An evolutionary study of legless skinks' (*Acontias* Cuvier, 1817) head and vertebrae morphology

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by

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Table of Contents

Declaration:

I hereby declare that the work presented here is my own. The only section that was not done by me is the intercept analysis. All subsequent analyses from this method were conducted by me. It is submitted as the requirement for the degree of Master of Science at Rhodes University, Makhanda, South Africa and has not been previously submitted for any other degree at any other university.

Date: 13 March 2021 Signature:  Location: Port Elizabeth

Summary

Environmental factors and/or processes can produce differences in general shape between individuals or particular parts of individuals. Examples of these biological processes may include ontogenetic development, adaptation to local geographic factors, or long-term evolutionary diversification. An organism is not likely to be able to optimise a single structure for multiple purposes and so trade-offs are likely to occur. An example of such a structure is the cranium, as it can be used for multiple activities such as defensive and sexual behaviour, locomotion, prey capture, and ingestion. Morphological characteristics have historically been used in the description of species. Genetic analyses have gained popularity as species delineation techniques and have been particularly useful in identifying cryptic species, especially among morphological conserved species like legless skinks of the subfamily Acontinae (e.g. *Acontias* Cuvier, 1817 and *Typhlosaurus* Weigmann, 1834). However, completely doing away with morphological techniques during species descriptions is not the best option. Therefore, novel methods to identify species, especially those with similar body plans, are needed. In this dissertation, we explore the links between head shape and vertebral number to environmental pressures to determine whether the evolutionary process is driven by environmental pressures (soil or biome) or is retained through ancestry. A novel species/clade delineation linked to vertebral number is also investigated. Head shape was expected to have a close link to the environment and the number of vertebrae was expected to have a closer link to ancestry. The first chapter investigates the drivers behind *Acontias* head shape evolution using geometric morphometric techniques. We found that environmental pressures did affect the evolution of head shape especially in the “soil” and “biome” categories but further investigation is advised. The second chapter explores the viability of using vertebral counts as a novel method for species and/or clade delineation in *Acontias* and to determine whether vertebral number can be linked to the environment. Delineating species based on vertebral count is likely not an option, however, delineating clades proved to show promising results. A link between vertebral count and environment was found in *Acontias* with larger bodied species occurring in different environments to smaller body species. In conclusion, the genus *Acontias* is difficult to delineate morphologically. Genetic sequence analyses can indicate differences and delineate the species. Even though there were differences in morphology based on environmental factors, it is not sufficient to delineate this subfamily alone. Further research is advised and this dissertation provides a good basis to work with.

Keywords: *Acontias*, Morphometrics, Phylogeny, Head shape, Computer tomography, Vertebral count

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Ethics

Ethics approval was obtained from the Rhodes University Animal Research Ethics Committee (Permit number: RU-DZE-2017-03-003) for use of specimens/tissues in this study.

List of Figures

List of Appendix Figures

List of Tables

List of Appendix Tables

Chapter 1: Introduction

Acontias tristis Werner, 1910 (Namaqualand dwarf legless skink; Photographer: Chad Keates).

Chapter 1

Introduction

Literature review

Taxonomy and cryptic species

The field of taxonomy, in general, has progressed substantially over the past few decades (Zachos 2018). This progression is mostly linked to the development of more cost-effective phylogenetic analyses based on genetic markers. This is generally most evident in studies on reptiles in recent years, especially in southern Africa (Bickford *et al.* 2007; Busschau *et al.* 2017; Conradie *et al.* 2018; Pietersen *et al.* 2018; Bauer *et al.* 2019; Keates *et al.* 2019; Portillo *et al.* 2019; Conradie *et al.* 2020; Hallermann *et al.* 2020). Phylogenetic analyses have also produced evidence of cryptic species in some reptile groups but further research is required to confirm if they can be described as full species (Medina *et al.* 2016; Main *et al.* 2018; Busschau *et al.* 2019; Kullenkampff *et al.* 2019; Busschau *et al.* 2020; Ceríaco *et al.* 2020). It should also be noted that as new species are being discovered, there are also species that were initially thought to be different being synonymised based on genetics (Prötzel *et al.* 2017; Šmíd *et al.* 2018; Taft 2018; Zhao *et al.* 2019).

Phylogenetic analyses using genetic data have been useful in delineating morphologically similar species and those with conserved body structures (e.g. legless lizards and snakes). Although cryptic species can be identified using genetic markers, species descriptions are still required to use traditional external morphological characters as diagnostic features. There is a need for new characters to be identified in order for such morphologically similar species to be delineated effectively. Phylogenetic analyses can be used to identify novel species and unique clades, and from there, new phenotypic characters can be assessed for species descriptions (Bickford *et al.* 2007; Cook *et al.* 2010). There is, however, no consensus on the concept of “species”. The paragraph following hopes to give a brief insight into some species concepts and the dilemma it comes with.

Species concepts

The concept of “species” is a fiercely debated one, with many ways to define a species (Yang 2014; Zachos 2016). Scientists use the concept of species to divide the natural world. Due to the debate of the species concept the natural world is divided in different, inconsistent ways. This is problematic as “species counts” is a method of measuring biodiversity and, if the counts vary according to the species concept used, then the biodiversity of the area will vary accordingly. It is important to be able to define species

because they are central for understanding the origin and dynamics of biological diversity. One of the main goals of evolutionary biology is to try and explain why lineages split into multiple distinct species (Barraclough 2019), and one of the major goals of systematics is species delimitation, with phylogenetic reconstruction being the other major goal (Yang 2014). Being able to shed light on the true biological significance of species; the question of "why categorise organisms into species?" is another reason. The answer to that question is that the organisation of the diversity of life into species allows these species to be protected, and in so doing protecting well-balanced, well-adapted gene pools (Meier and Willmann 2000).

There are currently ~32 different concepts, and counting, that are used to define a species, so there is no surprise that a consensus on the topic has not yet been reached. Yet, there are concepts that seem to be more popular than others, and these include: 1) the Morphological Species Concept (MSC), 2) the Biological Species Concept (BSC) and 3) the Phylogenetic Species Concept (PSC) (Zachos 2016, 2018). Whenever a taxonomic study is conducted, the researcher should at least have some idea of the higher taxonomic level of the study"s organism and an idea of which species concept to base the research on (Cook *et al.* 2010). This will help to give the researcher direction and know what to look out for in the results.

