Phylogenetic and morphological analysis of the *Afroedura nivaria* (Reptilia: Gekkonidae) species complex in South Africa

By

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Declaration

I hereby declare that the work contained in this dissertation is my own original work and that I have not previously, in its entirety or in part, submitted it for any degree or examination at any university.

…… ……

Buyisile Getrude Makhubo Date

Dedication

I dedicate this work to my loved ones who are forever with me in spirit; they have helped in making me the person that I am today. Marriet Victoria Mlangeni, you always had a positive outlook on life. Bessie Khumalo, mngomu you always told me to make use of the opportunities I get and to make the most of my life. To maThandi, thank you for being such a humble soul. Cedric Ndoda Mlangeni, I am grateful that you taught me I deserve only the best and that I should always do my best. To bhuti (Mbongeleni “grippa” Mzungu), I shall honour your last words “Mzoyi, please remain just as you are”.

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Ngiyabonga Mvelingqangi ngakho konke kuloluhambo

Psalm 23 *Proverbs 19 verse 21* *Psalm 139*

Abstract

The *Afroedura nivaria* complex is one of the six recognized species complexes within a southern African endemic genus, *Afroedura*. The *A. nivaria* complex is a morphologically conservative group of medium-sized geckos endemic to South Africa though they are unevenly distributed in the Eastern Cape, Free State and KwaZulu-Natal provinces. The complex comprises the following five species: *A. nivaria* (Boulenger 1894), *A. amatolica* (Hewitt 1925), *A. karroica* (Hewitt 1925), *A. tembulica* (Hewitt 1926) and *A. halli* (Hewitt 1935). These nocturnal and rupicolous geckos shelter in narrow rock crevices on outcrops. It is currently unknown whether a) the described species are valid and b) if additional lineages are present on isolated outcrops. I investigated the hypothesis that endemics with a narrow distribution, that is, *A. amatolica* and *A. tembulica* are valid species but that isolated populations in the widespread species (*A. nivaria*, *A. karroica* and *A. halli*) demonstrate genetic variation at the species level. Fragments of two mitochondrial genes (16S rRNA and ND4) and a single nuclear marker (KIAA) were sequenced and analysed using Bayesian inference, maximum parsimony and maximum likelihood. All analyses strongly supported the genetic distinctiveness of the described species. The *A. nivaria* complex is not monophyletic, *A. karroica* appeared to be outside the species complex and *A. pondolia* (thought to be outside the *A. nivaria* complex) consistently nested within *A. nivaria* complex. Additional clades recovered in the phylogeny within *A. halli* and *A. nivaria* had large genetic divergences and no spatial overlap. Narrowly distributed *A. amatolica* showed to have two highly diverged clades. Clades recovered in the phylogeny highlight geographical structuring. These findings suggest the existence of up to four additional cryptic lineages within the complex. I used morphometric data (ecologically relevant morphological traits) to investigate whether the genetic lineages would present morphological conservatism. Multivariate analyses of 19 variables showed variation within the *A. nivaria* species complex was accounted for mostly by differences in locomotor apparatus (limbs and feet) and head dimensions. These traits are mostly related to microhabitat usage and/or dietary specialization in lizards. There were no significant differences for body dimensions between species within the complex, indicative of morphological conservatism. It appears genetic divergence has been achieved among the different clades within *A. nivaria* complex, but with much similarity in phenotype being retained because of fragmented but similar habitats occupied.

Opsomming

Die *Afroedura nivaria* kompleks is een van ses herkende spesies komplekse binne die endemiese suidelike Afrika genus, *Afroedura*. Die *A. nivaria* kompleks is ‘n morfologiese konserwatiewe groep bestaande uit medium grootte geitjies endemies tot Suid Afrika, alhoewel hulle oneweredig verspreid is in die Oos Kaap, Vrystaat en Kwazulu-Natal provinsies. Die kompleks bestaan uit die volgende vyf spesies: *A. nivaria* (Boulenger 1894), *A. amatolica* (Hewitt 1925), *A. karroica* (Hewitt 1925), *A. tembulica* (Hewitt 1926) and *A. halli* (Hewitt 1935). Hierdie geitjies kom snags voor en skuil tussen nou skeure op klip koppies. Dit is tans onbekend of a) die beskryfde spesies geldig is en b) of die addisionele afstammelinge voorkom op geisoleerde koppies. Met die studie het ek die hipotese ondersoek dat endemiese spesies met ‘n noue verspreiding (*A. amatolica* en *A. tembulica*) geldige spesies is, maar dat spesies met ‘n wye verspreiding (*A. nivaria*, *A. karroica* and *A. halli*) genetiese variasie op spesie vlak wys. Fragmente van twee mitochondriale gene (16S rRNA and ND4) en ‘n enkele nuklêre merker (KIAA) se basispaaropeenvolgingsdata was verkry en geanaliseer deur Bayesian inferensie, maksimum parsimonie en maksimum waarskynlikheid. Alle analise het die genetiese kenmerkendheid van die beskryfde spesies sterk ondersteun. Die *A. nivaria* kompleks is monofileties, *A. karroica* het geblyk om buite die spesies kompleks voor te kom en *A. pondolia* (voorheen beskryf as buite die *A. nivaria* kompleks) het voortdurend binne die *A. nivaria* kompleks voorgekom. Addisionele klades afkomstig vanaf die filogenië van *A. halli* en *A. nivaria* het vir beide spesies groot genetiese divergensie met geen ruimtelike oorvleuling gewys. *Afroedura amatolica*, met sy noue verspreiding, het twee hoogs divergente klades getoon. Die klades onthul deur die filogenie beklemtoon ‘n geografiese struktuur. Hierdie bevindings blyk die bestaan van tot vier ekstra kriptiese afstammelinge binne die kompleks. Ek het morfometriese data (ekologiese relevante morfologiese eienskappe) gebruik om vas te stel of die genetiese afstammelinge morphologies konserwatief sal wees. Meerveranderlike analises op 19 veranderlikes het variasie binne die *A. nivaria* spesies kompleks getoon. Hierdie veranderinge was meestal gevind in die beweeglikheidsapparatuur (ledemate en voete) en kop dimensies. Die verskeie eienskappe hou meestal verband met die mikrohabitatte wat gebruik word en/of dieët spesialisering in akkedisse. Daar was geen noemenswaardige verskille in liggaamsdimensies tussen spesies in die kompleks nie, beduidend op ‘n konserwatiewe morfologie. Dit wil blyk of genetiese divergensie tussen die verskeie klades van die *A. nivaria* kompleks bewerkstellig is met ooreenstemming in die fenotipes as gevolg van gefragmenteerde maar soortgelyke habitat verbruik.

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Chapter 1

General Introduction

The molecular approach to systematics

Molecular data approaches play a fundamental role in ecological, evolutionary, population and conservation genetics studies. Genetic markers have shown to be excellent indicators of diversity in phylogeographic and biogeographic studies in a wide range of both vertebrates and invertebrates (Moritz *et al.* 1987, White *et al.* 2008) for example, birds (Warren *et al.* 2003, Bowie *et al.* 2004, 2005), mammals (Hayano *et al.* 2003), reptiles (Leaché & Reeder 2002, Tolley *et al.* 2004, 2006, Hasbun *et al.* 2005, Greenbaum *et al.* 2007, Swart *et al.* 2009), fish (Farias *et al.* 1999), and insects (Clark *et al.* 2001). Mitochondrial DNA (mtDNA) is particularly useful because it is easy to obtain large datasets using universal primers (Avise *et al.* 1987, Moritz *et al.* 1987, Kocher *et al.* 1989, Galtier *et al.* 2009) and there is little or no recombination in comparison to nuclear genes. In addition, high rates of mutation (in mtDNA) can reflect population histories over relatively short periods of time. Results obtained from such studies can be correlated to ecology or geography to map species histories. However, mtDNA has uniparental inheritance, and relying on this single marker to narrate a species history results in biased estimates of evolutionary relationships (Avise *et al.* 1987, Pinho *et al.* 2007). The use of genetic markers is not without shortcomings, in particular, the use of mtDNA. Mitochondrial DNA alone seems to underestimate genetic diversity and may not reveal evolutionary processes at population level or address factors such as population size, migration and/or dispersal rates of a species (Moritz 1994). Again, paternal leakage, recombination and heteroplasmy complicate interpretation of patterns (White *et al.* 2008) but these can be accounted for (Bermingham & Moritz 1998). Despite the drawbacks, mtDNA is useful in documenting genetic variation in groups of organisms, answering questions important to tracing species histories and resolving taxonomic conflicts (Pinho *et al.* 2007).

Phylogenies are widely used in evolutionary biology, as they are considered an approximation of species relationships. This approach has however, shifted in the last three decades from being based solely on morphological characters which can easily be subject to phenotypic plasticity to a more pluralistic approach. The incorporation of genetic markers has increased and the reliability of phylogenies has become an important criterion in clarifying species boundaries and identifying cryptic diversity thus, bringing an understanding of the mechanisms of evolution and history of organisms (Tamura *et al.* 2007). Essentially, molecular techniques provide a means of recognizing faunal diversity that can go undetected using traditional morphological analyses (Couper *et al.* 2005,

Rissler *et al.* 2006). Even Darwin himself came to the same, now widely accepted conclusion, that genealogies accurately reflect classification (Le Guyader & Combes 2009).

In some cases, where phylogenies reveal cryptic diversity, an extension to a phylogeographic approach that covers a larger geographic area is usually followed. Phylogeography originally referred to the gene genealogies linked to geographic distributions between species or closely-related species (Avise *et al.* 1987). Phylogeographic approaches are widely practiced for their ability to test for various speciation hypotheses and understanding processes that have led to the present state of divergence between populations of the same species (Bermingham & Moritz 1998). This approach also gives more insight on vicariance and dispersal or colonization events in a region (Swart *et al.* 2009) meaning a more in-depth understanding of the processes responsible for the origin and maintenance of species communities (or rather, speciation events). This again, also feeds in to the conservation management of either highlighted diversity hotspots or intraspecific lineages across taxa (Rissler *et al.* 2006) as conservation is dependent on up-to-date taxonomy. Of recent interest, it appears that species delimitation has become inter-connected with phylogeography studies because they deal with patterns and processes that occur at inter or intra-specific levels (Camargo *et al.* 2010).

Molecular systematics and 'species' definition

Species are the cornerstone of biology, particularly in the fields of ecology and conservation. Their correct delimitation is essential because when boundaries are properly estimated between a set of species, real entities in nature that are evolving individually, the number of extant species can be correctly inferred (Coyne & Orr 1998, Petit & Excoffier 2009). The topic of species delimitation and species concepts is widely debated and many species concepts exist (see de Queiroz & Donoghue 1988, Ferguson 2002, Hebert *et al.* 2003, de Queiroz 2005, 2007).

In herpetology, species concepts that are lineage-based have been accepted (Frost & Hillis 1990, Hebert *et al*. 2003), primarily with the use of the *evolutionary species concept* and the *phylogenetic species concept* for defining species (de Queiroz 2007). An *evolutionary species concept* defines a species as a single lineage of ancestor-descendant populations which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate (Wiley 1978). With the *phylogenetic species concept*, a species is a phylogenetic cluster (clade) of organisms that is diagnosably distinct from other such clusters, within which there is a parental pattern of ancestry and descent (Cracraft 1989). These species concepts mainly focus on species as evolutionary units. Adopting both concepts, a species can be defined as a group of individuals that share the same

recent common ancestor and are diagnosably distinct from other such clusters. Employing these species concepts has allowed systematists to elevate the status of many taxa once thought to be races to the species level or vice versa because of lack of genetic differences thereof. Therefore, owing to the recognition of more allopatric species, numbers of reptile fauna being recognized are on the rise each year (Branch *et al.* 2006).

Molecular approaches in taxonomic revisions

The two major goals of systematics are delimiting species and reconstructing their phylogenic relationships (Wiens & Penkrot 2002). Using mtDNA data for systematics is economical and phylogenies based on mtDNA sequence data have been very effective as first indicators of boundaries in species that have not been investigated or those that are contentious (Galtier *et al.* 2009, Rato & Harris 2008). Constructing molecular phylogenies is also helpful in supplementing and validating species-level taxonomies which were initially based on morphology only (Hillis 1987, Marais 2004, Jesus *et al.* 2005, Oliver *et al.* 2009). Examination of multiple genetic datasets combined with morphological or ecological information is now a standard for modern taxonomic revisions (e.g. Rawlings *et al.* 2008). Several studies show how this plurastic approach can be useful, that is, where traditional morphological analysis cannot resolve conflicts and complimentary molecular studies have been employed in answering many questions concerned with evolutionary biology or conservation biology (e.g. Bauer *et al.* 2003, Rawlings & Donnellan 2003, Mahoney 2004, Rawlings *et al.* 2008, Leaché *et al.* 2009, Doughty *et al.* 2010).

Modern taxonomic revisions have led to the recognition of numerous additional species because of the high number of cryptic species being identified especially with the southern African reptile fauna in the recent decades. The combination of different approaches such as morphology, gene sequences (e.g. allozyme analysis, SNPs, mtDNA, nuclear sequence data), ecology, geographic distribution, behaviour and so forth for delineating species is now widely accepted. Ideally, this allows evolutionary hypotheses to be formulated and tested revealing more accurate species relationships. This way, a stable alpha taxonomy system for southern African reptiles could well be established (Wiens & Penkrot 2002, Bauer *et al.* 2003, Branch *et al.* 2006). Thus, lineages which are reproductively isolated or monophyletic (i.e. they have exclusive DNA haplotype phylogenies relative to other such lineages) can be considered an evolving entity under the evolutionary and/or phylogenetic species concept (Wiens & Penkrot 2002, Bauer & Lamb 2005).

Species delineation therefore, is improved by an integrated approach of multiple independent datasets to help identify lineages (de Queiroz 2007) and define species boundaries in intricate

species complexes (Vences *et al.* 2004). This also aids in explaining the process of genetic differentiation between species and understanding dispersal mechanisms of species in a given region (Branch *et al.* 2006, Pinho *et al.* 2007). Modern taxonomic revisions especially in range restricted species continue to reveal the existence of species and/or overlooked species that are of possible conservation concern (Bauer *et al.* 2003).

Morphological analysis and taxonomy

Linear morphometrics (biometrical) and geometric morphometrics are powerful techniques for studying variation in form and size being very useful in purely morphological or functionally based studies (Adams & Rohlf 2000, Stayton 2005). Technological advances continue to show that morphometrics are also valuable in investigating morphological variation (linked to geography) in closely related populations and/or in supporting characters historically used to delimit, what is now known as morphotypes (infraspecific variation) and understanding ecological and historical causes (Bastos-Silveira & Lister 2007). Taking measurements of body size and shape from live animals or preserved museum specimens has been used to test various ecological and evolutionary hypotheses, such as ecological radiation (Knox *et al*. 2001), Bergmann's rule (e.g. Ashton & Feldman 2003), sexual selection (Zuffi *et al*. 2011) and character displacement amongst others. Previous researchers have shown that particular morphometric characters are indeed useful in distinguishing closely related species (Blair *et al*. 2009). Integrating multivariate and geometric morphometrics for investigating patterns of morphological variation can help determine evolutionary processes involved through the analysis of different morphological aspects (Kaliontzopoulou *et al*. 2007, Kaliontzopoulou 2011). The application of molecular techniques in conjunction with morphological examination provides insight into the taxonomic discrepancies, especially when dealing with the taxonomy of morphologically conservative and widespread groups (Vences *et al*. 2004).

Inputs toward species conservation

For conservation measures to be put in effect, conservation units first need to be identified. The phylogenetic approach has been widely used in studying species that are of conservation concern (species considered under threat because they are not recognized genetically or if their genetic diversity is threatened). Therefore, the use of molecular markers to identify lineages is encouraged but must be accompanied by taxonomic studies (morphological descriptions) in order to compile fully recognizable species lists that are applicable as units of conservation assessments (Carranza *et al.* 2000, Branch *et al.* 2006, Couper *et al*. 2008). Moritz (1994) explored the applications of genetic data and categorized them into two practical classes: 1) utilizing sequence data for gene

conservation that is, identifying and managing gene diversity inferred from phylogenetic data and 2) applying information obtained from the sequence data in molecular ecology that is derived mostly from allele frequencies for short-term management of populations. This is an intractable situation since different species behave differently and may require management at different levels of the taxonomic hierarchy. Molecular work has been helpful in identifying such species and as well as diversity hotspot regions where traditional taxonomy failed. Findings from such phylogenetic and/or phylogeographic studies can also lead to the actual naming of taxonomic units which can be used in conservation, land-use planning or legislation (Taberlet & Bouvet 1994, Pereira *et al.* 2002). Comparative studies are also another way of contributing to conservation through the identification of regions of high diversity and endemism and regions where evolutionary processes are likely to continue to operate (Davis *et al.* 2008).

A major problem for biodiversity conservation and management is that a significant amount of species diversity remains undocumented (Oliver *et al.* 2009, Gehring *et al*. 2012, Scheffers *et al*. 2012). This may be due to the fact that many species that have not yet been discovered are small, difficult to find or have small geographic ranges (Scheffers *et al*. 2012). One other challenge is that certain species are difficult to discriminate based solely on morphology. However, molecular phylogenetic studies continue to uncover cryptic lineages within recognized species though attempts to describing cryptic species based on molecular data only are rather thwarted because of a lack of diagnosable morphological differences (Herbet *et al*. 2004, Bickford *et al*. 2007). Hence, with the use of molecular techniques only, faunal diversity can be recognized under the phylogenetic context without being assigned to recognized taxonomic ranks. The shortcoming of this is that such lineages tend to be overlooked by conservation or land-use management authorities where fauna conservation priorities are linked to name-based lists (Couper *et al.* 2005). Advances in molecular data usage for example, using statistical phylogenetic methods such as *p*-distances, allow us to delimit such genetic lineages as operational taxonomic units (OTUs) even though taxonomical status remains unknown or the use of DNA barcoding e.g. Nagy *et al*. (2012) for species discovery and identification. Not only can this information be used to easily recognize undescribed diversity, effective priorities for conservation can also be set owing to the near-accurate species numbers and their known localities (Nagy *et al*. 2012, Scheffers *et al*. 2012).

Landscape changes in southern Africa

Climate is a dynamic variable that plays a major role in shaping the environment (Cowling *et al.* 1997). This probably drives lineage diversification for some taxa, as biologists continue to time events linked to notable shifts in climate (Bauer & Good 1996, Avise *et al*. 1998, Carranza *et al*. 2002,

Austin *et al*. 2004, Bauer & Lamb 2005, Gamble *et al*. 2008b, Swart *et al*. 2009). Climatic fluctuations are believed to be responsible for the genetic diversification and adaptation of species to new environments (Tolley *et al.* 2006, Rabosky *et al.* 2007). With more knowledge on the geological and climatic history of Earth, vicariance and dispersal hypotheses can also be tested with the use of dated molecular phylogenies. This approach is fundamental to understanding the evolution of ecologically differentiated species (Rundell & Price 2009). However, sudden changes in the environment are most likely to lead to changes or adaptations of species to newer ecological opportunities, a phenomenon known as species radiation. An ecological divergence in populations can in turn lead to reproductive isolation should conditions keep these populations separate. Species radiations have been discussed immensely with Darwin's finches as the model taxon. Some species do undergo adaptive radiations, that is, rapid lineage diversification accompanied by morphological changes and specialized ecological adaptation as a response to natural selection and ecological opportunity due to environmental changes (Ridley 2004, Glor 2010).

Prior to mid-Miocene, southern Africa was dominated by a mixture of forest vegetation. South African climate underwent major changes in the past five million years (Pliocene and Pleistocene periods) which influenced the structure and composition of South African vegetation (Mucina & Rutherford 2006). The late Pliocene came to an end with a major decline in temperature approximately 2.8 million years ago (MYA), a key climatic episode which was accompanied by the formation of grasslands (Cowling *et al.* 1997). The cooling trend of the Pliocene led to greater aridity in South Africa with the forest biome being less favoured. This shift from dense woodlands to more open vegetation is also indicated by the faunal changes ca. 2.8-2.5 MYA. From pollen analyses, it shows that grasslands have been essentially in place throughout the Holocene and they became more widespread during the Pleistocene. It appears that in some taxa, the genetic composition and geographical distribution may have been influenced by climatic changes during the Pliocene and Pleistocene (Cowling *et al.* 1997, Daniels *et al.* 2004, Tolley *et al.* 2006, 2008, Swart *et al.* 2009).

Reptile diversity in southern Africa

Squamates, that is snakes, lizards and amphisbaenians are very speciose and make up approximately 9500 living species forming the major part of the world's terrestrial diversity (Conrad 2008, Uetz 2010). Southern Africa is well known for having the richest reptile diversity in Africa with well over 500 reptile species, possibly approaching 600 species (Branch 1998, 1999). Lizards form a dominant component, at least 60%, of this reptile fauna (Branch 1999, Branch *et al.* 2006, Alexander & Marais 2007). Over the last three decades, taxonomy, molecular systematics and biogeographic studies have shown South Africa to be a global hotspot for reptile diversity. South Africa has the third richest

lizard fauna in the world with almost 300 species of which half of them are endemic (Branch 1998). Reptile diversity in this sub-region may be even higher than currently estimated, with projections of undescribed species in geckos, dwarf chameleons, larcetids, scincids and cordylids (Branch *et al.* 2006). The number of described of reptile species is on the rise every year, with 126 described in 2011 alone worldwide and 95 new species already described in 2012 (Uetz 2010). Branch and colleagues (2006) projected that geckos have the greatest numbers of known undescribed species and cryptic species, especially rupicolous geckos including *Afroedura*, *Lygodactylus* and *Pachydactylus*. Over 50 reptile species that had restricted distributions and could be of conservation concern were noted in Branch (1999). Consequently, answering one of the main questions in conservation biology of identifying what must be preserved at the intraspecific level could be of importance (Taberlet & Bouvet 1994).

Background on the study taxa, mountain flat geckos (Afroedura)

In the publication 'On the classification and evolution of geckos', Underwood (1954) compiled the first comprehensive gecko classification, marking the first attempt to understanding evolution, systematics and biogeography of this group of lizards. In this publication, three clusters or families were recognized: Eublepharidae, Gekkonidae (Diplodactylinae and Gekkoninae) and Sphaerodactylidae. These were later refined by Kluge (1967) forming a single family Gekkonidae with four subfamilies: Gekkoninae, Eublepharinae, Diplodactylinae and Sphaerodactylinae, still recognizing the same higher order scheme. These have since remained as stable units. Further studies continued to recognize higher order groups and re-arranging the taxonomy. Han *et al*. (2004) subdivided Pygopodidae into three highly divergent groups. Two recent molecular phylogenetic studies recognize seven families: Carphodactylidae, Diplodactylidae, Eublepharidae, Gekkonidae, Pygopodidae, Phyllodactylidae and Sphaerodactylidae (Gamble *et al.* 2008a, 2008b). Recent estimates of total diversity are over 1400 described species across 118 genera with Gekkonidae being the largest group comprising of more than 85% of the gekkotan genera (Kluge 2001, Bauer 2002, Pianka & Vitt 2003, Han *et al.* 2004, Uetz 2011). Vast majority of Gekkonidae genera are fairly recent or resurrected since 1954 (Feng *et al.* 2007).

Most early work on gecko systematics including most phylogenetic analyses was dominated by examination of morphological characters which included external features such as digital structures plus opthamological, osteological and mycological characters (Kluge 1983, Russell 1979, Russell & Bauer 1988). The monophyly of the living Gekkota is supported by numerous morphological characters and further supported by various molecular studies (Harris *et al.* 2001, Han *et al.* 2004, Feng *et al.* 2007). Phylogenetic reconstructions of the gekkotan lizards suggest that Gekkonidae and

Pygopodidae are monophyletic and basal among squamates (Han *et al.* 2004, Townsend *et al.* 2004, Feng *et al.* 2007, Vidal & Hedges 2009). Inter-generic relationships of the Gekkonidae have been more difficult to resolve than those within other gekkotan families (Jackman *et al.* 2008a). Madagascan and some southern African Gekkonidae genera e.g. *Pachydactylus* have received much attention through morphological and molecular studies (Kluge & Nassbaum 1995, Bauer *et al.* 2002, Bauer & Lamb 2002, Lamb & Bauer 2002, Arnold *et al.* 2008), and these few studies show that geckos have a tendency of housing high levels of cryptic diversity (see Oliver *et al.* 2009 for references). Molecular markers continue to show their usefulness for recovering relationships among animal taxa and have been employed in analysis of intrageneric and/or sister genera relationships among gekkotans (Carranza *et al.* 2000, Lamb & Bauer 2001, Bauer & Lamb 2002).

From Underwood's classification, four pad-bearing gekkotan genera were found taxonomically problematic and these were *Afroedura, Aristelliger, Calodactylodes* and *Paragehyra*. These groups appeared to be unrelated to one another and had no obvious affinities with previously discussed groups in Underwood's 1954 publication (Russell & Bauer 2002). This study focuses on one of the problematic groups, the mountain flat geckos, genus *Afroedura* (Gekkonidae). For a long time the southern African geckos in the genus *Afroedura* were placed with the Australian *Oedura* based simply on their similarity in appearance. It was Loveridge (1944) who initially separated *Afroedura* from the *Oedura* on the basis of the smaller number of adhesive pads and a verticillate tail of most of the African species. Underwood (1954) kept these genera in different subfamilies, Gekkoninae and Diplodactylinae, even though they had superficially similar appearance (Loveridge 1947). *Afroedura* and *Calodactylodes* were then grouped within the same digitally defined cluster (Russell 1972) but Russell & Bauer (1989) later concluded that *Afroedura* and *Calodactylodes* were more likely convergent than related and this was later supported by Feng *et al*. (2007). The genus *Afroedura* is restricted to southern Africa that is, from Mozambique southwards to northern and eastern South Africa to central Free State and towards the Western Cape and the Karoo and northwards to central Angola (Mouton & Mostert 1985). *Afroedura* differs from other gekkonids mainly by their anatomy of the digits: free, clawed, have a large pair of adhesive pads distally separated from two-three pairs of smaller adhesive pads proximally (Loveridge 1947). There are currently 15 recognized species within this genus (Hewitt 1937, Loveridge 1947, Onderstall 1984, Branch 1998). Despite some work having been done, species boundaries remain contentious. At least six species complexes are recognized within this genus since Onderstall (1984) who originally recognized only three major groups (Africana, Pondolia and Transvaalica) distinguished by nature of smaller digital adhesive pads, using the number and arrangement of scansors and the nature of the tail as separating characters (Onderstall 1984, Mouton & Mostert 1985).

In the Free State, Eastern Cape, KwaZulu-Natal and Lesotho, a species referable to the *Afroedura nivaria* complex requires further investigation because it is thought to be housing cryptic diversity. The *A. nivaria* species complex presently comprises of five species: *A. nivaria* Boulenger, 1894 (mountain flat gecko), *A. karroica* Hewitt, 1925 (karoo flat gecko), *A. amatolica* Hewitt, 1925 (Amatola flat gecko), *A. tembulica* Hewitt, 1926 (Tembu flat gecko) and *A. halli* Hewitt, 1935 (Hall's flat gecko) (Fig. 1.1). These geckos are strictly nocturnal lizards and rupicolous, inhabiting narrow rock crevices in rocky outcrops (koppies/inselbergs) that are scattered throughout the grassland biome occurring from sea-level to mountain tops (Pianka & Vitt 2003). They can withstand lower temperatures than most other lizards. They have large eyes, vertical slit-like pupils and their eyes are permanently open, they use their tongues to keep the eyes clean (Hewitt 1937). The tail is readily discarded as an escape technique and adults often have regenerated tails but quite different in shape and colour from the original ones. They shed their skin periodically including a thin film from their membrane covering the eye. Adult males can be distinguished from females by the presence of pre-anal pores. These geckos are insectivorous and their diet comprises of ants, beetles, grasshoppers, mosquitoes, sandflies, termites, and centipedes amongst other insects (Loveridge 1947, Branch 1998). Females usually lay two relatively medium to large hard-shelled eggs (oviparous) and may use communal egg-laying sites (Branch 1998). Eggs are soft and sticky when first laid but harden rapidly being firmly attached to rock surfaces under loose flakes. These geckos have strict habitat preferences linked to suitable rock outcrops (Hewitt 1923). Onderstall (1984) believed that their rupicolous nature accompanied by limited vagility is the main reason for their discontinuous or disjunct distribution often being restricted with no known instances of sympatry. Bates & Branch (*in prep*.) recently conducted a morphological study on this complex and they suggest that allopatric populations appear to be morphologically conservative but correspond with the five described species. They also suggested that there may be undescribed taxa in the complex.

