available (*B. sp. 1*: $Z = -8.685$, $P < 0.001$; *B. sp. 2*: $Z = -6.228$, $P < 0.001$), while chameleons in the closed habitat type utilise the habitat in a random fashion compared to those available ($Z = -1.905$; $P > 0.05$). Once separated by sex, perch use in *B. damaranum* males showed significant positive correlations with tail length ($R = 0.566$; $P = 0.003$) and femur length ($R = 0.599$; $P < 0.01$). Perch use in *B. damaranum* differed significantly from *B. sp. 1* ($F = 17.944$; $P < 0.001$) and *B. sp. 2* ($F = 9.049$; $P < 0.01$), with the forest habitat chameleons using wider perches on average (*B. damaranum*: 3.83 ± 1.87 mm; *B. sp. 1*: 2.61 ± 0.84 mm; *B. sp. 2*: 2.10 ± 0.77 mm). No variation in perch use, however, was found between *B. sp. 1* and *B. sp. 2* ($F = 2.22$; $P > 0.05$) as well as between sexes for all species (*B. damaranum*: $F = 0.79$, $P > 0.05$; *B. sp. 1*: $F = 0.61$, $P > 0.05$; *B. sp. 2*: $F = 0.36$, $P > 0.05$).

Performance of grip strength and sprint speed

Dowel size had a significant effect on hand grip strength for all species (*B. damaranum*: $F = 128.79$, $P < 0.001$; *B. sp. 1*: $F = 46.054$, $P \leq 0.001$; *B. sp. 2*: $F = 348.7$, $P < 0.001$), with chameleons exerting stronger grip forces on the narrow (5mm diameter) dowel (*B. damaranum*: 1.44 ± 0.55 N; *B. sp. 1*: 0.74 ± 0.19 N; *B. sp. 2*: 0.58 ± 0.12 N) versus the broad (10 mm diameter) dowel (*B. damaranum*: 0.19 ± 0.09 N; *B. sp. 1*: 0.12 ± 0.09 N; *B. sp. 2*: 0.05 ± 0.03 N). The effect of dowel size on tail

performance was significant for *B. sp. 2* ($F = 22.834$; $P < 0.001$), indicating stronger grip forces on the narrow dowel (Broad: 0.71 ± 0.25 N; Narrow: 1.12 ± 0.41 N). Dowel size, however, did not have an effect on tail grip forces for *B. damaranum* ($F = 2.782$; $P > 0.05$) and *B. sp. 1* ($F = 0.114$; $P > 0.05$).

When testing the differences in hand grip strength using hand size (MH) as the covariate, differences between species remained significant for the broad dowel (Table 5), with the forest habitat species being stronger on average (Figure 4a). On the narrow dowel, hand grip strength was only significant between *B. damaranum* and *B. sp. 2* (Table 5), with *B. damaranum* the stronger on average (Figure 4b). When tail length was introduced as the covariate, tail grip strength on the broad dowel remained significant for all interactions except for *B. damaranum* and *B. sp. 1*. On the narrow dowel all interactions were non-significant (Table 5). The forest habitat species was stronger on average on both dowel sizes (Figure 4c, d).

Sprint speed was correlated with femur length ($R = 0.83$; $P < 0.001$), tibia length ($R = 0.82$; $P < 0.001$), humerus length (0.82 ; $P < 0.001$) and radius length (0.78 ; $P < 0.001$) in *B. damaranum*. There were no correlations between morphological traits and sprint speed in both *B. sp. 1* and *B. sp. 2*. The ANOVAs revealed significant differences in sprint speed between *B. damaranum* and the fynbos species (*B. damaranum* vs. *B. sp. 1*: $F = 35.50$, $P < 0.001$; *B. damaranum* vs. *B. sp. 2*: $F = 12.47$, $P \leq 0.001$), with chameleons from the forest habitat running faster on average (Figure 5). There was no difference between *B. sp. 1* and *B. sp. 2* ($F = 0.002$; $P > 0.05$). The ANCOVAs, using tibia length as the covariate, revealed similar interactions (*B. damaranum* vs. *B. sp. 1*: $F = 28.41$, $P < 0.001$; *B. damaranum* vs. *B. sp. 2*: $F = 34.19$, $P < 0.001$; *B. sp. 1* vs. *B. sp. 2*: $F = 0.002$, $P = 0.970$).

Discussion

Operating under divergent selection, an adaptive radiation arises when lineages diversify into an array of phenotypic forms in response to both ecological and sexual selection (e.g. Schluter 1988; Schluter 1996; Streelman & Danly 2003). Consequently, the general outline for these radiations sees three sequential axes of divergence, namely divergence in habitat use, morphology and communication (Streelman & Danley 2003), a progression observed across a broad range of species (Endler 1983; Schluter & Grant 1984; Garland & Losos 1994; Wiens, Brandley & Reeder 2006). In accordance with this framework, our study provides empirical evidence of morphological divergence between *B. damaranum* and *B. sp. 1* which utilise different macro and microhabitat types. We compare these findings, using *B. sp. 1* and *B. sp. 2* which are not closely related, but are morphologically similar and live in similar habitats, as further reinforcement for the observed correlations between habitat use and morphological divergence.

Divergence in habitat structure plays a fundamental role during the early stages of vertebrate radiations as it promotes variation in habitat utilisation, the precursor to phenotypic change if habitat structure affects organismal performance. Variation in habitat structure was indeed evident between the two vegetation types, with the fynbos habitat type, defined by dense, low vegetation, possessing significantly narrower perches on average. Variation among sites was absent within both vegetation types, with the exception of a single fynbos site (Bosrug) where the perches were significantly smaller. This can, however, be attributed to variation in abiotic factors such as altitude, rainfall and soil; all of which are strong indicators of habitat structure in this environment (Cowling et al. 1997). Consequently, habitat utilisation varied between each habitat type, with *B. damaranum* selecting perches at random (from a pool of larger perches) and the two fynbos species selecting perches wider than those randomly available (from a pool of smaller perches); a trend similar to what was observed in *B. pumilum* (Herrel et al. 2011). The perches used did, however, differ between the two ecomorphs, with *B. damaranum* using wider perches on average. Furthermore, there were associations between habitat use and morphology, such as in *B. damaranum* males,

where tail and femur length were correlated with perch diameter. Interestingly, these associations were also observed in *B. pumilum* males. This suggests that tail length not only aids in support while perched, by wrapping their tail around branches (K. A. Tolley & G. J. Measey, pers. observ.), but may aid in support during competitive encounters with conspecifics, preventing displacement from their respective perches. Similarly, limb length (e.g. femur length) may promote rapid movement over horizontal perches, a mechanism which may be beneficial during these competitive encounters.

The differences in habitat structure and use, however, must impose selection pressures on ecologically relevant traits specific to that habitat type for there to be selection on morphology (e.g. Irschick & Losos 1998; Vanhooydonck, Herrel & Irschick 2006; Hopkins & Tolley 2011; Herrel et al. 2011). As observed in *B. pumilum*, both *B. sp. 1* and *B. sp. 2* possess smaller hands/feet and shorter tail, suggesting an adaptation to gripping the narrow perches associated with the fynbos habitat type, while *B. damaranum* possess larger hands/feet and a longer tail, which may assist in bridging gaps between perches or providing stability during perching activities. Head dimensions also varied between the two vegetation types with both *B. sp. 1* and *B. sp. 2* possessing a relatively wider head and a reduced casque.

However, to associate these divergences in morphology with the differences in habitat use, the selection pressures experienced must facilitate functional differences in ecologically relevant traits. For instance, the prehensile nature of chameleon's hands, feet and tail suggests that the ability to hold onto a perch is ecologically relevant. If so, then chameleons with larger hand, feet and longer tails, used to hold wider perches, should possess stronger grip forces than those with smaller hands, feet and shorter tails (Herrel et al. 2011). This indeed was the case as marked differences in gripping performances were found, with *B. damaranum* being stronger on average. This suggests the presence of different selection regimes in each habitat type and that selection on morphology is associated with differences in performance. Additionally, hand grip strength was shown to be

dependent on dowel diameter, with stronger forces measured on the narrower dowel (5mm diameter). However, only *B. damaranum* showed significant correlations between gripping performance and morphology on this dowel. Although reasons for this remain untested, the narrow dowel diameter does resemble the perch diameters utilised by *B. damaranum* more closely (Figure 4b; *B. damaranum* perch diameter: 3.83 ± 1.87 mm; *B. sp. 1* perch diameter: 2.61 ± 0.84 mm; *B. sp. 2* perch diameter: 2.10 ± 0.77 mm), suggesting that both *B. sp. 1* and *B. sp. 2* are in fact at a selective disadvantage in a forest habitat type, given their lower grip forces. Consequently, this further advocates the presence of an optimum perch diameter in both habitat types, something that has been observed in other chameleon species (Losos, Walton & Bennett 1993). To fully substantiate this, however, performance measurements on a dowel mimicking the perch diameters in the fynbos habitat type would have to be performed.

Sprint speed, another indicator of performance in lizards, also varied between vegetation types with *B. damaranum* running faster on average, an outcome reflecting what was observed in *B. pumilum* (Herrel et al. 2011). There was, however, no variation in limb length between chameleons in the two vegetation types, which differs from *B. pumilum*, where limb length was longer in the fynbos habitat type (Hopkins & Tolley 2011). This is interesting as limb length is a general indicator of sprint speed in lizards (e.g. Losos & Sinervo 1989; Losos 1990b; Bauwens et al. 1995). As sprint speed is usually associated with habitat openness (e.g. Losos 1990b; Mellville & Swain 2000; Herrel et al. 2002; Irschick et al. 2005), it was previously hypothesised that the closed vegetation type may in fact be spatially more “open” at the ground level for chameleons, with high sprint speeds potentially allowing rapid escape from ground-dwelling predators after a fall (Herrel et al. 2011). Instead, the differences in performance observed here may be due to the absence of any selection pressures for rapid movement in the fynbos habitat. The characteristic dense vegetation, composed of low grasses, brush and leaf litter, coupled with the short, narrow, vertical perches may not provide the spatial platform necessary for rapid movement. Instead, selection may favour slow movement, where limb length is advantageous for navigating through the dense ground-cover or for

bridging gaps between the vertical perches (Herrel et al. 2011). In the forest habitat, however, limb length may necessitate quicker movement along the frequently abundant, horizontal perches, advantageous during competitive encounters with conspecifics or to escape from predators.

The varying patterns of sexual dimorphism in each species further elucidate the different ecological pressures experienced in each habitat type. On the one hand, the forest habitat type, which offers some respite from predator detection, facilitates strong sexual dimorphism where selection acts on the development of secondary sexual characteristics, used for competitive signalling or as a mate attractant (Endler 1980; Zuk & Kolluru 1998; Stuart-Fox & Ord 2004, Dolman & Stuart-Fox 2009). In male *B. damaranum*, these characteristics include an enlarged body size, flamboyant coloration and strongly developed ornaments such as a high casque and large gular scales. By contrast, selection in the open fynbos habitat type favours a cryptic phenotype, as the cost of predator detection likely outweighs the benefit of enhanced reproductive performance (Stuart-Fox & Ord 2004; Dolman & Stuart-Fox 2009). Consequently, sexual dimorphism in this habitat is weak and in this case was restricted to tail length and head width *B. sp. 1* and *B. sp. 2*. Given the high probability of predator detection in this habitat type, contests between conspecifics in the form of close range displays are selected against, suggesting an increase in direct conflict and selection for a greater fighting performance. Although not thoroughly investigated, observational accounts have shown a higher frequency of bite-induced scarring on chameleons in open habitats (K.A. Tolley, pers. observ.). Therefore, male investment in a wider head, an indicator of bite force in open *Bradypodion* ecomorphs (Measey et al. 2009), may provide the necessary weapon required for these intrasexual encounters. The dimorphism in tail length, as discussed earlier in *B. damaranum*, may further aid such encounters by providing the necessary support needed to prevent displacement from their initial perch. Interestingly, alternate dimorphism in body size was observed between vegetation types, with males larger in the forest habitat and females larger in the fynbos habitat. Many reasons do exist; however, they are not mutually exclusive. For instance, a larger male body size in the forest habitat may be beneficial during territorial displays or social encounters (e.g. Rand

1967, Jenssen 1970), while a larger female body size in the fynbos habitat may provide the necessary body cavity size for egg production (Andrews & Rand 1976). Alternatively, difference in dimorphism between vegetation types may be due to the sex identification approach implemented, where females were judged as individuals larger than the smallest male that bears no hemipenes. Consequently, in a population where males possess a larger body size, females may be misidentified as juveniles (which are excluded from the analysis), inevitably resulting in a bias of large females. This could account for the inverted sexual dimorphism found in *B. sp. 1* and *B. sp. 2*.