The MSC is possibly one of the simpler concepts to understand as a layman. It is based on morphology; i.e., what the organism looks like (the study of shape). The idea on which this concept is based is that if it looks the same, then it must be the same. This is, of course, not simply a brief glance over the organism, but rather a study of mostly external features, by an individual with the required skill-set and expertise to draw conclusions. A more formal definition following Cronquist (1978) is as follows "*Species are the smallest groups that are consistently and persistently distinct and distinguishable by ordinary means*". From this definition, species recognition depends on what is viewed as ordinary means, which in today"s terms may include genomic data (Cronquist 1978; Zachos 2016). In other words, the MSC states that "a species is a community, or a number of related communities, the distinctive morphological characters of which are, in the opinion of a competent systematist, sufficiently definite to entitle it, or them, to a specific name" (Regan 1925).

In many groups of plants and animals, cryptic species have been discovered, many of which look extremely similar and almost indistinguishable from one another morphologically; e.g., *Liolaemus* genus of lizards in South America (Wollenberg Valero *et al.* 2019), and *Psammophylax kellyi* and *P. multisquamis* (Keates *et al.* 2019) in East Africa. Individuals of cryptic species may not interbreed where they coexisted (e.g., *Hakaria simonyi, Pristurus insignus*, *P. sokotranus* and four more lizard species from the Socotra

Archipelago (Vasconcelos *et al.* 2016)), thus maintaining the integrity of their respective gene pool. This is perhaps more common in animals than in plants, but the result is the same; it shows that a species concept based on morphology alone is problematic (Meier and Willmann 2000). Animals that looked very similar initially, turned out to be different when further studies included genetic aspects (Meier and Willmann 2000). This came across quite strongly when behavioural and ecological properties were taken into account.

The BSC, developed around mid-20th century by Ernst Mayr, relies on the reproductive capacity of organisms to define different species. Mayr defined biological species as groups of interbreeding, natural populations that are reproductively isolated from other such groups (Mayr 1942; Meier and Willmann 2000; Zink and Klicka 2018). Another way to put it is to say that a biological species is a reproductively cohesive assemblage of populations. The emphasis of the definition is whether individuals can reproduce successfully (by interbreeding) and the offspring produced are fertile, and not based on the degree of morphological difference. This concept goes against the presence of gene flow between groups, but this may simply reflect genetic divergence (genetic isolation) that does not preclude reproductive compatibility (Coyne 1992). This concept, however, is still a step forward from the MSC, where species were determined by their morphological differences. It should also be kept in mind that the status of a species is a property of its population and not individuals. If a single member of the population makes a „mistake" and hybridizes, it does not mean that the species status is now lost (Meier and Willmann 2000). A limiting factor for this concept is that it does not take into account any species that reproduce asexually, which narrows the number of organisms to which it can be applied.

The PSC is based on the tenet that there are distinct diagnosable characters between species, which are determined by the presence or absence of gene flow, meaning that it favours the side of genetics. The concept is based on the idea that species differ from each other absolutely (Groves 2017). According to Nixon and Wheeler (1990), a phylogenetic species that reproduce sexually and/or asexually is the smallest aggregation of populations or lineages, respectively.

Zachos (2016) splits the PSC into two versions. The first version, referred to as the Diagnosability Version, states along with Nixon and Wheeler"s (1990) definition that "*A species is the smallest population or group of populations within which there is a parental pattern of ancestry and descent*". Diagnosability does not necessarily mean that these characters are apomorphic. This brings us to the second version, the Monophyly Version, which states that "*A species is the least inclusive taxon recognized in a phylogenetic classification. As with hierarchical levels of taxa in such a classification, organisms are grouped into species because of evidence of monophyly. Taxa are ranked as species rather*

than some higher level because they are the smallest monophyletic groups deemed worthy of formal recognition, because of the amount of support for their monophyly and/or because of their importance in biological process operating on the lineage in question". Monophyly is a historical relationship, a community of descent comprising an ancestor and all its descendants. The character states thought to be apomorphic based on phylogenetic analysis are used to identify monophyly, not define them (Zachos 2016).

Despite these fiercely debated concepts, the field of taxonomy has advanced substantially over the last few decades (Zachos 2018). This advancement is linked to the development in the field of genetics where a number of cryptic species have been described (Bickford *et al.* 2007; Trontelj and Fišer 2009; Jörger and Schrödl 2013; Fišer *et al.* 2018; Struck *et al.* 2018) many amongst reptiles (Bickford *et al.* 2007; Busschau *et al.* 2017; Conradie *et al.* 2018; Pietersen *et al.* 2018; Bauer *et al.* 2019; Keates *et al.* 2019; Portillo *et al.* 2019; Conradie *et al.* 2020; Hallermann *et al.* 2020). Phylogenetic analysis has also produced evidence of cryptic species in reptile groups but further research is required to confirm if they can be described as full species (Medina *et al.* 2016; Main *et al.* 2018; Kullenkampff *et al.* 2019; Busschau *et al.* 2020). It should also be noted that as often as new species are being described, there are also species that were initially thought to be different being synonymised (Prötzel *et al.* 2017; Šmíd *et al.* 2018; Zhao *et al.* 2019).

As mentioned previously, these are three of the most common species concepts and one can see that none of them alone can define a species in all taxa. Hull (1997, 1999) noted that scientists would like their concepts to ideally be as general, applicable, and theoretically significant as possible (Hull 1997, 1999; Templeton 2001). However, when it comes to species concepts, these outlines are often in conflict with each other, which lead to the conclusion that there is no single ideal species concept and that a combination is likely required (Hull 1997, 1999). In an attempt to construct an all-encompassing species concept, De Queiroz developed the Unified Species Concept (USC) (de Queiroz 2005, 2007). The USC works on the basis that as soon as a population fragments, the lineage that arises becomes a new species (de Queiroz 2005). This, of course, also has arguments for and against it. At the end of the day, there is still no consensus on what a species is; in fact, a paper by Mishler and Wilkins (2018) suggested that the focus should move from "species" to "clade", which should solve the problem of defining a species (Mishler and Wilkins 2018). That, however, is another topic and not part of this study. Despite no consensus being met, most biologists acknowledge that species are real and that species delimitation is a meaningful and important exercise (Yang 2014).

Species descriptions

Before we move on to how species descriptions were/are/will potentially be conducted, we need to know what a species is. This is not in the context of the various species concepts, but rather from the description of the species and what overall criteria should be looked at. A species needs to be identifiable and unique (Fišer *et al.* 2018). It must have a unique name under its respective code, where the name needs to be linked to a type specimen lodged in a museum (Cook *et al.* 2010). This is not to say that DNA sequencing cannot be used but rather that it cannot be conducted by any taxonomist; some level of skill or experience in this field is required to achieve correct results (Cook *et al.* 2010).