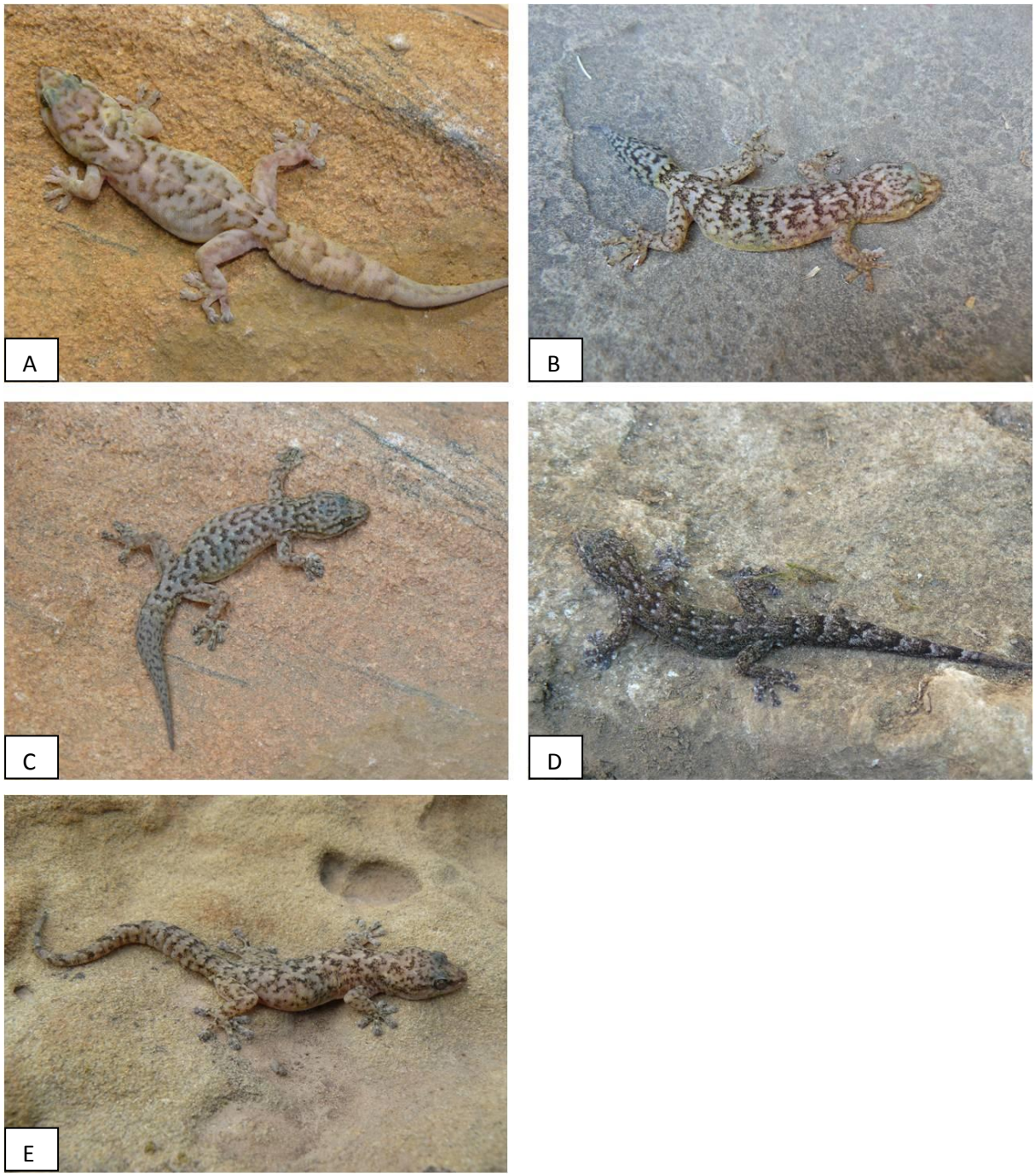


Figure 1.1 Photograph of a) *A. nivaria* (Platberg), b) *A. halli* (Dordrecht), c) *A. karroica* (near Cradock), d) *A. amatolica* (Hogsback), and e) *A. tembulica* (Cofimvaba). Photographs taken by M.F. Bates.

Background on the study area

The grassland biome in South Africa mainly occurs on the high central plateau (highland), the inland areas of the eastern seaboard, the mountainous areas of KwaZulu-Natal and the central parts of the Eastern Cape (Mucina & Rutherford 2006). Within the grassland biome, the distribution of flat geckos, *A. nivaria* species complex, falls within two bioregions namely, the Drakensberg and the Sub-Escarpment grassland bioregions as outlined in Mucina & Rutherford (2006). The Drakensberg Grassland Bioregion occurs on the Lesotho highlands and immediate surrounds KwaZulu-Natal stretching southwards along the high lying areas of the escarpment in the Eastern Cape Province to reach the Stormberg and Amathole mountains. This bioregion has the least number of vegetation types meaning there is less plant diversity compared to the other bioregions in the area. It borders the Sub-Escarpment Grassland Bioregion that occurs at low altitudes on the foothills of the Drakensberg and eastern escarpment from around Volksrus to the Queenstown area.

Aims and Objectives

Despite some work having been done, species boundaries within the genus *Afroedura* remain contentious. This group of geckos is identified as one of the taxonomically problematic groups in South African reptiles (Branch *et al*. 2006). In the Free State, Eastern Cape, KwaZulu-Natal and Lesotho, a species complex referable to the *Afroedura nivaria* complex requires further investigation because it is thought to be housing cryptic diversity (Bates & Branch *in prep*). Bates & Branch (*in prep*.) recently conducted a morphological study on this complex and they suggest that allopatric populations appear to be morphologically conservative but correspond with the five described species and they believed that there may be more undescribed taxa hidden in the complex. The aims of this study are to test species boundaries of the *Afroedura nivaria* species complex in South Africa using molecular markers, to construct a phylogeny and to examine whether morphological characters distinguish the lineages or if the lineages would demonstrate morphological conservatism. Currently, it is unknown whether 1) the described species are valid in a phylogenetic context, 2) whether geckos on the numerous isolated outcrops are distinct genetic lineages and 3) if the *A. nivaria* species complex houses cryptic diversity. Several hypotheses will be tested to address the aims of this study.

Hypotheses:

- There are at least five recognized species which are distinct evolutionary lineages (*A. nivaria*, *A. karroica, A. amatolica, A. tembulica and A. halli*).
- Populations on numerous isolated outcrops of the three widespread species (i.e. *A*. *nivaria*, *A*. *karroica* and *A*. *halli*) comprise distinct genetic lineages. High genetic variation and reciprocal monophyly will indicate that these lineages represent cryptic species rather than populations of the same species.
- Well defined genetic lineages cannot be distinguished based on morphological traits that are ecologically relevant, due to their presumed conservative morphologies. In cases where the morphology is similar despite the large genetic differences, this will suggest the existence of cryptic species and morphological conservatism due to similar environments.

The findings of this study will be used to update taxonomy in an evolutionary context for this species complex. This marks the first phylogenetic study looking specifically into this species complex and incorporating morphometric analysis using ecologically relevant morphological variables to examine morphological differentiation within this group of endemic geckos.

Chapter 2

Phylogenetic relationships among members of the *Afroedura nivaria* species complex

INTRODUCTION

Molecular systematics and phylogenetics

Over the decades, the incorporation of genetic markers has vastly increased and the reliability of phylogenies has become an important criterion in clarifying species boundaries and identifying cryptic diversity (Tamura *et al*. 2007). Molecular approaches allow us, among other things, to quantify genetic diversity, characterize new species, retrace historical patterns of dispersal and track the movements of individuals within populations, and to resolve taxonomic conflicts (Avise 1994, Pinho *et al*. 2007). The use of mitochondrial gene markers have proven useful because of their overall high mutation rate therefore, coalesce more quickly than nuclear genes providing the ability to detect evolutionary changes that may have occurred over short periods of time (Blackburn & Measey 2009). The simplicity of inheritance is yet another advantage for mitochondrial genes (Avise *et al*. 1987, White *et al*. 2008, Freeland 2005). Mitochondrial DNA shows relatively high levels of intraspecific polymorphism and therefore, will often reveal multiple genetic lineages both within and among populations and on most cases, genealogies have accurately reflected classification (e.g. Guyader & Combes 2009) recognizing faunal diversity that can go undetected using traditional character-based phylogenies (Couper *et al*. 2005, Rissler *et al*. 2006). Again, the incorporation of molecular techniques in taxonomic revisions has helped determine species boundaries in contentious species complexes (Bauer & Lamb 2002, Vences *et al*. 2004, Bauer & Lamb 2005), and also identifying distinct lineages that can be fully recognized in species lists applicable as valid taxonomic units of conversation assessments (Carranza *et al*. 2000, Branch *et al*. 2006). However, nuclear gene markers have also shown to be excellent for higher-level systematic studies that require slowly evolving genes because mitochondrial genes may be evolving too rapidly for effective studies looking at ancient evolution of a species, and can provide a robust phylogeny for deep divergences (e.g. Groth & Barrowclough 1999).

Species, fundamental units of comparison in nearly all fields of biology, derive their importance from their significance in systematics, an old discipline of science responsible for the taxonomic framework largely used in biology (de Queiroz 2005), has historically been focused on the concept of species. Properly estimated species boundaries that is, individually evolving entities in nature, often

mean that the number of extant species can be correctly inferred providing a practical up-to-date taxonomy for our reptile diversity (Coyne & Orr 1998, Petit & Excoffier 2009). The levels of distinctness for recognizing species differ widely between different taxonomic groups (Johns & Avise 1998), hence many species concepts exist. Species concepts that are lineage-based are becoming dominant (de Queiroz & Donoghue 1988, Frost & Hillis 1990, Ferguson 2002, Hebert *et al*. 2003, de Queiroz 2005, 2007), primarily with the use of the *evolutionary species concept* and the *phylogenetic species concept* for defining species (de Queiroz 2007). Adopting both these concepts, a species can be defined as a group of individuals that share the same recent common ancestor and are diagnosably distinct from other such clusters. Systematists have been able to elevate the status of many taxa to species level or vice versa because of lack of genetic diversity, e.g. to morphotypes and the recognition of cryptic diversity in other taxa (Tolley *et al*. 2004, Lehtinen *et al*. 2007, Pepper *et al*. 2006, Pinho *et al*. 2007, Nielsen *et al*. 2011). Ultimately, the correct delimitation of species, giving an indication to evolutionary management units, is essential in conservation biology as well.

Taxonomic history of the study taxa (Afroedura)

The genus *Afroedura* Loveridge, 1944 was formerly referred to the Australian genus *Oedura* Gray, 1842. Loveridge (1944) later realized that the African species formed a fairly homogenous group distinguished by having one to three pairs of scansors (adhesive toepads) beneath the fourth toe and a verticillate tail. Hence, the genus was erected to accommodate this group of African geckos. Fitzsimons (1943) stated that femoral pores were lacking in all the African species he examined and were present in males of all Australian species. Loveridge's (1944) separation was apparent to Underwood (1954) who placed the genera *Afroedura* and *Oedura* under different subfamilies (Gekkoninae and Diplodactylinae respectively) although the validity of his use of ophthalmological characters was doubtful (Cogger 1964). In 1972, Russell grouped *Afroedura* and *Calodactylodes* within the same digitally defined cluster and Russell & Bauer (1989) concluded that *Afroedura* and *Calodactylodes* were more likely convergent than related and this was later supported by Feng *et al*. (2007). Numerous studies have looked at higher order relationships between these two genera (Loveridge 1944, Cogger 1964, Russell & Bauer 1990) but the genus *Afroedura* has not received much attention on the species-level taxonomy. From Branch *et al*. (2006), it was projected that geckos have the greatest numbers of known undescribed species and cryptic species especially rupicolous geckos including *Afroedura*, *Lygodactylus* and *Pachydactylus* in southern Africa. Thus, species-relationships within a taxonomically problematic group, *Afroedura* were examined.

Currently, fifteen species are recognized within the genus *Afroedura* all occurring within southern Africa and northwards into Angola (Hewitt 1937, Loveridge 1947, Onderstall 1984, Branch 1998). At

least six species complexes are recognized within this genus since Onderstall (1984) who originally recognized only three major groups, i.e. *A. pondolia* group, *A. transvaalica* group and the *A. africana* group. In the Free State, Eastern Cape, KwaZulu-Natal and Lesotho, a species complex referable to the *Afroedura nivaria* complex (separated from the *A. africana* group) is believed to be housing cryptic diversity, and merits further investigation. The *A. nivaria* species complex presently comprises of five species: *A. nivaria* (Boulenger 1894), *A. karroica* (Hewitt 1925), *A. amatolica* (Hewitt 1925), *A. tembulica* (Hewitt 1926) and *A. halli* (Hewitt 1935). These endemic geckos are primarily nocturnal and rupicolous (Branch 1998, Pianka & Vitt 2003), inhabiting narrow rock crevices in rocky outcrops that are scattered throughout the grassland biome. They have strict habitat preferences linked to suitable rock outcrops (Fig. 2.2). Owing to that, these species have disjunct distribution often being restricted with no known instances of sympatry (Onderstall 1984).

Distribution of Afroedura nivaria species complex

Members of the *A. nivaria* species complex are found in the Eastern Cape, Free State and KwaZulu-Natal provinces in South Africa extending into Lesotho (Fig. 2.1). The widely distributed *A. nivaria* is found on the Drakensberg mountain range of Lesotho adjacent KwaZulu-Natal extending to the eastern Free State. This species prefers large sandstone rock faces on mountain summits, and its type locality is the Drakensberg mountain range (Hewitt 1927, 1937). *A. halli* is another widely distributed species which was first described from Telle Junction, Herschel District (Power 1939, Loveridge 1947) at a height of 1371 m. This species appears to be restricted to the southern Drakensberg, the Maluti mountains and the Stormberg; range: mountains on the north of Eastern Cape adjacent western Lesotho and southern Free State (Hewitt 1937, Branch 1998). Another widely distributed species is *A. karroica* found on the inland mountains of Eastern Cape on rock outcrops in montane grassland (Loveridge 1947, Branch 1998) from the Albany District (type locality), Cradock District, Graaf Reneit, and Tarkastad towards slopes of Winterberg. A narrowly distributed *A. amatolica* only occurs on the Amatola and Katberg mountains south to Fish River, Eastern Cape; type locality near Hogsback (Hewitt 1927, Loveridge 1947). This species prefers rock outcrops on montane grassland and dry thicket. *Afroedura tembulica* has a very restricted distribution. It is known to occur on the mountains from Cofimvaba, Imvani, Tembuland to the Queenstown District, Eastern Cape.

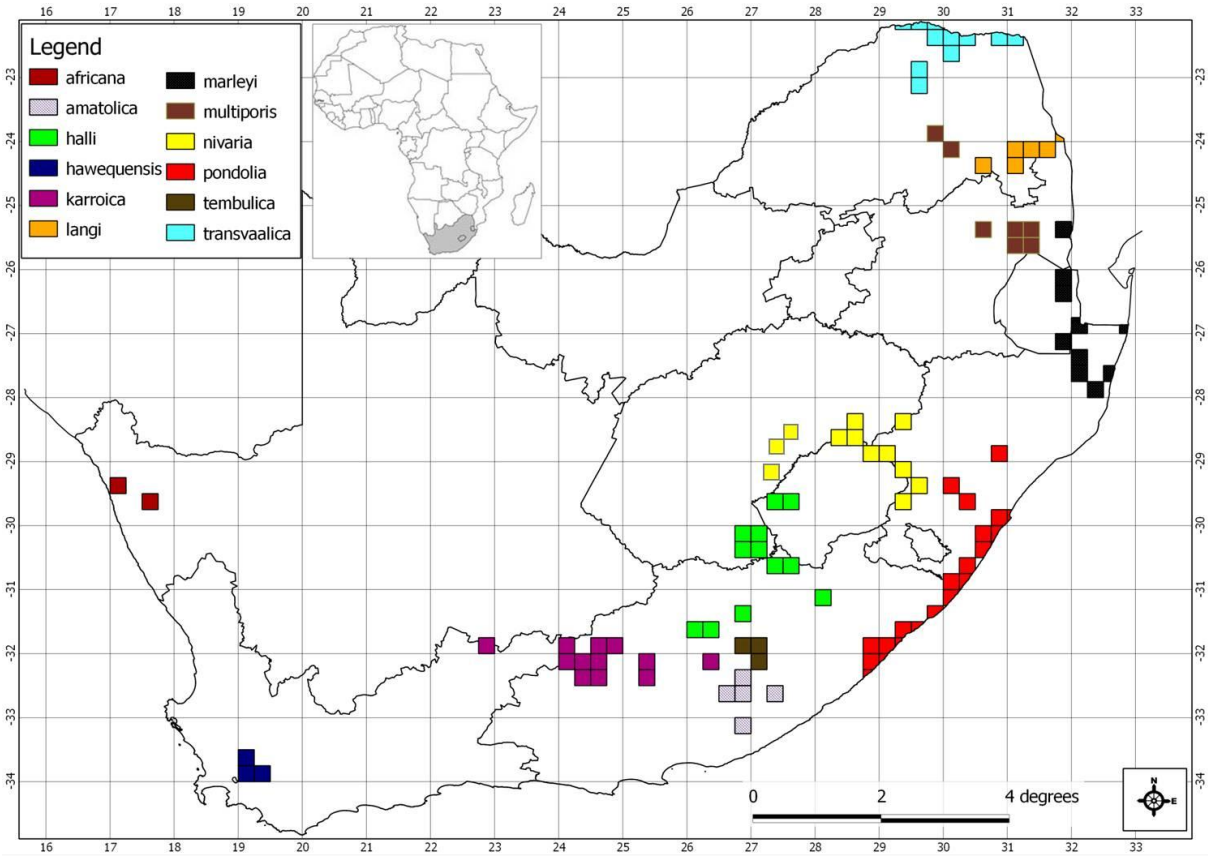


Figure 2. 1 Distribution map of the *Afroedura* species considered for this study, showing the known areas in which these species occur in South Africa. South African Reptile Conservation Assessment, Animal Demography Unit (http://sarca.adu.org.za).

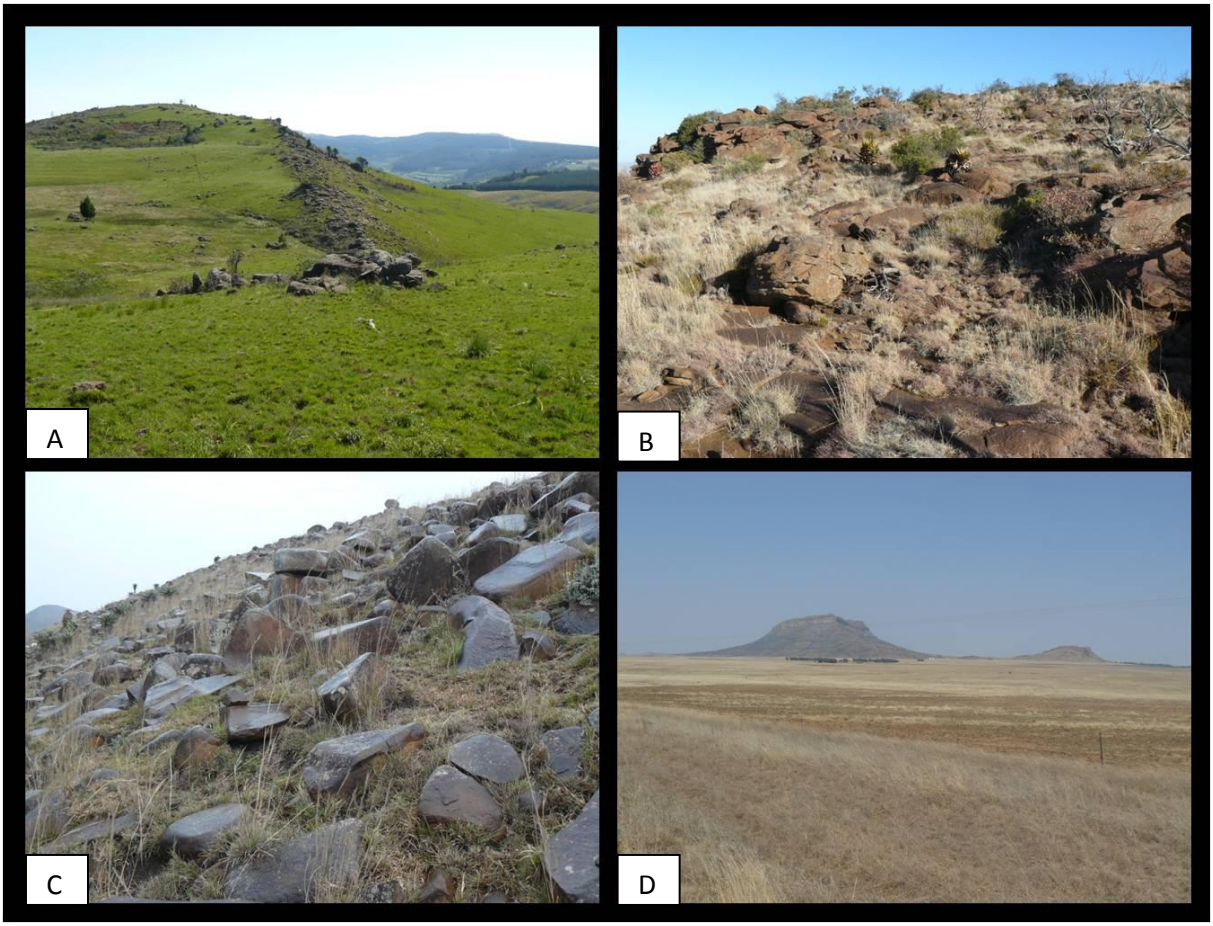


Figure 2. 2 Photographs showing habitat of the *Afroedura nivaria* species complex, South Africa. A) *A. amatolica*, Hogsback. B) *A. karroica*, Buffelskop near Cradock. C) *A. tembulica*, Cofimvaba. D) *A. halli*, Thaba Phatswa (outcrop around grassland). Photographs taken by M.F. Bates.

Although phylogenetic approaches have been used with great success in resolving contentious species boundaries, testing biographic hypothesis, examining speciation patterns and their ability to reveal high occurrences of cryptic diversity among geckos, no studies to date have addressed the genetic assessment for the five species in the *A. nivaria* group. A recent morphological study on the *A. nivaria* species complex conducted by Bates & Branch (*in prep*.) suggested that allopatric populations appear to be morphologically conservative but correspond with the five described species and there may be undescribed taxa in the complex. Using a phylogenetic framework, I hypothesized that 1) the five taxonomically recognized species are distinct genetic lineages; 2) the *A. nivaria* species complex is a monophyletic group and, 3) populations of the more widespread species, i.e. *A. nivaria*, *A. halli* and *A. karroica* occurring on isolated outcrops comprise several distinct lineages. In the present study, two different datasets were employed to test the hypotheses, first a mitochondrial DNA dataset (16S and ND4) for all samples and secondly, a sub-set of samples were chosen from each of the recovered lineages (mtDNA phylogeny) to compile a nuclear (nucDNA) gene dataset; this was to ensure the robustness of the phylogeny at the deeper nodes. The inclusion of gene fragments from various molecular markers that evolve at different rates is likely to increase the accuracy of the resulting phylogeny at both the deeper branches and tips.

MATERIALS AND METHODS

Sampling

Sampling took place by active search, catching the geckos by hand (between and under rocks), during 2010-2011, with samples supplemented by those already available at the South African National Biodiversity Institute (SANBI). For species with large distributions, sampling was more spread out covering as many outcrops as possible. Where possible, a maximum of six individuals were collected as representatives of populations in each of the outcrops visited. Tail clips from live specimens or liver tissue from voucher specimens were taken for each individual and stored in 99% ethanol for later extraction, and live ones were then released. A limited number of voucher specimens per site were deposited at the National Museum, Bloemfontein.

A total of 135 samples, including eight representatives from other complexes within the genus, were used for the phylogenetic analyses (Appendix C). There were 33 samples from eight sites for *A. nivaria*, 29 samples from six sites for *A. karroica*, 37 samples from 10 sites for *A. halli*, eight samples from three sites for *A. amatolica* and seven samples from a single known locality of *A. tembulica* (Fig. 2.3).

PCR amplification, DNA sequencing and alignment

Two mitochondrial gene fragments were selected for this study for their relatively high rate of evolution with little or no recombination and their ability to reflect sufficient population variation over short periods of time as compared to nuclear genes. These are the widely used 16S ribosomal RNA (16S rRNA; Palumbi *et al*. 1991) and the protein-coding nicotinamide adenine dinucleotide dehydrogenase (NADH) subunit 4 (ND4; Arevalo *et al*. 1994, Jackman *et al*. 2008b). However, mitochondrial genes have uniparental inheritance (maternal only) and may give biased estimates of evolutionary relationships (Avise *et al*. 1987). Thus, the nuclear gene, KIAA (Portik *et al.* 2011) which was shown to be a variable marker which can be incorporated in squamate phylogenetic and phylogeographic studies was included in this study.

Total genomic DNA was extracted from tissue samples according to standard procedures with a proteinase-K digestion followed by a salt extraction protocol (Aljanabi & Martinez 1997). Where tissue samples were small, the Qiagen DNeasy tissue kit was used (Valencia, CA, USA) to extract DNA. Polymerase Chain Reaction (PCR) was used to amplify each of the markers selected using published primer pairs (Table 2.1). For amplification, approximately 10-30 ng/µl of DNA template was added to make up a 25 µl PCR reaction mixture (Table 2.2). Samples that proved problematic for

amplification were treated on a case by case basis. In some cases, 0.2 µl bovine serum albumin (BSA) was added (regarding ND4) to the reaction mixture to enhance the amplification process. Primers for the genes were optimized to the specificity of the targeted species. The PCR cycling profile included an initial denaturation step at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 49 °C for 30 s and extension at 72 °C for 30 s for 16S and 40 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 45 s and extension at 72 °C for one minute for ND4 with a final extension at 72 °C for eight minutes for both of them. For KIAA, the cycling profile included an initial denaturation step at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 45 s with a final extension at 72 °C for eight minutes. When necessary, annealing temperatures were adjusted to increase specificity on a case by case basis. PCR product (2-3 µl) was visualized with 1% agarose gel (0.8 g agarose powder in 80 ml 1.0 X TBE stained with GoldView™ or ethidium bromide) electrophoresis. Thereafter, PCR products were sent to Macrogen Inc. (Seoul, Korea) for sequencing. Geneious version 5.4 (Drummond *et al*. 2011) (Biomatters Ltd 2010) was used to edit and align the DNA sequences. The protein coding genes, ND4 and KIAA, were translated to amino acid sequences to check for premature stop codons and confirm the preservation of the amino acid reading frame.

Phylogenetic analysis

Phylogenetic analysis was carried out using two different datasets, first a mitochondrial (16S and ND4) dataset for all samples and secondly, a sub-set of samples chosen from each clade (2-3 samples) recovered from the mtDNA phylogeny was used to compile a combined mitochondrial and nuclear dataset. *Afrogecko porphyreus* was chosen as an appropriate outgroup taxon for this study because it was found to be a sister group to *Afroedura* within the same family (Han *et al*. 2004, Feng *et al*. 2007). Several other taxa within *Afroedura* but outside the *A. nivaria* complex were included in order to ensure that the complex is placed in context within the whole genus (i.e. *A. bogerti*, *A. hawequensis*, *A. langi*, *A. marleyi*, *A. multiporis multiporis*, *A. m. haackei*, *A. pondolia* and *A. transvaalica*). Samples of these taxa were available at SANBI.