In conclusion, the patterns of divergence described in this study are consistent with the general framework of adaptive divergence (Streelman & Danley 2003). The structure of both the fynbos and forest habitat types differed significantly, leading to differences in habitat utilisation between *B. damaranum* and *B. sp. 1*. Within the fynbos habitat, however, the similar structural pressures drove similar patterns of habitat utilisation between *B. sp. 1* and *B. sp. 2*. The ecologically relevant morphological differences observed suggest an adaptive response to the difference in habitat structure. Finally, the differences in the functionality of these morphological traits provide the proximate reason for selection on morphology associated with differences in habitat use and structure. Moreover, the difference in visibility by predators in the different habitats probably drives the levels of sexual dimorphism observed. Overall, the patterns described here for *B. damaranum* and *B. sp. 1* are similar to those previously documented for *B. pumilum* populations living in divergent habitats (Herrel et al. 2011; Hopkins & Tolley 2011). This not only suggests the presence of distinct ecomorphological forms, but proposes repeated and independent evolution of morphology associated with habitat type across the *Bradypodion* lineage. Furthermore, the observed similarity in functional morphology between *B. sp. 1* and *B. sp. 2* introduces a distinct fynbos phenotype, further demonstrating the effect similar ecological pressures have on driving the evolution of similar morphological forms.

Chapter 3: Population genetics of recently diverged dwarf chameleon ecomorphs in adjacent habitat types

Introduction

Adaptive radiation is one of the most recognised and studied themes in evolutionary biology with many examples across a broad range of species (e.g. Grant 1976; Schluter 1988; Burns et al. 2002; Albertson 2003; Young et al. 2009; Losos et al. 1998). These radiations are governed by mechanisms such as natural selection and gene flow which mediate the degree of differentiation observed between populations (Endler 1977). Under ecological theory, divergent natural selection, working in combination with either the allopatric or sympatric model of speciation, can restrict gene flow in response to the synergism between historical and ecological progressions (Schluter 1988; Schluter 1996; Rundle & Nosil 2005). Consequently, these divergent pressures can manipulate the functionality of morphological traits in specific environments, providing the necessary platform for reproductive isolation (e.g. Losos et al. 1998; Albertson 2003; Herrel et al. 2011).

The general outline for vertebrate radiations sees the presence of several ecological stages where diversification occurs through divergences in habitat use, trophic morphology and communication (Streelman & Danley 2003). Given that these stages can occur in any geographical context, the geographic progressions experienced are nonetheless influential as they shape the ecological platform for divergent pressures (Wiens 2004). These extrinsic progressions, in the form of barriers of unsuitable habitat (e.g. mountain; river) or through alterations in the preferred distributions via climatic shifts, can cause fragmentation of species ranges, allowing distinct ecological factors to further diminish or restrict gene flow between them. For instance, the structural habitat can reduce the fitness of immigrants from an alternative environment (Mayr 1947; Nosil, Vines & Funk 2005). These divergent selection pressures impact ecologically relevant traits which increase the fitness of an individual in a specific habitat type. For example, *Anolis* lizards with large toe-pads possess greater adhesive forces, a mechanism used for clinging to perches in the forest canopy.

Consequently, these anoles possess the selective advantage for clinging ability over anoles with small toe-pads in this specific microhabitat (Elstrott & Irschick 2004). Gene flow could also be restricted through different sexual selection pressures, where traits used in communication and mate attraction may diverge. This was observed in mature male *Gallotia galloti* where ultra-violet markings were linked to assortative mating patterns, restricting gene flow between various populations (Thorpe & Richard 2001). Non-ecological factors such as genetic drift and the founder effect, among other, could also influence these speciation events.

Classifications of these recently diverged lineages, however, are not without taxonomic discrepancies, often through the lack of congruence between genetic and morphological analyses (e.g. Normark & Lanteri 1998; Heckman et al. 2006; Köhler & Deen 2010; Baldwin et al. 2011). These discrepancies may be due to processes such as phenotypic plasticity, introgression and inadequate or biased sampling, among others. For example, analyses on limb length in *Anolis sagrei* hatchlings, a trait used extensively in defining anole radiations (Irschick & Losos 1998), facilitated longer limb development on broader substrates, indicative of a plastic response (Losos et al. 2000). Consequently, these common-garden or breeding experiments are important in defining radiations as they differentiate plasticity from ecological adaptation. Alternatively, introgression events can fuel taxonomic discrepancies in various ways: 1) Two species may be perceived as one through shared mitochondrial DNA haplotypes (e.g. Seehausen et al. 1997); 2) genetic swamping of one species by another may reinforce reproductive isolation between partially isolated species (Dowling et al. 1997; Turelli et al. 2001); or 3) adaptive evolution may be promoted via interspecific gene transfer (Grant & Grant 1992). Many additional factors or alternative explanations, contributing to phenotypic variation, could confound taxonomy.

A phylogeny showing signs of an adaptive radiation is the southern African endemic Dwarf Chameleons, genus *Bradypodion*, where divergent natural selection pressures are hypothesised to have propagated new lineages into an array of structural habitat types (Chapter 2; Measey et al.

2009; Herrel et al. 2011; Hopkins & Tolley 2011). Within the Cape Floristic Region (CFR) their allopatric distributions are consistent with the climatic shifts experienced during the mid-Miocene and Plio-Pleistocene period (Tolley et al. 2006; Tolley et al. 2008). Uprising of the Benguela current, through Antarctic glaciation events, fashioned the modern semi-arid environment along the south-western coastline of Africa (Udeze & Oboh-Ikuenobe 2005). This restricted the Afromontane forest vegetation to refugia along the south coast, sustained by orographic rainfall, and allowing the establishment of the fynbos biome (Scott et al. 1997; Chase & Meadows 2007). This transition between habitat types is hypothesised to have propagated new *Bradypodion* radiations into the CFR with the older lineages persisting in the forest habitats (Tolley et al. 2006; Tolley et al. 2008).

One such species, *Bradypodion damaranum*, characterised morphologically by a high casque, long tail and brightly coloured flanks (Figure 1a; Tolley et al. 2004; Tolley et al. 2006), resides in the fragmented forest patches on the south-facing slopes of the Outeniqua and Tsitsikamma Mountains, South Africa. Phylogenetic studies revealed *B. damaranum* and an undescribed species (*Bradypodion* species 1, or *B. sp. 1*; Tolley et al. 2004; Tolley et al. 2006), distributed in the adjacent fynbos vegetation, to cluster together, forming a recently diverged (± 3 Ma), well-resolved monophyletic relationship (Figure 2b; Tolley et al. 2004; Tolley et al. 2006). Despite their recent divergence and geographically adjacent distributions, both lineages show distinct morphological divergences, with *B. sp. 1* distinguishable by smaller feet, a reduced casque, plain coloration and a short tail (Chapter 2; Tolley et al. 2006; Tolley & Burger 2007). A second undescribed species (*Bradypodion* species 2, or *B. sp. 2*; Tolley et al. 2004; Tolley et al. 2006), closely related to *Bradypodion ventrale*, neighbours *B. sp. 1* in its distribution and also occurs in the fynbos habitat type (Figure 1c). Morphologically, both *B. sp. 1* and *B. sp. 2* are similar, however, genetically they are highly divergent, occupying a separate clade with an ancestral split dating back to approximately 14.1 million years ago (Tolley et al. 2006; Tolley et al. 2008). Patterns of chameleon morphological variation observed are highly correlated with environmental variables (e.g. perch width), causing notable differences in performance between habitat types. For example, both limb

and tail length correlated with perch diameter, an environmental variable which differed significantly between habitat types (Chapter 2). In a habitat characterised by narrow perches (fynbos), both *B. sp. 1* and *B. sp. 2* evolved smaller hands and a shorter tail, indicating both the ecological relevance of perch use and that difference in performance does facilitate morphological convergence (Chapter 2). This not only elucidates the presence of ecomorphs but demonstrates the existence of different selective regimes in each habitat type (Chapter 2). Contrasts in sexual selection pressures also seem to be evident between habitat types, with males from the forest habitat possessing enhanced body size, colouration and ornamental characters; traits used for sexual or competitive signalling (Darwin 1859; Lappin & Husak 2005; Stuart-Fox et al. 2006; Measey et al. 2009, Herrel et al. 2011). In the fynbos habitat type, however, sexual dimorphism is weak and mostly restricted to tail length and head width in males (Chapter 2). Given the high probability of predator detection in this habitat type, close range displays are selected against, possibly limiting these interactions to aggressive conflicts. The nature of these selective regimes coupled with the chameleon's strong reliance on vegetation type (Tolley et al. 2006; Stuart-fox & Moussalli 2007; Hopkins & Tolley 2011; Herrel et al 2011; Measey 2011) may be enough to promote genetic differentiation between the two habitat types, providing a mechanism for limiting gene flow and promoting reproductive isolation leading to speciation.

Hence, to provide insights into the evolution of ecomorphs in *Bradypodion*, fast evolving microsatellite markers in combination with mitochondrial markers were used to test fine-scale patterns of gene flow both within and between the ecomorph populations occupying the forest and fynbos habitat types. The functional morphological divergence in response to alternate habitat types should create a strong barrier to gene flow, facilitating strong genetic structure between the two macrohabitats (*B. damaranum* vs. *B. sp. 1*). Within each macrohabitat, however, historical and anthropogenic sources of habitat fragmentation should influence population structure, with patterns of isolation by distance governing gene flow. Lastly, despite the unequivocal similarity in functional morphology between *B. sp. 1* and *B. sp. 2* (Chapter 2), higher levels of genetic

divergence and population structure should persist between the two fynbos species, consistent with that of other known ecomorphological forms (e.g. Losos et al. 1998).

Materials and Methods

Sample collection and DNA extraction

Tissue samples from chameleons were obtained from multiple localities representing both *B. damaranum* and *B. sp. 1* distributions (Figure 6). As a number of samples were readily available, additional field work targeted areas where sampling required improvement. For *B. damaranum*, this included the forested areas on the south-facing slopes of the Outeniqua and Tsitsikamma Mountains (Bloukrans pass, Garden of Eden, Tsitsikamma and Keurbooms Nature Reserve). For *B. sp. 1*, additional samples were obtained from the fynbos on north-facing slopes of the Tsitsikamma (Joubertina, Louterwater farm and Grootnek Farm) and the Kouga Mountains (Geelhoutbos). Samples of *B. sp. 2* were obtained from the fynbos on the Baviaanskloof Mountains (Bosrug) which is separated from the Kouga Mountains by a river valley dominated by thorn acacia. Tail clippings (± 3 mm) were taken from each individual and stored in 100% ethanol. Males were identified by the presence of hemipenal bulge, while females were identified when animals larger than the smallest male bear no hemipenes (snout-vent length ≥ 45 mm, Jackson 2007). The coordinates (latitude/longitude) of all captures were recorded and chameleons were returned to their initial perch. DNA extractions commenced using the DNeasy blood and tissue extraction kit (Qiagen Inc.) following manufacturer's instructions.

Microsatellite and mitochondrial DNA protocol

The presence of genetic structure within and between ecomorphs was evaluated using a combination of twelve microsatellite markers and one mitochondrial marker (Table 6). This allowed for assessment of levels of divergence, estimates of gene flow and levels of genetic isolation both within and between habitat types. All sample sites were included into the mtDNA study,

representing the entire known distribution of both *B. damaranum* and *B. sp. 1* (Figure 6). For comparative purposes, representatives of *B. sp. 2* were also included from the Baviaanskloof Mountains (Bosrug). The sites with larger sample sizes (≥ 4 specimens) were incorporated into the microsatellite analysis, including from Joubertina, Louterwater farm, Grootnek Farm and Geelhoutbos for *B. sp. 1*; Garden of Eden, Bloukrans pass and Plaatbos for *B. damaranum* (Figure 7). The general lab protocol for microsatellite amplification proceeded as follows (Feldheim et al. 2010): The forward primer of each locus was 5'-end labelled with one of four fluorescent dyes (6-FAM, HEX, PET, NED). Those exhibiting the same label and expressing no signal inhibition of PCR products were amplified simultaneously using a Multiplex PCR kit (Qiagen). A final PCR reaction volume of 10 μ l included: 6 μ l of 2x Qiagen Multiplex Master Mix, 1 μ l of primer mix (2 mM), 1 μ l water and 2 μ l of template DNA. PCR conditions comprised of initial denaturation for 15min at 95 °C followed by 34 amplification cycles of denaturation for 30 s at 94 °C, annealing for 90 s at locus specific temperature (Table 1) and extension for 50 s at 72 °C. Final extension occurred for 30 min at 60 °C. Subsequently, 15 μ l of deionised formamide and 2 μ l of FS500LIZ size standard (Applied Biosystems) were added to the PCR products. Genotyping was carried out on an ABI 3130 Prism (Applied Biosystems, Foster City, California, USA) and the raw data was scored using Genemapper software v 3.7 (Applied Biosystems).