Species descriptions in the past few centuries, before the aid of relatively fast and inexpensive DNA-sequencing technology, relied primarily on the appearance of the species. This idea ties strongly with the MSC. This concept seemed to make sense and was accepted for many years, but it does not uncover the existence of cryptic species, which are for the most part morphologically indistinguishable (Bickford *et al.* 2007; Cook *et al.* 2010; Struck *et al.* 2018). The reason for species mainly being delineated based on their morphology, behaviour, and locality, is because those were most likely the only data available to taxonomists at the time (Cook *et al.* 2010). Even with the development of genetic identification through DNA sequencing, it is still required that the taxonomist has some idea of what the species could be, at least at a higher taxon level. This is because of the different oligonucleotide primers that are used in the process. It is no good using primers designed for mammals when your study animal is a reptile, for example.

Currently, the use of DNA sequences to delineate species is common and it is almost the norm to use DNA analyses alongside other traditional techniques. DNA barcoding is a technique that has been used successfully to identify different species (DeSalle *et al.* 2005; Miller *et al.* 2016; Kullenkampff *et al.* 2019), but it is met with much criticism when it comes to describing new species (Dunn 2003; DeSalle *et al.* 2005; Jörger and Schrödl 2013). While it performs fairly well on pre-existing taxa (DeSalle *et al.* 2005), one of the criticisms is that the barcoding technique only uses the cytochrome *c* oxidase subunit I gene (COI) (DeSalle *et al.* 2005), which leads many to question its reliability. More recently, however, more genes have been used in the barcoding field and no longer solely rely on *COI* or are at least used in conjunction with *COI* (Aliabadian et al. 2009).

The future of species descriptions should depend on a combination of techniques (Padial *et al.* 2010; Jörger and Schrödl 2013). The genetic sequencing (using a minimum of three genes, ideally including slower evolving nuclear and fast evolving mitochondrial genes, as this resolves the issue mentioned with a single barcode gene) should be used alongside

differences in morphology, ecology, biology, and distribution (Padial *et al.* 2010). This will provide stronger evidence to describe a species as new. This does mean that a lot more effort and time needs to go into describing the species. Over the past decade, genomic sequencing has become more affordable and thus more accessible to a wider spectrum of scientists (Eriksson *et al.* 2020). For example, with the invention of nanopore sequencing, a researcher can obtain a machine from $1000 (Oxford Nanopore Technologies 2020). This machine can weigh less than 450g and is powered through a USB port. This grants the researcher the ability to sequence and analyse whole genomes in real time anywhere in the world. If this cost-effective trend continues, and this technology becomes even more affordable, scientists will not only have type specimens to describe species, but also easy access to whole genome sequences for their research. Using genome-scale datasets with a thorough and diverse sampling of taxa is also proposed as the way forward and has already been implemented in recent studies (Krehenwinkel *et al.* 2019; Burbrink *et al.* 2020). If this trend continues there could be more dramatic changes than expected in the future of species description.

In the end, whichever delineation method/s you choose, the one aspect that is crucial for all of them is that the taxonomist/geneticist must have a species concept in mind to guide the delineation process. By having a species concept in mind, it gives an idea for what sort of data can be used to delineate a species (Cook *et al.* 2010). Without one, there are no grounds to say whether or not the specimen is a new species or not.

There is still a need/use for morphological characters as delineation methods for species, especially if used in conjunction with genetic methods. A common morphological character used in studies delineating snakes and other limbless species include snout-vent length, tail length, head length, head height, etc. These among others were used in a study by Busschau *et al.* (2020) when delving deeper into the taxonomy of *Leptotyphlops* complex (Busschau *et al.* 2020). Hohl *et al.* (2017) also used morphological measurements of the body and skull in when comparing two shovel-headed species of *Amphisbaena* (Hohl et al. 2017). Keeping morphological characters as a delineation method also aids in the education of students and volunteers (citizen scientists) that are able to conduct or assist with research. Explaining key morphological characters to them is a much easier and more logical starting point that trying to start with explaining genetic sequencing (Dunn 2003). This will allow for faster in-field identification of species based on specific morphological characters. This is more efficient for species that are not morphologically similar. For morphologically similar species a genetic confirmation will need to be used as well to confirm the identification (as would be the case for many fossorial reptiles), however, morphological characters can still be used for initial identification (Dunn 2003).

Fossorial reptiles

Organisms with traits best suited to survive the environmental/selective pressures experienced are those that will successfully reproduce and thus pass on those traits (genes) to offspring, portraying the idea of the species "adapting" (Aerts *et al.* 2000). Evolution towards a fossorial lifestyle has led to many shared characteristics, such as the reduction, and even complete loss, of limbs in some species, reduced eyes, and elongation of the body; examples of which include worm lizards (Amphisbaenidae) and limbless skinks (Scincidae) (Wiens and Slingluff 2001; Kazi and Hipsley 2018; Bergmann and Morinaga 2019; Bergmann *et al.* 2020). This means that many of the ornamental characteristics that are used for delineation in other genera are of no use for these genera as they lack any external ornamentation.

Squamate reptiles (group containing snakes and lizards) are characterized by either a lizard-like body form, with four limbs consisting of five digits each, or a snake-like body form, with elongated bodies with reduced or no limbs (Pough *et al.* 2004; Bergmann and Morinaga 2019). The elongate snake-like body form has evolved independently at least 26 times (Wiens and Slingluff 2001). The snake body shape that we all know today arose from the most successful of these body forms. Modern snakes consist of approximately 3000 species and amphisbaenians (which also arose from the same body form) consist of roughly 167 species (Kazi and Hipsley 2018; Uetz *et al.* 2020). However, the snake-like body form also evolved independently in seven phylogenetically diverse squamate families, including Scincidae (Wiens and Slingluff 2001; Wiens *et al.* 2006; Uetz *et al.* 2020). This body form consisted of two ecomorphs: the surface dwelling ecomorph consists of a long, thin tail, and the burrowing ecomorph consists of a short, almost stubby tail (Wiens *et al.* 2006; Brandley *et al.* 2008). This is thought to be because the surface dwelling ecomorph undergoes tail elongation while the burrowing ecomorph undergoes trunk elongation (Brandley *et al.* 2008). Brandley *et al*. (2008) also shows that the estimated time for acontine skinks to have transitioned from a pentadactyl (five digits on each foot), tetrapod lizard-like ancestor to a limbless snake-like morphology that lacks all limbs took ca. 68.7 million years (Brandley *et al.* 2008). By not having any limbs, fossorial species have moved from a walking method of locomotion to one of slithering, and with no limbs to dig through the soil, they use their heads to create burrows.