The number of parsimony informative and uninformative sites was estimated in MEGA v. 5.0 (Tamura *et al*. 2007). Sequence data were also used to compute sequence divergences as uncorrected *p*-distances with missing data deleted in pairwise comparison between and within species within this complex. The saturation of the codon positions for the ND4 gene was assessed with Dambe v. 5.3.5 (Xia 2000). No codon position was found to be saturated; all codons were included in analysis.

A partition homogeneity test was run in PAUP* v. 4.0b10 for the combined mtDNA (16S and ND4) dataset (dataset 1) and then for the mitochondrial (16S/ND4)-nuclear (KIAA) sequence data (dataset 2) to ensure that there was no conflict between the different genes and datasets could be combined for analyses. The Akaike Information Criterion (AIC) in ModelTest v. 3.06 developed by Posada and Crandall (1998) was used to find the evolutionary model that best fit the dataset (for each dataset separately) for the subsequent model based analyses. Results of the ModelTest identified the general time-reversible model as the best fit for the separate and combined mitochondrial gene datasets (Rodríguez *et al*. 1990), incorporating a gamma shape distribution for variable sites and a proportion of invariant sites (GTR + I + G). The same model was also the best fit for the combined nuclear-mitochondrial dataset.

Phylogenetic reconstruction was carried out using three methods: maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). Phylogenetic analyses were first conducted on the mtDNA dataset only (dataset 1) to identify major clades within this complex. The two mitochondrial genes, 16S and ND4, could be combined into a single dataset, a partition homogeneity test found them to be congruent. Parsimony phylogenies were constructed using PAUP* 4.0b10 (Swofford 2002) for the individual markers and the combined markers. The heuristic search algorithm was executed with the following conditions: tree-bisection-reconnection branch swapping, equal character weighting, 100 replicates of random taxon addition, and gaps treated as missing data. To asses node support in resulting topologies (the reliability of the resulting inferred tree), a non-parametric bootstrap test was conducted of 1000 pseudoreplicates with 25 random additions of sequences per replicate. Bootstrap values above 75% were considered to indicate strong support.

Bayesian inference was conducted in MrBayes v. 3.1.0 (Ronquist & Huelsenbeck 2003) with default priors incorporating selected models for the separate datasets. The number of rate parameters set were “lset Nst = 6” with invariant sites and a gamma distribution, “rates = invgamma”. The mtDNA dataset was partitioned into two markers (16S/ND4) and the combined dataset into three markers (16S/ND4/KIAA). The analyses were initiated with random starting trees; the MCMC (one cold, three heated chains) were run with two parallel runs for 10,000,000 generations each and trees sampled every 1000 generations. Trees generated prior to reaching stationarity were discarded as burn-in. Burn-in was determined by examining the standard deviation of split frequencies below 0.01 and the effective sampling size (ESS) of all parameters (ESS > 200) using Tracer v. 1.5 (Rambaut & Drummond 2007). Generally, stationarity is consistently reached within the first 10% (up to 15%) of the total number of generations. A 50% majority rule tree was obtained from the remaining trees (excluding burn-in). Node support was assessed based on the Bayesian posterior probabilities (PP) with PP ≥

0.95 considered strong support for the nodes. All trees were visualised using Figtree version 1.3.1 (Rambaut 2009).

Maximum likelihood analyses (ML) were implemented in RAxML v. 7.2.8 (Stamatakis 2006) at the CIPRES Science Gateway (www.phylo.org/sub_sections/portal/). Datasets were partitioned by marker, incorporating a GTR model and implementing the automatic halting of bootstrapping for the analysis (Stamatakis *et al*. 2008).

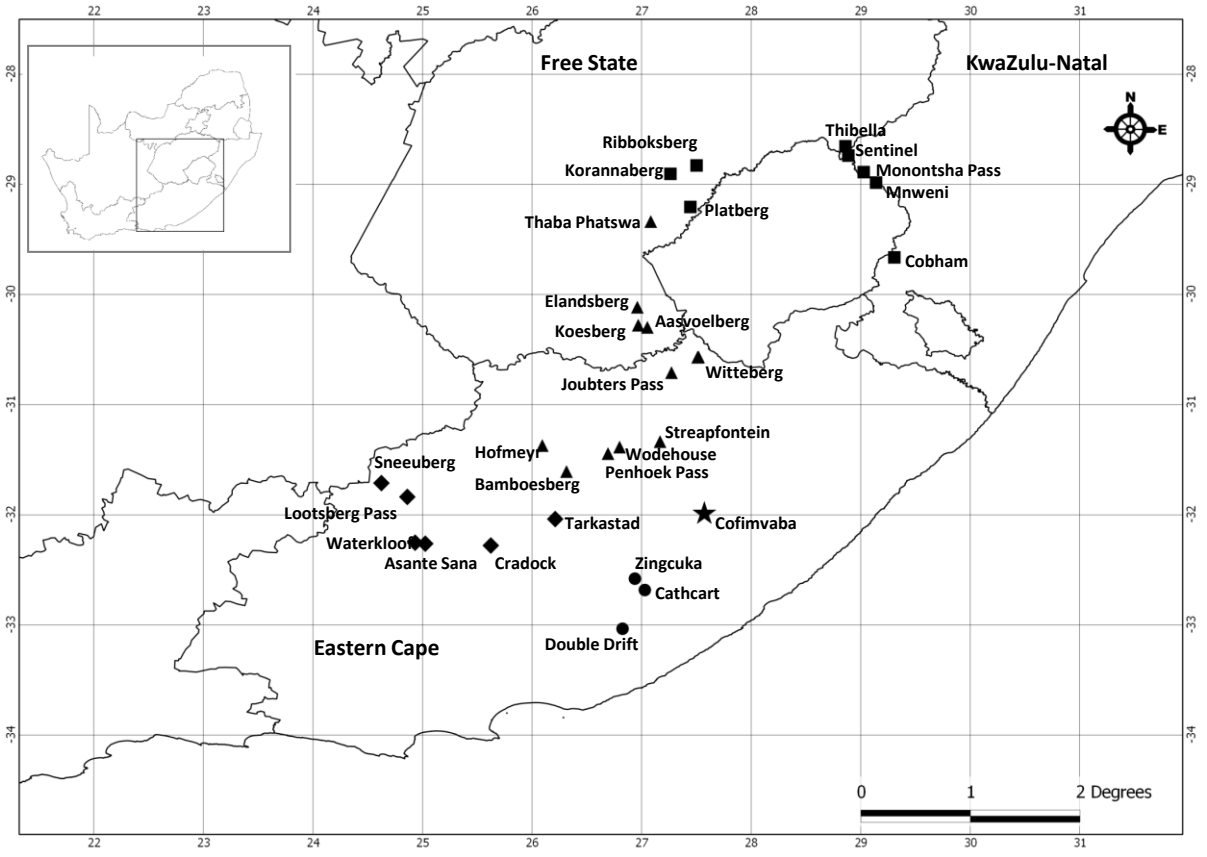


Figure 2. 3 Map of KwaZulu-Natal, Free State and Eastern Cape provinces in South Africa showing sampling localities of each of the five species sequenced for this study. Key to map: square = *A. nivaria*; triangle = *A. halli*; diamond = *A. karroica*; circle = *A. amatolica*; star = *A. tembulica*.

Table 2. 1 A list of genes and associated primers used in this study.

Gene	Primer	Reference	Primer sequence
16S rRNA	16Sa	Palumbi *et al*. 1991	5'-CGCCTGTTTATCAAAAACAT-3'
	16Sb		5'-CCGGTCTGAACTCAGATCACGT-3'
ND4	Leu-tRNA	Arevalo *et al*. 1994,	5'-CATTACTTTTACTTGGATTTGCACCA-3'
	ND4-R4	Jackman *et al*. 2008b	5'-GCAAATACAAACTAYGAACG-3'
	F3		5'-TGACTACCAAAAGCTCATGTAGAAGC-3'
KIAA	F2	Portik *et al*. 2011	5'-TTGGAAAACTACTTCCTGAA-3'
	R 2		5'-AAAATGACCTCCTCCTGGCAA-3'

Table 2. 2 PCR recipes used to amplify target gene regions. The total PCR reaction mixture equals 25 µl (± 30 ng/µl of DNA template). All reagents were measured in micro liters (µl).

REAGENT	**16S rRNA**	**ND4(STT)**	**ND4(GoTaq)**	**KIAA**
ddWater	16.8	16.6	16.6	16.6
STT Buffer	2.5	2.5		
STT $MgCl_2$	2.5	2.5		
Reaction Buffer			5.0	5.0
Primer F	0.3	0.4	0.4	0.4
Primer R	0.3	0.4	0.4	0.4
dNTPs	0.4	0.4	0.4	0.4
BSA		0.2		
SuperTherm Taq	0.2	0.2		
GoTaq			0.2	0.2
Temp °C	49	47-50	52-54	54-60
Cycles	35	40	40	35
Size bp (approx.)	600	900	900	>1000

RESULTS

Sequence variation

Each sample was sequenced in the forward direction except for a few samples which were sequenced using the reverse primer because amplification using the forward primer was unsuccessful. Sequence alignments and complementary sequences were performed in Geneious v. 5.4 (Drummond *et al*., 2011) using default parameters. Where there were obvious mismatches due to alignment errors, adjustments were made by eye.

One hundred and twenty five samples from 21 localities of *A. nivaria*, *A. halli*, *A. karroica*, *A. amatolica* and *A. tembulica* plus representative species outside the *A. nivaria* complex, but within the genus, were sequenced for the 16S rRNA gene fragment. A total of 427 base pairs (bp) were aligned, of which 152 sites (35.6%) were variable among the *A. nivaria* species complex (54.3% including outgroups) and 126 (29.5%) were parsimony informative (38.9% including outgroups). The highly variable and difficult to align section of the 16S rRNA gene was excluded from the analysis (total of 106 bp). There were 59 unique sequences within the dataset. The 16S distances for pairwise comparisons between described taxa and/or clades (not described) ranged from 6-16% (Table 2.3). The greatest divergence was observed among the *A. nivaria* clades with approximately 12% divergence between *A. nivaria* (*sensu stricto*) clade versus *A.* cf. *nivaria* clade B and *A.* cf. *nivaria* clade C.

For the ND4 gene fragment, total fragment length obtained for 117 samples was 733 bp and only 596 bp were used for analysis; the associated t-RNAs were excluded from further analyses due to ambiguity and sequence length variability (different primer pairs were used to amplify the gene). The gene fragment comprised 191 conserved sites and of the 405 variable sites, 308 (51.7%) were parsimony informative (57.4% including outgroups). The translated sequence, 198 amino acids in length, began coding at the third base. There were 134 variable sites, of which 82 (41.4%) were parsimony informative (45.5% including outgroups). Within this dataset, 96 sequences were found unique. The observed *p*-distances for the ND4 comparisons were considerably high with certain values exceeding 25%, ranging between 11% and 29% (Table 2.3). *A. amatolica* cf. clade D (Double Drift Nature Reserve) appears to be the most divergent from the other species. Intraspecific sequence divergence values were generally low and ranged from 0% to 3% for both mtDNA markers.

The combined mitochondrial gene dataset (dataset 1) comprised 125 individuals. The fragment length was 1023 bp of which 407 sites were conserved and of the 616 variable sites, 508 sites were parsimony informative and 108 singletons.

For the KIAA nuclear gene, total fragment length obtained for 15 samples was 621 bp with 557 conserved sites and 25 parsimony informative sites of the 64 sites that were variable.

Table 2. 3 Pairwise genetic distance values (uncorrected *p*-distance) within and among main the mtDNA clades for 16S rRNA (below diagonal) and ND4 (above diagonal) gene sequences. Intraclade sequence diversity separated for each gene is shown in bold on the last column.

	1	2	3	4	5	6	7	8	9	10	16S	ND4
1 *A.* cf. *amatolica* clade D	--	**0.27**	0.28	0.28	0.27	0.29	0.27	0.26	0.28	0.26	**0.00**	**0.02**
2 *A. amatolica*	**0.08**	--	0.22	0.22	0.21	0.23	0.22	0.19	0.17	0.20	**0.00**	**0.00**
3 *A.* cf. *halli* clade A	0.10	0.10	--	**0.13**	0.21	0.22	0.22	0.21	0.23	0.24	**0.01**	**0.00**
4 *A. halli*	0.10	0.10	**0.06**	--	0.21	0.20	0.21	0.22	0.23	0.24	**0.01**	**0.00**
5 *A. karroica*	0.10	0.11	0.14	0.13	--	0.23	0.22	0.21	0.22	0.20	**0.02**	**0.01**
6 *A.* cf. *nivaria* clade B	0.10	0.10	0.09	0.09	0.14	--	**0.11**	**0.24**	0.23	0.23	**0.01**	**0.01**
7 *A.* cf. *nivaria* clade C	0.12	0.12	0.11	0.12	0.16	**0.06**	--	**0.24**	0.21	0.23	**0.03**	**0.00**
8 *A. nivaria*	0.08	0.07	0.10	0.12	0.11	**0.11**	**0.12**	--	0.21	0.17	**0.02**	**0.01**
9 *A. tembulica*	0.07	0.09	0.12	0.11	0.12	0.11	0.12	0.09	--	0.20	**0.00**	**0.00**
10 *A. pondolia*	0.10	0.09	0.12	0.13	0.12	0.11	0.13	0.09	0.11	--	**0.01**	**0.00**

Phylogenetic analysis

The mitochondrial phylogeny and the individual mitochondrial gene trees were largely similar to each other when considering relationships with high support (≥ 0.95 PP and ≥ 75% bootstrap support values), and consistent with the partition homogeneity test results. The individual mtDNA gene trees did not conflict (Figs. 2.4 & 2.5). For the combined mitochondrial datasets, all three methods (maximum parsimony, maximum likelihood and Bayesian inference) produced very similar topologies (Appendix A), as did the combined mitochondrial and nuclear analyses (Figs. 2.6 & 2.7; Appendix B).

All the five described species within the *A. nivaria* complex were well supported in every analysis (Fig. 2.6). Some of the samples which were originally identified as one of the species are shown here to be distinct sub-clades, possibly representing cryptic species. In particular, the widespread species *A. halli* and *A. nivaria* showed geographic structuring. In *A. halli*, two clades were recovered (1.0 PP; 100% bootstrap support): *A. halli* sensu stricto and *A.* cf. *halli* clade A, whereas in *A*. *nivaria*, three clades were recovered in the phylogeny with strong support: *A. nivaria* sensu stricto, *A.* cf. *nivaria* clade B and *A.* cf. *nivaria* clade C. From the phylogenies, three major clades in the putative *A. nivaria* species complex were recovered with nine sub-clades within these three larger clades. Of the three main clades, the inland clade included a close relationship of *A. halli*, *A.* cf. *halli* clade, *A.* cf. *nivaria* clade B and *A.* cf. *nivaria* clade C, while the south-eastern clade comprised of *A*. *tembulica*, *A*. *amatolica*, *A*. *nivaria* sensu stricto and *A*. *pondolia* (outside the *A*. *nivaria* complex). The Karoo clade comprised of *A. karroica* together with the other representative taxa from outside the *A*. *nivaria* complex. A rather unexpected result was that of a narrowly distributed *A*. *amatolica* which showed to have two genetically distinct clades, *A. amatolica* sensu stricto and *A.* cf. *amatolica* clade D with strong support (1.0 PP; 84% bootstrap support) and a clear divergence of 8% for 16S and 27% for ND4.

The analyses however, could not support the *A. nivaria* species complex as monophyletic even though the representative taxa from other species complexes were placed outside the *A. nivaria* complex. Mitochondrial markers were surprisingly consistent with the basal placement of *A. karroica* as well as a surprise inclusion of a population of *A. pondolia* nested with *A. nivaria* sensu stricto (1.0 PP; 96% bootstrap support) from the Drakensberg escarpment and surrounds, a described type locality for this species. Interestingly, *A. karroica* appears to be nested outside the complex forming a polytomy with *A. bogerti*, *A. langi*, *A. marleyi*, *A. multiporus*, *A. pondolia* and *A. transvaalica*, species that belong to other species complexes within the genus, even though the relationship received poor support (0.84 PP; 64% bootstrap support) suggestive that the *A*. *nivaria* species

complex might not be monophyletic. The mitochondrial phylogeny supported the sister relationship of *A. hawequensis* to all the other species with high support.

The combined mitochondrial and nuclear DNA phylogeny failed to recover significant support for the deeper nodes but is largely congruent with the mitochondrial phylogeny when comparing major relationships (Fig. 2.7). Nonetheless, the relevant consistencies between analyses identified the *A. nivaria* complex not to be monophyletic as *A. karroica* appeared nested outside the species complex and because *A*. *pondolia* was nested within the *A. nivaria* complex. Overall, sequence divergences among the clades were higher than within the clades (Table 2.3).



Figure 2. 4 Maximum parsimony (MP) phylogram produced from 16S rRNA mtDNA sequences. Bootstrap support values (1000 replicates) are shown at the corresponding nodes. Bootstrap support values below 50% are not shown.



Figure 2. 5 Maximum parsimony (MP) phylogram produced from ND4 mtDNA sequences. Bootstrap support values (1000 replicates) are shown at the corresponding nodes. Bootstrap support values below 50% are not shown.

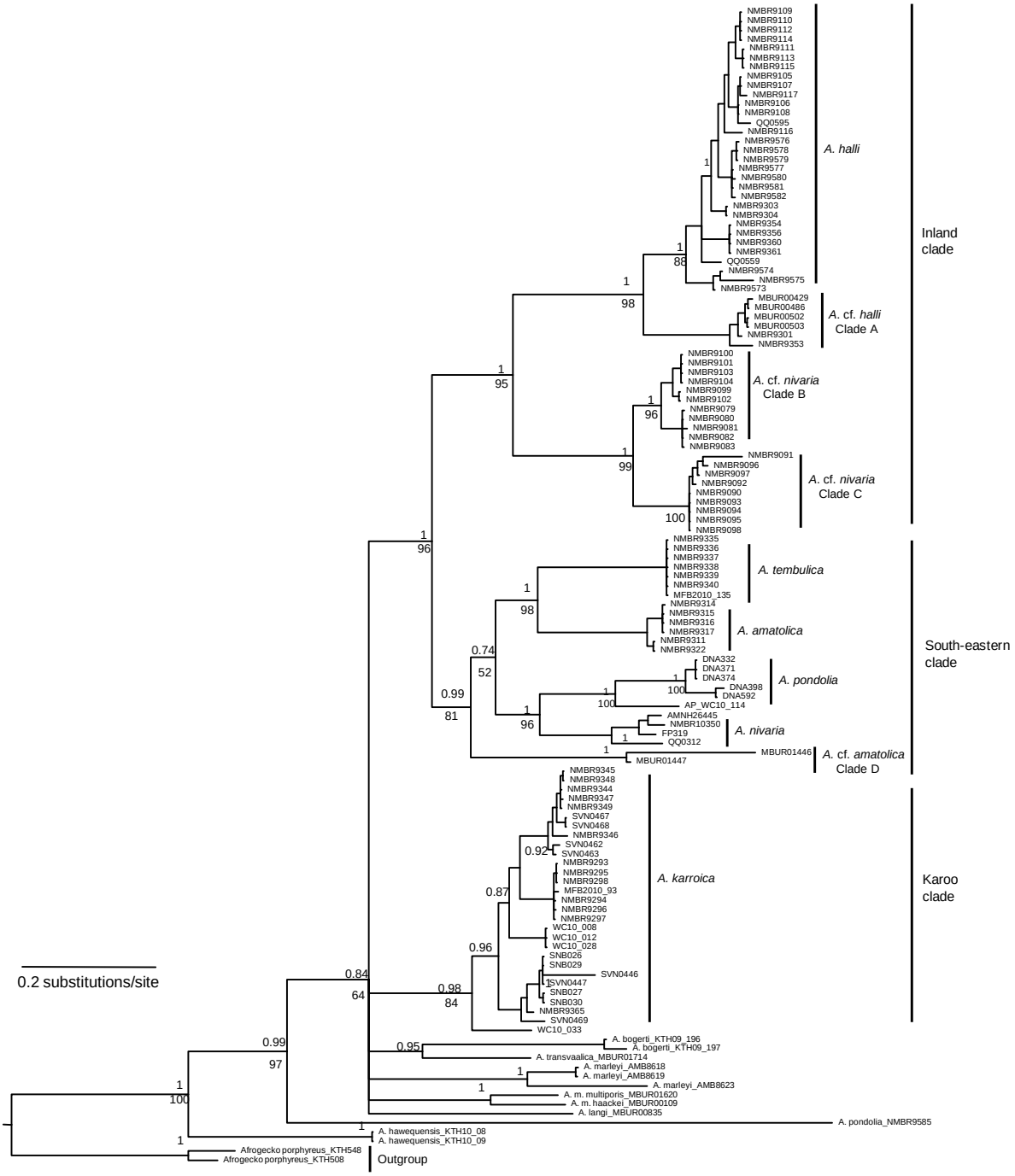


Figure 2. 6 Bayesian 50%-majority-rule consensus phylogram of the combined mtDNA data (16S and ND4) with branch lengths drawn proportionally to the number of site changes. Posterior probabilities are shown above branches and likelihood bootstrap values (1000 replicates) below branches. The tree was rooted with *Afrogecko porphryeus* as outgroup.



Figure 2. 7 Bayesian 50%-majority-rule consensus phylogram based on sequences of the mitochondrial (16S and ND4) and nuclear (KIAA) genes (1622 bp aligned length). Posterior probabilities are shown above branches and likelihood bootstrap values (1000 replicates) below branches. *Afrogecko porphryeus* was used as outgroup (not shown).

DISCUSSION

Taxonomic implications and biogeography

The distinctiveness of the five described species was well supported and phylogenetic analyses revealed three major clades within the *A. nivaria* complex: the inland clade which consisted of *A. halli* and *A.* cf. *nivaria*; the south-eastern clade included *A. tembulica*, *A. amatolica*, *A. nivaria* sensu stricto and *A. pondolia*; and the Karroo clade consisted of *A. karroica*. It appears that *A. karroica* is outside the species complex suggesting that the *A. nivaria* complex is not monophyletic. Additional clades recovered in the phylogeny, which were originally identified as one of the species, are shown here to be new clades because they were monophyletic in the tree and had high sequence divergences. There is no spatial overlap between these clades. Discontinuous and often restricted occurrences have been suggested for this group of geckos as sympatry has not been recorded (Onderstall 1984). Clearly distinguished clades that represent the five described species recovered from the phylogeny disagree with Onderstall (1984), who suggested that the five taxa (*A. nivaria*, *A. halli*, *A. karroica*, *A. amatolica*, and *A. tembulica*) could probably be subspecies of *A. nivaria*.

Although an explicit phylogenetic hypothesis depicting relationships within the *A. nivaria* complex has not been previously proposed, certain statements addressing overall similarities or particular morphological characters are consistent with these findings. For example, some authors have considered *A. halli* to closely resemble *A. nivaria* while *A. amatolica* was closely allied to *A. tembulica* based on traditional morphology analysis (Hewitt 1937, Fitzsimons 1943), and this reflects relationships that were recovered in the present study.

Interestingly, *A. halli* and *A.* cf. *nivaria* clades formed a well supported clade i.e. inland clade (1.0 PP, 96% bootstrap support). This clade was further subdivided into two diverged groups 1) *A. halli* sensu stricto (specimens from the type locality, Telle Junction are within this group) with *A.* cf. *halli* clade A, and 2) *A.* cf. *nivaria* clade B and *A.* cf. *nivaria* clade C. Following a morphological analysis of this group, Bates & Branch (*in prep*.) proposed the possibility of a relationship between *A. nivaria* and *A. halli* on the eastern Free State and indeed, *A. halli*, *A.* cf. *halli* clade A, *A.* cf. *nivaria* clade B, and *A.* cf. *nivaria* clade C have shown to be closely related phylogenetically. Considering the distribution of these two species, this relationship is also supported by geographic proximity of these clades (Figs. 2.3 & 2.8). The distribution of *A.* cf. *nivaria* is continuous on the west of the Drakensberg Mountains but the habitat surrounding outcrops may have not been favourable for dispersal, and possibly be an important historic interruption of the distribution of *A. nivaria*. Previous authors found *A. nivaria* to be undoubtedly occurring around the Drakensberg from the east in KwaZulu-Natal around to the

Free State and specimens examined were evidently not different morphologically hence, described as a single species. Similarly, the distribution of *A*. *halli* is considered to be continuous, from the Stormberg through to southern Drakensberg and the Maluti Mountains (north of Eastern Cape adjacent western Lesotho and southern Free State (Fitzsimons 1943, Loveridge 1947, Onderstall 1984, Branch 1998) and thus, considered a single species (Fig. 2.8).

The nucleotide sequence divergence (uncorrected *p*-distance) between the *A. nivaria* clades varied between 9-12% for 16S and 20-22% for ND4 while the two *A. halli* clades had lower divergence levels (6% for 16S and 13% for ND4). These levels of divergence overlapped much with divergences among the recognized species in this complex. These sequence divergence values compare with those reported between species for reptiles and are higher or equivalent to those observed among distinct species of geckos (Bauer & Lamb 2002, Jesus *et al*. 2005, Rocha *et al*. 2005, 2009, Glaw *et al*. 2010). Interestingly, although these clades are molecularly distinct (e.g. ~12% for 16S and 24% for ND4 genetic distances between *A. nivaria* sensu stricto from the other two undescribed clades), they show no obvious pattern of morphological variation. On the basis of this data, the undescribed clades of *A. nivaria* may need to be treated as full species or at least recognize the monophyletic groups as distinct operational taxonomic units (de Queiroz & Gauthier 1990), but this awaits a detailed examination.

Recent molecular studies have revealed that different species may not differ conspicuously in morphology yet they can be separated by extremely large genetic differences indicating a long history of isolation (Pepper *et al*. 2006, Oliver *et al*. 2009, Couper *et al*. 2008, Doughty *et al*. 2008). Many such species have been described or re-described following modern taxonomic revision approaches that incorporate molecular phylogenies. Similar to these cases are *A.* cf. *nivaria* and *A. halli* clades recovered from the phylogeny. These clades do not differ overtly in the standard morphological characters e.g. nature of tail shape, colouration and body size. Thus, the phylogenetic pattern observed here may indicate a history of isolation and highlights the lack of continuity in geographic distribution between the clades. This is typical of cryptic species; large genetic divergence with no apparent morphological difference because of environmental pressures presented by similar habitats occupied by such species, as may be the case with *A. halli* and *A. nivaria*. A larger dataset utilizing various nuclear markers for a clearer resolution on the deeper nodes (e.g. Pinho *et al*. 2007) may be needed to infer a more robust phylogenetic hypothesis for the *A. nivaria* species complex.