The laboratory protocol for the NADH dehydrogenase subunit 4 (ND4) mitochondrial marker proceeded as follows: A final PCR reaction volume of 25 μ l included: 3 μ l of a 1 mM dNTP solution, 3 μ l of 25 mM MgCl₂, 0.2 μ l of both forward and reverse primer, 3 μ l of Mg₂₊ free buffer solution, 0.1 μ l Taq polymerase, 15.5 μ l dH₂O and 1-2 μ l of genomic DNA. The PCR thermal cycling procedure included: Initial denaturation for 4 min at 94°C followed by 34 amplification cycles of denaturation for 30 s at 94 °C, annealing for 40 s at 55 °C and extension for 40 s at 72 °C. Final extension occurred for 4 min at 72 °C. PCR products were run on a 1% agarose gel and visualised under a UV light to verify amplification. Following purification, using the SV-Gel and PCR clean-up system (Promega), amplicons were sequenced directly using the forward primer

ND4-F3. DNA sequencing was performed under standard conditions: 25 cycles of 10 s at 96 °C, 5 s at 50 °C and 4 min at 60 °C. Sequences were edited and aligned using Geneious software v 4.7 (Drummond et al. 2009).

Mitochondrial DNA analysis

To investigate the levels of polymorphism and haplotype uniqueness, all mtDNA data were screened for both nucleotide (π) and haplotype (h) diversity (Nei 1987), respectively. Sequence divergence, between *B. damaranum*, *B. sp. 1* and *B. sp. 2*, was estimated in MEGA v.5.1 (Tamura et al. 2011) using the Tamura-Nei model of evolution. This model assumes equivalent substitution rates between both populations and takes into account differences in transitional and transversional substitutions (Nei & Kumar 2000). A spatial analysis of molecular variance (SAMOVA) was carried out to determine the clustering patterns of sampled sites for all *B. damaranum* and *B. sp. 1* individuals. This analysis takes the spatial location of each site into consideration and bases the clustering patterns on the number of groups ($K = 2 - 19$) that maximise the proportion of variance amongst groups (Dupanloup et al. 2002). To assess whether isolation by distance is present within and between habitat types (*B. damaranum* and *B. sp. 1*), a Mantel test (IBDSW v. 3.23; Jensen et al. 2005) was run using pairwise F_{ST} comparisons between individuals as well as between sites (10 000 permutations). A haplotype network was then constructed to assess the relationship between haplotypes (TCS; Clement et al. 2000).

Microsatellite analyses

Microsatellite data were screened for genotyping errors and the possible presence of null alleles (Microchecker 2.2.3; van Oosterhout et al. 2004), linkage disequilibrium and Hardy-Weinberg equilibrium (Genepop v 4.1.4; Raymond and Rousset 1995; Rousset 2008). General statistics (observed and expected heterozygosity, number of alleles) were calculated for each population representing each ecomorph (Genepop v 4.1.4; Raymond and Rousset 1995; Rousset 2008). Furthermore, F-statistics (F_{ST} and F_{IS}) were estimated between each site sampled, and by grouping

chameleons according to vegetation type (by ecomorph) using Genepop v 4.1.4 (Raymond and Rousset 1995; Rousset 2008), respectively. Parameters for the Markov Chain were set at default with 1000 de-memorization steps including 100 batches of 5000 iterations.

To determine the levels of genetic structure both within and between the two ecomorphs, a Bayesian individual-based clustering (IBC) method was implemented in Geneland v. 4.0.2 (Pritchard et al. 2000; Falush et al. 2003). This IBC method incorporates multi-locus genotype data to detect genetic structure under the assumptions of both Hardy-Weinberg and linkage equilibrium. This, along with the incorporation of spatial data, allows for fine-scale testing of genetic differentiation. The total number of clusters (K) was assumed to vary between 1 and 10, with 10 independent runs per K-value. Under the uncorrelated allele frequency model, runs included 500,000 iterations, extracting data (thinning) every 100th iterations. Both the maximum number of nuclei (300) and the maximum rate of Poisson processes (100) were left at their default values. All geographic coordinates were included into the analysis. Finally, levels of isolation by distance both within and between the two habitat types were estimated using IBDSW v. 3.23 (Jensen et al. 2005). Pairwise F_{ST} comparisons per individual as well as per site were incorporated. Given the limited number of inter-site comparisons within the forest and fynbos, isolation by distance was estimated in each habitat type using individual pairwise F_{ST} comparisons only.

To evaluate migration rates both within and between ecomorph populations a maximum-likelihood coalescence approach was implemented in Migrate-n v. 3.3.2 (Beerli & Felsenstein 1999). Using a combined dataset (microsatellites and mtDNA), default parameters with constant mutation rates among loci were implemented under the stepwise mutation model. Starting estimates based on F_{ST} calculations were used with a burn-in period of 20 000; number of short chains = 10 000 trees; and long chains = 100 000 trees. However, due to inconsistent results across multiple runs the analyses was removed from the study.

Results

Mitochondrial DNA analysis

The haplotype network, based on 841 bp of the ND4 mitochondrial marker, revealed two distinct clades separated by 8-9% sequence divergence (Figure 8). One of the clades (Clade A) comprised haplotypes from *B. sp. 2* only (green clade; Figure 8). The second clade (Clade B) can be further subdivided into two sub-clades, *B. damaranum* and *B. sp. 1*, separated by 12 mutational steps and 1.8% sequence divergence (Figure 8). No haplotypes were shared among any clades or sub-clades. Within each habitat type, the SAMOVA revealed structuring patterns consistent with geological progression and patterns of habitat fragmentation. For *B. sp. 1*, individuals sampled on the north-facing slopes of the Tsitsikamma Mountains (light red clade; Figure 8) and Kouga Mountains (dark red clade; Figure 8) clustered into two groups, respectively; while for *B. damaranum*, individuals from the Tsitsikamma (Light and intermediate blue clades; Figure 8) and Knysna (Dark blue clade; Figure 8) forests showed distinct clustering patterns. Analysis of molecular diversity indices for these two ecomorph clades revealed a greater nucleotide (π) and haplotype (h) diversity in *B. damaranum* (Table 7). Between habitat types, the Mantel test revealed no significant patterns of isolation by distance between individuals ($r = 0.24$; $p < 0.05$; Figure 9a) or between sites ($r = 0.24$; $p < 0.05$; Figure 9b). Similarly, no significant isolation by distance was found between individuals (forest: $r = 0.37$; $p > 0.05$; fynbos: $r = 0.42$; $p > 0.05$) or sites (forest: $r = 0.25$; $p > 0.05$; fynbos: $r = 0.58$; $p > 0.05$) within both vegetation types.

Microsatellite analyses

Across both habitat types, all twelve loci proved to be polymorphic with between 4 and 26 observed alleles per locus, per sample site (Table 8). The most polymorphic loci across all sample sites were Bth93 (mean = 14.14 alleles) and Bpu9 (mean = 12.71 alleles). No deviations from linkage equilibrium expectations as well as no evidence of null alleles, stuttering or large allele dropout were found. Tests for departures from HWE detected significant heterozygote deficiencies ($p <$

0.05) for six loci in two of the sample sites, namely Garden of Eden and Plaatbos. In global tests, pooling across sample sites revealed increasing numbers of departures from HWE, with an increasing proportion of the heterozygote deficiencies remaining significant after Bonferroni correction. These deviations were substantiated by the positive F_{IS} values found across all sample sites, indicative of patterns of inbreeding, with the highest occurring at both Garden of Eden and Plaatbos (Table 8). Across macrohabitats, F_{ST} values indicated significant differences between *B. damaranum* and *B. sp. 1* ($F_{ST} = 0.067$; $P < 0.001$). Within each macrohabitat, significant differences were found between all pairwise sample site comparisons, with the comparison between Bloukrans pass and Plaatbos being the only exception (Table 8).

The Bayesian clustering analysis using microsatellite loci estimated a total of four clusters across both vegetation types. Individuals from both *B. sp. 1* and *B. damaranum* were clustered separately; each with two clusters, respectively (Figure 9). For *B. sp. 1*: All sample sites on the north-facing slopes of the Tsitsikamma Mountains (Joubertina; Grootnek; Louterwater) were grouped together to form cluster 1, while individuals from the Kouga Mountains (Geelhoutbos) formed cluster 2. For *B. damaranum*: Individuals from Plaatbos and Bloukrans pass were grouped together, while individuals from Garden of Eden formed the last remaining cluster (Figure 9). Between habitat types, no significant patterns of isolation by distance were observed between individuals ($r = 0.27$, $p > 0.5$) or between sites ($r = 0.22$; $p > 0.05$). Significant correlations between genetic and geographic distance did, however, persist within both the fynbos ($r = 0.31$; $p < 0.001$; Figure 10a) and forest ($r = 0.55$; $p < 0.001$; Figure 10b) vegetation types.

Discussion

For chameleons from forest and fynbos vegetation types, the presence of genetic structuring (microsatellites and mtDNA) suggests that habitat plays a major role in shaping genetic diversity across the landscape. There is congruence between morphological forms and the strong genetic

structure found between *B. sp. 1* and *B. damaranum*. Within each habitat, both mtDNA and microsatellite analysis reveal similar structuring patterns. These patterns, however, are not governed by barriers to gene flow, but by isolation by distance. Furthermore, mtDNA data confirms that *B. sp. 2* is indeed highly divergent despite its morphological similarity to *B. sp. 1* (chapter 2).

Across southern Africa, dwarf chameleons show diversification patterns consistent with the major climatic and geological events during the mid-Miocene and Plio-Pleistocene periods (Tolley et al. 2006; Tolley et al. 2008). Along the South-western coastline, shifts in habitat type propagated *Bradypodion* to radiate into the newly established fynbos biome with the older lineages persisting in the preceding Afromontane forest habitats, now constrained to refugia along the southern coastline and Drakensberg escarpment (Tolley et al. 2006; Tolley et al. 2008). In response to varying local natural and sexual selection pressures, it is hypothesised that rapid morphological differentiation occurred between these recently diverged lineages; a phenomenon which is well-documented in a variety of species (e.g. Grant 1976; Schluter 1988; Losos et al. 1998; Burns et al. 2002; Albertson 2003; Young et al. 2009).

Indeed, sequence divergence estimates between *B. sp. 1* and *B. damaranum* indicate a recent divergence between the two lineages, consistent with that of the proposed divergence during the Plio-Pleistocene period, but lower than what is usually found between chameleon species (Tolley et al. 2006; Tolley et al. 2008; Tolley et al. 2011; Gehring et al. 2011). Regardless, the high levels of genetic structure found by the Bayesian and SAMOVA clustering analyses, coupled with the lack of any shared mtDNA haplotypes, suggests an absence of gene flow between the forest and fynbos habitat types. Under the ecological speciation model, such pre-zygotic isolation mechanisms may arise following divergent ecological selection pressures, caused by differences in habitat structure (Funk 1998; Via et al. 2000; Nosil 2004; Nosil, Vines & Funk 2005). These divergent pressures facilitate the functional optimisation of specific ecologically relevant traits to maximise an organism's relative fitness in a specific habitat type (Losos et al. 1990; Irschick & Losos 1998;

Herrel et al. 2011; Chapter 2). Consequently, given the chameleon's strong reliance on vegetation type (Tolley et al. 2006; Stuart-fox & Moussalli 2007; Hopkins & Tolley 2011; Herrel et al 2011; Measey 2011), this would suggest that in a given habitat, any immigrant specialised to a structurally different habitat type would experience a reduction in fitness (Mayr 1947; Nosil, Vines & Funk 2005). For instance, the larger hands and longer tail characteristic of *B. damaranum* allows for effective movement across the wider perches associated with the forest habitat type (Chapter 2). Therefore, in this specific microhabitat (substrate) *B. damaranum* has a selective advantage over *B. sp. 1*, which has smaller hands and a shorter tail (Chapter 2). This 'selection against immigrants' is a strong barrier to gene flow as migration may never occur between habitats, providing a platform for the evolution of other isolating mechanisms to govern the ecological speciation processes (reviewed in Rundle & Nosil 2005).