Locomotion is important when it comes to finding and obtaining prey and for avoiding predators (Edwards 2014), and for a fossorial species that burrows, limbs may in fact pose a hindrance. The convergent evolution that led to the loss of limbs in some animals, such as snakes and legless lizards, has also resulted in the elongation of the body (trunk) and or tail (Woltering 2012). This elongation of the trunk or tail can be due to increasing the number of

vertebrae or the length of the vertebrae in that region (Bergmann and Irschick 2012). In terms of increasing the number of vertebrae, this occurs during the developmental stages of the animal (Woltering 2012). Somatic growth and *Hox* gene expression are primary factors driving the formation of the vertebrate body axis (Richardson *et al.* 1998; Muller *et al.* 2010). The rate of vertebrae development depends on what is known as the „somitogenesis clock". This clock essentially determines the rate at which somites are produced. The faster the „clock" ticks, the more somites are produced and thus, the more vertebrae develop (Woltering 2012). Thus, elongation of a body through either vertebral lengthening or increased vertebral count could affect burrowing ability and locomotion in a fossorial, limbless animal, and an assessment of which developmental path led to the body elongation in groups could be useful to understanding the biology and evolution of the species.

In amphisbaenians, for example, there are three distinct burrowing movements that correlate to the snout shape. The round-headed forms (e.g., *Zygaspis* (Mendes 2017) and *Blanus* (Kazi and Hipsley 2018) genera) use a forward driving stroke to penetrate and compact the soil, while the keel-headed and spade-snouted forms (e.g., *Monopeltis* (Hohl *et al.* 2017), and *Cadea* (Kazi and Hipsley 2018) genera) swing their snouts laterally from side to side and up and down in a seemingly random motion (Gans 1974). The movement is of course not random but a two-cycle movement to widen the anterior end of the tunnel slightly. The final head shape is the wedge shape (e.g. *Agamodon* (Gans 1974) that move their heads in an oscillating motion. The animal rotates its head about its long axis and then reverses it immediately with a simultaneous twisting of the head. This motion scrapes the sharp-edged lateral canthus across the tunnel thus shaving off sand grains. This loosened material is then compacted into the tunnel walls by the side of the head. Individuals with narrower heads have been shown to be more effective at digging but have a weaker bite force, thus potentially limiting their range of prey (Kazi and Hipsley 2018; Le Guilloux *et al.* 2020). These conflicting selective pressures are what are thought to limit the evolution of the skull shape in head-first burrowers as it imposes a trade-off between burrowing efficiency (locomotion) and prey size (diet) (Vanhooydonck *et al.* 2011). The aforementioned groups are fossorial and thus are thought to have limited dispersal ability as they have no limbs or wings to travel great distances (Daniels *et al.* 2005; Albert *et al.* 2007; Lee *et al.* 2013). This could lead to populations becoming isolated. Population isolation is one of the largest causal mechanisms to lead to speciation (Coyne 1992). Populations that are isolated due to a geographic barrier will lead to divergence either due to natural selection or genetic drift, which over time will lead to speciation (Coyne 1992). It is thus expected that fossorial lizard groups may be quite speciose, if the group is widely distributed. With a widely distributed species they are likely to be found in a variety of different habitats/environments which could also lead to different adaptations.

Environmental characteristics of South Africa

South Africa is a diverse region in many respects. It is a region that has varying rainfall levels and differences in rainfall seasonality (Grab and Knight 2015). In addition, there is also variability in the landscape in terms of elevation, with mountain ranges, and the escarpment, which forms a sharp upward elevation gradient from the coastal regions to the interior of the country (Grab and Knight 2015). This heterogeneity of climatic and geological features in South Africa is a driving force in the development of the diverse assemblage of macro-habitats, from tropical forest to desert, classified into biomes. Southern Africa consists of nine biomes; these are the Albany Thicket, Desert, Fynbos, Forest, Grassland, Indian Ocean Coastal Belt, Nama-Karoo, Savanna, and Succulent Karoo Biome (South African National Biodiversity Institute 2006-2018). Each biome has a set of characters (highlighted in Table A1) in relation to rainfall, climate, etc. that together create the general environment/climate of the biome. The constituent diversity of micro-habitat structures within each macro-habitat (biome), including different substrates and vegetation organization, contributes to the complexity of each macro-habitat (biome), and variety at both scales may be a strong factor in producing the high diversity and endemism of faunal species.

South Africa has a wide variety of geologies and thus has many types of soil (Fey 2010). The different soil types form a part of the microhabitat and so it would be expected that the animals, in this case of fossorial species, would be adapted to these environments (Kazi and Hipsley 2018). In this study, soil was divided into five categories (Organic, Orthic, Humic, Melanic and Vertic) (Fey 2010). Each soil type, because they are so broad, have a variety of densities and characteristics of which some may overlap. The soil types will also compose of many grain sizes and not just a single size. The soil density range can, however, give an indication to the range of grain size as smaller grain sizes are usually able to be more compacted. Other factors such as moisture, clay content, etc. also contribute to the density of the soil (Fey 2010).

Organic soils often have a low bulk density (0.05 to 0.15 Mg m^3) and are associated with swamps, marshes, and vleis where rainfall is high, temperatures are cool or cold and the ground is almost permanently wet (Fey 2010). Humic soils contain a marked accumulation of humus in a mineral surface horizon. They are well drained soils even though there is high rainfall in the area (Fey 2010). Humic soils are mostly found in the provinces of Mpumalanga and KwaZulu Natal‟s Grassland Biome in South Africa. The properties of vertic soils are perhaps the most well-known of the top soils in South Africa known for their blocky structure and presence of slickensides. Vertic soils have some of the highest clay content

out of the top soils with over 50% clay content in most cases. This makes the soils quite dense and subject to much expansion and contraction from temperature and moisture (Fey 2010). This soil type can be found in the Highveld areas of Limpopo, Free State and Mpumalanga, North West and KwaZulu Natal. The biomes in these areas are predominantly grassland and savanna. Melanic soils are often black or dark coloured. They have a well-developed blocky structure although not as strong as the vertic soils and retain water well. Melanic soils mainly occur in areas with semi-arid climates including northern Eastern Cape and Free State (Fey 2010). This is again part of the Grassland Biome. All other top soil types not mentioned fall into the orthic category. This makes this category very broad and spread across multiple biomes. The biomes that host this soil type include Succulent Karoo, Nama-Karoo, Fynbos and Savanna Biomes in the Western and Northern Cape provinces.