According to the phylogenetic analyses, *A. nivaria*, type locality known from the highest point of the Drakensberg Mountain, clearly groups well with *A. pondolia* and was consistently placed within the

closely related *A.* amatolica/A*. tembulica* group. *Afroedura nivaria* sensu stricto and *A. pondolia* differed by 9% and 17% for 16S and ND4, respectively which was lower compared to divergence values between *A. nivaria* sensu stricto and *A*. cf. *nivaria* clades (11% 16S; 24% ND4). Specimens of *A. pondolia* that nested with *A. nivaria* sensu stricto were from Hluleka Nature Reserve, Dwesa Nature Reserve and a single sample from Mkambathi Nature Reserve and these are known localities for *A. pondolia* (SARCA: http://sarca.adu.org.za). It is possible though that this may be a case of misidentification but unlikely because no overlap in distribution has been reported for *A. pondolia* and *A. nivaria* (Onderstall 1984). Eliminating the possibility of misidentification, the relationship can be explained by a historically shared ancestry. Because both *A. nivaria* and *A. pondolia* are nested within a clade that included *A. amatolica* and *A. tembulica*, the position of *A. pondolia* may be correctly reflected (Fig. 2.8). Taking the distribution of the south-eastern clade into consideration, *A. amatolica* and *A. tembulica* occur along the south-eastern coast and to east of the Drakensberg escarpment, and *A. pondolia* is mainly coastal and fairly widespread consisting of scattered relic populations (Onderstall 1984). Geographic proximity also supports the sister relationship between *A. pondolia* and *A. nivaria* shown here. Again, a large distributional interruption exists between *A.* tembulica/A*. amatolica* and *A. nivaria* sensu stricto (Fig. 2.1) and thus, the inclusion of this population of *A. pondolia* in the south-eastern clade may partly illustrate a historic connection of the species' distribution on the south east of South Africa.

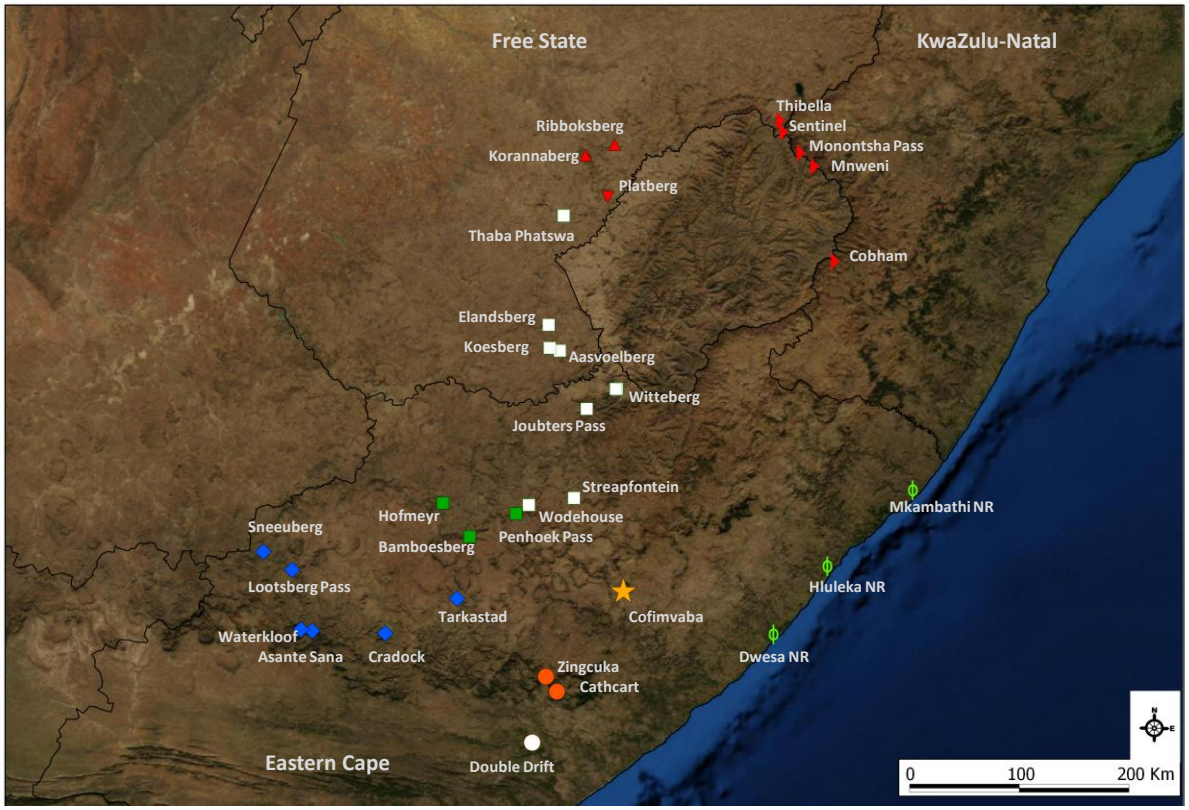


Figure 2. 8 Map of KwaZulu-Natal, Free State and Eastern Cape provinces in South Africa showing sampling localities of each of the five species sequenced for this study. Key to map: ▲ = *A.* cf. *nivaria* clade B; ▼= *A.* cf. *nivaria* clade C;▶= *A. nivaria*; ■ = *A. halli*; □ = *A.* cf. *halli* clade A;◆ = *A. karroica*;●= *A. amatolica*; ○= *A.* cf. *amatolica*; ★= *A. tembulica*; ϕ = *A. pondolia*.

It was Onderstall (1984) who recognized three distinct species groups within the genus *Afroedura*, based on the number and arrangement of scansors (adhesive digital pads) and the nature of the tail as distinguishing characters. Because one character is shared among the species groups, Onderstall's (1984) use of only two characters to distinguish the different species groups had the following implications: 1) the Africana and Transvaalica groups (verticillate flattened tail), and the Pondolia and Transvaalica groups (two pairs of scansors) are sister species, and 2) because no characters are shared between the Africana and the Pondolia groups, Onderstall (1984) suggested that these groups are not closely related even though they are geographically in broad contact in the Cape provinces (Fig. 2.1). *Afroedura pondolia*, which belongs to the Pondolia group, is widely distributed along the south-east coast of South Africa comprised of relic populations. It is shown here to be closely related to *A. nivaria* (Africana group), a relationship which is not in precise agreement with the current taxonomy. Because only a few samples were included in analysis, the relationship shown here could be an effect of a small sampling size and hence, be biased but further investigation using a larger dataset may be helpful in resolving what appears to be contentious species complexes boundaries. Conversely, in his survey of the former Transvaal, Jacobsen (1992) uncovered numerous new populations of flat geckos that did not easily fit into the existing taxonomic arrangement of the *A. pondolia* complex. This could indicate that the Pondolia group is problematic taxonomically as a whole.

The phylogeny showed additional clades that do not correspond to the described species. *A. amatolica* was one such example, having two genetically distinct clades, a consistent finding that was well supported in all analyses (0.99 PP; 81% bootstrap support). Although geckos often present very high degrees of mtDNA sequence divergence (Lamb & Bauer 2000, Harris *et al*. 2002, Jesus *et al*. 2002, Lamb & Bauer 2002, Harris *et al*. 2004b), the two *A. amatolica* clades displayed a relatively high divergence value (8% for 16S, 27% for ND4) within the ranges reported from other reptile studies. Sequence divergence for ND4 is higher compared to 8-12% divergence values typical for species recognition (Pinho *et al*. 2007) and several-fold higher than previously accepted divergences of 2-5.4% for defining species boundaries in squamates (e.g. Hasbun *et al*. 2005). These values show that these clades have been probably evolving in allopatry for a long time. Historic geographic separation is mostly likely to be responsible for the distinctiveness of the two clades of *A. amatolica*. The most likely scenario is a physical barrier to gene flow such as unfavourable habitats surrounding outcrops thus inhibiting dispersal or changes in vegetation linked to pre-historic climatic oscillations and subsequent habitat exclusiveness. Double Drift Game Reserve, where *A*. cf. *amatolica* clade D samples were collected, is one of the three game reserves that form the Great Fish River Reserve and lies at the valley of the Great Fish River. The reserve is almost 100 km away from the Amatole

Mountains (sampling localities of *A. amatolica* sensu stricto), leaving a stretch of unsuitable habitat in between Double Drift and the Amatole which could have led to allopatric speciation and thus, promoting reproductive isolation. The relationship of these distinct clades deserves further investigation.

In agreement with the current taxonomic arrangement, the five described species included in this study were all found to be genetically distinct lineages but phylogenetic evidence did not support the monophyly of the *A. nivaria* complex. However, four of the five species of the *A. nivaria* complex formed a monophyletic group leaving out *A. karroica* even though the relationship was not well supported (0.84 PP; 64% likelihood bootstrap). This was a consistent finding in all analyses (ML, MP and Bayesian inference). Because the *A. nivaria* complex was formerly placed in the *A. africana* complex (Onderstall 1984), it is well possible that *A. karroica* belongs to the Africana species group. The transfer of *A. karroica* from the *A. nivaria* complex to the *A. africana* complex would result in monophyly of the *A. nivaria* species complex. The only other common connection between *A. africana* and *A. karroica* in the Africana group would be geographic occurrence of the two species throughout the Karoo (an extension of dolerites from Angola right through the Western Cape to north Eastern Cape) (Onderstall 1984, Branch 1998). The pattern of relatively short and poorly resolved branches at the base of the tree is suggestive of relatively rapid radiation.

Mouton & Mostert (1985) placed *A. hawequensis* within the Africana group based on morphology, and this appeared to be correctly placed being outside the *A. nivaria* complex. Suggesting that *A. karroica* be transferred to the Africana group has one drawback. Logically, it would have been expected that at least *A. karroica* be a sister taxon to *A. hawequensis* since both species were previously proposed to be in the Africana group (Onderstall 1984, Mouton & Mostert 1985) but it was not the case here. Mouton & Mostert (1985) further suggested that *A. hawequensis* could be the “distributional gap filler” between western and south-eastern species of the Africana group but might be a separate unit that has been isolated for long periods of time and does not geographically form part of the Great Escarpment. Having samples from other taxa in the Africana group would have helped place these species in context of the *A. africana* complex. However, this is beyond the scope of this study and would warrant further investigation.

In the absence of fossil data for this group with which to test biogeography hypotheses, calibrating rates of molecular evolution would have been difficult. Hence, no attempt was made to apply a molecular clock to the data to infer the possible historical events that could have led to the current patterns of speciation within the *A. nivaria* species complex. However, geographic subdivision among some reptile taxa in the eastern southern Africa has been attributed to Plio-Pleistocene

changes in the extent and consolidation of the Kalahari sands, which are believed have isolated rupicolous forms thereby promoting cladogenesis (Broadley 1978). Under such a scenario, Jacobsen (1989) proposed a link between the minor interspecific differentiations in morphology observed in the genus *Afroedura*, flat geckos, to substrate limitation. This scenario was also proposed for the flat lizards, *Platysaurus* (Broadley 1978, Jacobsen 1994). Therefore, it is possible that divergence between *A. karroica* and a common ancestor of the *A. africana* complex reflects isolation between the sandy substrates of the Kalahari Basin and rocky, uplifted substrate of the Great Escarpment as the *A. africana* complex occupies mostly the Karoo and this was also proposed for members of the *Pachydactylus capensis* complex (Bauer & Lamb 2002). In turn, speciation in the *A. nivaria* complex probably reflects vicariance along the escarpment itself with *A. halli* and *A.* cf. *nivaria* occupying the north-western side (from Bamboesberg to Ribboksberg; Figs. 2.3 & 2.8) and *A. amatolica*, *A. tembulica* and *A. pondolia* occupying the coastal and lowveld areas to the east with *A. nivaria* sensu stricto occupying the higher elevations of the Drakensberg escarpment. Changes in climate and substrate availability probably acted as a major cause of isolation and may have aided speciation among the flat geckos in South Africa as is the case with *Palmatogecko* (Bauer 1999).

Avise *et al*. (1998) have shown Pleistocene to have had a considerable impact on the phylogeographic patterns within and among closely related species in several vertebrates. Older divergences in other lizard groups have been associated with Miocene climatic events (e.g. Daniels *et al*. 2002, Rawlings & Donnellan 2003, Schulte *et al*. 2003, Matthee *et al*. 2004, Tolley *et al*. 2011, Townsend *et al*. 2009). In Bauer & Good (1996), they found the separation of genus *Rhoptropus* from *Pachydactylus* to be 56.5 million years ago (MYA) and 86 MYA from *Tarentola* but the dating was based on immunological distance and on a 92 MYA estimate for the separation of Africa and South America. However, given the sequence divergences between the clades/species of up to 16% (16S) and 29% (ND4), these exceed values that have been observed for divergences of reptiles that have occurred during the Plio-Pleistocene period (Matthee & Flemming 2002; Tolley *et al*. 2004, 2006, 2010; Bauer & Lamb 2005, Swart *et al*. 2009) suggesting that divergences within the *A. nivaria* species complex may be much older. Assuming that the molecular rate is the same for ND2 and ND4, divergence values of ~20% have been associated with mid-Miocene divergence, suggesting very old lineages dating back to Miocene/Oligocene as shown within a diverse genus of East African chameleons, *Kinyongia* (Tolley *et al*. 2011). It may be likely that the latest episodes of climate cycling of the Plio-Pleistocene would have been responsible for initiating intraspecific divergence (clades with short branch lengths) within this complex (e.g. Tolley *et al*. 2008) and probably divergences during Miocene or older could be shown by long branches in the phylogeny. The proposed long

history dating back to Miocene is yet another possibility for the divergences within the *A*. *nivaria* species complex.

Markers evolving at different molecular rates: mtDNA vs. nucDNA

The nuclear gene tree supported the major splits observed in mtDNA analyses showing the presence of several well-differentiated entities. These clades correspond not only to the fully recognized species of *A. nivaria* species complex but also to several forms within some of the described species, all of which have a similar level of genetic differentiation to that observed between the acknowledged species. However, relationships between the undescribed forms are well supported both with mtDNA and nuclear data analyses suggesting a scenario of an ancient diversification because if it were rapid diversification, relationships would have been weakly supported with very short internal nodes and short branch lengths, and low sequence divergences (e.g. Pinho *et al*. 2007). Some studies have shown that in testing species boundaries, the use of multiple nuclear markers to determine if gene flow is absent among the mtDNA groups is subsequently an important step in resolving the systematics of complex species complexes (e.g. Leaché *et al*. 2009).

In light of the results obtained, it is not surprising that given such structure and high genetic divergence between recovered mitochondrial clades, that they are supported by the nucDNA gene tree. Such results suggest complete lineage sorting of this marker which would mean that, in addition to the highly divergent mtDNA clades, the nuclear genome is as distinct in the different clades (Rato *et al*. 2010). KIAA has shown to be a variable marker and can be useful for reptile phylogenetic studies. High levels of intraspecific genetic variation for mtDNA have already been described for other geckos (Arnold *et al*. 2008, Austin *et al*. 2004, Jesus *et al*. 2005, Perera & Harris 2010, Rato & Harris 2008), and when comparisons have been possible, variation within nuclear markers has been also remarkable (Carranza *et al*. 2002, Harris *et al*. 2004a, Rocha *et al*. 2005, Rato & Harris 2008). Some studies have found nuclear markers to have little variation which can be accounted for as a result of incomplete lineage sorting in the selected genes (e.g. Arnold *et al*. 2008, Austin *et al*. 2004).

Overall, the analysis of variation in a slower evolving nuclear gene (KIAA) gave a basic picture of relationships between the *A. nivaria* species complex for the deeper nodes. These findings highlight the importance of evaluating multiple independent data sources prior to defining taxonomic units and in particular the difficulties of determining species boundaries in this species complex. Several studies have shown how a plurastic approach can be useful where traditional morphological analysis fail to resolve conflicts and how molecular studies have been employed to answer many questions

concerned with evolutionary and/or conservation biology (e.g. Bauer *et al.* 2003, Rawlings & Donnellan 2003, Mahoney 2004, Rawlings *et al.* 2008, Leaché *et al.* 2009, Doughty *et al.* 2010). However, a large-scale survey of nuclear variation within this group to corroborate what the nuclear subset revealed may prove useful in understanding the processes responsible for speciation events such as vicariance and dispersal or colonization events (e.g. Swart *et al.* 2009). It has also been hypothesized that geckos may have a relatively faster rate of mtDNA evolution (Chiari *et al*. 2009, Harris *et al*. 2004a, Jesus *et al*. 2005) and some authors have found that cryptic species are often overlooked especially with geckos because they appear more morphologically conservative than other reptile taxa and may be the case with the phylogenetic relationships recovered of the *A. nivaria* complex (Harris *et al*. 2004a, 2004b, Perera & Harris 2010).

Genetic divergences to identify or delimit species

Molecular divergence and the topology of the recovered trees demonstrate that each of the mtDNA clades are deeply divergent from each other and are currently referred to *A. nivaria* complex. Based on levels of genetic divergence (Table 2.3; Fig. 2.6), I found considerable evidence for additional unrecognized clades/species in the *A. nivaria* species complex and similarly deep divergences have been recorded for species with narrow geographical ranges (e.g. salamanders; Moritz *et al*. 1992, Mahoney 2004). The use of sequence data in species delimitation has been particularly controversial and some authors have argued that species should not be delimited based on these data alone (Moritz *et al*. 1992, Wiens & Penkrot 2002). Sequence divergences are applied here with caution and are not regarded as absolute values for species relationships but indicators of relatedness of the sequence data (Daniels *et al*. 2002). Hence, studies of the ecology, a detailed morphological examination and potential interactions between these evolutionary divergent, but similar sized ecologically comparable flat rupicolous geckos might prove rewarding (Oliver *et al*. 2012).

Species delimitation and species concepts

It is known that gecko systematics has traditionally relied heavily on digital structure and much attention of the systematic history has focused on species groups within this genus, centering on species descriptions and species boundaries using traditional morphological characters and geographical distributions (Onderstall 1984, Mouton & Mostert 1985, Jacobsen 1992). Subsequently, Bates & Branch (*in prep*.) conducted a morphological analysis of this complex in which they recovered the five described species and additional subtle differences in other populations of the described species.

Incongruence between genetic and traditional morphological borders suggests longer periods of separation and no gene flow among discreet lineages of *A. nivaria* complex. Restricted ranges of these species are largely concordant with other southern African lizards with low vagility or high substrate specificity (Mouton & van Wyk 1994). However, molecular boundaries are largely congruent with geographical breaks in this species complex. These findings suggest that species of the *A. nivaria* complex have restricted dispersal capabilities and possibly historical isolation. The separation between rock outcrops and the development of intervening flatland areas seem more likely as a possible barrier to gene flow as suggested for *Agama atra* (Matthee & Flemming 2002). The genetic subdivision shown here and the lack of morphological differentiation within the *A. nivaria* species complex, suggest the presence of cryptic diversity, of which has been observed in other species of geckos. This is typical of cryptic species to retain their morphological appearance due to similar selective pressures experienced in occupying similar habitats although geographically separated, restricting gene flow. It is for the same reason that no morphological variation has been recorded for additional/new clades within some of the described species. Thus, lineages which are reproductively isolated or monophyletic (i.e. they have exclusive DNA haplotype phylogenies relative to other such lineages) can be considered separate evolving entities under the evolutionary and/or phylogenetic species concept (Wiens & Penkrot 2002, Bauer & Lamb 2005). In addition to a robust analysis of morphological characters, incorporating molecular techniques that take into account the genetic differences among species in systematic studies is an approach that fulfills the phylogenetic species concept. This marks the first attempt to evaluate patterns of intra/interspecific diversity in *A. nivaria* species complex.

Chapter 3

Morphometric variation of the *Afroedura nivaria* species complex

INTRODUCTION

Background on the evolution of morphological variation

An organism's phenotypic appearance can be influenced to some extent by natural selection. Natural selection, a concept coined by Charles Darwin (1859) as an explanation for adaptation and speciation, is a gradual process by which different forms of a character if associated with fitness are preserved in a population (Ridley 2004). Thus, individuals that are best adapted to their environments are more likely to survive and reproduce. Adaptation and speciation however, are affected by numerous factors such as geographic isolation by an extrinsic barrier or behavioural isolation in which reproductive isolation can be achieved (little or no genetic flow). Habitat structure plays a major role in the early stages of vertebrate radiations (Streelman & Danley 2003), divergence in habitat can result into differing degrees of crypsis and/or selection on morphological characteristics that enhances a species performance in a particular habitat (e.g. Herrel *et al*. 2012). Therefore, changes in specific ecological niches and the environment may cause species to respond to selective pressures through morphological differentiation (i.e. adaptation). Lizards being widely distributed and covering a wide range of habitats reflect this very well through a large range of morphological diversity of the general body form (Zaaf *et al*. 1999).

Shifts to a fundamentally new habitat are likely to be accompanied by different adaptive character sets in a species. It has been shown that a relationship between morphological and ecological variation of an organism exists (Losos 1990b), a concept known as ecomorphology. Correlations between body form and utilization of habitat are key examples of this concept. In lizards, links between habitat use and limb proportions are well demonstrated, suggesting that morphological variation is adaptive in the context of microhabitats (Losos 1990b, Zaaf *et al*. 1999, Danley & Kocher 2001, Leal *et al*. 2002, Johnson *et al*. 2005). In the case of geckos, the relationship between digital form and habitat type has been explored (Russell & Bauer 1989, Gamble *et al*. 2012). However, it is not always the case where morphometric analyses would show that morphological variation of a species corresponds to the clear phylogenetic structure especially if the species have undergone recent genetic differentiation, or if there is strong selection on the body form/morphology which would cause the phenotype to adapt to the environment (Streelman & Danley 2003, Guillaume *et al*. 2006).

Variation in habitat use and morphology may be strongly correlated among species independent of their phylogenetic relatedness (Harvey & Pagel 1991, Wainwright & Reilly 1994), which suggests an important role of adaptation to the success of species occupying specific niches and thus, natural selection. Geographical or ecological barriers play a major role in inhibiting gene flow (which may lead to reproductive isolation) and thus genetic divergence can occur, but with similarity in phenotype retained because of the similar selective pressures due to occupying fragmented but similar habitats and/or environments. Species that occupy evolutionarily stable habitats tend to be remarkably similar morphologically despite millions of years of genetic separation, typical of cryptic species, and this has been observed in several lizard groups (Smith *et al*. 2001, Glor *et al*. 2003, Leaché *et al*. 2009, Swart *et al*. 2009). Molecular studies have shown that different species, although separated by a large genetic distances, they can often be confused becaue of their conservative morphology, as observed in the *Pachydctylus serval*/*weberi* groups (Bauer *et al*. 2006). This can mislead taxonomy in species that exhibit comparable patterns (Oliver *et al.* 2009). In a nutshell, morphological conservatism explains the similarity in morphology within a taxonomic group which has had a fragmented geographic distribution but different species are still in similar environments. Thus, there is no gene flow but because species are still in similar habitats, they retain their morphological similarity (as the common ancestor).

As much as similarities in the environment can induce morphological conservatism, differences in environment can also lead to phenotypic divergences of related species if occupying different niches. Occupying similar niches can also lead to phenotypic convergence in unrelated taxa, for example, convergence to a similar body form due to similarities in the habitat occupied. Convergent evolution, also called parallel/repeated evolution of traits, has long been used to explain the independent evolution of similarity in morphological traits between separate evolutionary lineages/unrelated species, due to selection pressures on the phenotype (increased fitness to the same environments) in response to local environmental conditions (Kearney & Stuart 2004, Harmon *et al*. 2005, and references herein). Convergence between lineages is often seen as evidence of adaptation through natural selection or of developmental constraints that limit or bias morphological evolution (Losos 2011). Convergent evolution clearly illustrates the degree of common response to fundamental biological challenges imposed on different species by the environment (Gamble *et al*. 2012). For example, similarities in morphology have been observed in several unrelated lizard groups, but which are found in similar habitats (e.g. Russell & Bauer 1990, Whiting *et al*. 2003, Kearney & Stuart 2004, Harmon *et al*. 2005, Revell *et al*. 2007, Tolley *et al*. 2008). However, similarity alone does not necessarily indicate convergence because similarity in morphology can also be a result of shared ancestry (plesiomorphy), a concept known as exaptation (Revell *et al*. 2007, Wake *et al*. 2011).

Convergence or conservatism of morphological traits has demonstrated the difficulty in relying only on morphology for systematic purposes, particularly at higher levels of inclusiveness (Kluge 1983, Loveridge 1944, Russell 1979, Russell & Bauer 1989, 2002).

Study taxa

Afroedura is a genus of geckos found in southern Africa, comprised of 15 described species within six species complexes and approximately 13 new species awaiting description. The five species of the *A. nivaria* species complex are found in the Eastern Cape, KwaZulu-Natal and Free State, South Africa. They are medium sized geckos characterized by having a depressed, verticillate tail, digits free, clawed and dilated with three pairs of scansors beneath the toes. Their tail is readily discarded as an escape technique (autotomy) and adults often have regenerated tail but quite different in shape and colour from the original one and they do shed their skin periodically. Adult males can be distinguished from the females by the presence of pre-anal pores and these in *A. nivaria*, *A. amatolica*, *A. tembulica*, and *A. halli* males form an angular series but in *A. karroica* these are arranged in a transverse series. All species are rupicolous utilizing a range of rocky substrates. For example, *A. karroica* prefers small sandstone rock outcrops in broken ground, *A. nivaria* is found in rock crevices under loose boulders lying on bedrock at very high altitudes (above 2750 m) whereas *A. halli*, a rather solitary species compared to other species in the complex, often only a single individual or a pair is found under suitable rock flakes on the west side of large overhanging boulders of weathered sandstone. The Amatola and Tembu flat geckos seem to be tolerant of conspecifics; up to 10 individuals may be found in suitable rock crevices on granite outcrops. They are strictly nocturnal and insectivorous, diet comprised of ants, beetles, grasshoppers, mosquitoes, sandflies, and termites amongst other insects (Hewitt 1937, Fitzsimons 1943, Loveridge 1947, Branch, 1998).

Members of this species complex appear morphologically similar, and are difficult to identify. Even though they appear similar, there are some characters that set the species apart taxonomically. For example, presence of internasals, type of dorsal scales, midbody scale rows, number of scales between the eye and nasals s, or pre-anal pores count have been taxonomically diagnostic, but their body shapes seem similar and this could represent a case of morphological conservatism (Table 3.1). For instance, *A. nivaria* closely resembles *A. halli* but in *A. nivaria* the rostral scale borders the nostril, scales on the back are juxtaposed, granular and more rounded in *A. nivaria* but more flattened in *A. halli* (Fitzsimons 1943, Loveridge 1947, Branch 1998).

Taking into consideration the phylogenetic results of the *A. nivaria* species complex (Chapter 2), clades recovered in the phylogeny, some of which are already described as species, were subject to

morphometric analysis. Because these species look similar, ecologically relevant traits rather than traditional traits were used to examine whether there is morphological variation among the clades uncovered genetically or if the clades are similar and therefore morphologically conservative. It is hypothesized that well defined genetic lineages recovered from the phylogeny (results; Chapter 2) might be difficult to distinguish morphometrically and thus, display morphological conservatism. To test this hypothesis, data from external linear morphological measurements of museum specimens (all deposited in the National Museum, Bloemfontein) was analyzed using multivariate statistical methods.

Table 3. 1 Distinguishing characteristics between members of the *Afroedura nivaria* species complex.

Species	SVL (mm)	Tail	Pre-anal pores	Scales on back	Head scales	Chin shields	Colour	Habitat	Range
A. nivaria	50-55	Segmented, slightly depressed	9-15 pores angular series	Granular, rounded and juxtaposed	Rostral borders nostril	~6 flattened polygonal	Light brown with dark motlings and transverse bands	Large sandstone rock faces at high altitudes	Drakensberg Mt entering Free State & KZN
A. halli	50-60	Segmented, depressed	6-8 pores angular series	Granular and juxtaposed	Rostral separated from nostril	>12 enlarged polygonal	Pale grey to greyish-brown with irregular brown crossbands	Rock flakes of weathered sandstone	Western Lesotho adjacent Free State&NE Cape
A. karroica	40-50	Segmented, much depressed	6-8 pores transverse series	Flattened, juxtaposed to subimbricate	Rostral entering nostril	not definite	Greyish with scattered blotches	Sandstone outcrops in broken ground	Inland Mts of Eastern Cape
A. tembulica	50-55	Segmented, much depressed	6-9 pores angular series	Granular	Rostral entering nostril	small/5-6 moderate polygonal	Greyish-brown with dark mottlings	Granite outcrpos	Mts around Queenstown E. Cape
A. amatolica	50-60	Segmented	10-12 pores slightly angular row	Flattened and imbricate	Rostral entering nostril	enlarged	Brownish-grey with zig-zag brown bands	Granite outcrops on montane grassland and dry thicket	Amatola Mountain range

MATERIALS AND METHODS

Data collection

All individuals measured were museum specimens from the National Museum Bloemfontein (Table 3.2). Morphometric measurements for 224 individuals were taken using a set of digital calipers with a resolution of 0.01 mm (Fig. 3.1). Following the measurements used in Vences *et al*. (2004), Harmon *et al*. (2008) and Herrel *et al*. 2012; the following external morphological measurements were taken: snout-vent-length (SVL), tail length (TL), head length (HL), head width (HW), head height (HH), lower jaw length (LJL), snout-eye distance (CT), snout-orbital length (QT), humerus length (HM), radius length (RD), hand length (HAND), carpal length (CP), finger length (FN), femur length (FM), tibia length (TB), foot length (FOOT), tarsal length (TR), toe length (TOE), interlimb length (ILL), body height (BH), body width (BW) , and hemipenis width (HPW). A set of digital photographs were again taken for each individual on 1 cm^2 grid paper to ensure correct identification post reference. Limb measurements were made on the left hand side of the animal unless bones were abnormal or broken (or missing with regards to toes). All measurements were taken by the same person to minimize measurement error.