Such isolating mechanisms may include variation in sexual selection pressures, where divergent selection regimes impact dimorphic characters used for communication. Correlations between sexual signals and habitat structure can promote assortative mating patterns between habitats, impacting either male traits (e.g. predator pressures) or female preferences (e.g. signalling); both intrinsic barriers to gene flow (e.g. Ryan & Wilczynski 1991; Thorpe & Richard 2001). For example, on the one hand, *B. damaranum* males are expected to utilise their large body size, flamboyant coloration and ornamental characters (high casque and large gular scales) to attract a mate or to engage in competitive signalling (Endler 1980; Zuk & Kolluru 1998; Stuart-Fox & Ord 2004, Dolman & Stuart-Fox 2009; Chapter 2). By contrast, the fynbos habitat favours the more cryptic phenotype, as the cost of predator detection outweighs the benefit of reproductive performance in open habitats (Stuart-Fox & Ord 2004; Dolman & Stuart-Fox 2009). Communication in the form of signals is therefore selected against with any ensuing encounters mostly restricted to physical contests.

On a macrohabitat scale, historical patterns of habitat fragmentation are prevalent within both the forest and fynbos habitat types. This can inevitably create ecological barriers to gene flow across both species ranges (Capula 1996; Templeton et al. 2001; Williams, Brawn & Paige 2003). Indeed both the Bayesian and SAMOVA clustering analyses revealed similar patterns of population structuring in both habitat types. *B. damaranum* clustering was restricted to the fragmented patches of the Tsitsikamma (South-facing slopes) and Knysna forests, while *B. sp. 1* clustering was confined to the isolated mountain fynbos patches along the Kouga and the north-facing slopes of the Tsitsikamma Mountains. Consequently, these patterns of structuring, possibly caused by habitat fragmentation and loss, may disrupt the gene flow – genetic drift equilibrium on a local scale, potentially facilitating patterns of isolation-by-distance within habitat types (e.g. Hutchison & Templeton 1999; Keller & Largiadèr 2003; Willi et al. 2007). Such patterns of isolation by distance were prevalent in both the fynbos and forest habitats, as indicated by microsatellite analysis. Interestingly, no isolation by distance was observed in mtDNA analysis. This lack of congruence between marker types may be due to the nature of microsatellites, where their higher resolution allows for more fine scale inferences regarding population structure and gene flow (Bossart & Prowell 1998; Balloux & Lugon-Moulin 2002). Alternatively, the lack of congruence may be due to alternate sampling schemes for each marker type. For microsatellite analyses, the frequency of sites sampled were few and spread widely, aimed for population based evaluation, while for mtDNA analyses the number of sites sampled closely represents the total distribution of both *B. damaranum* and *B. sp. 1* ranges. This may create a situation where the effects of genetic drift seem stronger between the more widely distributed sampling schemes. Nevertheless, microsatellite analysis revealed a greater degree of genetic differentiation per unit distance in *B. sp. 1*. This may be indicative of the fire prone nature of the fynbos habitat, where large-scale demographic crashes, as a result of natural fires, could severely affect chameleon populations (M. Burger & A. A. Turner pers. comm.). Populations that undergo these extreme fluctuations are probably prone to repeated bottleneck and founder events, which can result in higher genetic differentiation between

populations over shorter geographic distances. This would also account for the lower nucleotide and haplotype diversities observed in the fynbos habitat. The elevated levels of genetic drift within both habitats may, however, be indicative of the pronounced levels of inbreeding, by virtue of the elevated positive F_{IS} estimates per population sampled, inevitably resulting in the significant heterozygote deficiency observed among loci. Although these levels are not necessarily detrimental, drift can outweigh natural selection in sufficiently small populations, leading to a loss of adaptive genetic variation and aid to the accumulation of deleterious alleles (Lande 1988; Lynch & Gabriel 1990; Lynch, Conery & Bürger 1995). This coupled with the increase in direct (e.g. urban expansion, agriculture) and indirect (e.g. invasive species, inappropriate management) anthropogenic pressures, which have caused severe habitat loss across both ranges (e.g. Rebelo 1992; Cushman 2006) can further limit gene flow between these isolated patches, providing a platform for the evolution of such non-ecological progressions.

In conclusion, the divergent nature of the selective regimes between the fynbos and forest habitat types (Chapter 2), coupled with the chameleon's strong reliance on vegetation type (Tolley et al. 2006; Stuart-fox & Moussalli 2007; Hopkins & Tolley 2011; Herrel et al 2011; Measey 2011), facilitated a process of natural selection against immigrants, where the functional optimisation of ecologically relevant traits in one habitat type reduces relative fitness in another, creating a strong extrinsic barrier to gene flow. This lack of migration allows for other mechanisms, such as sexual selection and drift, to isolate populations further, aiding in the ecological speciation process. Within each habitat, however, severe habitat fragmentation and loss negate the levels of population structuring observed, with patterns of isolation by distance governing gene flow.

Chapter 4: Conclusion

An adaptive radiation arises when lineages diversify into an array of phenotypic forms in response to divergences in habitat use, morphology and communication (Streelman & Danley 2003). In accordance with this concept, the present study provides empirical evidence of a progressive adaptive divergence in morphology between two recently diverged Dwarf Chameleon lineages, *B. damaranum* and *B. sp. 1*, residing in forest and adjacent fynbos habitats, respectively. Furthermore, fine scale genetic analyses suggests that adaptation of ecologically relevant traits to each habitat type has created an extrinsic barrier to gene flow between the two lineages, which is possibly reinforced by the development of intrinsic barriers such as differing sexual selection pressures. Within each macrohabitat, however, the levels of structuring observed are not governed by barriers to gene flow, but rather by patterns of isolation by distance.

Differences in habitat structure, an important component during the initial stages of the radiation process, were explored by examining the habitat relevant to chameleons (i.e. perch diameter). A significant difference in this microhabitat was identified, with the fynbos habitat having narrower perches on average. This facilitated divergence in chameleon habitat utilisation in each vegetation type, with *B. damaranum* selecting perches at random and *B. sp. 1* selecting perches wider than those randomly available. Given the chameleon's strong reliance on vegetation type (Tolley et al. 2006; Stuart-Fox & Moussalli 2007; Tolley & Burger 2007; Measey et al. 2009), the differences in habitat structure and use imposed selection pressures on ecologically relevant traits specific to each habitat type, particularly those related to locomotion (limb length; tail length) and bite force (head width). To associate such divergences in morphology with the differences in habitat use, however, selection must facilitate the functional optimization of these ecologically relevant traits. Indeed, both gripping forces (hand and tail) and sprint speed differed with *B. damaranum* being faster and stronger on average, respectively. This suggests the presence of alternate selection regimes operating in each habitat type and that selection on morphology is associated with differences in performance.

Furthermore, these functional differences would suggest that, in a given habitat, any immigrant specialised to an alternate habitat should experience a reduction in fitness (Mayr 1947; Nosil, Vines & Funk 2005). For instance, only *B. damaranum* showed significant correlations between gripping force and morphology on the narrow dowel used. The dowel diameter, however, resembled the perch diameters utilised by *B. damaranum* more closely, placing *B. sp. 1* at a selective disadvantage for gripping in this forest habitat type. These patterns of ‘selection against immigrants’ across macrohabitats facilitated the strong genetic structure observed between *B. damaranum* and *B. sp. 1*. Known to be a strong extrinsic barrier, patterns of migration may be completely restricted between habitat types, allowing for the evolution of intrinsic mechanisms to further govern the ecological speciation processes (reviewed in Rundle & Nosil 2005).

Such intrinsic mechanisms may include differences in sexual selection pressures, where characters associated with communication diverge. Enabled by the alternate selection regimes operating in each habitat type, this may create an intrinsic barrier to gene flow between *B. damaranum* and *B. sp. 1*, given the known effects that assortative mating patterns have on reinforcing reproductive isolation (e.g. Ryan & Wilczynski 1991; Thorpe & Richard 2001). On the one hand, the forest habitat type, which offers some relief from predator detection, facilitates strong sexual dimorphism where selection acts on the development of secondary sexual characteristics, used for competitive signalling or as a mate attractant (Endler 1980; Zuk & Kolluru 1998; Stuart-Fox & Ord 2004, Dolman & Stuart-Fox 2009). In male *B. damaranum*, this includes enlarged body size, flamboyant coloration and strongly developed ornaments such as a high casque and large gular scales. By contrast, in the fynbos habitat, where sexual dimorphism is weak, selection acts towards a more cryptic phenotype, as the cost of predator detection outweighs the benefit of reproductive performance in open habitats (Stuart-Fox & Ord 2004; Dolman & Stuart-Fox 2009). Communication in the form of signals is therefore selected against, restricting any subsequent encounters to physical contests. Consequently, male investment in a wider head in this habitat, an

indicator of bite force in *Bradypodion* (Measey et al. 2009), may provide the necessary weapon required for such encounters.

In conclusion, the patterns of morphological divergence described in this study are consistent with the general framework describing an adaptive radiation (Streelman & Danley 2003). This specialisation to each habitat type has created an effective barrier to gene flow between habitats, consistent with a true allopatric diversification. Furthermore, the patterns of phenotypic change described for both *B. damaranum* and *B. sp. 1* resemble those previously described in their allopatric neighbour, *B. pumilum*, closely, suggesting not only the presence of distinct ecomorphological forms but the occurrence of independent repeated evolution of phenotypes within the *Bradypodion* lineage.

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Addenda

Addendum 1: Descriptive statistics (in millimetres) for all morphometric characters used for female *B. sp. 1*.

Character	Mean	Variance	Standard Deviation	Minimum	Maximum
SVL	59.22	38.41	6.20	47.76	69.77
TL	41.98	15.57	3.94	36.09	52.12
AHL	13.89	0.84	0.91	12.04	15.59
KHL	16.96	1.44	1.20	14.35	20.04
HW	5.79	0.45	0.67	4.66	7.21
HW2	7.94	0.41	0.64	6.66	8.95
KHH	6.17	0.19	0.43	5.32	6.99
AHH	7.59	0.34	0.59	6.44	8.70
LJL	12.61	0.87	0.92	10.35	14.26
CT	9.58	0.63	0.79	7.88	10.79
QT	11.64	0.99	0.99	9.56	13.86
KCH	10.88	0.97	0.98	7.98	11.65
ACH	5.65	0.31	0.55	4.60	6.49
FM	10.51	1.38	1.17	8.06	12.95
TB	9.02	1.35	1.17	6.67	10.89
MF	3.38	0.27	0.52	2.27	4.23
LF	4.41	0.35	0.59	3.30	5.87
HM	10.92	1.89	1.37	8.61	13.99
RD	9.12	1.40	1.18	6.36	11.14
MH	3.16	0.35	0.59	2.02	4.56
LH	4.39	0.48	0.69	2.90	5.81

Addendum 2: Descriptive statistics (in millimetres) for all morphometric characters used for male *B. sp. 1*.

Character	Mean	Variance	Standard Deviation	Minimum	Maximum
SVL	51.95	21.15	4.60	39.91	59.31
TL	42.52	23.42	4.84	30.25	52.89
AHL	13.29	0.97	0.99	10.62	14.92
KHL	16.07	1.50	1.22	13.13	18.32
HW	5.36	0.12	0.33	4.80	5.99
HW2	7.44	0.23	0.48	6.08	8.28
KHH	5.92	0.21	0.45	4.79	6.67
AHH	7.29	0.60	0.78	5.15	8.84
LJL	11.84	1.14	1.07	8.90	13.82
CT	9.16	0.63	0.79	6.89	10.54
QT	10.98	1.11	1.06	8.50	12.80
KCH	9.48	0.99	0.99	6.70	11.01
ACH	5.33	0.41	0.64	4.22	6.52
FM	9.33	1.32	1.16	6.48	11.22
TB	8.18	1.02	1.02	5.89	10.42
MF	3.05	0.15	0.38	2.42	3.89
LF	4.02	0.30	0.54	3.09	5.26
HM	9.57	0.76	0.87	8.08	11.16
RD	8.37	0.89	0.94	6.17	10.46
MH	3.01	0.28	0.53	2.24	4.48
LH	3.98	0.23	0.48	3.16	4.86

Addendum 3: Basic descriptive (in millimetres) statistics for all morphometric characters used for female *B. sp. 2*.