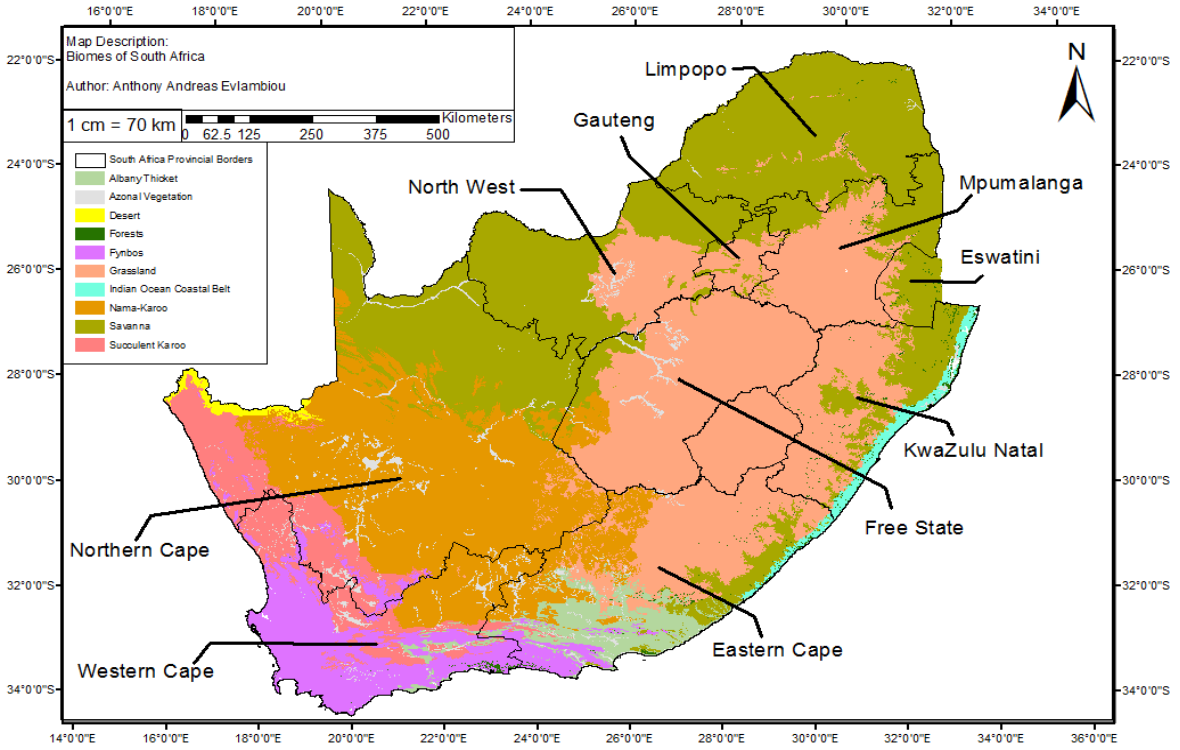


Figure 1.1: Map of South Africa indicating the provincial boundaries and biomes found in the country. Data was obtained from the Vegetation Map of South Africa (VegMap 2018) project run by SANBI (South African National Biodiversity Institute 2006-2018)

Study animals

Acontinae initially contained three genera: *Acontias, Acontophiops* and *Typhlosaurus* (FitzSimons 1943; Branch 1998; Lamb *et al.* 2010). *Microacontias* was a later addition, which represented the smaller bodied (SVL<150 mm) *Acontias* species (Daniels *et al.* 2006;

Lamb *et al.* 2010), though it, along with *Acontophiops*, has subsequently been synonymised with *Acontias* based on phylogenetic analysis (Lamb *et al.* 2010). Should all of the variability found in Daniels *et al.* (2006) be taken into consideration, and to be recognised as its own genus, a total of 10 genera would be required, which is disruptive. Thus Lamb *et al.* (2010) proposed a much less disruptive approach that still maintains strictly monophyletic units, in which only two genera are recognised as part of the Acontinae subfamily. These are *Typhlosaurus* (scales covering eyes), corresponding to the West Coast *Typhlosaurus*, and *Acontias,* which comprise the remaining acontines. The West Coast *Typhlosaurus* are associated with much sandier soils compared to most *Acontias* species (Branch 1998; Alexander and Marais 2007; Lamb *et al.* 2010).

Acontias are limbless, burrowing, scincid lizards, with shiny, overlapping and tight-fitting scales (Branch 1998; Alexander and Marais 2007) and are found across most of southern and eastern Africa (Broadley and Greer 1969; Branch 1998; Spawls *et al.* 2018), in a variety of different habitats and soil types. Some species of *Acontias* are known to live in the leaf litter or between the roots of trees and shrubs. The genus was thought to be genetically depauperate, and species-poor, due to the fact that their body shapes are relatively conservative and lack all external ornamentation. This makes delineating species within this genus very difficult based on morphological data alone. The description of cryptic or previously undescribed species in the genus of legless skinks, *Acontias*, are amongst those that benefited from advancement in phylogenetic analysis (Conradie *et al.* 2018). Phylogenetic analysis based on genetic markers has helped in delineating species within this genus (Daniels *et al.* 2002; Daniels *et al.* 2005; Daniels *et al.* 2006; Daniels *et al.* 2009; Engelbrecht 2012; Busschau *et al.* 2017; Conradie *et al.* 2018; Pietersen *et al.* 2018; Zhao *et al.* 2019). It should, however, be noted that delineating *Acontias* species using the current morphological characters is not adequate enough, especially in the *Acontias meleagris* group (Daniels *et al.* 2002; Daniels *et al.* 2005; Daniels *et al.* 2006; Daniels *et al.* 2009; Engelbrecht 2012).

Only three species of *Acontias* were initially recognised (Hewitt 1938); these were *A. lineatus, A. plumbeus,* and *A. meleagris*. These three species each had many forms within them, including *A. meleagris lineicauda, A. m. meleagris, A. m. orientalis, A. plumbeus, A. plumbeus namaquensis, A. p. gracilicauda, A. p. tasmani* and *A. lineatus orangensis*. *Acontias lineatus* was seen as sharply different from the other two species; there are three supraciliary scales and a single large and elongate supraocular followed by another elongate scale of smaller size (Hewitt 1938). All other forms were classified on the basis of tail characteristics. Those with tapering tails fell under *A. plumbeus* and those with more or less cylindrical tails fell under *A. meleagris.* This form of classification was purely

based on morphology and so followed the Morphological Species Concept. Geographical distribution played a minor role in this case and genetic analyses were not available. In more recent phylogenetic analyses, the legless skink species were, however, found to be paraphyletic (Lamb *et al.* 2010; Wagner *et al.* 2012; Engelbrecht *et al.* 2013; Busschau *et al.* 2017; Pietersen *et al.* 2018). From an original eight species and nine subspecies described for the genus (Broadley and Greer 1969; Branch 1998), current taxonomic assessments describe a number of new species, bringing the total close to 30 species and subspecies for the genus (Conradie *et al.* 2018). The delineation and classification of species has historically relied on the use of morphological characters, however recently genetic markers are included in the identification of genetically disparate groups, and ultimately species (DeSalle *et al.* 2005; Conradie *et al.* 2018; Zhao *et al.* 2019). Many of the recent evolutionary studies (Daniels *et al.* 2009; Busschau *et al.* 2017) used genetic analyses alongside morphometric evidence to delineate species.