Only adult specimens (snout-vent length greater than 30 mm for females and 37 mm for males) were used in the morphometric analyses. Preceding analyses, all variables were log-transformed to normalize the data. Data (log-transformed values) was then screened for outliers using summary statistics and graphic displays (box plots and histograms) for all variables with a pairwise exclusion of missing values. All statistical tests were carried out in the Statistical Package for the Social Sciences (SPSS) v. 15.0 software package for Windows (SPSS Inc., Chicago, IL, USA). Tail length was excluded from further analyses because of the inconsistency in the measurements due to autotomy.

Sexual dimorphism

Due to a small sampling size (Table 3.2), only data from two species, *A*. *nivaria* and *A*. *halli*, sub-divided according to clades recovered from the phylogeny (Chapter 2), could be examined for sexual dimorphism (n > 20 per sex). Log-transformed values were re-screened for outliers separately for each dataset by using summary statistics. To remove the effect of size, all variables were regressed with log-SVL as a covariate and the unstandardized residuals were saved and used as input data for subsequent analyses. To examine that SVL was an appropriate covariate and that the assumptions of analysis of covariance (ANCOVA) were not violated, equality of slopes was investigated using a custom general linear model (GLM).

Potential differences between sexes were evaluated using the principal components analysis (PCA), a multivariate analysis that summarizes variance to resultant principal components (PC) identifying sets of variables that contribute to the overall morphological variation. Unstandardized residuals were used in the PCA to produce linear combinations of morphological characteristics which were compared between sexes. The correlation model of PCA was used because all variables were single-dimensional, linear and measured using the same scale (Pimentel 1979). Thus, a PCA on the correlation matrix of the residuals was conducted; varimax rotation method with Kaiser Normalization and the resulting principal component scores were saved. The Kaiser-Meyer-Olkin (KMO) test ensured that sampling adequacy was sufficient to proceed with the PCA. Only principal components with eigenvalues greater than one were extracted. Using the principal component scores, one-way analysis of variance (ANOVA) was run to examine the principal components for significant differences between sexes with sex as the fixed factor for the two species. A single principal component (FM, TB and HW) was significantly different between the sexes for *A. halli* and explained 10% of the total variation (see Results). Because this dimorphism is relatively minor is scope, data from both sexes was combined into a single dataset for further analyses thereby increasing statistical power of the dataset.

Species level morphological analysis

The dataset included all five species within the *A*. *nivaria* species complex but subdivided according to the genetic clades recovered from the phylogeny (Chapter 2). To size correct, all variables (log-transformed values) were regressed with log-SVL as a covariate and the unstandardized residuals were saved and used as input data for subsequent analyses. To examine that SVL was an appropriate covariate and that the assumptions of analysis of covariance (ANCOVA) were not violated, equality of slopes between clades was investigated using a custom general linear model (GLM). Because this comparison consisted of a series of tests, the Bonferroni correction was applied in order to minimize the possibility of Type I errors (Rice 1989).

To identify sets of variables that contributed to the overall morphological variation between clades/species, a PCA on the residuals as input variables using the correlation matrix model was conducted; varimax rotation method with Kaiser Normalization and the resulting principal component scores were saved. The correlation model (method) of PCA was used because all variables were single-dimensional, linear and measured using the same scale (Pimentel 1979). The Kaiser-Meyer-Olkin (KMO) test ensured that sampling adequacy was sufficient to proceed with the PCA. Only principal components with eigenvalues greater than one were considered. Using the

saved principal component scores, one-way analysis of variance (ANOVA) was run to examine the significance between lineages of the extracted PCs with lineages as the fixed factor.

The PCA was also run on a dataset which included additional specimens from outside the *A*. *nivaria* species complex (*A. pondolia*, *A. langi* and *A. transvaalica*) to put the *A. nivaria* complex in context with the other species complexes in the genus *Afroedura*. Following the same procedure detailed for the *A*. *nivaria* complex above, residuals were used as input data in a PCA and with the resulting principal component scores; a one-way ANOVA was run to examine the significance of the extracted PCs.

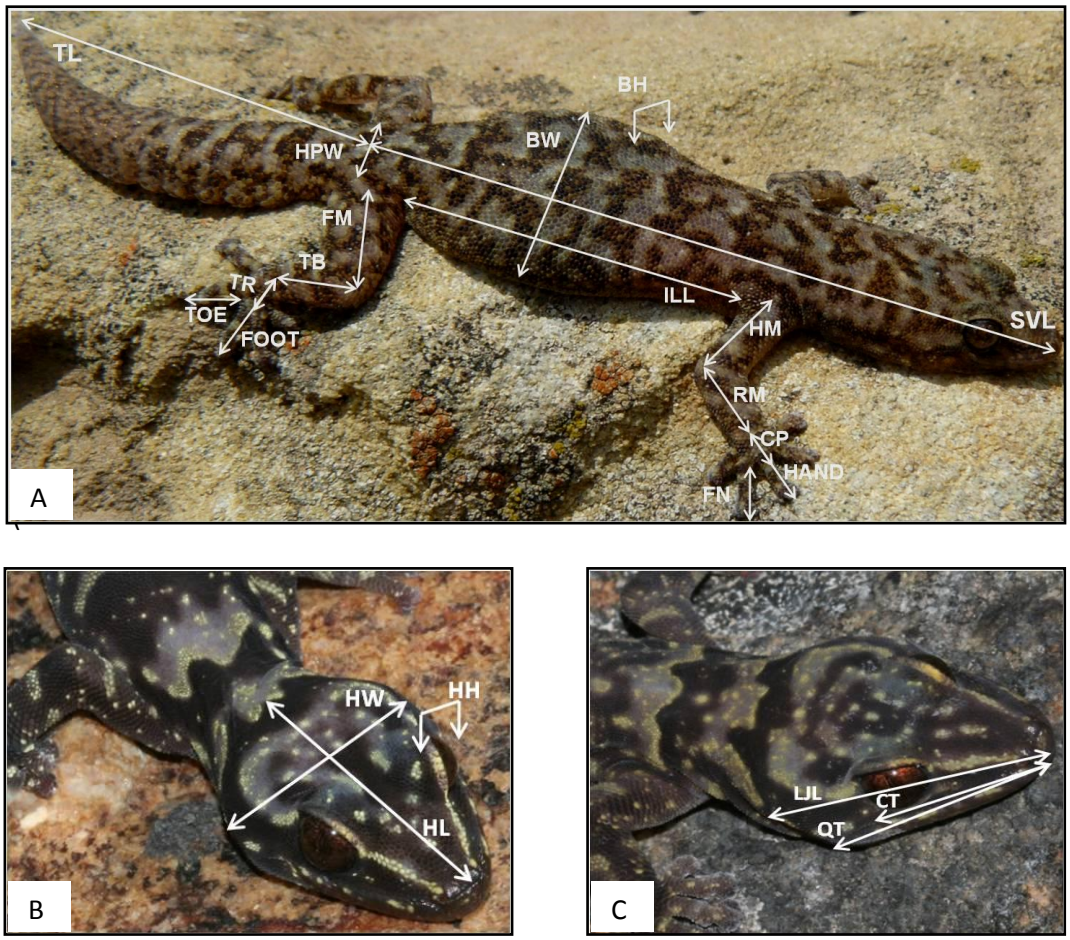


Figure 3. 1 Morphometric measurements taken for museum specimens of Afroedura: a) snout-vent-length (SVL), tail length (TL), humerus length (HM), radius length (RD), carpal length (CP), finger length (FN), hand length (HAND), femur length (FM), tibia length (TB), tarsal length (TR), toe length (TOE), foot length (FOOT), body width (BW), body height (BH), hemipenis width (HPW), and interlimb length (ILL); b) head length (HL), head width (HW), and head height (HH); c) lower jaw length (LJL), snout-eye distance (CT), and snout-orbital length (QT).

Table 3. 2 Specimens used in the morphometric analysis of the *Afroedura nivaria* species complex (denoted with *) plus representative taxa from other species complexes within the genus.

Number	Species	Sampling localities	Sample size (*N*) Males	Sample size (*N*) Females
1	*A. nivaria**	4	13	17
2	*A.* cf. *nivaria* clade B*	3	12	12
3	*A.* cf. *nivaria* clade C*	3	7	7
4	*A. halli**	12	24	47
5	*A*. cf. *halli* clade A*	-	-	-
6	*A. karroica**	5	10	8
7	*A. amatolica**	3	3	8
8	*A.* cf. *amatolica* clade D*	-	-	-
9	*A. tembulica**	1	3	2
10	*A. pondolia*	6	1	12
11	*A. langi*	1	3	4
12	*A. transvaalica*	2	5	8

RESULTS

Sexual dimorphism

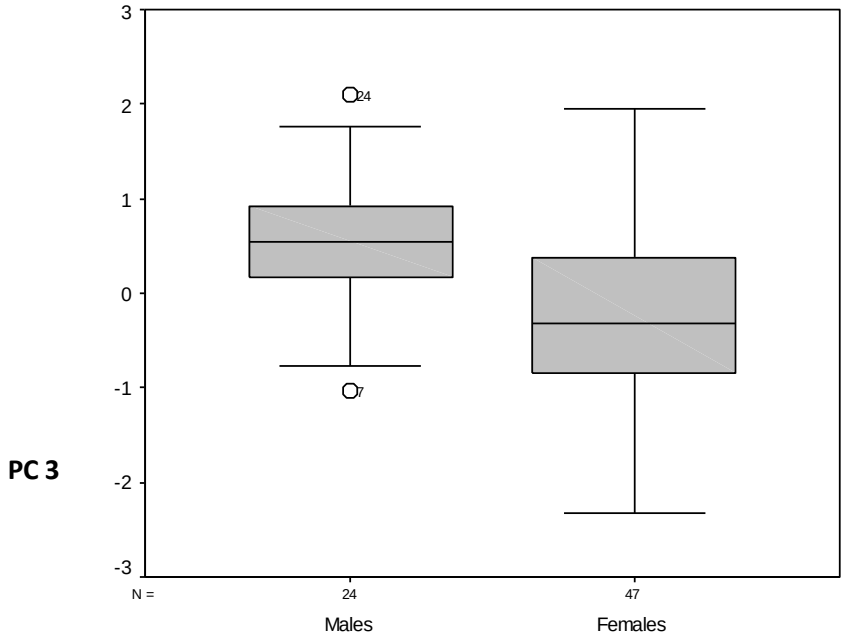
Of the 222 individual specimens measured (Appendix D), a total of 206 specimens were used in analyses (Table 3.2). Following screening of the dataset, some individuals were excluded because they were considered juveniles based on their body size. For the analysis of sexual dimorphism, only *A. halli* sensu stricto and *A. nivaria* sensu stricto had adequate sampling (KMO ≥ 0.5) for the analysis and thus, sexual dimorphism was only explored for these two species. PCA on *A. halli* sensu stricto extracted seven PCs with eigenvalues > 1.0 which explained 69.29% of the total variance (Table 3.3). A single PC was found significant by analysis of variance ($p < 0.01$; ANOVA) which correlated with forelimbs (FM, TB) and head width (HW). Males had significantly longer hindlimbs and a wider head than females (Fig. 3.2). Although *A. nivaria* sensu stricto had an adequate sampling size (KMO = 0.508), rotation (PCA) could not converge hence, failing to show significant variation with the variables included in analysis, an effect of small sampling size.

Table 3. 3 Results of the analysis of variance on the principal components extracted in *Afroedura halli* (by sex), with the percentage of variance explained by each component. Sizable correlations are bolded for factor loadings > 0.5. NS = not significant.

	Principal component						
Variable	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**
LJL	**0.814**	0.083	0.055	-0.058	0.054	-0.069	0.087
HL	**0.795**	0.158	0.105	0.101	0.116	0.175	-0.081
HH	**0.645**	-0.040	-0.202	0.092	0.116	-0.464	-0.109
QT	**0.626**	0.072	0.312	0.100	0.098	0.239	0.377
CT	**0.519**	0.200	0.103	-0.089	-0.035	0.179	0.506
FN	0.104	**0.840**	0.098	0.004	0.126	-0.064	-0.011
TOE	0.145	**0.787**	-0.013	0.156	0.050	0.013	-0.050
FOOT	0.002	**0.613**	-0.306	0.080	0.007	0.479	0.104
FM	-0.080	-0.056	**0.711**	0.249	-0.060	0.124	0.125
HW	0.213	0.188	**0.705**	-0.049	0.073	-0.146	-0.001
TB	0.101	-0.119	**0.665**	0.191	-0.025	0.040	-0.267
RD	0.076	0.043	0.171	**0.889**	0.063	0.023	0.107
HM	-0.004	0.158	0.121	**0.859**	-0.078	-0.005	-0.106
BW	0.097	0.015	0.049	-0.131	**0.752**	0.053	0.076
BH	0.143	0.222	-0.324	0.219	**0.683**	-0.051	0.214
HAND	0.129	0.396	0.190	0.082	**0.521**	-0.040	-0.385
ILL	0.015	0.179	-0.181	0.044	-0.307	**-0.660**	0.275
TR	0.300	0.245	-0.206	0.108	-0.390	**0.653**	0.034
CP	-0.055	0.100	0.096	-0.036	-0.136	0.162	**-0.802**
Eigenvalue	3.601	2.245	1.968	1.676	1.477	1.169	1.028
% Exp.	13.74	11.41	10.43	9.40	8.66	7.93	7.72
Cum. %	13.74	25.15	35.58	44.98	53.64	61.57	69.29
F	1.023	1.102	12.183	0.286	0.454	0.704	0.144
p	0.315	0.298	0.001*	0.594	0.503	0.404	0.706

*$P < 0.01$

Figure 3. 2 A graphical representation of variation for PC3, which was the only significantly different principal component between sexes of *Afroedura halli*, with the mean and standard error (bars) shown. PC3: hindlimbs and head width. Open circles indicate outliers found after analyses.



Species level morphological analysis

Two multivariate analyses were run to assess variation within *A. nivaria* species complex, one with all the variables included and the other which excluded sexually dimorphic variables (i.e. FM, TB, HW) to ensure that dimorphism would not confound the analysis.

A PCA using the size corrected residuals (all variables included) extracted seven principal components with eigenvalues greater than 1.0. Total variance explained by the extracted PCs was 65.90%. All variables showed to be reliable contributors to the analysis (communalities > 0.5). The KMO test indicated that sampling was adequate for the dataset (KMO = 0.703). An ANOVA on the seven principal components scores showed four PCs to be significantly different between the clades examined ($p < 0.05$). Principal components that showed significant differences correlated with fore and hind feet (PC1), head length (PC2), hindlimbs and head width (PC3), and head height (PC5) (Table 3.4; Fig.3.3). When the sexually dimorphic variables were excluded in the PCA, correlation of variables to principal components was not much changed and the results obtained were similar to the one with all the variables included. From principal components analyses, with or without dimorphic variables, principal components that correlated with body height and width were not significantly different between the clades. Therefore, sexual dimorphism was considered to have little or no effect on the outcome of the results.

Not all genetic clades could be represented in the morphometric dataset, some clades, that is, *A.* cf. *halli* clade A and *A.* cf. *amatolica* clade D were excluded because there were no specimens available. From the variation observed for the *A. nivaria* species complex, *A. amatolica* was found to have significantly smaller hands and feet in relation to size compared to the rest of the clades in the complex (Fig. 3.3, PC1) while *A. karroica* appears to have a much more depressed head (Fig. 3.3, PC5). The only significant difference between *A. halli* and *A. nivaria* was hind limbs and head height (Fig. 3.3, PC 3; PC 5). Body dimensions did not show any significant difference for any of the clades (Table 3.4). Between the three well defined genetic clades of *A. nivaria*, it was clear that *A. nivaria* sensu stricto is significantly different, whereas *A*. cf. *nivaria* clade B and C were not significantly different in morphology despite being genetically divergent (Fig. 3.3). For example, *A. nivaria* sensu stricto appeared to have a significantly shorter head but larger feet compared to *A.* cf. *nivaria* clade B and *A.* cf. *nivaria* clade C. Post-hoc pairwise comparisons are shown in Table 3.5.

Table 3. 4 Principal component (PC) loadings for each of the original variables measured (residuals) of the *Afroedura nivaria* complex. Sizeable correlations are bolded for principal components that were significantly different between species (rotated matrix). PC: principal components, % Exp.: percentage of variation explained, Cum. %: cumulative percentage variation. Abbreviations: head length (HL), head width (HW), head height (HH), lower jaw length (LJL), snout-eye distance (CT), snout-orbital length (QT), humerus length (HM), radius length (RD), hand length (HAND), carpal length (CP), finger length (FN), femur length (FM), tibia length (TB), foot length (FOOT), tarsal length (TR), toe length (TOE), interlimb length (ILL), body height (BH), and body width (BW).

	Principal component						
Variable	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**
FOOT	**0.764**	-0.093	0.014	0.152	0.187	0.056	0.296
FN	**0.743**	0.192	0.120	0.087	-0.046	-0.013	-0.109
HAND	**0.728**	0.166	0.175	0.079	-0.113	0.097	-0.183
TOE	**0.688**	0.021	-0.014	0.126	0.176	0.026	0.064
CP	**0.443**	-0.140	0.263	0.116	-0.243	0.113	0.153
LJL	-0.049	**0.795**	0.065	-0.094	0.142	0.088	0.026
HL	0.016	**0.782**	-0.070	0.024	-0.176	0.019	-0.214
QT	0.180	**0.671**	0.323	0.130	0.144	0.092	0.173
CT	0.216	**0.561**	0.124	0.165	0.265	-0.059	0.264
TB	0.065	0.048	**0.815**	0.029	0.038	-0.086	-0.119
FM	0.075	0.012	**0.763**	0.212	0.041	0.035	0.091
HW	0.178	0.278	**0.597**	-0.064	0.143	0.120	0.136
RD	0.111	0.031	0.074	**0.856**	0.160	-0.040	0.059
HM	0.270	0.049	0.121	**0.853**	0.030	0.012	0.064
HH	0.034	0.154	0.130	0.163	**0.798**	-0.054	-0.085
BW	0.088	0.129	-0.015	-0.074	-0.111	**0.890**	0.011
BH	0.088	-0.007	0.114	0.102	0.588	**0.643**	-0.034
ILL	0.089	0.003	-0.024	-0.180	0.212	-0.071	**-0.767**
TR	0.384	0.136	0.066	-0.149	0.207	-0.175	**0.501**
Eigenvalue	4.158	2.068	1.524	1.328	1.309	1.098	1.035
% Exp.	14.30	11.82	10.00	9.09	7.44	6.92	6.33
Cum. %	14.30	26.12	36.12	45.21	52.65	59.57	65.90
F	10.711	3.129	3.667	1.668	11.965	0.959	1.609
p	**< 0.001**	**0.006**	**0.002**	0.132	**< 0.001**	0.455	0.148

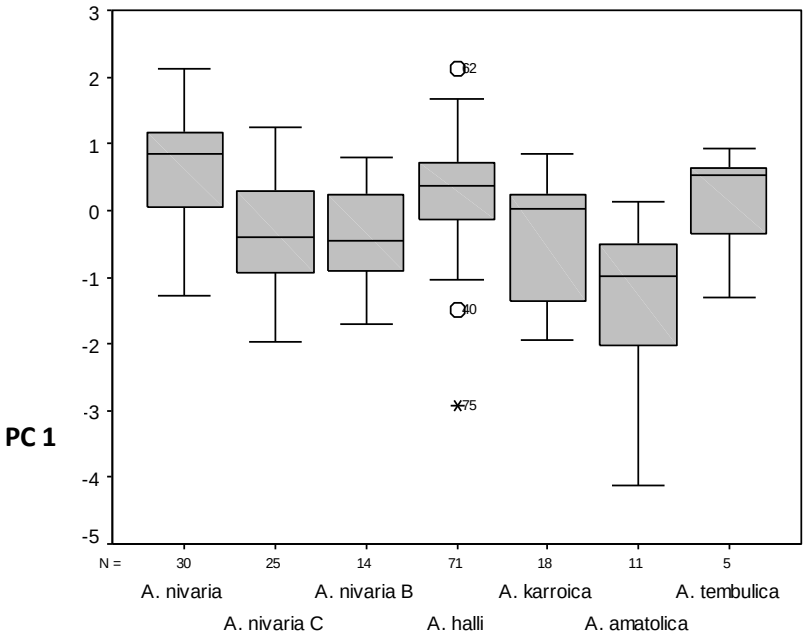
F- and *P*-values are given for the principal components that were significantly different between species by analysis of variance on the principal component scores.
Significant at $p < 0.05$

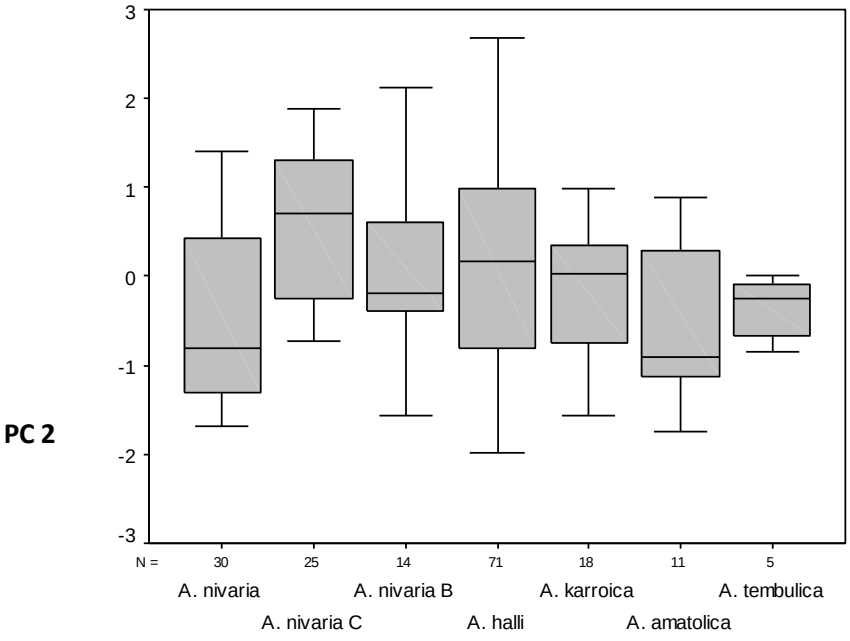
Table 3. 5 Results of the Bonferroni corrected post-hoc pairwise comparisons on the significant principal components (PC) for each of the clades of the *Afroedura nivaria* complex. PC1: fore and hind feet; PC2: head length; PC3: hind limbs and head width; PC5: head height.

Dependent Variable	(I) clade	(J) clade	Mean Difference (I-J)	Std. Error	Sig.
PC 1	*A. nivaria*	*A. nivaria* C	0.942	0.23	0.002
		A. nivaria B	1.028	0.28	0.007
		A. karroica	1.059	0.26	0.001
		A. amatolica	2.050	0.31	0.000
	A. nivaria C	*A. nivaria*	-0.942	0.23	0.002
		A. amatolica	1.108	0.31	0.011
	A. nivaria B	*A. nivaria*	-1.028	0.28	0.007
	A. halli	*A. karroica*	0.721	0.23	0.039
		A. amatolica	1.711	0.28	0.000
	A. karroica	*A. nivaria*	-1.059	0.26	0.001
		A. halli	-0.721	0.23	0.039
	A. amatolica	*A. nivaria*	-2.050	0.31	0.000
		A. nivaria C	-1.108	0.31	0.011
		A. halli	-1.711	0.28	0.000
		A. tembulica	-1.531	0.47	0.026
	A. tembulica	*A. amatolica*	1.531	0.47	0.026
PC 2	*A. nivaria*	*A. nivaria* C	-0.986	0.26	0.005
	A. nivaria C	*A. nivaria*	0.986	0.26	0.005
PC 3	*A. nivaria*	*A. halli*	0.668	0.21	0.034
		A. tembulica	1.525	0.46	0.025
	A. nivaria C	*A. tembulica*	1.462	0.47	0.045
	A. halli	*A. nivaria*	-0.668	0.21	0.034
	A. tembulica	*A. nivaria*	-1.525	0.46	0.025
		A. nivaria C	-1.462	0.47	0.045
PC 5	*A. nivaria*	*A. nivaria* B	0.851	0.28	0.049
		A. halli	1.216	0.19	0.000
		A. karroica	1.842	0.25	0.000
		A. tembulica	1.653	0.41	0.002
	A. nivaria C	*A. karroica*	1.209	0.26	0.000
	A. nivaria B	*A. nivaria*	-0.851	0.28	0.049
		A. karroica	0.991	0.30	0.028
	A. halli	*A. nivaria*	-1.216	0.19	0.000
	A. karroica	*A. nivaria*	-1.842	0.25	0.000
		A. nivaria C	-1.209	0.26	0.000
		A. nivaria B	-0.991	0.30	0.028
		A. amatolica	-1.102	0.33	0.019
	A. amatolica	*A. karroica*	1.102	0.33	0.019
	A. tembulica	*A. nivaria*	-1.653	0.41	0.002

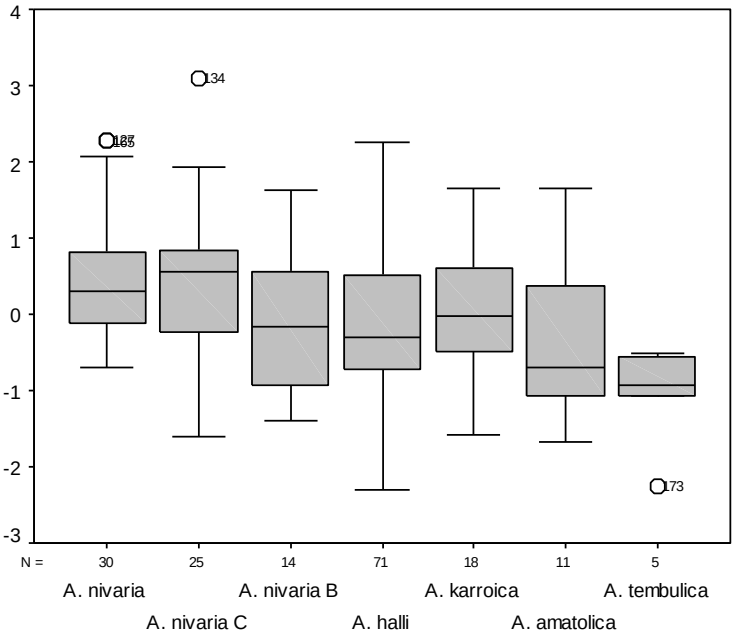
The mean difference is significant at the 0.05 level

Figure 3. 3 A graphical representation of variation for significantly different principal components between species of *Afroedura nivaria* complex with the mean and standard error (bars) shown. PC1: fore and hind feet; PC2: head length; PC3: hind limbs and head width; PC5: head height. Open circles show outliers and asterisk indicates extreme values.