Character	Mean	Variance	Standard Deviation	Minimum	Maximum
SVL	63.66	18.65	4.31	55.11	70.42
TL	45.09	8.33	2.87	40.78	49.88
AHL	14.43	0.58	0.76	13.20	15.90
KHL	17.77	0.65	0.80	16.25	19.03
HW	6.41	0.05	0.21	6.05	6.70
HW2	8.26	0.47	0.68	7.15	9.15
KHH	6.47	0.14	0.38	5.93	7.24
AHH	8.14	0.46	0.68	7.16	9.24
LJL	13.49	0.28	0.52	12.28	14.24
CT	10.81	0.57	0.76	8.91	11.14
QT	12.63	0.41	0.65	11.66	13.74
KCH	10.55	0.74	0.87	9.55	12.39
ACH	6.28	0.94	0.70	5.30	7.32
FM	11.61	1.57	1.25	8.72	13.54
TB	10.21	0.49	0.70	8.85	11.23
MF	4.02	0.53	0.73	3.01	5.82
LF	5.53	0.42	0.65	4.65	6.81
HM	11.71	1.56	1.25	10.07	14.29
RD	10.57	0.91	0.94	9.04	12.08
MH	3.37	0.13	0.36	2.88	3.93
LH	4.52	0.52	0.72	2.90	5.57

Addendum 4: Basic descriptive (in millimetres) statistics for all morphometric characters used for male *B. sp. 2*.

Character	Mean	Variance	Standard Deviation	Minimum	Maximum
SVL	53.41	26.78	5.17	46.84	60.18
TL	42.57	40.69	6.37	36.49	52.35
AHL	13.42	0.59	0.76	12.58	14.49
KHL	16.42	0.79	0.89	15.18	17.66
HW	5.87	0.19	0.44	5.19	6.46
HW2	7.72	0.11	0.34	7.20	8.13
KHH	6.40	0.10	0.32	5.98	6.82
AHH	7.42	0.39	0.63	6.46	8.11
LJL	12.64	0.73	0.86	11.42	13.28
CT	8.96	0.88	0.93	7.38	9.90
QT	11.27	0.48	0.69	10.42	12.06
KCH	9.76	0.67	0.82	8.89	10.85
ACH	5.72	0.41	0.64	4.87	6.58
FM	9.87	1.25	1.12	8.60	11.11
TB	8.51	1.31	1.13	6.77	9.63
MF	3.22	0.35	0.59	2.66	3.99
LF	4.77	0.21	0.46	4.41	5.70
HM	10.27	1.52	1.24	8.28	11.28
RD	9.22	0.67	0.81	8.33	10.33
MH	3.00	0.27	0.52	2.50	3.78
LH	4.64	0.33	0.58	3.99	5.41

Addendum 5: Descriptive statistics (in millimetres) for all morphometric characters used for female *B. damaranum*.

Character	Mean	Variance	Standard Deviation	Minimum	Maximum
SVL	57.40	16.26	12.75	41.82	82.46
TL	64.39	23.35	15.28	46.54	90.23
AHL	14.58	8.28	2.87	11.02	20.78
KHL	19.84	15.69	3.96	14.65	26.88
HW	6.05	1.52	1.23	4.26	8.10
HW2	8.24	2.27	1.49	6.38	11.30
KHH	6.53	0.98	0.98	5.20	8.28
AHH	8.07	1.99	1.41	6.54	10.58
LJL	13.58	6.02	2.45	10.71	18.76
CT	10.05	3.53	1.87	7.55	13.39
QT	12.14	4.60	2.14	9.50	16.19
KCH	12.22	8.06	2.83	9.48	17.77
ACH	6.88	2.17	1.47	5.31	10.01
FM	10.17	6.75	2.59	7.25	14.92
TB	9.28	5.13	2.26	6.31	13.76
MF	3.91	1.74	1.35	1.26	5.37
LF	5.36	1.82	1.41	1.90	6.62
HM	10.52	2.03	2.79	2.90	8.20
RD	9.13	7.79	2.07	6.74	15.86
MH	3.68	4.29	1.16	6.12	12.47
LH	5.13	1.35	1.61	2.19	5.77

Addendum 6: Descriptive statistics (in millimetres) for all morphometric characters used for male*B. damaranum*.

Character	Mean	Variance	Standard Deviation	Minimum	Maximum
SVL	67.02	16.67	8.16	53.26	83.60
TL	84.71	20.47	14.40	62.63	117.30
AHL	17.31	3.17	1.78	14.79	20.46
KHL	23.81	7.02	2.65	19.44	28.40
HW	6.71	0.71	0.83	4.97	8.10
HW2	9.88	1.13	1.06	7.79	11.48
KHH	7.67	0.72	0.84	6.26	9.40
AHH	9.43	1.12	1.06	7.54	11.60
LJL	15.88	2.74	1.65	13.31	19.20
CT	11.67	1.20	1.09	9.22	14.00
QT	14.49	2.98	1.72	11.18	17.90
KCH	15.30	4.27	2.06	11.46	18.91
ACH	8.62	1.31	1.14	6.31	10.52
FM	12.38	4.13	2.03	9.05	16.50
TB	11.07	2.81	1.67	7.94	14.70
MF	4.92	0.96	0.98	3.24	6.40
LF	6.47	1.27	1.12	3.45	8.00
HM	12.22	3.05	1.75	9.24	16.30
RD	11.41	2.08	1.44	8.78	13.60
MH	4.96	1.36	1.17	2.01	7.05
LH	6.34	1.18	1.08	4.40	8.33

Addendum 7: Descriptive statistics (in millimetres) for the random perch diameters (RPDs) measured at each site in the fynbos habitat type.

Site	Mean	Minimum	Maximum	Variance	Standard Deviation
Geelhoutbos	1.30	0.16	3.61	0.67	0.82
Grootnek	1.52	0.24	3.84	0.57	0.75
Joubertina	1.49	0.15	3.09	0.44	0.66
Louterwater	1.42	0.17	4.10	0.87	0.93
Bosrug	1.02	0.09	2.86	0.39	0.62

Addendum 8: Descriptive statistics (in millimetres) for the random perch diameters measured at each site in the forest habitat type.

Site	Height (m)	Mean	Minimum	Maximum	Variance	Standard Deviation
Garden of Eden	1.60	2.4480	0.20	5.56	1.370	1.1705
	2.25	2.7095	0.21	6.45	2.469	1.5714
Plaatbos	1.60	2.6841	0.38	6.25	1.867	1.3663
	2.25	3.2801	0.03	8.49	4.340	2.0832
	3.50	2.8778	0.72	5.71	1.633	1.2777

Addendum 9: Descriptive statistics (in millimetres) for perches chosen by each lineage in their respective habitat type (*B. sp. 1* and *B. sp. 2* in the fynbos; *B. damaranum* in the forest).

Lineage	Sex	Mean	Minimum	Maximum	Variance	Standard Deviation
<i>B. sp. 1</i>	Female	2.47	1.25	3.97	0.67	0.82
	Male	2.76	1.15	4.43	0.72	0.85
<i>B. sp. 2</i>	Female	3.96	1.37	8.90	4.72	2.17
	Male	3.55	2.20	5.00	1.21	1.10
<i>B. damaranum</i>	Female	1.96	0.75	3.20	0.64	0.80
	Male	2.19	0.82	3.99	0.55	0.74

Addendum 10: Descriptive statistics (in Newton's) for both hand and tail grip strength for *B. damaranum*, *B. sp. 1* and *B. sp. 2* on the narrow (5 mm) and broad (10 mm) dowel diam

Lineage	Grip Trait	Dowel	Mean	Variance	Standard Deviation	Minimum	Maximum
<i>B. damaranum</i>	Hand	Narrow	1.44	0.29	0.54	0.74	2.39
		Broad	1.91	0.01	0.09	0.06	0.44
	Tail	Narrow	1.80	0.35	0.59	0.91	3.40
		Broad	2.04	0.40	0.63	1.04	3.31
<i>B. sp. 1</i>	Hand	Narrow	0.74	0.04	0.18	0.54	0.98
		Broad	0.11	0.01	0.07	0.06	0.30
	Tail	Narrow	1.34	0.21	0.46	0.54	1.88
		Broad	1.19	0.13	0.36	0.75	1.71
<i>B. sp. 2</i>	Hand	Narrow	0.58	0.14	0.12	0.43	0.77
		Broad	0.05	0.01	0.03	0.02	0.16
	Tail	Narrow	1.12	0.16	0.41	0.50	1.85
		Broad	0.71	0.06	0.25	0.33	1.37

Addendum 11: Descriptive statistics (in centimetres per second) for sprint speed for each respective lineage.

Lineage	Mean	Minimum	Maximum	Variance	Standard Deviation
<i>B. sp. 1</i>	8.87	5.43	15.67	3.73	1.92
<i>B. sp. 2</i>	8.59	5.24	13.23	4.20	2.05
<i>B. damaranum</i>	13.08	5.81	25.00	15.49	3.93

Tables

Table 1: Principal component (PC) loadings for all variables (scaled to lower jaw length) for *B. damaranum*, *B. sp. 1* and *B. sp. 2* (separated by sex), with the percentage variation for each component explained. The correlations in bold represent the principal components which differed significantly between ecomorphs. Variables not included indicated by dash.

Trait	Females			Males			
	Principal Components			Principal Components			
	1	2	3	1	2	3	4
AHL	-	-	-	0.52	0.44	0.36	0.20
KHL	0.79	0.14	0.19	0.85	0.18	0.15	0.11
HW	-0.02	0.76	-0.04	0.11	0.04	0.01	0.93
HW2	0.03	0.79	0.18	-	-	-	-
AHH	0.14	0.05	0.85	0.29	0.28	0.68	-0.17
KHH	0.17	0.20	0.82	0.11	-0.08	0.90	0.14
QT	-	-	-	0.17	0.84	-0.04	-0.21
CT	0.12	0.67	0.16	0.08	0.78	0.15	0.34
KCH	0.85	0.17	0.10	0.83	0.28	0.16	-0.11
ACH	0.82	-0.17	0.11	0.86	-0.06	0.12	0.13
% Variance	33.74	20.29	12.74	39.65	13.66	12.09	11.16

Table 2: Principal component (PC) loadings for all body variables (scaled to snout-vent length) in *B. damaranum*, *B. sp. 1* and *B. sp. 2* (separated by sex), with the percentage variation for each component explained. Correlations in bold represent the principal components which differed significantly between ecomorphs.

Trait	Females		Males	
	Principal Components		Principal Components	
	1	2	1	2
TL	0.78	0.09	0.76	0.08
FM	-0.18	0.83	-0.02	0.85
TB	0.34	0.69	0.08	0.73
MH	0.76	0.23	0.65	0.03
LH	0.71	0.05	0.81	0.02
HM	0.05	0.76	0.12	0.71
RD	0.23	0.55	0.46	0.53
MF	0.76	0.09	0.58	0.17
LF	0.77	0.07	0.70	0.11
% Variance	37.34	19.45	34.39	18.60

Table 3: The analysis of variance (MANOVA), mean differences as well as the multiple comparisons (t-test) between species for each principal component. F- and P-values are given for the principal components showing significance between morphs. NS, not significant. (1), (2) and (3) represent *B. damaranum*, *B. sp. 1* and *B. sp. 2* respectively.

Sex	Principal Component	MANOVA		Mean difference (mm) \pm SD			Multiple comparisons (LSD)		
		F	P	(1) – (2)	(2) – (3)	(1) – (3)	(1) – (2)	(2) – (3)	(1) – (3)
<i>Body allometry (SVL)</i>									
Female	PC1	41.86	< 0.001	1.71 \pm 0.18	-0.40 \pm 0.22	1.31 \pm 0.25	< 0.001	NS	< 0.001
	PC2	0.14	NS	0.14 \pm 0.27	-0.47 \pm 0.33	-0.32 \pm 0.37	NS	NS	NS
Male	PC1	10.63	< 0.001	1.05 \pm 0.21	-0.60 \pm 0.38	0.45 \pm 0.37	< 0.001	NS	NS
	PC2	0.8	NS	0.07 \pm 0.26	-0.57 \pm 0.46	-0.50 \pm 0.45	NS	NS	NS
<i>Head allometry (LJL)</i>									
Female	PC1	37.9	< 0.001	1.62 \pm 0.20	0.15 \pm 0.23	1.76 \pm 0.26	< 0.001	NS	< 0.001
	PC2	1.97	NS	-0.58 \pm 0.30	0.11 \pm 0.35	-0.48 \pm 0.38	NS	NS	NS
	PC3	2.9	NS	-0.65 \pm 0.27	0.17 \pm 0.32	-0.49 \pm 0.35	NS	NS	NS
Male	PC1	14.08	< 0.001	0.90 \pm 0.21	0.70 \pm 0.37	1.60 \pm 0.36	< 0.001	NS	< 0.001
	PC2	6.72	< 0.01	-0.47 \pm 0.24	1.42 \pm 0.41	0.96 \pm 0.40	NS	< 0.01	< 0.01
	PC3	0.9	NS	-0.36 \pm 0.27	0.07 \pm 0.45	-0.28 \pm 0.44	NS	NS	NS
	PC4	0.59	NS	0.26 \pm 0.28	-0.38 \pm 0.48	-0.12 \pm 0.47	NS	NS	NS

Table 4: Comparisons between random perch diameters (RPD's) of sampled localities in the fynbos habitat type. Geelhoutbos, Grootnek, Joubertina and Louterwater represent the localities for *B. sp. 1*, while Bosrug represents the locality for *B. sp. 2*. Values below the diagonal represent the Z-statistics while the values above the diagonal represent the ensuing probability values.