The *Acontias* genus forms two distinct clades according to Lamb *et al.* (2010); the first clade comprises large (>200 mm SVL) to very large (>300 mm SVL) stout-bodied species (*A. plumbeus*, including *A. poecilus*), *A. aurantiacus*, *A. cregoi* and *A. rieppeli*. The second clade comprises of three groups: 1) intermediate-sized (~180 mm SVL) species (A. *gariepensis, A. jappi,* and *A. lineatus*), 2) *Acontias lineatus* and *A. litoralis*, and 3) several intermediate (~150 mm SVL) to large (>200 mm SVL) sized *Acontias* (e.g., *A. meleagris*, *A. occidentalis* and *A. gracilicauda* groups).

For *Acontias* specifically, common species description factors include colouration ventral, midbody and subcaudal scale counts and scale positioning, scales around the eye, and whether scales are fused in particular areas (Broadley and Greer 1969). The aforementioned examples all fall under the morphological method of species delineation. Since the morphologies are all relatively similar, we can no longer delineate similar looking species based on external morphology alone, and we need to look further, perhaps into their anatomy, distribution, habitat, behaviour, or genetic relatedness.

The genus *Acontias* has a strong association with a fossorial lifestyle (Conradie *et al.* 2018; Zhao *et al.* 2019). Their body plan is rather uniform and lacks any ornamentation or appendages (limbs). Without any limbs to burrow, they instead use their heads pushing through the soil in an almost serpentine like motion, throwing their bodies from side to side in many horizontal travelling waves flowing from head to tail (Heideman 1989).

Rationale

The delineation and classification of species has historically relied on the use of morphological characters, and recently have begun relying on genetic markers to identify genetically disparate groups, and ultimately species. However, although genetics have been useful in delineating species, and the identification of cryptic species, morphological analyses are still beneficial to understand the phenotypic differences between species and to investigate the selective forces that shaped the phenotype of species. Thus, an understanding of how phenotypes are shaped, and which environmental forces may in fact be shaping species in the South African region, can provide an understanding of whether species will ultimately be able to adapt to a changing climate. For organisms that are relatively immobile, or that do not readily disperse in their habitat, the environmental characteristics of the region that they inhabit are paramount to their survival. It is assumed that these organisms will have adapted to local and regional environments and that their phenotype will reflect these adaptations. Burrowing lizards, therefore, will be adapted to particular soil characteristics (looseness, humidity, mineral content, etc.), and will have adapted to those local conditions. Soils, and indeed most environmental characteristics, differ between the various biomes of South Africa (Mucina *et al.* 2006; Grab and Knight 2015), and it is expected that the burrowing species of *Acontias* will show phenotypic adaptations to the various environmental characteristics of the biome that the species inhabit.

Acontias have fewer diagnostic characters compared to most other lizards, as they lack limbs, and a novel diagnostic system is proposed to aid in species descriptions, namely using vertebral counts as diagnostic characters. This kind of diagnostic system has been employed in species descriptions in snakes (Weinell *et al.* 2020) and in some species of *Acontias* (Wagner *et al*. 2012; Conradie *et al.* 2018), and due to the similarity in body plan, it is expected to be an effective diagnostic tool in the legless skinks. Wagner *et al.* (2012) used vertebral counts to determine the genus of a legless lizard, which was described as *A. schmitzi.* Conradie *et al.* (2018) used vertebral counts as a descriptive statistic to aid in the description of two new *Acontias* species (*A. albigularis* and *A. wakkerstroomensis).*

Additionally, we expect that there will be differences in phenotypic characteristics (namely, head shape) within the genus that are linked with biome characteristics. No published studies to date have specifically looked at interspecific head shape differences in this genus. With this study, we aim to investigate the use of a novel character for clade delineation (vertebral count), and the link between phenotype and environment in a charismatic group of lizards.

One of the greatest benefits of computed tomography (CT) scanning for herpetology is that the specimen can be examined in a relatively non-invasive and non-destructive manner, there is no need to dissect or destroy the specimen (Broeckhoven and du Plessis 2018) thus maintaining museum specimens in good condition. This means that specimens can be kept and used again for other studies. CT-scanning also produces high resolution images which you can adjust and manipulate as needed to get the best image for the study (du Plessis *et al.* 2016; du Plessis *et al.* 2017). If vertebral counts can be used to delineate species (Wagner *et al.* 2012; Conradie *et al.* 2018), or at least part of the delineation process, the specimens can be sent to be computed tomography scanned (CT- or CAT-scanned).