PC 3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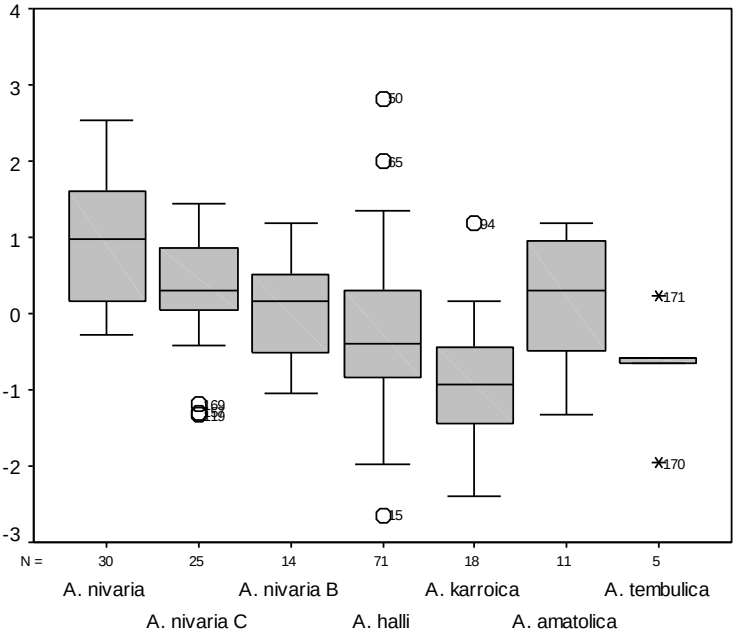
A. nivaria B
A. karroica
A. tembulica
A. nivaria C
A. halli
A. amatolica

PC 5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A. nivaria B
A. karroica
A. tembulica
A. nivaria C
A. halli
A. amatolica

Morphological analysis including species outside the A. nivaria species complex

For the multivariate analysis that included species outside the *A. nivaria* species complex, the principal component analysis using residuals (17 variables; tarsal (TR) and carpal (CP) had communalities < 0.5 and were excluded from further analyses) extracted six principal components with eigenvalues greater than 1.0. These extracted PCs explained 66.75% of the total variance (Table 3.6). All variables included in analysis showed to be reliable contributors to overall variation (communalities > 0.5) and the KMO test indicated that sampling was adequate for the dataset (KMO = 0.749). An ANOVA using the principal component scores (Table 3.6) showed that all six PCs had significant differences between species/clades (p < 0.01; Fig. 3.4). Principal components were correlated with head size and shape, feet, forelimbs, hindlimbs, body structure and interlimb length.

Genetic clades from the phylogeny were again taken into consideration for this analysis. From the principal components, it appears that the three species, *A*. *pondolia, A. langi* and *A. transvaalica* may have smaller heads given their body sizes and proportionally longer limbs (*A. langi* had the longest limbs; PC3 & PC4) compared to the species within the *A. nivaria* complex (Fig. 3.4, PC1). Again, *A*. *pondolia* and *A. langi* appear to have significantly longer interlimb lengths (Table 3.7). Conversely, the *A. nivaria* species complex appears to have significantly larger heads than the other species. Between the three well defined genetic clades of *A. nivaria*, no variation was observed when considering head dimensions, limbs and the interlimb length (Table 3.7). However, *A. nivaria* sensu stricto had a significantly larger body structure in comparison to the other two genetic clades, which did not show any significant differences. On the other hand, *A. amatolica*, *A. karroica* and *A. tembulica* showed to have smaller limb lengths and interestingly, with much variation from the feet measurements (Fig. 3.4, PC2), *A. nivaria* sensu stricto was significant for bigger feet when compared to all the species.

Table 3. 6 Principal component (PC) loadings for each of the original variables measured (residuals) of the *Afroedura nivaria* complex including additional species from other species complexes within the genus. Sizeable correlations are bolded for principal components that were significantly different between species (rotated matrix). PC: principal components, % Exp.: percentage of variation explained, Cum. %: cumulative percentage variation. Abbreviations: head length (HL), head width (HW), head height (HH), lower jaw length (LJL), snout-eye distance (CT), snout-orbital length (QT), humerus length (HM), radius length (RD), hand length (HAND), finger length (FN), femur length (FM), tibia length (TB), foot length (FOOT), toe length (TOE), interlimb length (ILL), body height (BH), and body width (BW).

	Principal component					
Variable	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**
LJL	**0.839**	-0.035	-0.026	0.011	0.024	-0.008
HL	**0.759**	0.041	-0.110	-0.026	-0.025	-0.039
QT	**0.691**	0.188	0.242	0.198	0.077	-0.038
CT	**0.605**	0.282	0.244	0.093	0.067	-0.049
HW	**0.522**	0.334	-0.154	0.317	0.195	-0.258
HH	**0.477**	0.162	0.296	-0.191	0.278	0.307
FN	0.186	**0.805**	0.001	0.087	-0.034	0.011
HAND	0.139	**0.758**	-0.084	0.125	0.006	0.038
TOE	0.106	**0.748**	0.172	-0.066	0.086	-0.054
FOOT	-0.035	**0.695**	0.301	0.025	0.186	-0.061
RD	0.157	0.065	**0.830**	0.095	0.037	-0.036
HM	-0.019	0.159	**0.791**	0.249	-0.051	-0.040
TB	0.129	0.052	0.070	**0.812**	-0.003	0.143
FM	0.014	0.069	0.257	**0.806**	0.070	-0.051
BH	0.011	0.091	0.206	0.022	**0.857**	0.158
BW	0.176	0.075	-0.318	0.079	**0.693**	-0.231
ILL	-0.092	-0.046	-0.093	0.105	-0.003	**0.902**
Eigenvalue	4.180	1.992	1.649	1.263	1.223	1.041
% Exp.	16.30	15.14	11.21	9.48	8.19	6.43
Cum. %	16.30	31.44	42.65	52.13	60.32	66.75
F	8.586	11.774	6.262	6.128	4.071	3.916
p	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**

F- and *P*-values are given for the principal components that were significantly different between species by analysis of variance on the principal component scores.
Significant at $p < 0.05$

Table 3. 7 Results of the Bonferroni corrected post-hoc pairwise comparisons on the significant principal components (PC) for each of the clades of the *Afroedura nivaria* complex including additional species from other species complexes within the genus. PC1: head dimension, PC2: fore and hind feet, PC3: forelimbs, PC4: hindlimbs, PC5: body dimension and PC6: interlimb length.

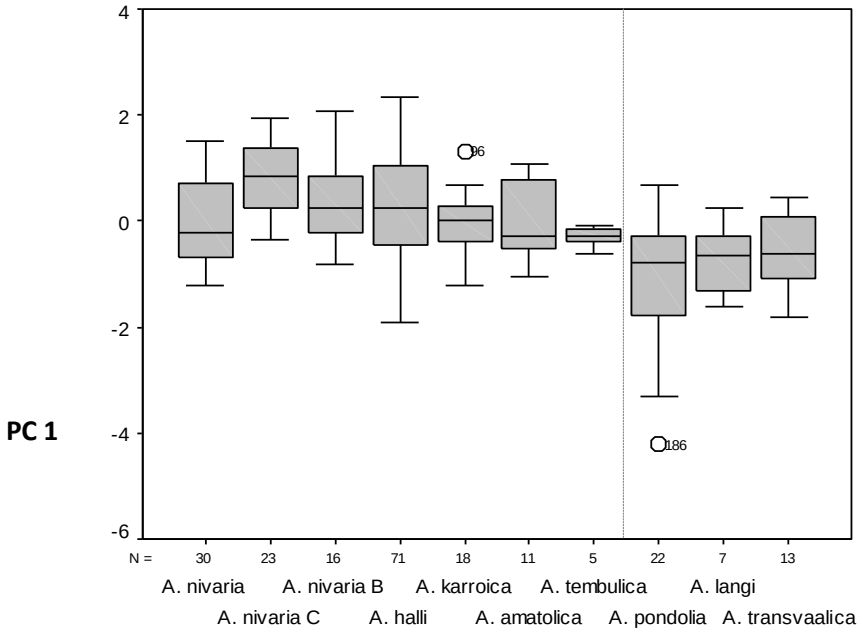
Dependent Variable	(I) clade	(J) clade	Mean Difference (I-J)	Std. Error	Sig.
PC 1	*A. nivaria*	*A. nivaria* C	-0.799	0.24	0.050
		A. pondolia	1.143	0.24	0.000
	A. nivaria C	*A. nivaria*	0.799	0.24	0.050
		A. pondolia	1.941	0.26	0.000
		A. langi	1.536	0.38	0.003
		A. transvaalica	1.372	0.30	0.000
	A. nivaria B	*A. pondolia*	1.545	0.29	0.000
	A. halli	*A. pondolia*	1.381	0.21	0.000
	A. karroica	*A. pondolia*	1.058	0.28	0.008
	A. amatolica	*A. pondolia*	1.186	0.32	0.013
	A. pondolia	*A. nivaria*	-1.143	0.24	0.000
		A. nivaria C	-1.941	0.26	0.000
		A. nivaria B	-1.545	0.29	0.000
		A. halli	-1.381	0.21	0.000
		A. karroica	-1.058	0.28	0.008
		A. amatolica	-1.186	0.32	0.013
	A. langi	*A. nivaria* C	-1.536	0.38	0.003
	A. transvaalica	*A. nivaria* C	-1.372	0.30	0.000
PC 2	*A. nivaria*	*A. nivaria* C	1.167	0.23	0.000
		A. nivaria B	0.914	0.26	0.021
		A. karroica	1.145	0.25	0.000
		A. amatolica	2.161	0.29	0.000
		A. pondolia	1.054	0.23	0.000
		A. transvaalica	1.815	0.28	0.000
	A. nivaria C	*A. nivaria*	-1.167	0.23	0.000
		A. halli	-0.784	0.20	0.005
	A. nivaria B	*A. nivaria*	-0.914	0.26	0.021
		A. amatolica	1.247	0.33	0.008
	A. halli	*A. nivaria* C	0.784	0.20	0.005
		A. karroica	0.763	0.22	0.027
		A. amatolica	1.779	0.27	0.000
		A. pondolia	0.672	0.20	0.048
		A. transvaalica	1.433	0.25	0.000
	A. karroica	*A. nivaria*	-1.145	0.25	0.000
		A. halli	-0.763	0.22	0.027
	A. amatolica	*A. nivaria*	-2.161	0.29	0.000
		A. nivaria B	-1.247	0.33	0.008

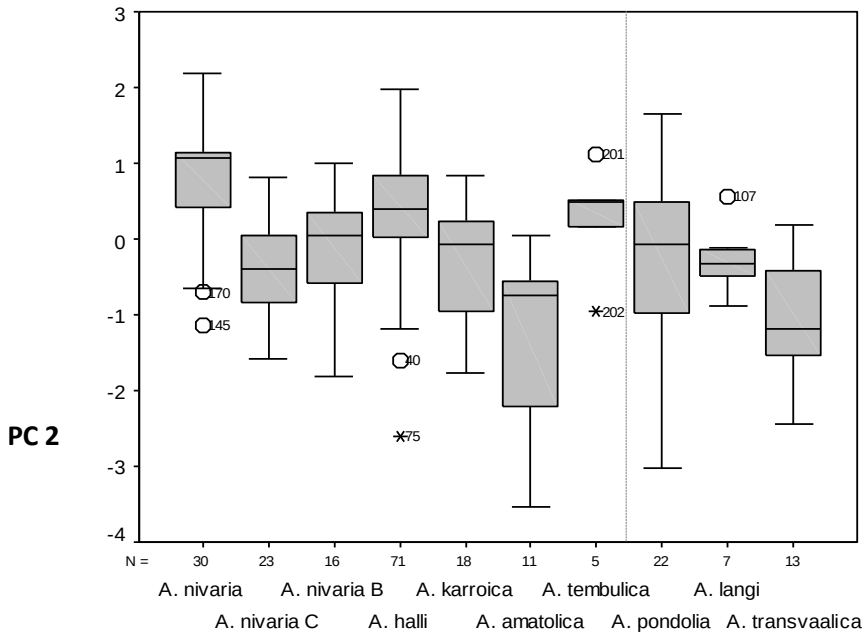
Dependent Variable	(I) clade	(J) clade	Mean Difference (I-J)	Std. Error	Sig.
	A. amatolica	*A. halli*	-1.779	0.27	0.000
		A. tembulica	-1.640	0.45	0.014
		A. pondolia	-1.107	0.31	0.017
	A. tembulica	*A. amatolica*	1.640	0.45	0.014
	A. pondolia	*A. nivaria*	-1.054	0.23	0.000
		A. halli	-0.672	0.20	0.048
		A. amatolica	1.107	0.31	0.017
	A. transvaalica	*A. nivaria*	-1.815	0.28	0.000
		A. halli	-1.433	0.25	0.000
PC 3	*A. nivaria*	*A. karroica*	1.365	0.27	0.000
	A. nivaria C	*A. karroica*	0.968	0.28	0.037
		A. langi	-1.467	0.39	0.010
	A. nivaria B	*A. langi*	-1.642	0.41	0.004
	A. halli	*A. karroica*	0.962	0.24	0.004
		A. langi	-1.473	0.36	0.003
	A. karroica	*A. nivaria*	-1.365	0.27	0.000
		A. nivaria C	-0.968	0.28	0.037
		A. halli	-0.962	0.24	0.004
		A. pondolia	-1.200	0.29	0.002
		A. langi	-2.434	0.40	0.000
	A. amatolica	*A. langi*	-2.100	0.44	0.000
	A. pondolia	*A. karroica*	1.200	0.29	0.002
	A. langi	*A. nivaria* C	1.467	0.39	0.010
		A. nivaria B	1.642	0.41	0.004
		A. halli	1.473	0.36	0.003
		A. karroica	2.434	0.40	0.000
		A. amatolica	2.100	0.44	0.000
		A. transvaalica	1.979	0.42	0.000
	A. transvaalica	*A. langi*	-1.979	0.42	0.000
PC 4	*A. nivaria* C	*A. langi*	-1.305	0.39	0.046
	A. halli	*A. langi*	-1.628	0.36	0.000
		A. transvaalica	-1.048	0.27	0.008
	A. amatolica	*A. langi*	-2.095	0.44	0.000
		A. transvaalica	-1.515	0.37	0.003
	A. tembulica	*A. langi*	-2.273	0.53	0.001
		A. transvaalica	-1.693	0.48	0.022
	A. pondolia	*A. langi*	-2.008	0.39	0.000
		A. transvaalica	-1.428	0.32	0.001
	A. langi	*A. nivaria* C	1.305	0.39	0.046
		A. halli	1.628	0.36	0.000
		A. amatolica	2.095	0.44	0.000
		A. tembulica	2.273	0.53	0.001
		A. pondolia	2.008	0.39	0.000

Dependent Variable	(I) clade	(J) clade	Mean Difference (I-J)	Std. Error	Sig.
	A. transvaalica	*A. halli*	1.048	0.27	0.008
		A. amatolica	1.515	0.37	0.003
		A. tembulica	1.693	0.48	0.022
		A. pondolia	1.428	0.32	0.001
PC 5	*A. nivaria*	*A. nivaria* C	0.903	0.26	0.029
		A. halli	0.884	0.20	0.001
		A. karroica	1.012	0.28	0.017
		A. tembulica	1.552	0.45	0.035
		A. pondolia	1.221	0.26	0.000
		A. transvaalica	1.204	0.31	0.007
	A. nivaria C	*A. nivaria*	-0.903	0.26	0.029
	A. halli	*A. nivaria*	-0.884	0.20	0.001
	A. karroica	*A. nivaria*	-1.012	0.28	0.017
	A. tembulica	*A. nivaria*	-1.552	0.45	0.035
	A. pondolia	*A. nivaria*	-1.221	0.26	0.000
	A. transvaalica	*A. nivaria*	-1.204	0.31	0.007
PC 6	*A. nivaria* C	*A. halli*	0.760	0.23	0.042
	A. halli	*A. nivaria* C	-0.760	0.23	0.042
		A. pondolia	-0.974	0.23	0.002
	A. pondolia	*A. halli*	0.974	0.23	0.002

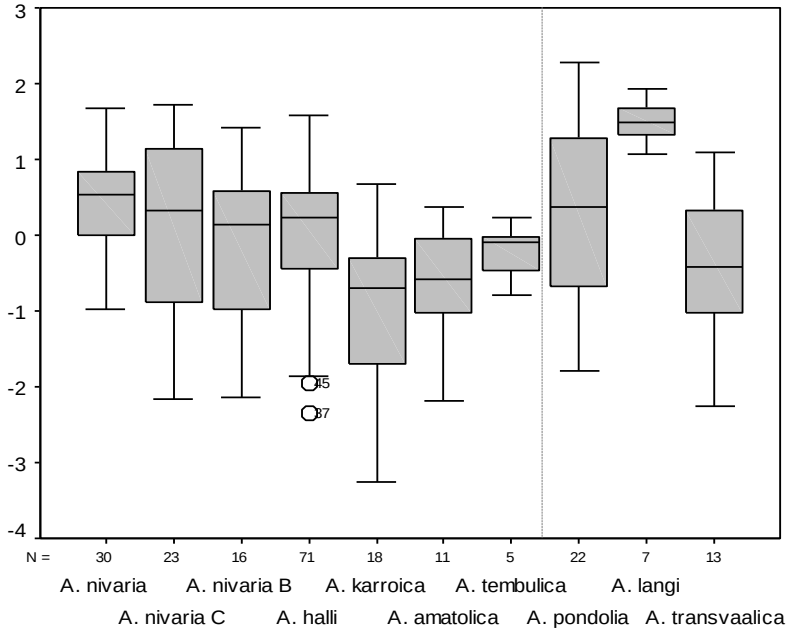
The mean difference is significant at the 0.05 level

Figure 3. 4 A graphical representation of variation for significantly different principal components between eight species included in the principal components analysis of the *Afroedura nivaria* complex with the mean and standard error (bars) shown. PC1: head dimension, PC2: fore and hind feet, PC3: forelimbs, PC4: hindlimbs, PC5: body size and PC6: interlimb length. Open circles show outliers and asterisk indicates extreme values. The *Afroedura nivaria* complex is displayed to the left of the vertical line and other representative species to the right.





PC 3


3
2
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-1
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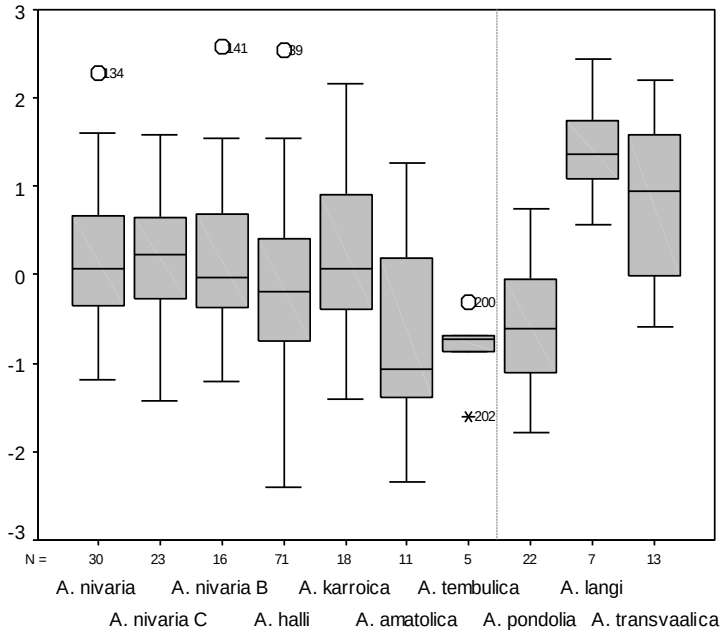
-3
-4
45
37
N =
30
23
16
71
18
11
5
22
7
13
A. nivaria
A. nivaria B
A. karroica
A. tembulica
A. langi
A. nivaria C
A. halli
A. amatolica
A. pondolia
A. transvaalica

PC 4


3
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-1
-2
-3
141
139
134
200
202
N =
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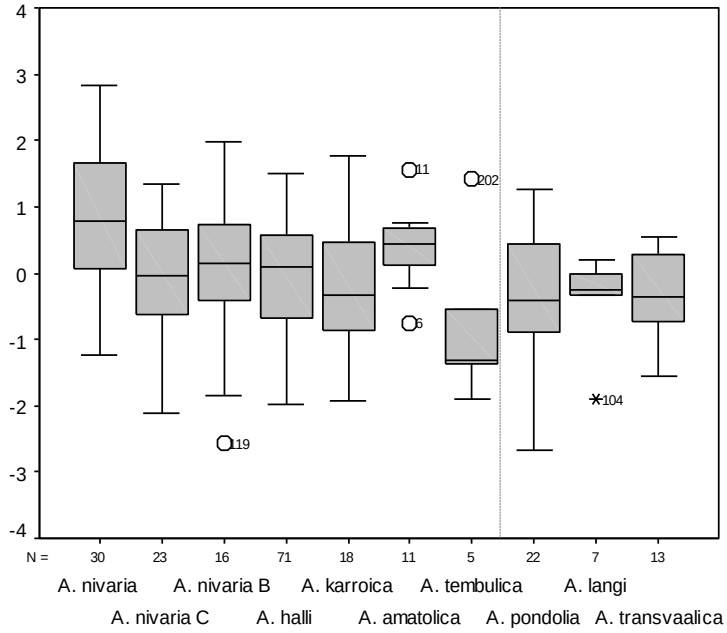
23
16
71
18
11
5
22
7
13
A. nivaria
A. nivaria B
A. karroica
A. tembulica
A. langi
A. nivaria C
A. halli
A. amatolica
A. pondolia
A. transvaalica

PC 5


4
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2
1
0
-1
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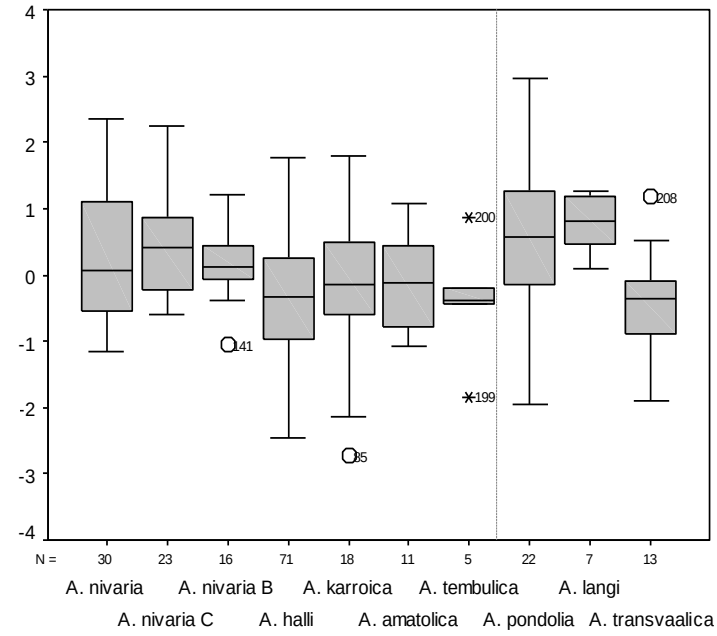
-4
11
202
6
104
119
N = 30 23 16 71 18 11 5 22 7 13
A. nivaria
A. nivaria B
A. karroica
A. tembulica
A. langi
A. nivaria C
A. halli
A. amatolica
A. pondolia
A. transvaalica

PC 6


4
3
2
1
0
-1
-2
-3
-4
200
208
141
199
85
N = 30 23 16 71 18 11 5 22 7 13
A. nivaria
A. nivaria B
A. karroica
A. tembulica
A. langi
A. nivaria C
A. halli
A. amatolica
A. pondolia
A. transvaalica

DISCUSSION

Morphological variation

Results of the morphometric analyses showed that there is variation within *A. nivaria* species complex with differentiation between clades/species found for locomotor apparatus (limbs and feet) and head dimensions. In lizards, these traits are mostly related to diet or crevice utilization (head shape) and locomotion (limbs) and in geckos, digital form being related to habitat type (feet) suggesting high habitat specialization (Russell & Bauer 1989). No differences were found for body dimensions for any of the clades within the *A. nivaria* complex. Because only a few pairwise comparisons were signicantly different between some of the clades/species this may help explain why they are hard to tell apart with much of their phenotypic similarities retained. This is the first study to examine morphometric data for the *A. nivaria* species complex.

For species that live on almost vertical surfaces, it has been suggested that having a relatively flat head is beneficial as it helps center the mass of the animal close to the substrate to prevent the tendency of toppling backward away from the surface (Vanhooydonck & Van Damme 1999, Vanhooydonck *et al*. 2002). In addition, flat heads and a generally minimized body height, are also convenient for lizards that frequently make use of small crevices in rocks or walls to hide from predators or as a place of rest for the night (Herrel *et al*. 2001, Verwajien *et al*. 2002), a true statement for species in the *A. nivaria* complex (Hewitt 1937, Onderstall 1984, Branch 1998). Even though *Afroedura* in general are distinguished from other geckos by having relatively flat heads, *A. karroica* appeared to have a significantly much more depressed head compared to the other species in the complex. Hewitt (1937) did note that *A. karroica* was very much flattened. While members of the *A. nivaria* complex are all rupicolous and inhabit rock cracks, the differentiation of *A. karroica* could be explained as adaptation to a different microhabitat altogether because its distribution spans the Karoo biome; the other four species occur mostly in the grassland biome. Branch (1998), mentions that *A. karroica* prefers small rock outcrops in broken ground and having a flattened body form may have the further advantage of making it less conspicuous on flat surfaces.

The relationship between dietary and phenotypic specialization has been well documented for vertebrates. Only a few cases of this general trend can be established for lizards and this is often considered to be due to a lack of dietary specialization in many lizard groups (Metzger & Herrel 2005). Some studies that have investigated the correlation between cranial form and dietary niche include Metzger & Herrel (2005), Stayton (2005), Vidal *et al*. (2005) and Kaliontzopoulou *et al*. (2008). It has been suggested that lizards feeding on large or harder prey e.g. hard-shelled beetles as

is for insectivorous species, are more likely to have larger heads and body sizes or vice versa. *A. nivaria* sensu stricto has a significantly smaller head length than *A. nivaria* clade C but overall, had a significantly wider head. The larger head width might relate to muscle mass for bite force (Measey *et al*. 2009), and might indicate that *A. nivaria* sensu stricto may have the need for a high bite force and thus, feed on harder prey while *A. nivaria* clade C might be efficient in capturing softer prey. It suggests that there may be some inclination for dietary specialization between the clades within the *A. nivaria* complex. However, the analysis of diet preference data would be needed to conclusively establish the relationship.

In lizards, differences in limb morphology which reflect differences between locomotor capacities have been related to differences in microhabitat use (Garland & Losos 1994). Various researchers have attempted to correlate habitat types and limb proportions. These studies have explored whether or not morphological characteristics are adaptations to the demand of the species' microhabitats (Glor *et al*. 2003, Johnson *et al*. 2005) and substrate specialization may have been an important causal factor in the radiation of African geckos (Bauer 1993). Various conclusions have been reached regarding the correlation of limbs to microhabitat. For example, in chameleons, longer limbs are associated with more dense habitats where movement between the vegetation requires a long reach for maneuvering tree branches (Hopkins & Tolley 2011). Unfortunately, such a comparison could not be made in the present study because no measurements of habitat or performance were taken.

Genetic divergence has been found among the different clades within *A. nivaria* complex, but with some similarity in phenotype being retained. Even though these species occupy fragmented habitats, selective pressures they are exposed to remain the same because of the similarity of stable ecological niches exploited (Smith *et al*. 2001, Glor *et al*. 2003, Leaché *et al*. 2009, Oliver *et al.* 2009). Statistically, body dimensions were found to be not significantly different for any of the clades/species in the *A. nivaria* complex. Having similar body sizes would indicate that all species within the complex share the same general body plan. Some lizard groups are generally restricted to isolated outcrops and demonstrate substrate specialization (e.g. Bauer *et al*. 1996) and in the case of the *A. nivaria* complex, species show to have strict habitat preferences tied to suitable rock outcrops (Onderstall 1984). This may be the contributing factor to their conserved morphologies since they occupy similar habitats.