	Geelhoutbos	Grootnek	Joubertina	Louterwater	Bosrug
Geelhoutbos	-	0.001	0.001	0.321	P < 0.001
Grootnek	-3.396	-	0.764	0.318	P < 0.001
Joubertina	-3.234	-0.764	-	0.086	P < 0.001
Louterwater	-0.922	-1.717	-2.037	-	P < 0.001
Bosrug	-3.946	-6.993	-7.031	-3.763	-

Table 5: Interspecific comparisons between the hand and grip strengths on two different dowel sizes (broad: 10 mm; narrow: 5 mm) using an analysis of covariance (ANCOVA) which incorporates the best trait correlated with performance as the covariate (MH). P-values are given for significant relationships only, NS is non-significant.

Dowel Size (Hand/tail)	Comparison	ANCOVA	
		F	P
Broad (Hand)	<i>B. sp. 1</i> vs. <i>B. sp. 2</i>	9.01	< 0.01
	<i>B. sp. 1</i> vs. <i>B. damaranum</i>	7.62	< 0.05
	<i>B. sp. 2</i> vs. <i>B. damaranum</i>	21.71	< 0.001
Narrow (Hand)	<i>B. sp. 1</i> vs. <i>B. sp. 2</i>	3.96	NS
	<i>B. sp. 1</i> vs. <i>B. damaranum</i>	3.09	NS
	<i>B. sp. 2</i> vs. <i>B. damaranum</i>	12.35	0.001
Broad (Tail)	<i>B. sp. 1</i> vs. <i>B. sp. 2</i>	15.12	0.001
	<i>B. sp. 1</i> vs. <i>B. damaranum</i>	1.12	NS
	<i>B. sp. 2</i> vs. <i>B. damaranum</i>	13.96	0.001
Narrow (Tail)	<i>B. sp. 1</i> vs. <i>B. sp. 2</i>	3.96	NS
	<i>B. sp. 1</i> vs. <i>B. damaranum</i>	0.15	NS
	<i>B. sp. 2</i> vs. <i>B. damaranum</i>	0.40	NS

Table 6: Summary of microsatellite and mitochondrial markers used in this study.

Locus	Species	Repeat Motif	Primer sequence (5'-3')	Allele Size	Label
<i>Microsatellites</i>					
Bth10	<i>Bradypodion thamnobates</i>	(TC) ₃ (AC) ₅ (TC) ₁₄ (AC) ₂₆	F: TGG AGT AGA GAC TGC GCT TG R: TGT GGA TAC CCA TTT CAC CA	120-228	HEX
Bth76	<i>Bradypodion thamnobates</i>	(ATAG) ₃₇	F: TTG TGG TTA GAG GGG CAT TG R: CCC CAA TCT CGT TGT TCT GT	100-233	HEX
Bth93	<i>Bradypodion thamnobates</i>	(ATAG) ₂₄	F: AAG GGC ACA TCA CTG AAT CC R: CGC CAG AGA TGA TGG AAT TT	87-227	6-FAM
Bme58	<i>Bradypodion melanocephalum</i>	(AG) ₃₂	F: TTG AAG CAA TGC ACA CAC AC R: GCA CCG GTT CTT TAG CTT TG	135-208	HEX
Bme128	<i>Bradypodion melanocephalum</i>	(AC) ₂₈	F: TCT GTT CTG TTG CTT TTC CTC R: CCC CAA TGA TCT CTC AAT GT	135-195	6-FAM
Bme176	<i>Bradypodion melanocephalum</i>	(TATG) ₁₉	F: TCC TGA CTG ACG GTC GAA TA R: TGC ACC TCT CTT AAC AGC TTA CA	146-287	NED
Bpu9	<i>Bradypodion pumilum</i>	(TATG) ₁₉ (TAAG) ₇	F: GGG AAT AGT GTG GTG CTT GC R: CAG CAA CCA CTG GAG AGA CA	122-297	PET
Bpu113	<i>Bradypodion pumilum</i>	(TATC) ₂₀	F: AAT TTT GTT GTT CCC GCA AG R: ACA CAA CCG AGG CTC AAC TC	114-241	NED
Bpu115	<i>Bradypodion pumilum</i>	(TAGA) ₁₄	F: GCT GTG ATA TGT AAA TTC AGG G R: CAC TTT GTT TTG GTC TCC CAC T	119-239	PET
Bpu507	<i>Bradypodion pumilum</i>	(TG) ₂₃	F: AAT CCC TCA CCT TCA CAT GC R: CCA GGT TCA AAA TCC CAT CA	187-231	6-FAM
Bpu26	<i>Bradypodion pumilum</i>	(TTAC) ₂₆	F: TGA AAT CTC GCT ATC CTT GT R: CTT TCG AGT AAG GGA GAC CT	128-263	HEX
Bpu132	<i>Bradypodion pumilum</i>	(TATG) ₂₇	F: CGC TAT TTC CCC TCA AAA TC R: TGG CTC CAT ATA GCA ACA CG	158-259	6-FAM
<i>Mitochondrial DNA</i>					
ND4	<i>Bradypodion pumilum</i>		F: TGA CTA CCA AAA GCT CAT GTA R: CAT TAC TTT TAC TTG GAT TTG C		

Table 7: Nucleotide and haplotype diversities (\pm SD) of the *B. sp. 1* and *B. damaranum* clades.

Ecomorph	Nucleotide diversity (π)	Haplotype diversity (h)
<i>B. sp. 1</i>	0.0031 \pm 0.0019	0.784 \pm 0.065
<i>B. damaranum</i>	0.0041 \pm 0.0024	0.872 \pm 0.042

Table 8: Descriptive statistics of each sample population (N) of *B. sp. 1* and *B. damaranum*.

Included are number of alleles (N_A); observed (H_O) and expected (H_E) heterozygosity; inbreeding coefficient (F_{IS}) as well as pairwise F_{ST} (above diagonal) and Nei's genetic distance (below diagonal) estimates. The localities include: (GHB) Geelhoutbos; (JB) Joubertina; (GN) Grootnek; (LW) Louterwater; (GOE) Garden of Eden; (BP) Bloukrans pass and (P) Plaatbos.

Site	N	N_A	H_O	H_E	F_{IS}	Pairwise F_{ST} /Nei's genetic distance						
						GHB	JB	GN	LW	GOE	BP	P
GHB	16	9.42	0.69	0.78	0.14	-	0.081	0.083	0.091	0.121	0.13	0.132
JB	18	10.17	0.69	0.76	0.09	0.082	-	0.021	0.015	0.127	0.106	0.095
GN	4	4.17	0.63	0.64	0.13	0.084	0.029*	-	0.025	0.118	0.144	0.137
LW	20	10	0.76	0.79	0.05	0.09	0.017	0.032*	-	0.115	0.089	0.092
GOE	40	18.5	0.75	0.88	0.17	0.118	0.123	0.129	0.11	-	0.025	0.053
BP	5	7.33	0.83	0.82	0.09	0.129	0.111	0.14	0.095	0.02	-	0
P	28	18	0.73	0.86	0.17	0.129	0.094	0.135	0.088	0.045	0.022**	-

All F_{ST} and F_{IS} values are significantly different from 0 ($P < 0.001$) except those marked with * ($P < 0.05$) and ** (NS)

Figures

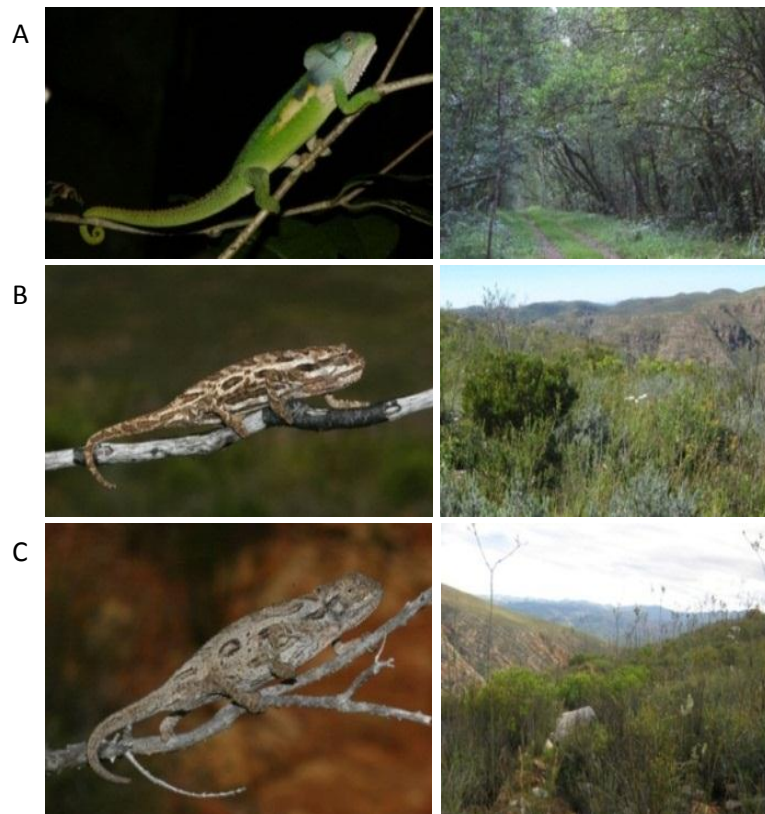


Figure 1: (A), an example of a closed habitat ecomorph (left) and its associated habitat type (forest). (B), (C) are open habitat ecomorphs *B. sp. 1* and *B. sp. 2* (left) and their associated habitat types (fynbos), respectively.

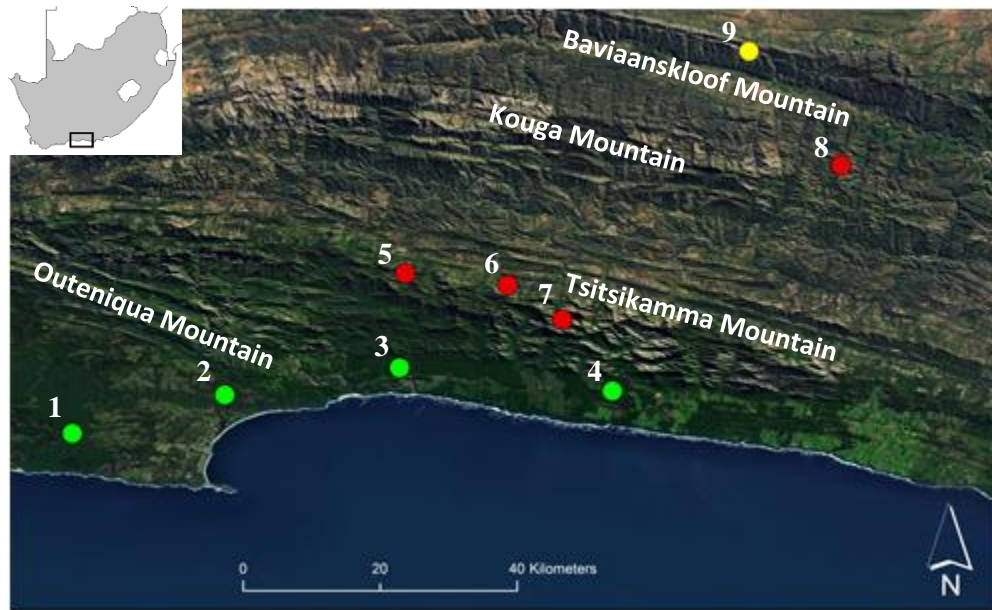


Figure 2: Localities of individuals sampled from *B. damaranum* (green), *B. sp. 1* (red) and *B. sp. 2* (yellow) for morphometric analysis. (1) Garden of Eden; (2) Keurbooms; (3) Bloukrans pass; (4) Plaatbos; (5) Louterwater; (6) Grootnek; (7) Joubertina; (8) Geelhoutbos; (9) Bosrug.