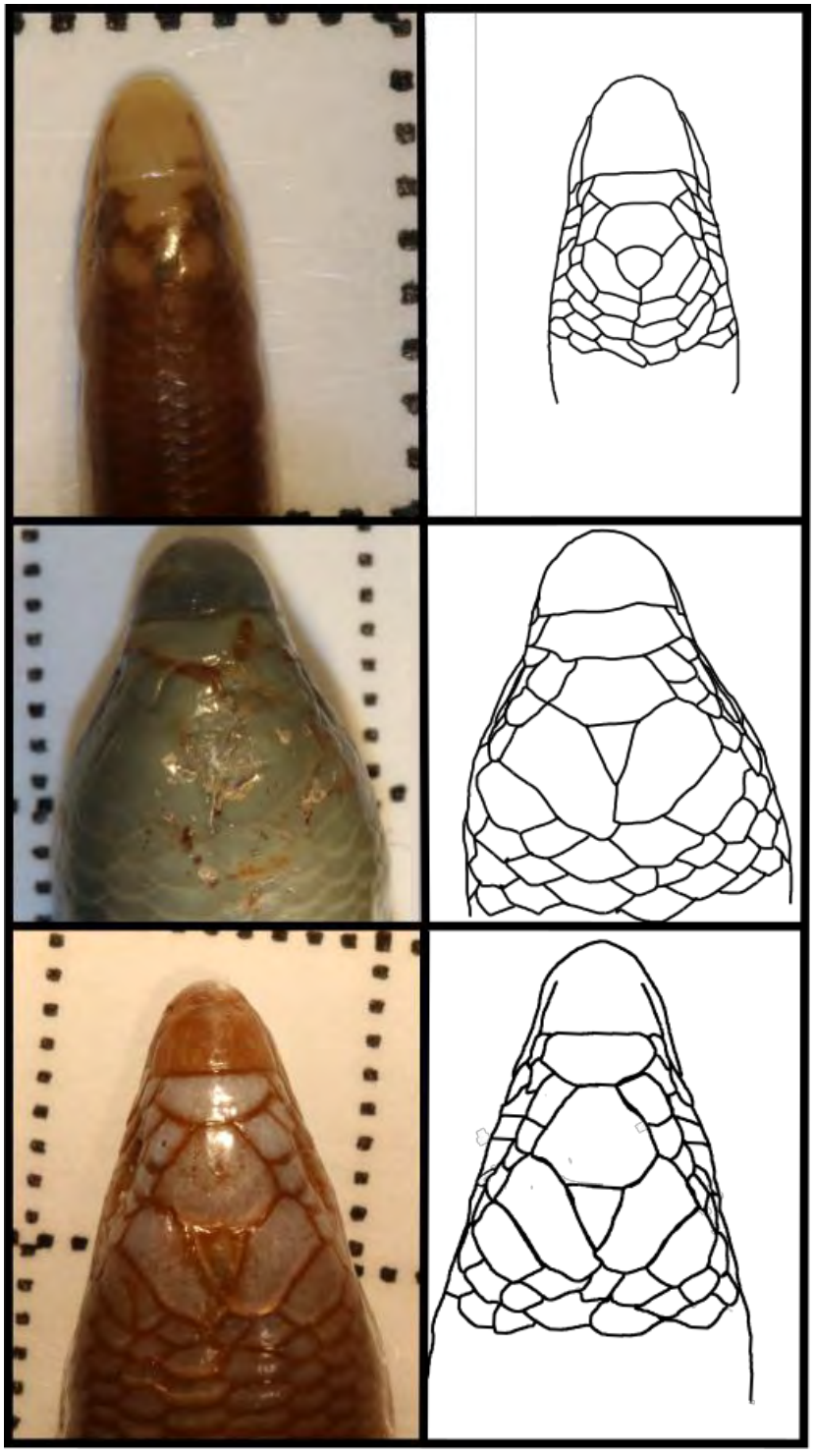
Research questions

Molecular work has provided a fairly detailed phylogeny of *Acontias* with some groups consisting of morphologically similar cryptic species whilst other species are morphologically variable. This indicates that external morphology is being driven by factors other than ancestry. Here, we test whether head shape is linked with ancestry or is more closely related to the environment (soil and biome). Although genetics are good species delineation methods, species descriptions are still routinely done using morphological character descriptors. It is for this reason that we would like to test a new set of characters (vertebral count) to assess its usefulness as a clade delineator in this group.

In the first data chapter we will investigate if *Acontias* head shape differs between species and if so, in what way. We will also investigate what the driver of *Acontias* head shape is. Is it retained from an ancestor (ancestry) or has it formed due to the influence of environmental factors (soil and biome)? In order to do this, traditional and phylogenetic comparative analyses were conducted.

In the second data chapter, we will investigate whether there is a link between the number of vertebrae and environmental factors (soil and biome) in *Acontias* or if the link is due to ancestry. We will also investigate if difference in vertebral counts can be used as a novel species/clade delineation method among *Acontias* species.

Chapter 2: Now that's using your head!: a morphometric study on whether there is a link between head shape and environment in legless skinks (*Acontias* Cuvier, 1817) .



Photographs and line drawings indicating different head shapes that can be found in the genus *Acontias*. *Acontas litoralis* (top), *Acontias occidentalis* (middle) and *Acontias gracilicauda* (bottom).

Chapter 2

Now that's using your head!: a morphometric study on whether there is a link between head shape and environment in legless skinks (Acontias Cuvier, 1817).

Introduction

Environmental factors and/or processes can produce differences in general shape between individuals or particular parts of individuals (Aerts *et al.* 2000; Vanhooydonck *et al.* 2000; Herrel *et al.* 2001; Edwards *et al.* 2012; Fabre *et al.* 2016). Examples of these biological processes may include ontogenetic development, adaptation to local geographic factors, or long-term evolutionary diversification (Openshaw and Keogh 2014). Morphology is one of the main components of an organism"s phenotype (Kaliontzopoulou 2011), which is often dependent on more than one aspect or response to selective pressures, especially if the structure in question is used for multiple purposes. The main problem is that the organism is most likely not able to optimise the structure for all functions simultaneously. It will likely reflect trade-offs between the adaptive responses to the selective pressures (Herrel *et al.* 2007; McBrayer and Corbin 2007; Barros *et al.* 2011). These selective pressures can come in a variety of forms. These shape changes may signal different functional roles played by the same parts, or different responses to the same selective pressures, or even differences in selective pressures themselves, to name a few (Zelditch *et al.* 2004; Fabre *et al.* 2014). The cranium makes for a good example for functional trade-offs and selective pressures as it can be used for activities such as defensive and sexual behaviour, locomotion, prey capture, and ingestion (Herrel *et al.* 2001; Herrel *et al.* 2007; McBrayer and Corbin 2007; Kaliontzopoulou *et al.* 2008; Barros *et al.* 2011; Stepanova and Bauer 2021).

In head-first burrowing reptiles, energy constraints associated with a fossorial lifestyle limits the overall body size (Navas *et al.* 2004; Wu *et al.* 2015) and head shape (Navas *et al.* 2004; Barros *et al.* 2011). Burrowing reptiles tend to exhibit limb reduction, body elongation (Navas *et al.* 2004), strengthening of the cranium (Lee 1998), shortening of the head and lower rostral angulation (Barros *et al.* 2011), shortening of the tail, and streamlining of scales (Wu *et al.* 2015). More specialised fossorial reptiles display reduction of eyes and ears, fixed lower eyelid, and a greater number of vertebrae and ribs associated with body elongation. Fossorial lizards and snakes generally follow this elongated body plan with short narrow heads and reduced limbs to lower energy expenditure during burrowing (Navas *et al.* 2004; Wu *et al.* 2015).

The functional trade-offs that affect the evolution of the cranium can, for example, be related to the response to selective pressures that involve locomotion in different microhabitats. This can be important because locomotory performance affects fitness (Aerts

et al. 2000) and is a key factor in predator escape, foraging, and territorial defences. The relationship between head morphology and locomotion may differ among species that occupy different microhabitats, as the effects of head shape and size on locomotor performance may differ (Barros *et al.* 2011; Vanhooydonck *et al.* 2011; Bergmann *et al.* 2020).