Relative to other species within the genus, *Afroedura*, multivariate analyses were in agreement with the findings of the analyses that included members of the *A. nivaria* complex only. There were little or no differences between the *A. nivaria* clades B and C but *A. nivaria* sensu stricto showed to have a

larger body structure compared to the other two genetic clades and *A*. *amatolica*, *A*. *karroica*, and *A*. *tembulica* had significantly shorter limb lengths. It is typical of rock-dwelling species to have increased limb lengths and reduced head depth (Revell *et al*. 2007). The three species outside the *A. nivaria* complex, that is *A*. *langi*, *A*. *pondolia* and *A*. *transvaalica* had significantly smaller heads but longer limbs and greater interlimb length suggesting that they may take longer strides when moving between rocks or may attain maximum speeds on open horizontal surfaces (Losos 1990a). As shown by *A. halli*, *A. karroica*, *A. amatolica* and *A. tembulica*, having shorter limbs could be an added benefit to maintaining stability on smooth substrates linked to more dense rocky habitats (Fischer *et al*. 2010). In the case of *A. karroica*, having significantly smaller feet than the rest of the species in the *A. nivaria* complex may suggest it occupies a much more cluttered habitat, typical of the Karoo biome characterized by exposed rock outcrops (small boulders tightly packed to each other) and sparse vegetation (Mucina & Rutherford 2006). The exception was *A. nivaria*, which had relatively longer limbs and larger feet suggestive that it may be occupying rather more open rocky habitats, that is, moving across loose boulders at very high altitudes as opposed to closely packed rocks. Characters related to the locomotory system have also been shown to be relatively larger in open than closed habitat morphs indicative that feet may be associated with adaptation to the habitat (e.g. chameleons; Hopkins & Tolley 2011).

The inclusion of other representative taxa in the multivariate analyses (putting the *A. nivaria* complex in context with other species in the genus) did not reveal much morphometric variation between clades/species of the *A. nivaria* complex. This further highlighted that similar environments that they inhabit with similar ecological pressures may be the causal factor for a lack of phenotypic variation by examining ecologically relevant variables. Little is known regarding locomotor performance in geckos and it may prove useful to employ such techniques e.g. looking at the inclination of the geckos' substrates or investigating maximum speeds and acceleration as shown in Higham & Russell (2010) and to see if these may relate to morphological diversity or conversely lack of.

The subject of sexual dimorphism has not received much attention in geckos despite being such as diverse groups of lizards (Zuffi *et al*. 2011). Sexual dimorphism is explained as the evolutionary result of selection operating on the body sizes of males and females. Previous studies have found morphological differences observed between sexes to be directly related to the type of microhabitat occupied (e.g. Butler *et al*. 2000, Hopkins & Tolley 2011). Geographic ranges that overlap between closely related species and the increased likelihood of interspecific competition may lead to selection for different body sizes and thus the exploitation of different niches (Pounds 1988).

Similarly, within species, sexual dimorphism may evolve to help avoid competition between the sexes. Previous authors have argued that sexual dimorphism in lizards has evolved due to 1) sexual selection which favours a relatively larger size in males because it offers an advantage in intrasexual mate competition and usually results in greater mating success or 2) natural selection for a greater size in females if it favours increased fecundity (Cox *et al*. 2003). Generally, males tend to be relatively larger than females for some traits, in the majority of the lizard groups (e.g. Hopkins & Tolley 2011) but it is not uncommon that absolute sexual size dimorphism be female-biased. Even though sexual dimorphism has been found in other lizard groups (e.g. Butler *et al*. 2000, Kratochvil & Frynta 2002, Kaliontzopulou *et al*. 2007, Oraie *et al*. 2011), it is not always strictly present in other species (e.g. Blaire *et al*. 2009, Zuffi *et al*. 2011). Sexual dimorphism is not apparent in the *A. nivaria* complex but it is likely that males may be slightly larger than females, when extrapolating from the results of *A*. *halli*. Relatively small sample sizes per clade and sex have limited these morphometric analyses. However, lack of sexual dimorphism in the *A. nivaria* complex may be the evolutionary result of selective pressures that do not vary among different populations and/or species in similar habitat types or dietary needs that do not exactly require male and/or female body sizes to differ because there is little or no competition for resource types, a strong show for conservatism in morphology (Butler *et al*. 2000, Oliver *et al*. 2007, 2009).

Environmental or ecological factors may also influence sexual dimorphism. For example, in chameleons, dimorphism was recorded to be more pronounced in morphs occupying a closed habitat, whereas there was little or no dimorphism in the more open habitat (Hopkins & Tolley 2011). Because there lowered risks in more dense habitats than in open habitats, thus species in more open habitats cannot afford to be flamboyant, risk attracting predators. This warrants an extensive investigation into sexual size dimorphism given that the present data was not sufficient to fully explore sexual dimorphism in this study.

Recognizing cryptic species

Cryptic species are defined as two or more distinct lineages classified as a single species based on their morphological similarities. Research on cryptic species has increased over the past two decades increased in large part by the increasing availability and accuracy of DNA sequence data (Bickford *et al*. 2007). Recent molecular research has revealed that different species may not differ conspicuously in morphology, yet be separated by extremely large genetic divergences indicative of a long history of isolation (Oliver *et al*. 2007, Couper *et al*. 2008, Doughty *et al*. 2008, Oliver *et al*. 2009, Nielsen *et al*. 2011). Morphometrics and geometric morphometrics continue to show differences in closely related species where traditional morphology failed resolve to contentious

species boundaries, by instead looking at ecologically relevant traits (i.e. traditional morphometrics) or variables that may be directly under selection.

Although there are some comparisons that show differences among the clades/species of the *A. nivaria* complex but in the overall morphometric scheme, these are for a few characters and also only a few of the pairwise comparisons showed significant variation. The variation found in this study is minor, and this lack of morphometric variation strongly suggests morphological conservatism within the genetically distinct clades recovered in the phylogeny (see Results; Chapter 2) and therefore, the presence of cryptic species.

Chapter 4

Conclusion

Molecular analyses were able to unambiguously differentiate between the described species and recognize additional genetically distinct clades within the originally identified species. These clades are shown here to be new evolutionary units considering that there were large genetic divergences with no spatial overlap between any of the genetic clades. The monophyly of the *A. nivaria* complex could not be supported because 1) *A. karroica* appears to be outside the complex, and 2) *A. pondolia* consistently nested with *A. nivaria* in the phylogeny.

Average percentage divergence between the five species, including the additional clades, ranged from 6-16% and 11-29% across the two mitochondrial genes 16S and ND4, respectively (Table 2.3). Many comparisons among clades overlap broadly to those between acknowledged species. These large divergences among clades and the geographical structure of the mtDNA clades can be indicative of historical isolation among populations within the different species of the *A. nivaria* species complex. Relatively deep divergences across narrow geographical areas have been observed in other lizards as well and the deep phylogenetic structure may be linked to vicariance events and/or climatic oscillations providing a biogeographic hypothesis for this complex (e.g. Moritz *et al*. 1992, Mahoney 2004). Some studies have shown that different species may have large genetic differences yet be inconspicuous in their morphology (Pepper *et al*. 2006, Oliver *et al*. 2009, Couper *et al*. 2008, Doughty *et al*. 2008). This observation is typical for cryptic diversity and the *A. nivaria* species complex shows to demonstrate this. However, it is important to note that sequence divergences were applied with caution in this study and not regarded as absolute values for species relationships.

As mentioned earlier, an explicit phylogenetic hypothesis depicting relationships of the *A. nivaria* complex has not been previously proposed, but certain statements regarding overall similarities or particular morphological characters and geography (from literature) are consistent with the findings of this study. Thus, I propose that the status of *A. karroica* within the *A. nivaria* complex and the association *of A. pondolia* with *A. nivaria* be revised. I argue that *A*. cf. *nivaria* and *A*. *nivaria* sensu stricto be treated as separated lineages, with *A*. cf. *nivaria* being elevated to species-level, but this awaits a detailed morphological analysis. Another species, *A*. *amatolica*, showed to have two distinct clades and possibly a phylogeographic study using a larger sampling data may be warranted.

Results of the morphometric data analysis showed that variation within *A. nivaria* species complex was accounted for by locomotor apparatus (limbs and feet) and head dimensions. Differences in

limb morphology and locomotor capacities may be related to differences in microhabitat use (Garland & Losos 1994). From the findings, *A. karroica* is clearly very much flattened than the other species, having a significantly much more depressed head as an adaptation to a specific microhabitat (Hewitt 1937). Although head size may be linked to utilization of crevices in rocky habitat, an alternative explanation is dietary specialization among some of the clades within the *A. nivaria* complex. For example, multivariate analyses showed *A. nivaria* sensu stricto to have a larger head and may possibly feed on harder prey while *A.* cf. *nivaria* could be efficient in capturing softer prey. However, statistical analyses failed to show any significant differences for body dimensions between species which could indicate morphological conservatism in the complex.

Overall, it appears that genetic divergence has been achieved among the different clades within *A. nivaria* complex, but with much similarity in phenotype being retained. These species occupy fragmented but similar habitats, and it may be the contributing factor to their conserved morphologies because of similar pressures experienced within the similar habitats they occupy. Sexual dimorphism is another field yet to be explored within the *A. nivaria* species complex, to help understand if the processes of sexual selection or natural selection are in play for this complex. Therefore, additional clades recovered from the phylogeny may well have achieved reproductive isolation (Fig. 2.7) even though there is little or no phenotypic differentiation. These divergent clades can be considered separate evolving entities under the evolutionary and/or phylogenetic species concept (Wiens & Penkrot 2002, Bauer & Lamb 2005) and/or be identified as operational taxonomic units (OTUs).

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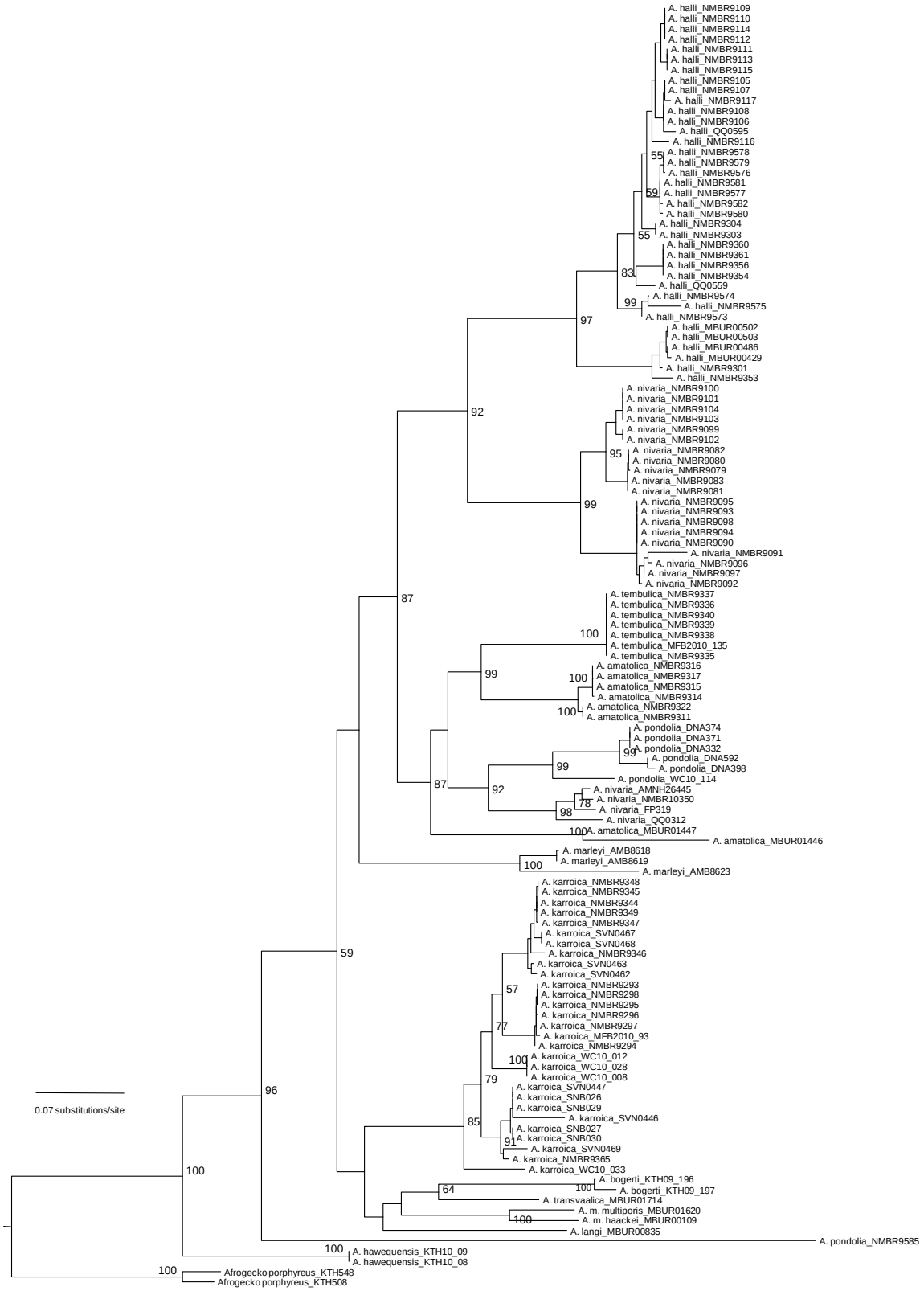
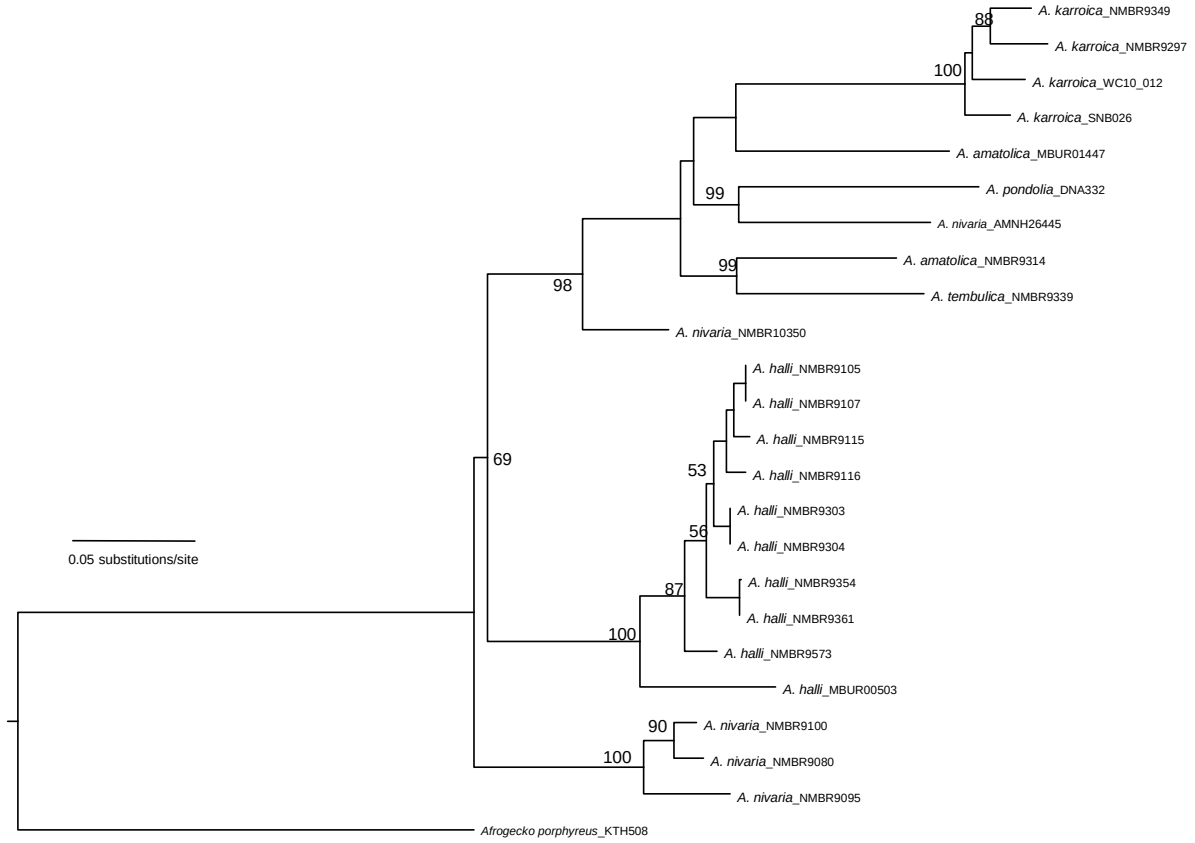
APPENDIX A Maximum likelihood tree of the combined mtDNA data (16S and ND4) with likelihood bootstrap values (1000 replicates) shown on branches. *Afrogecko porphyreus* was used as outgroup.



APPENDIX B Maximum likelihood tree based on sequences of the mitochondrial (16S and ND4) and nuclear (KIAA) genes (1622 bp aligned length). Likelihood bootstrap values (1000 replicates) are shown on the branches. *Afrogecko porphryeus* was used as outgroup.



APPENDIX C Geographical data of flat gecko individuals used for the phylogenetic analysis in this study.

GENUS	SPECIES	FIELD NO.	VOUCHER NO.	VOUCHER MUSEUM	LATITUDE	LONGITUDE	LOCALITY
Afroedura	*amatolica*	MBUR 01446			33°2′4′′	26°49′35′′	Double Drift Game Reserve
Afroedura	*amatolica*	MBUR 01447			33°2′4′′	26°49′35′′	Double Drift Game Reserve
Afroedura	*amatolica*	MFB 2010.105	NMB R9311	National Museum, BFN	32°40′58′′	27°1′47.2′′	Zingcuka, Amatole MTNS
Afroedura	*amatolica*	MFB 2010.108	NMB R9314	National Museum, BFN	32°34′39.9′′	26°56′35.9′′	Farm No. 18, Amatole MTNS
Afroedura	*amatolica*	MFB 2010.109	NMB R9315	National Museum, BFN	32°34′46.5′′	26°56′21.5′′	Farm No. 18, Amatole MTNS
Afroedura	*amatolica*	MFB 2010.110	NMB R9316	National Museum, BFN	32°34′46.9′′	26°56′19.9′′	Farm No. 18, Amatole MTNS
Afroedura	*amatolica*	MFB 2010.111	NMB R9317	National Museum, BFN	32°34′46.9′′	26°56′19.9′′	Farm No. 18, Amatole MTNS
Afroedura	*amatolica*	MFB 2010.116	NMB R9322	National Museum, BFN	32°40′53.7′′	27°1′38.9′′	Zingcuka, Amatole MTNS
Afroedura	*bogerti*	KTH09-196			16°12′2.2′′	12°22′6.6′′	Omuha Lodge
Afroedura	*bogerti*	KTH09-197			16°12′2.2′′	12°22′6.6′′	Omuha Lodge
Afroedura	*halli*	MBUR 00429			31°36′39′′	26°18′53′′	Farm
Afroedura	*halli*	MBUR 00486			31°36′52′′	26°18′50′′	
Afroedura	*halli*	MBUR 00502			31°36′39′′	26°18′53′′	Farm
Afroedura	*halli*	MBUR 00503			31°36′39′′	26°18′53′′	Farm
Afroedura	*halli*	MFB 2010.102	NMB R9304	National Museum, BFN	29°20′20.6′′	27°5′2.4′′	Thaba Phatshwa, Ladybrand District
Afroedura	*halli*	MFB 2010.103	NMB R9303	National Museum, BFN	29°20′16.8′′	27°5′2.3′′	Thaba Phatshwa, Ladybrand District
Afroedura	*halli*	MFB 2010.148	NMB R9354	National Museum, BFN	31°23′8.4′′	26°47′56.8′′	Farm No. 147, Wodehouse District
Afroedura	*halli*	MFB 2010.150	NMB R9356	National Museum, BFN	31°23′1′′	26°48′0.0′′	Farm No. 147, Wodehouse District
Afroedura	*halli*	MFB 2010.154	NMB R9360	National Museum, BFN	31°23′7.9′′	26°47′57.8′′	Farm No. 147, Wodehouse District
Afroedura	*halli*	MFB 2010.155	NMB R9353	National Museum, BFN	31°22′18.3′′	26°5′37.9′′	Farm: Kruis Fontein, Hofmeyr District
Afroedura	*halli*	MFB 2010.156	NMB R9361	National Museum, BFN	31°23′8.4′′	26°47′56.8′′	Farm No. 147, Wodehouse District
Afroedura	*halli*	MFB 2010.58	NMB R9105	National Museum, BFN	30°17′55.8′′	27°3′12.4′′	Aasvoelberg, Zastron District
Afroedura	*halli*	MFB 2010.59	NMB R9106	National Museum, BFN	30°17′55.7′′	27°3′9.9′′	Aasvoelberg, Zastron District
Afroedura	*halli*	MFB 2010.61	NMB R9107	National Museum, BFN	30°17′55.7′′	27°3′9.9′′	Aasvoelberg, Zastron District
Afroedura	*halli*	MFB 2010.62	NMB R9108	National Museum, BFN	30°18′3.3′′	27°3′9.8′′	Aasvoelberg, Zastron District
Afroedura	*halli*	MFB 2010.64	NMB R9109	National Museum, BFN	30°16′47.9′′	26°58′9.6′′	Koesberg, Zastron District

Afroedura	*halli*	MFB 2010.65	NMB R9110	National Museum, BFN	30°16′46.5′′	26°58′9.0′′	Koesberg, Zastron District
Afroedura	*halli*	MFB 2010.66	NMB R9111	National Museum, BFN	30°16′46.3′′	26°58′9.4′′	Koesberg, Zastron District
Afroedura	*halli*	MFB 2010.67	NMB R9112	National Museum, BFN	30°16′44.8′′	26°58′11.4′′	Koesberg, Zastron District
Afroedura	*halli*	MFB 2010.68	NMB R9113	National Museum, BFN	30°16′44.8′′	26°58′11.4′′	Koesberg, Zastron District
Afroedura	*halli*	MFB 2010.69	NMB R9114	National Museum, BFN	30°16′44.8′′	26°58′11.4′′	Koesberg, Zastron District
Afroedura	*halli*	MFB 2010.70	NMB R9115	National Museum, BFN	30°16′44.8′′	26°58′11.4′′	Koesberg, Zastron District
Afroedura	*halli*	MFB 2010.80	NMB R9116	National Museum, BFN	30°6′56.6′′	26°57′40.9′′	Elandsberg, Zastron District
Afroedura	*halli*	MFB 2010.81	NMB R9117	National Museum, BFN	30°6′54.8′′	26°57′41.7′′	Elandsberg, Zastron District
Afroedura	*halli*	MFB 2010.99	NMB R9301	National Museum, BFN	31°26′41.4′′	26°41′37.3′′	Penhoek Pass, Wodehouse District
Afroedura	*halli*	QQ0559		National Museum, BFN	31°20′11′′	26°69′67′′	Jamestown Rd near Streepfontein
Afroedura	*halli*	QQ0595		National Museum, BFN	30°30′36.8′′	27°′5′95.7′′	Aasvoelberg, Zastron District
Afroedura	*halli*		NMB R9574	National Museum, BFN	30°42′36′′	27°16′23′′	Jobert's Pass, Lady Grey District
Afroedura	*halli*		NMB R9575	National Museum, BFN	30°42′36.4′′	27°16′22.1′′	Jobert's Pass, Lady Grey District
Afroedura	*halli*		NMB R9576	National Museum, BFN	30°34′4.4′′	27°31′13′′	Witteberg MTNS, Herschel District
Afroedura	*halli*		NMB R9577	National Museum, BFN	30°34′4.4′′	27°31′13′′	Witteberg MTNS, Herschel District
Afroedura	*halli*		NMB R9573	National Museum, BFN	30°42′36.3′′	27°16′22′′	Jobert's Pass, Lady Grey District
Afroedura	*halli*		NMB R9578	National Museum, BFN	30°34′11.1′′	27°30′42.7′′	Witteberg MTNS, Herschel District
Afroedura	*halli*		NMB R9579	National Museum, BFN	30°34′11.1′′	27°30′42.7′′	Witteberg MTNS, Herschel District
Afroedura	*halli*		NMB R9580	National Museum, BFN	30°33′36.4′′	27°31′41.1′′	Witteberg MTNS, Herschel District
Afroedura	*halli*		NMB R9581	National Museum, BFN	30°33′36.4′′	27°31′41.1′′	Witteberg MTNS, Herschel District
Afroedura	*halli*		NMB R9582	National Museum, BFN	30°31′59.8′′	27°32′52.8′′	Mfinci, Witteberg MTNS, Herschel
Afroedura	*hawequensis*	KTH10-08			33°40′59.2′′	19°5′44.4′′	Limietberg
Afroedura	*hawequensis*	KTH10-09			33°40′59.2′′	19°5′44.4′′	Limietberg
Afroedura	*karroica*	MFB 2010.139	NMB R9344	National Museum, BFN	32°2′18.6′′	26°12′41.8′′	Farm No. 124, Tarkastad District
Afroedura	*karroica*	MFB 2010.140	NMB R9345	National Museum, BFN	32°2′19.3′′	26°12′42′′	Farm No. 124, Tarkastad District
Afroedura	*karroica*	MFB 2010.141	NMB R9346	National Museum, BFN	32°2′19.3′′	26°12′42′′	Farm No. 124, Tarkastad District
Afroedura	*karroica*	MFB 2010.142	NMB R9347	National Museum, BFN	32°2′19.3′′	26°12′42′′	Farm No. 124, Tarkastad District
Afroedura	*karroica*	MFB 2010.143	NMB R9348	National Museum, BFN	32°2′20.4′′	26°12′41.2′′	Farm No. 124, Tarkastad District
Afroedura	*karroica*	MFB 2010.144	NMB R9349	National Museum, BFN	32°2′23.6′′	26°12′40.2′′	Farm No. 124, Tarkastad District
Afroedura	*karroica*	MFB 2010.90	NMB R9293	National Museum, BFN	32°16′44.5′′	25°37′23.6′′	Buffelskop, Cradock District