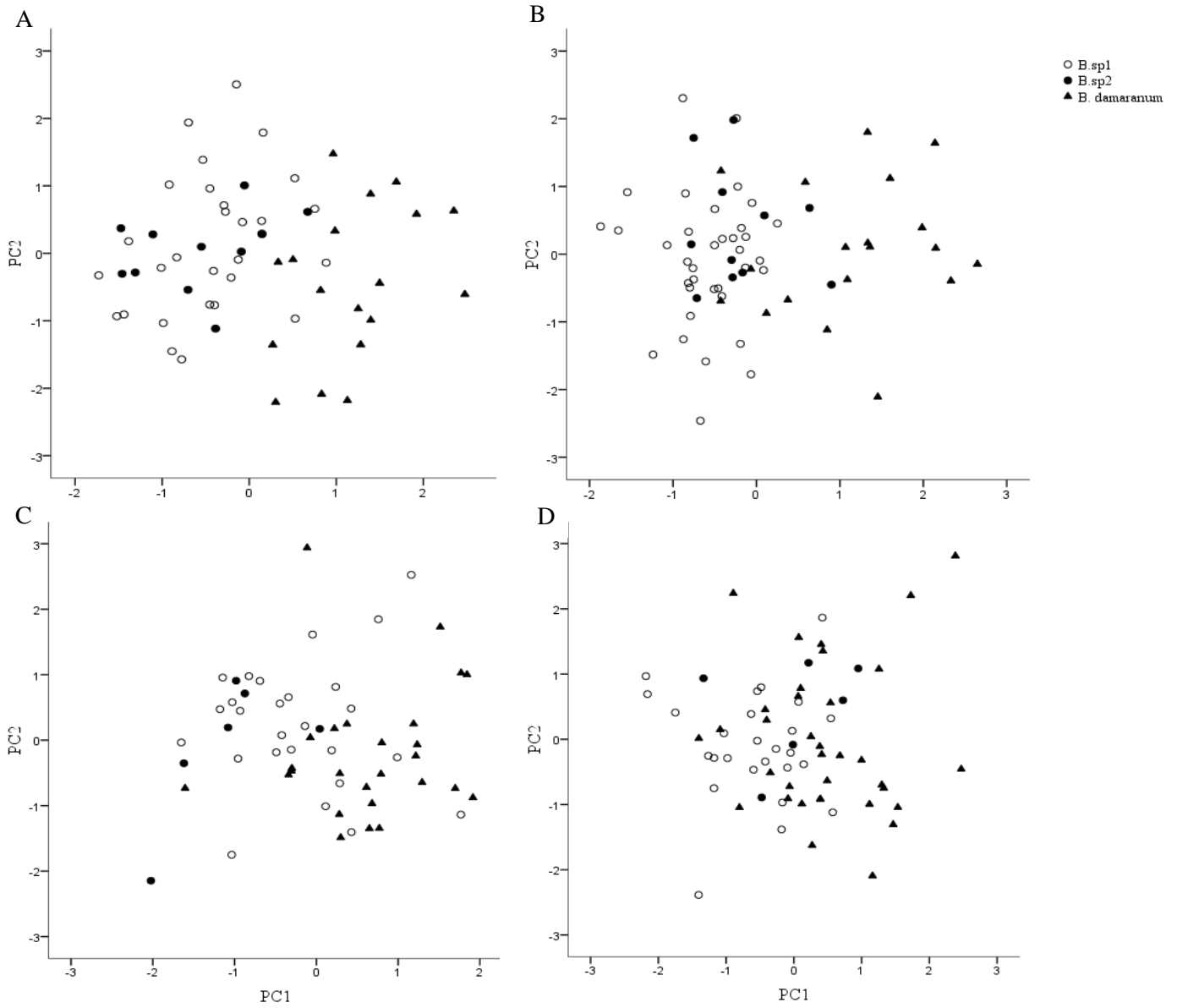


Figure 3: Scatterplots of the principal components that showed significant differences between species (separated by sex). (A) and (B) represent the relationship between PC1 and PC2 for the head and body allometries of females, respectively. Similarly, (C) and (D) represent the relationship between PC1 and PC2 for the head and body allometries of males, respectively.

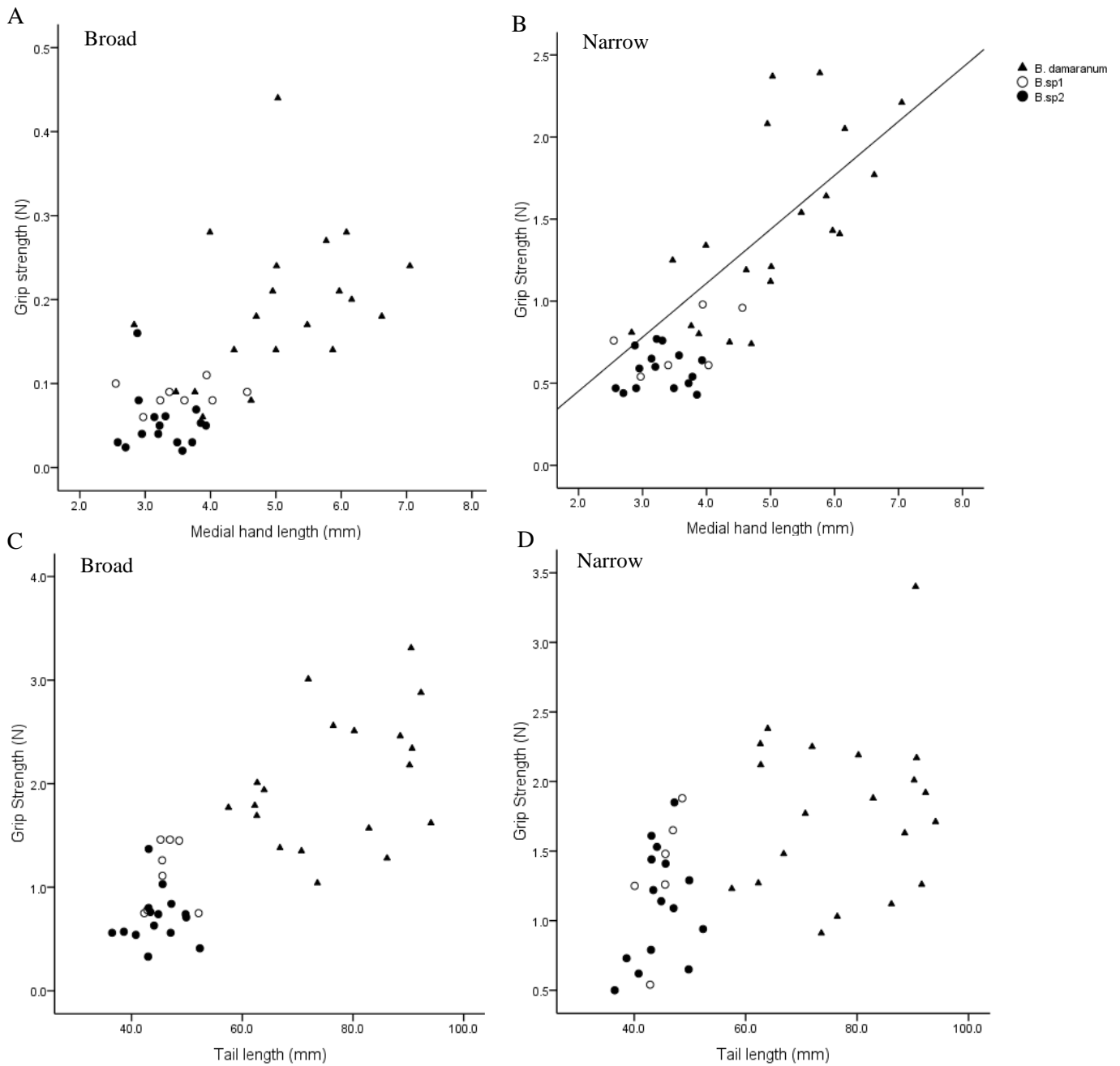


Figure 4: The relationship between grip strength and morphology on different sized dowels. Hand grip strength on the broad and narrow dowels is depicted in (A) and (B), while tail grip strength on the broad and a narrow dowel is depicted in (C) and (D), respectively. Hand grip strength was only correlated for *B. damaranum* on the narrow dowel.

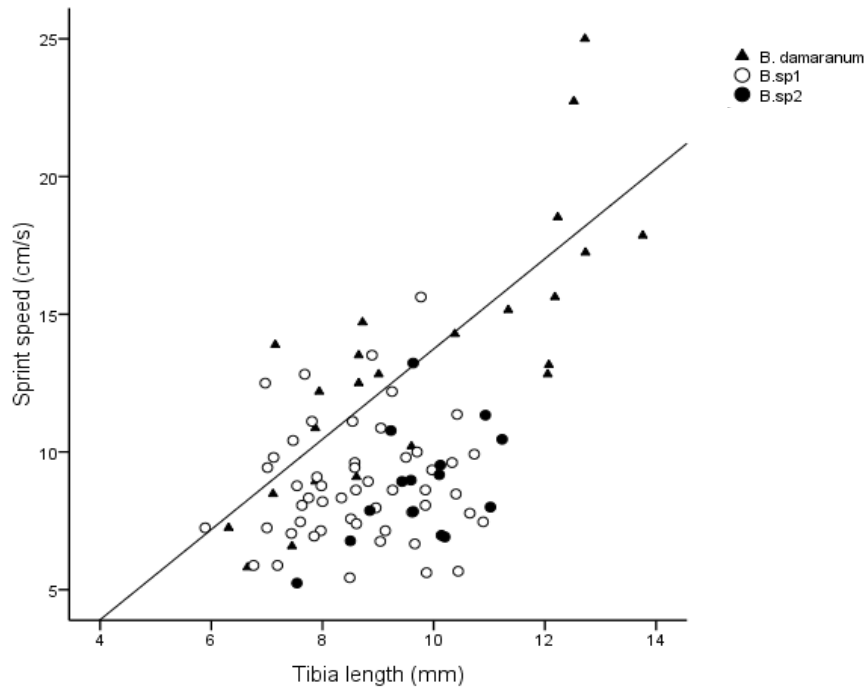


Figure 5: A correlation between tibia and sprint speed for *B. damaranum*, *B. sp1* and *B. sp. 2*. Although there was no correlation between morphology and sprint speed for both species residing in the fynbos habitat type this was not the case for chameleons from the forest habitat type. Their relationship is represented by the trend line.

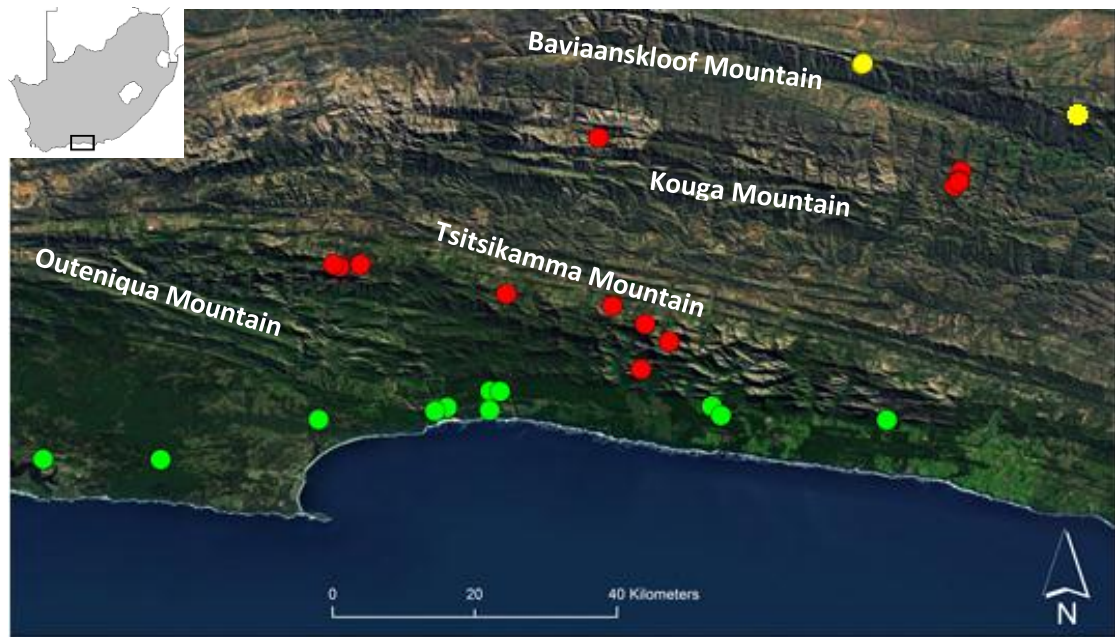


Figure 6: The localities of individuals from *B. damaranum* (green), *B. sp. 1* (red) and *B. sp. 2* (yellow) used for mtDNA analysis. This dataset includes additional sample localities from available samples.