Afroedura	*karroica*	MFB 2010.91	NMB R9294	National Museum, BFN	32°16′44.4′′	25°37′23.7′′	Buffelskop, Cradock District
Afroedura	*karroica*	MFB 2010.92	NMB R9295	National Museum, BFN	32°16′43.8′′	25°37′26.3′′	Buffelskop, Cradock District
Afroedura	*karroica*	MFB 2010.93	tail clip		32°16′44.5′′	25°37′26.8′′	Buffelskop, Cradock District
Afroedura	*karroica*	MFB 2010.95	NMB R9297	National Museum, BFN	32°16′41.5′′	25°37′26.6′′	Buffelskop, Cradock District
Afroedura	*karroica*	MFB 2010.96	NMB R9298	National Museum, BFN	32°16′41.5′′	25°37′26.6′′	Buffelskop, Cradock District
Afroedura	*karroica*	MRB 2010.94	NMB R9296	National Museum, BFN	32°16′42.5′′	25°37′26.3′′	Buffelskop, Cradock District
Afroedura	*karroica*	SNB-026			31°42′42.6′′	24°37′30.6′′	Hartbeesfontein, Sneeuberge
Afroedura	*karroica*	SNB-027			31°42′43′′	24°37′31′′	Hartbeesfontein, Sneeuberge
Afroedura	*karroica*	SNB-029			31°42′43′′	24°37′31′′	Hartbeesfontein, Sneeuberge
Afroedura	*karroica*	SNB-030			31°42′43′′	24°37′31′′	Hartbeesfontein, Sneeuberge
Afroedura	*karroica*	SVN 446			31°42′43′′	24°37′31′′	Sneeuberg near Suurfontein farm
Afroedura	*karroica*	SVN 447			31°42′43′′	24°37′31′′	Sneeuberg near Suurfontein farm
Afroedura	*karroica*	SVN 462			31°16′43′′	25°37′26′′	~20km NE Cradock
Afroedura	*karroica*	SVN 463			31°16′43′′	25°37′26′′	~20km NE Cradock
Afroedura	*karroica*	SVN 467					rd btw Tarkastad & Commando Drift
Afroedura	*karroica*	SVN 468					rd btw Tarkastad & Commando Drift
Afroedura	*karroica*	SVN 469					~5km N of Middelburg
Afroedura	*karroica*	WC10-008			32°17′9′′	25°3′3.0′′	Zuurkloof, Asante Sanna
Afroedura	*karroica*	WC10-012			32°15′44′′	25°1′33′′	Zuurkloof, Asante Sanna
Afroedura	*karroica*	WC10-028			32°16′0.0′′	21°1′46′′	Zuurkloof, Asante Sanna
Afroedura	*karroica*	WC10-033			32°25′30.7′′	24°93′30′′	Top of Waterkloof
Afroedura	*karroica*		NMB R9365	National Museum, BFN	31°50′15′′	24°51′42.5′′	Lootsberg Pass, Graaf Reinet District
Afroedura	*langi*	MBUR 00835			24°0′29′′	31°12′24′′	Cleveland Nature Reserve
Afroedura	*m. haackei*	MBUR 00109			25°27′31′′	30°41′48′′	
Afroedura	*m. multiporis*	MBUR 01620			24°0′22′′	30°0′5.0′′	Wolkberg Wilderness Area
Afroedura	*marleyi*	AMB 8618			25°28′36′′	31°58′15′′	The Hippos
Afroedura	*marleyi*	AMB 8619			25°28′36′′	31°58′15′′	The Hippos
Afroedura	*nivaria*	AMNH 26445	NMB R10196	National Museum, BFN	28°53′18′′	29°1′39′′	Top of Mnweni cutback
Afroedura	*nivaria*	FP319			28°44′19′′	28°53′15.2′′	The Sentinel
Afroedura	*nivaria*	MFB 2010.16	NMB R9090	National Museum, BFN	29°11′24′′	27°25′53′′	Platberg, Ladybrand District

Afroedura	*nivaria*	MFB 2010.17	NMB R9091	National Museum, BFN	29°11′27′′	27°26′3′′	Platberg, Ladybrand District
Afroedura	*nivaria*	MFB 2010.18	NMB R9092	National Museum, BFN	29°11′27′′	27°26′3′′	Platberg, Ladybrand District
Afroedura	*nivaria*	MFB 2010.22	NMB R9093	National Museum, BFN	29°12′18′′	27°26′44′′	Platberg, Ladybrand District
Afroedura	*nivaria*	MFB 2010.23	NMB R9094	National Museum, BFN	29°12′18′′	27°26′44′′	Platberg, Ladybrand District
Afroedura	*nivaria*	MFB 2010.24	NMB R9095	National Museum, BFN	29°12′18′′	27°26′44′′	Platberg, Ladybrand District
Afroedura	*nivaria*	MFB 2010.25	NMB R9096	National Museum, BFN	29°12′17′′	27°26′46′′	Platberg, Ladybrand District
Afroedura	*nivaria*	MFB 2010.26	NMB R9097	National Museum, BFN	29°12′17′′	27°26′46′′	Platberg, Ladybrand District
Afroedura	*nivaria*	MFB 2010.27	NMB R9098	National Museum, BFN	29°12′17′′	27°26′46′′	Platberg, Ladybrand District
Afroedura	*nivaria*	MFB 2010.28	NMB R9099	National Museum, BFN	28°49′41′′	27°30′7′′	Ribboksberg, Clocolan District
Afroedura	*nivaria*	MFB 2010.29	NMB R9100	National Museum, BFN	28°49′41′′	27°30′7′′	Ribboksberg, Clocolan District
Afroedura	*nivaria*	MFB 2010.30	NMB R9101	National Museum, BFN	28°49′41′′	27°30′7′′	Ribboksberg, Clocolan District
Afroedura	*nivaria*	MFB 2010.31	NMB R9102	National Museum, BFN	28°49′41′′	27°30′7′′	Ribboksberg, Clocolan District
Afroedura	*nivaria*	MFB 2010.32	NMB R9103	National Museum, BFN	28°49′41′′	27°30′7′′	Ribboksberg, Clocolan District
Afroedura	*nivaria*	MFB 2010.33	NMB R9104	National Museum, BFN	28°49′41′′	27°30′7′′	Ribboksberg, Clocolan District
Afroedura	*nivaria*	MFB2012.iii.6	NMBR10350	National Museum, BFN	28°59′2′′	28°68′26′′	Monontsha Pass, Phuthaditjhaba
Afroedura	*nivaria*	Middeldeel 1	NMB R9230	National Museum, BFN	29°14′45′′	27°30′15′′	Farm Erfpacht, Ladybrand District
Afroedura	*nivaria*	QQ0312		National Museum, BFN	29°39′46′′	29°18′26′′	Lakes District of Cobham NR
Afroedura	*nivaria*		NMB R9082	National Museum, BFN	28°54′18′′	27°15′41′′	Korannaberg, Excelsior District
Afroedura	*nivaria*		NMB R9083	National Museum, BFN	28°54′17′′	27°15′43′′	Korannaberg, Excelsior District
Afroedura	*nivaria*		NMB R8396	National Museum, BFN	28°39′15′′	28°51′40′′	Thibella village, Harrismith district
Afroedura	*nivaria*		NMB R9079	National Museum, BFN	28°54′15′′	27°15′48′′	Korannaberg, Excelsior District
Afroedura	*nivaria*		NMB R9080	National Museum, BFN	28°54′15′′	27°15′48′′	Korannaberg, Excelsior District
Afroedura	*nivaria*		NMB R9081	National Museum, BFN	28°54′15′′	27°15′48′′	Korannaberg, Excelsior District
Afroedura	*pondolia*	AMB 8623			27°19′21′′	31°25′59′′	Godlwayo Hill
Afroedura	*pondolia*	DNA-332			31°49′28.5′′	29°18′10.1′′	Chalet nr. 4, Hluleka Nature Reserve
Afroedura	*pondolia*	DNA-371			31°49′28.5′′	29°18′10.1′′	Chalet nr. 4, Hluleka Nature Reserve
Afroedura	*pondolia*	DNA-374			31°49′28.5′′	29°18′10.1′′	Hluleka Nature Reserve
Afroedura	*pondolia*	DNA-398			22°12′53′′	29°24′49′′	Dwesa conference room, Dwesa NR
Afroedura	*pondolia*	DNA-592			22°12′53′′	29°24′49′′	Dwesa conference room, Dwesa NR
Afroedura	*pondolia*	WC10-114			32°18′34.7′′	28°49′37′′	Mkambati Nature Reserve

Afroedura	*pondolia*		NMB R9585	National Museum, BFN	32°18′33′′	28°49′39′′	Dwesa-Cwebe Nature Reserve
Afrogecko	*porphryeus*	KTH508			33°38′40′′	19°43′59′′	Naudesberg near Montagu
Afrogecko	*porphryeus*	KTH548			33°44′32′′	20°1′51′′	Goedemoed, Langeberg
Afroedura	*tembulica*	MFB 2010.130	NMB R9335	National Museum, BFN	31°59′20.3′′	27°34′23.9′′	Tshangana, Cofimvaba District
Afroedura	*tembulica*	MFB 2010.131	NMB R9336	National Museum, BFN	31°59′22.1′′	27°34′22.3′′	Tshangana, Cofimvaba District
Afroedura	*tembulica*	MFB 2010.132	NMB R9337	National Museum, BFN	31°59′21.5′′	27°34′22.4′′	Tshangana, Cofimvaba District
Afroedura	*tembulica*	MFB 2010.133	NMB R9338	National Museum, BFN	31°59′21.5′′	27°34′22.4′′	Tshangana, Cofimvaba District
Afroedura	*tembulica*	MFB 2010.134	NMB R9339	National Museum, BFN	31°59′21.5′′	27°34′22.4′′	Tshangana, Cofimvaba District
Afroedura	*tembulica*	MFB 2010.135	tail clip	National Museum, BFN	31°59′21.5′′	27°34′22.4′′	Tshangana, Cofimvaba District
Afroedura	*tembulica*	MFB 2010.136	NMB R9340	National Museum, BFN	31°59′22.6′′	27°34′23′′	Tshangana, Cofimvaba District
Afroedura	*transvaalica*	MBUR 01714			22°12′53′′	29°24′49′′	

APPENDIX D Individual samples of *Afroedura nivaria* species complex including representatives outside the complex that were used for morphometric analysis in this study.

GENUS	SPECIES	ID	LOCALITY	SEX	SVL
Afroedura	*amatolica*	NMBR8236	Waterfall farm,Cathcart	M	54.57
Afroedura	*amatolica*	NMBR8235	Waterfall farm,Cathcart	M	50.96
Afroedura	*amatolica*	NMBR8237	Waterfall farm,Cathcart	F	51.28
Afroedura	*amatolica*	NMBR8241	Farm 32, Cathcart	F	42.78
Afroedura	*amatolica*	NMBR8238	Waterfall farm,Cathcart	F	37.73
Afroedura	*amatolica*	NMBR9314	Farm 18, Cathcart	F	44.83
Afroedura	*amatolica*	NMBR9316	Hogsback	F	48.51
Afroedura	*amatolica*	NMBR8234	Waterfall farm,Cathcart	F	39.65
Afroedura	*amatolica*	NMBR9311	Zingcuka	F	33.75
Afroedura	*amatolica*	NMBR9322	Zingcuka	M	46.3
Afroedura	*amatolica*	NMBR9317	Farm 18, Cathcart	F	34.07
Afroedura	*nivaria*	NMBR9081	Korannaberg	F	48.47
Afroedura	*nivaria*	NMBR9095	Platberg	F	58.26
Afroedura	*nivaria*	NMBR5878	Clocolan	M	58.37
Afroedura	*nivaria*	NMBR6139	Ladybrand	M	62.78
Afroedura	*nivaria*	NMBR5309	Ladybrand	M	58.74
Afroedura	*nivaria*	NMBR5877	Clocolan	M	54.29
Afroedura	*nivaria*	NMBR5876	Clocolan	F	58.52
Afroedura	*nivaria*	NMBR4532	Ladybrand	M	45.9
Afroedura	*nivaria*	NMBR9091	Ladybrand	M	56.61
Afroedura	*nivaria*	NMBR9099	Ribboksberg	M	59.53
Afroedura	*nivaria*	NMBR9100	Ribboksberg	F	57.75
Afroedura	*nivaria*	NMBR9079	Korannaberg	F	54.5
Afroedura	*nivaria*	NMBR9104	Ribboksberg	F	44.46
Afroedura	*nivaria*	NMBR9093	Platberg	M	44.44
Afroedura	*nivaria*	NMBR9103	Ribboksberg	M	53.22
Afroedura	*nivaria*	NMBR9102	Ribboksberg	M	57.18
Afroedura	*nivaria*	NMBR9082	Korannaberg	F	37.14
Afroedura	*nivaria*	NMBR9230	Platberg		36.76
Afroedura	*nivaria*	NMBR9080	Korannaberg	M	53.74
Afroedura	*nivaria*	NMBR9090	Platberg	M	49.13
Afroedura	*nivaria*	NMBR5311	Ladybrand	F	54.91
Afroedura	*nivaria*	NMBR9096	Platberg	M	55.9
Afroedura	*nivaria*	NMBR9094	Platberg	M	59.96
Afroedura	*nivaria*	NMBR6138	Ladybrand	F	58.1
Afroedura	*nivaria*	NMBR5313	Ladybrand	M	56.21
Afroedura	*nivaria*	NMBR5315	Ladybrand	F	53.89
Afroedura	*nivaria*	NMBR5314	Ladybrand	F	59.13
Afroedura	*nivaria*	NMBR9097	Platberg	M	47.53
Afroedura	*nivaria*	NMBR4534	Ladybrand	F	52.87
Afroedura	*nivaria*	NMBR5875	Clocolan	F	61.34
Afroedura	*nivaria*	NMBR4533	Ladybrand	M	54.58
Afroedura	*nivaria*	NMBR9101	Ribboksberg	M	59.11
Afroedura	*nivaria*	NMBR9098	Platberg	F	45.11
Afroedura	*nivaria*	NMBR9092	Platberg	F	41.65
Afroedura	*nivaria*	NMBR9083	Korannaberg		32.3
Afroedura	*nivaria*	NMBR5316	Ladybrand	F	47.02
Afroedura	*nivaria*	NMBR5312	Ladybrand	M	43.17

Afroedura	*nivaria*	NMBR5310	Ladybrand	F	46.21
Afroedura	*nivaria*	NMBR6136	Ladybrand	M	44.74
Afroedura	*nivaria*	NMBR7055	Silasberg, Harrismith	F	61.63
Afroedura	*nivaria*	NMBR7058	Silasberg, Harrismith	M	57.26
Afroedura	*nivaria*	NMBR7056	Silasberg, Harrismith	M	57.57
Afroedura	*nivaria*	NMBR7059	Silasberg, Harrismith	M	58.8
Afroedura	*nivaria*	NMBR8396	Thibela village, Qwaqwa	F	47.7
Afroedura	*nivaria*	NMBR7057	Silasberg, Harrismith	F	32.31
Afroedura	*halli*	NMBR5842	Thaba Nchu	M	62.85
Afroedura	*halli*	NMBR5806	Thaba Nchu	F	53.91
Afroedura	*halli*	NMBR5215	Thaba Phatswa	M	60.37
Afroedura	*halli*	NMBR9303	Ladybrand	M	63.65
Afroedura	*halli*	NMBR5841	Thaba Nchu	M	61.2
Afroedura	*halli*	NMBR5223	Thaba Phatswa	M	59.44
Afroedura	*halli*	NMBR5843	Thaba Nchu	F	42.2
Afroedura	*halli*	NMBR5216	Thaba Phatswa	F	56.74
Afroedura	*halli*	NMBR5218	Thaba Phatswa	F	56.2
Afroedura	*halli*	NMBR5804	Thaba Nchu	F	54.64
Afroedura	*halli*	NMBR5219	Thaba Phatswa	M	58.19
Afroedura	*halli*	NMBR5226	Thaba Phatswa	M	53.54
Afroedura	*halli*	NMBR5224	Thaba Phatswa	F	50.23
Afroedura	*halli*	NMBR5227	Thaba Phatswa	F	53.66
Afroedura	*halli*	NMBR0097	Thaba Phatswa	F	57.56
Afroedura	*halli*	NMBR1141	Thaba Nchu	M	52.66
Afroedura	*halli*	NMBR5217	Thaba Phatswa	M	54.04
Afroedura	*halli*	NMBR5222	Thaba Phatswa	F	54.52
Afroedura	*halli*	NMBR5225	Thaba Phatswa	M	59.27
Afroedura	*halli*	NMBR0891	Spitskop, Zastron	F	50.31
Afroedura	*halli*	NMBR5805	Thaba Nchu	F	53.87
Afroedura	*halli*	NMBR1140	Thaba Phatswa	M	57.47
Afroedura	*halli*	NMBR5220	Thaba Phatswa	F	51.75
Afroedura	*halli*	NMBR2727	Thaba Phatswa	F	53.82
Afroedura	*halli*	NMBR5221	Thaba Phatswa	F	44.74
Afroedura	*halli*	NMBR9580	Herschel	M	57.76
Afroedura	*halli*	NMBR9575	Joubert's Pas	M	63.75
Afroedura	*halli*	NMBR9577	Herschel	M	56.51
Afroedura	*halli*	NMBR9576	Herschel	F	56.86
Afroedura	*halli*	NMBR9573	Joubert's Pas	F	55.51
Afroedura	*halli*	NMBR9581	Herschel	F	58.54
Afroedura	*halli*	NMBR6157	Joubert's Pas	F	59.96
Afroedura	*halli*	NMBR6156	Joubert's Pas	F	58.24
Afroedura	*halli*	NMBR9356	Wodehouse	M	59.48
Afroedura	*halli*	NMBR9578	Herschel	F	50.71
Afroedura	*halli*	NMBR9360	Wodehouse	F	51.87
Afroedura	*halli*	NMBR9361	Wodehouse	F	38.39
Afroedura	*halli*	NMBR9579	Herschel	F	39.07
Afroedura	*halli*	NMBR9574	Joubert's Pas	F	35.6
Afroedura	*halli*	NMBR6158	Joubert's Pas	F	34.33
Afroedura	*karroica*	RY1085	Lootsberg Pass, Middelburg District	F	50.05
Afroedura	*karroica*	NMBR9348	Tarkastad	F	43.71
Afroedura	*karroica*	RY980	Valley of Desol.Upper View Site, Graaff-Reinet	F	47.66
Afroedura	*karroica*	NMBR9344	Tarkastad	F	46.53
Afroedura	*karroica*	NMBR9365	Lootsberg Pass, Graaf Reinet	F	51.71

Afroedura	*karroica*	NMBR9345	Tarkastad	M	46.25
Afroedura	*karroica*	NMBR9293	Buffelshoek, Cradock	M	42.04
Afroedura	*karroica*	NMBR9298	Buffelshoek, Cradock	F	48.21
Afroedura	*karroica*	NMBR9296	Buffelshoek, Cradock		36.27
Afroedura	*karroica*	NMBR9297	Buffelshoek, Cradock	F	40.02
Afroedura	*karroica*	RY981	Valley of Desol.Upper View Site, Graaff Reinet	M	44.45
Afroedura	*tembulica*	NMBR9338	Cofimvaba	M	53.02
Afroedura	*tembulica*	NMBR9337	Cofimvaba	F	51.72
Afroedura	*tembulica*	NMBR9340	Cofimvaba	M	48.34
Afroedura	*tembulica*	NMBR9339	Cofimvaba	M	45.95
Afroedura	*tembulica*	NMBR9335	Cofimvaba	F	34.17
Afroedura	*karroica*	NMBR7235	unknown	M	45.35
Afroedura	*karroica*	NMBR7237	unknown	M	44.44
Afroedura	*karroica*	NMBR7236	unknown	F	44.4
Afroedura	*karroica*	NMBR7232	unknown	M	46.61
Afroedura	*karroica*	NMBR7234	unknown	M	41.71
Afroedura	*karroica*	NMBR7233	unknown	M	41.57
Afroedura	*karroica*	NMBR7239	unknown	M	45.27
Afroedura	*karroica*	NMBR7238	unknown	M	37.76
Afroedura	*nivaria*	NMBR3348	Sentinel Peak	F	58.7
Afroedura	*nivaria*	NMBR6298	Klavervlei, Harrismith	M	61.38
Afroedura	*nivaria*	NMBR6292	Klavervlei, Harrismith	F	53.04
Afroedura	*nivaria*	NMBR6297	Klavervlei, Harrismith	M	57.37
Afroedura	*nivaria*	NMBR6301	Klavervlei, Harrismith	F	53.1
Afroedura	*nivaria*	NMBR6378	Harrismith	M	56.29
Afroedura	*nivaria*	NMBR6377	Harrismith	F	55.3
Afroedura	*nivaria*	NMBR3345	Sentinel Peak	F	57.15
Afroedura	*nivaria*	NMBR6293	Klavervlei, Harrismith	F	54.71
Afroedura	*nivaria*	NMBR6741	General Will, Harrismith	M	55.23
Afroedura	*nivaria*	NMBR3350	Sentinel Peak	F	56.51
Afroedura	*nivaria*	NMBR6296	Klavervlei, Harrismith	M	44.5
Afroedura	*nivaria*	NMBR6380	Harrismith	F	58.42
Afroedura	*nivaria*	NMBR3349	Sentinel Peak	F	58.61
Afroedura	*nivaria*	NMBR3347	Sentinel Peak	F	50.76
Afroedura	*nivaria*	NMBR652	Monontsha, Qwaqwa	F	54.66
Afroedura	*nivaria*	NMBR3351	Sentinel Peak	F	41.9
Afroedura	*nivaria*	NMBR3352	Sentinel Peak	M	42.61
Afroedura	*nivaria*	NMBR6379	Harrismith	M	58.13
Afroedura	*nivaria*	NMBR751	Harrismith	M	53.99
Afroedura	*nivaria*	NMBR3346	Sentinel Peak	M	53.25
Afroedura	*nivaria*	NMBR6262	Bethlehem	F	42.74
Afroedura	*nivaria*	NMBR3359	Monontsha Pass	M	43.71
Afroedura	*nivaria*	NMBR6742	General Will, Harrismith	F	50.64
Afroedura	*nivaria*	NMBR6263	Bethlehem	F	35.03
Afroedura	*halli*	NMBR9110	Koesberg, Zastron	F	57.13
Afroedura	*halli*	NMBR7282	Mabula Mtn, Ladybrand	F	56.13
Afroedura	*halli*	NMBR9108	Aasvoelberg, Zastron	M	51.34
Afroedura	*halli*	NMBR7240	Koesberg, Zastron	F	55.25
Afroedura	*halli*	NMBR9109	Koesberg, Zastron	M	46.67
Afroedura	*halli*	NMBR9117	Elandsberg, Zastron	M	60.08
Afroedura	*halli*	NMBR9112	Koesberg, Zastron	F	53.85
Afroedura	*halli*	NMBR7283	Mabula Mtn, Ladybrand	M	54.79
Afroedura	*halli*	NMBR7223	Zastron	F	55.42

Afroedura	*halli*	NMBR7285	Mabula Mtn, Ladybrand	F	51.15
Afroedura	*halli*	NMBR7288	Mabula Mtn, Ladybrand	F	56.48
Afroedura	*halli*	NMBR7231	Aasvoelberg, Zastron	M	53.52
Afroedura	*halli*	NMBR7250	Elandsberg, Zastron	M	51.98
Afroedura	*halli*	NMBR7222	Zastron	F	57.58
Afroedura	*halli*	NMBR7230	Aasvoelberg, Zastron	F	53.03
Afroedura	*halli*	NMBR7229	Aasvoelberg, Zastron	M	63.63
Afroedura	*halli*	NMBR7284	Mabula Mtn, Ladybrand	F	55.14
Afroedura	*halli*	NMBR7251	Elandsberg, Zastron	F	47.7
Afroedura	*halli*	NMBR7221	Zastron	F	47.68
Afroedura	*halli*	NMBR7286	Mabula Mtn, Ladybrand	F	47.27
Afroedura	*halli*	NMBR9114	Koesberg, Zastron	F	40.12
Afroedura	*halli*	NMBR9108	Aasvoelberg, Zastron	M	50.87
Afroedura	*halli*	NMBR9115	Koesberg, Zastron	F	37.04
Afroedura	*halli*	NMBR7244	Genadeberg, Zastron	F	48.52
Afroedura	*halli*	NMBR7281	Mabula Mtn, Ladybrand	M	64.93
Afroedura	*halli*	NMBR9105	Aasvoelberg, Zastron	F	55.66
Afroedura	*halli*	NMBR7290	Mabula Mtn, Ladybrand	F	36.78
Afroedura	*halli*	NMBR7289	Mabula Mtn, Ladybrand	F	50.23
Afroedura	*halli*	NMBR9107	Aasvoelberg, Zastron	F	42.07
Afroedura	*halli*	NMBR7291	Mabula Mtn, Ladybrand	F	37.71
Afroedura	*halli*	NMBR7220	Zastron	F	29.11
Afroedura	*halli*	NMBR7249	Elandsberg, Zastron	F	36.01
Afroedura	*pondolia*	NMBR6106	Vernon Crookes, Natal	F	51.21
Afroedura	*pondolia*	NMBR6060	Vernon Crookes, Natal	F	53.97
Afroedura	*pondolia*	NMBR6062	Vernon Crookes, Natal	M	53.73
Afroedura	*pondolia*	NMBR6061	Vernon Crookes, Natal	F	53.97
Afroedura	*pondolia*	NMBR5456	Marina Beach, Natal	M	51.14
Afroedura	*pondolia*	NMBR6721	Vernon Crookes, Natal	F	47.94
Afroedura	*pondolia*	NMBR6063	Vernon Crookes, Natal	F	55.18
Afroedura	*pondolia*	NMBR6059	Vernon Crookes, Natal	F	49.93
Afroedura	*pondolia*	NMBR9585	DwesaCwebe Nature Reserve, KZN	M	49.6
Afroedura	*pondolia*	NMBR5455	Marina Beach, Natal	M	51.6
Afroedura	*pondolia*	NMBR6057	Vernon Crookes, Natal	F	47.17
Afroedura	*pondolia*	NMBR6058	Vernon Crookes, Natal	F	49.68
Afroedura	*pondolia*	NMBR6720	Vernon Crookes, Natal		37.71
Afroedura	*pondolia*	NMBR6715	Vernon Crookes, Natal	F	33.86
Afroedura	*pondolia*	NMBR6712	Vernon Crookes, Natal	F	38.72
Afroedura	*pondolia*	NMBR5458	Marina Beach, Natal	M	46.89
Afroedura	*transvaalica*	NMBR7266	Zimbabwe	F	65.2
Afroedura	*transvaalica*	NMBR7265	Zimbabwe	F	60.12
Afroedura	*transvaalica*	NMBR7267	Zimbabwe	M	54.77
Afroedura	*transvaalica*	NMBR7264	Zimbabwe	F	62.26
Afroedura	*transvaalica*	NMBR7268	Zimbabwe	M	52.75
Afroedura	*transvaalica*	NMBR9493	Vele coal mine, Limpopo	M	57.02
Afroedura	*transvaalica*	NMBR9505	Vele coal mine, Limpopo	M	58.35
Afroedura	*transvaalica*	NMBR9496	Vele coal mine, Limpopo	F	55.49
Afroedura	*transvaalica*	NMBR9508	Vele coal mine, Limpopo	F	53.1
Afroedura	*transvaalica*	NMBR9494	Vele coal mine, Limpopo	M	56.04
Afroedura	*transvaalica*	NMBR9495	Vele coal mine, Limpopo	F	65.66
Afroedura	*transvaalica*	NMBR9506	Vele coal mine, Limpopo	F	53.18
Afroedura	*transvaalica*	NMBR9507	Vele coal mine, Limpopo	F	49.73
Afroedura	*langi*	NMBR6069	Hoedspruit, Limpopo	M	54.7
Afroedura	*langi*	NMBR6070	Hoedspruit, Limpopo	F	55.3

Afroedura	*langi*	NMBR6071	Hoedspruit, Limpopo	F	43.7
Afroedura	*langi*	NMBR6072	Hoedspruit, Limpopo	M	45.86
Afroedura	*langi*	NMBR6073	Hoedspruit, Limpopo	F	40.89
Afroedura	*langi*	NMBR6074	Hoedspruit, Limpopo	F	43.77
Afroedura	*langi*	NMBR6068	Hoedspruit, Limpopo	M	44.88
Afroedura	*pondolia*	RY1039	Ntshongweni Dam, Camperdown District	F	50.26
Afroedura	*pondolia*	RY448	Spioenkop, Farm No.7583, Impendle	M	55.13
Afroedura	*pondolia*	RY450	Spioenkop, Farm No.7583, Impendle	M	52.59
Afroedura	*pondolia*	RY1041	Ntshongweni Dam, Camperdown	M	52.1
Afroedura	*pondolia*	RY1040	Ntshongweni Dam, Camperdown	F	49.24
Afroedura	*pondolia*	RY449	Spioenkop, Farm No.7583, Impendle	F	49.75
Afroedura	*pondolia*	RY905	Nhlosane Mount, Farm: Welton (2108), Impendle	F	39.54
Afroedura	*pondolia*	RY908	Nhlosane Mount, Farm: Welton (2108), Impendle		35.44
Afroedura	*nivaria*	AMNH26445	Top of Drakensberg	F	50.07