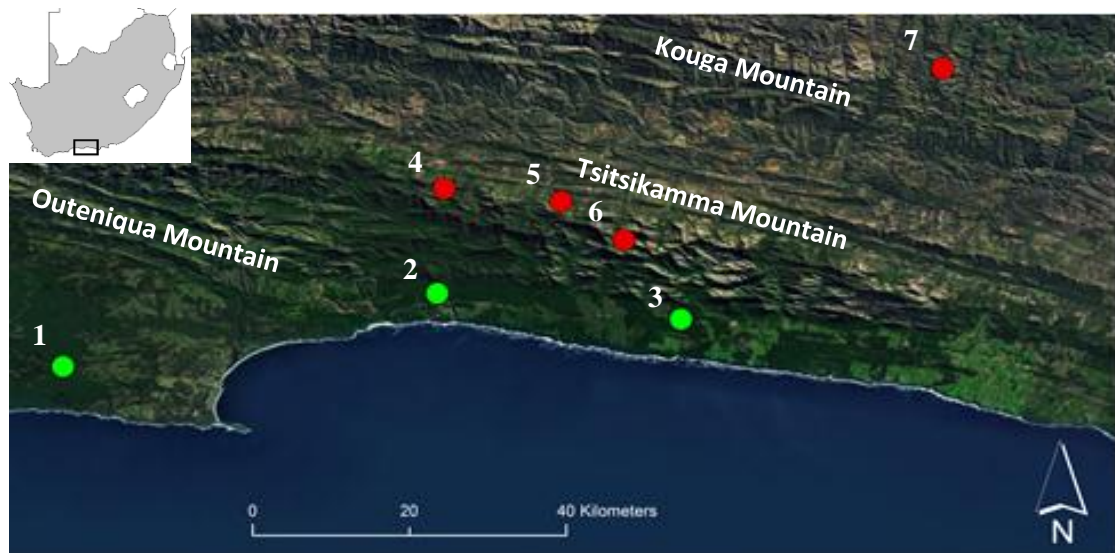


Figure 7: The localities of individuals from *B. damaranum* (green) and *B. sp. 1* (red) used in the microsatellite analyses. This is a reduced dataset using sites only from (1) Garden of Eden; (2)

Bloukrans pass; (3) Plaatbos; (4) Louterwater farm; (5) Grootnek farm; (6) Joubertina and (7) Geelhoutbos.

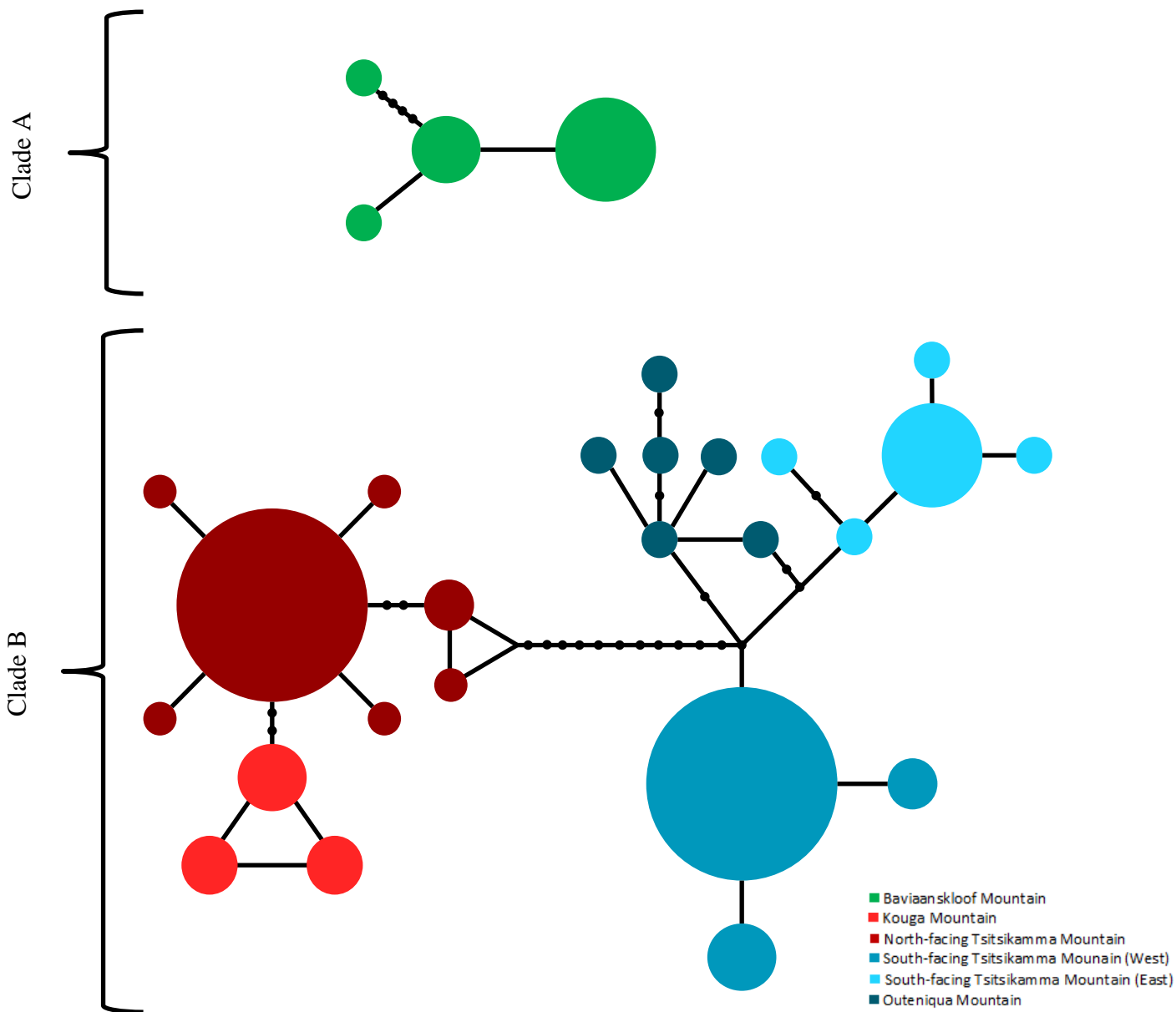


Figure 8: A haplotype network illustrating the two clades (A and B) following TCS analysis. Clade A is comprised of *B. sp. 2* (green) completely. Clade B shows the relationship between the *B. sp. 1* (red) and *B. damaranum* (blue) clades. The sizes of the circles indicate the frequency of the haplotype. Each line represents a mutational step with additional steps depicted as small circles on the branch. The patterns of structuring (SAMOVA) within each clade are depicted by various shades of each colour.

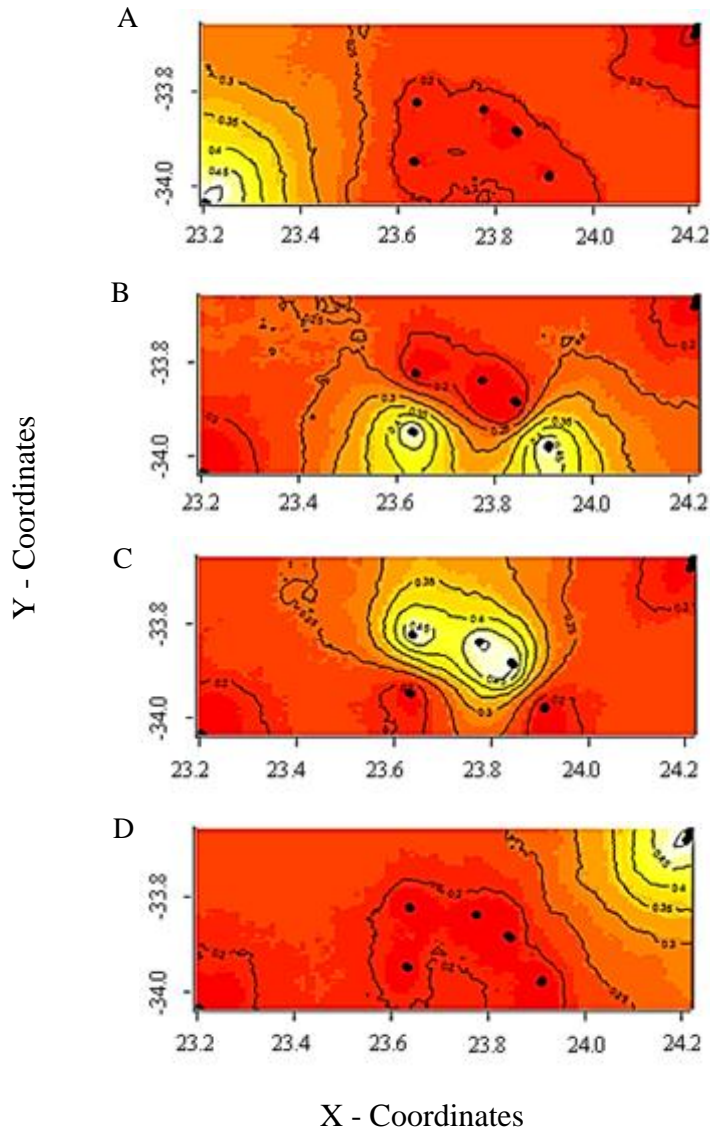


Figure 9: A Geneland probability map of the four spatially derived genetic clusters for *B. damaranum*: (A) Garden of Eden, (B) Bloukrans pass and Plaatbos; and *B. sp. 1*: (C) Joubertina, Grootnek and Louterwater, (D) Geelhoutbos. Each diagram represents the exact same geographic area and axes indicate latitude (X - Coordinates) and longitude (Y - Coordinates). The lighter areas correspond to higher probabilities of belonging to a specific cluster. Sampled sites are indicated by black dots. For further reference, refer to figure 7.

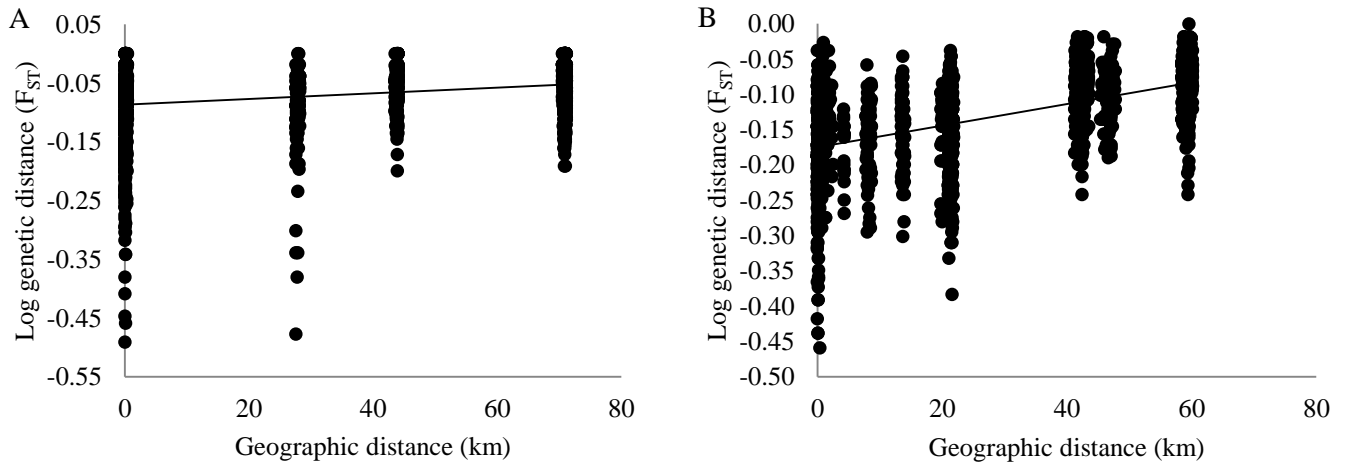


Figure 10: Significant ($p < 0.05$) patterns of isolation by distance, based on microsatellite analysis, observed within the forest (A) and fynbos (B) habitat types, respectively.