For example, Australian agamid lizards" (Amphibolurinae) skull shape (based on CT scans) differs according to the habitat in which they are found (Gray *et al.* 2019). It was found that tree-dwelling agamas (e.g. *Lophosaurus spinipes* and *L. boydii*) have longer skulls and snouts, while terrestrial living species (e.g. *Tympanocryptis* spp., *Ctenophorus isolepis* and *C. gibba*) have shorter, blunt skulls, and saxicolous species (e.g. *Ctenophorus fionni, C. ornatus* and *C. decresii*) have dorsoventrally flattened skulls. These skull characteristics are likely a result from trade-offs to optimise functional capabilities (Barros *et al.* 2011; Gray *et al.* 2019) that are linked to the habitat in which the species are found and/or the diet. Another example is a study conducted by Barros *et al.* (2011) that investigated the roles of diet and locomotion in the evolution of cranial design in gymnophthalmid lizards (Gymnophthamlidae), with a specific focus on the head-first burrowers. Head shape for the head-first burrowers was found to be influenced by their evolutionary history (ancestry) and microhabitat usage, independent of the prey consumed. The head-first burrowers have shorter heads with lower rostral angulation and the shape resembles that of the round-headed amphisbaenians of the genus *Blanus* (Gans 1969; Gans and Montero 2008; Kazi and Hipsley 2018).

Fossorial amphisbaenians or „worm lizards" (*Amphisbaena*) of the Caribbean are another good example of head-first burrowers. Amphisbaenians are a clade of predominantly limbless, burrowing squamates that live underground in the loose and sandy soils of the tropical and subtropical regions of the world (Gans 1969; Kazi and Hipsley 2018). Unlike the anoles in the same system that rapidly diversified both taxonomically and phenotypically to fill various niches, amphisbaenians remained very similar (Kazi and Hipsley 2018). Their morphology is thought to be due to the adaptations related to the stresses associated with a subterranean lifestyle. These adaptations include the elongate body and robust skull, with distinct snout shapes (shovel, spade, keel and round) corresponding to specific burrowing behaviours, a high degree of interdigitisation among dermal roofing bones, and reduction or loss of the eyes and ears (Gans and Montero 2008; Muller *et al.* 2016; Kazi and Hipsley 2018). The previously mentioned features indicate strong selective pressures related to microhabitat use. Since they are a group of burrowing organisms, it suggests that the variation in the soil type or other ecological variables may drive the observed difference in skull shape across species. However, at the same time, a stabilising

selection to maintain adequate digging performance throughout ontogeny is thought to constrain their allometry (Hipsley *et al.* 2016; Kazi and Hipsley 2018). It was found that the shape of the snout in amphisbaenians is highly correlated with specific burrowing motions. The round-snouted forms (e.g., the genus *Blanus*) used forward-driving strokes to penetrate and compress the soil, while the keel-headed forms (e.g., the genus *Cadea*) swing their snouts laterally side to side. It was also shown that individuals that had narrower heads were more efficient at burrowing than those with broader heads. Other species that have shown to have head shape differences due to habitat differences include caecilians (Herrel and Measey 2010; Sherratt *et al.* 2014), eels (De Schepper *et al.* 2005), and snakes (Shine *et al.* 2006; Da Silva *et al.* 2018; Keates *et al.* 2019).

The Scincidae genus *Acontias,* comprising of around 30 species, is indigenous to southern and eastern Africa (Branch 1998; Spawls *et al.* 2018) and has a strong association with a fossorial lifestyle (Branch 1998; Alexander and Marais 2007). Their body plan is rather uniform and lacks any ornamentation or appendages (limbs). Without any limbs to burrow, they instead use their heads, and bodies to push through the soil in an almost serpentine-like motion (Heideman 1989). The Acontinae subfamily initially contained three genera: *Acontias, Acontophiops* and *Typhlosaurus. Microacontias* was a later edition, which described the smaller-bodied *Acontias* species (Daniels *et al.* 2006; Lamb *et al.* 2010), though it, along with *Acontophiops*, has subsequently been synonymised with *Acontias* (Lamb *et al.* 2010). *Acontias* are found across southern and eastern Africa in many different soil types and biomes; i.e., in many different environments. Although the overall body shape of *Acontias* is very conservative, based on the studies mentioned in the previous section, one could expect that species found in different environments might have adaptations that make them better suited to that environment. As head-first burrowers, the differences could be expected to be found in the head-shapes of members of this genus.

A study by Heideman *et al.* (2008) indicated that tentatively there does seem to be a relationship between head shape and the environment in Acontinae. Heideman *et al.* (2008) mentioned that the heads of the smaller bodied taxa, such as *Acontias litoralis,* tend to be flatter, narrower, and more pointed, which is apparently a more energy efficient and robust shape for burrowing through sand according to previous studies (Gans 1960, 1969; Gil *et al.* 1993). Wide, and higher heads, such as in *A. gracilicauda,* are presumably not as well adapted to move through sand but energetically less demanding in other media such as grass and more compact soil (Gans 1960, 1969; Gil *et al.* 1993; Heideman *et al.* 2008). This does not mean that the different species are able to burrow at the same rate through the substrate. Vanhooydonck *et al.* (2010) noted that the wider the head of a head-first burrowing species, such as *Acontias,* the more time is needed to burrow into the substrate,

such as soil. These two head shapes link to the size of the *Acontias* species with the larger bodied species exhibiting rounder, higher heads and the smaller bodied species exhibiting sharper, flatter heads (Heideman *et al.* 2008). Branch 1998 and Bates *et al.* (2014) also noted that *Acontias grayi, A. litoralis, A. lineatus* and *A. tristis* all occur along the South African west coast. This region of the country mostly comprises of the Fynbos, Succulent Karoo, Nama Karoo, and Desert biomes. Other *Acontias* species such as *A. meleagris, A. orientalis* and *A. gracilicauda* have a wider distribution along the west and south coasts and the interior, respectively, thus associating them with a more Fynbos-Savanna-Grassland type biomes (Branch 1998; Bates *et al.* (eds). 2014 (reprint 2014)).

Aims and Objectives

Aim: The aim of this study is to investigate *Acontias* head shape in relation to environmental factors.

Objective: I intend to investigate this aim by employing two-dimensional (2D) geometric morphometric techniques, adjusted for phylogenetic relatedness and environmental factors such as soil and biome type, to determine whether the head shape is retained due to ancestry or influenced by the environmental factors.

Question: Is *Acontias* head shape retained due to ancestry or linked to environmental factors?

Hypothesis: Species with more pointed heads will occur in looser soils mostly along the West coast, and those with more rounded heads will occur in more compact soils.

Methods

Specimen sampling

Nineteen species of *Acontias* and three outgroup species of *Typhlosaurus* were used in this study (*Typhlosaurus lomiae* was omitted from the morphometric dataset as landmarks could not be placed with high confidence on the dorsal side of the head, all *Typhlosaurus* species were included in the lateral dataset). Specimens were obtained from the Port Elizabeth Museum"s (PEM) herpetological collection, Eastern Cape, South Africa (Table A2). The number of individuals from each species varies from one to 18, depending on their availability in the museum collection (Fig. 2.1; Table A2).

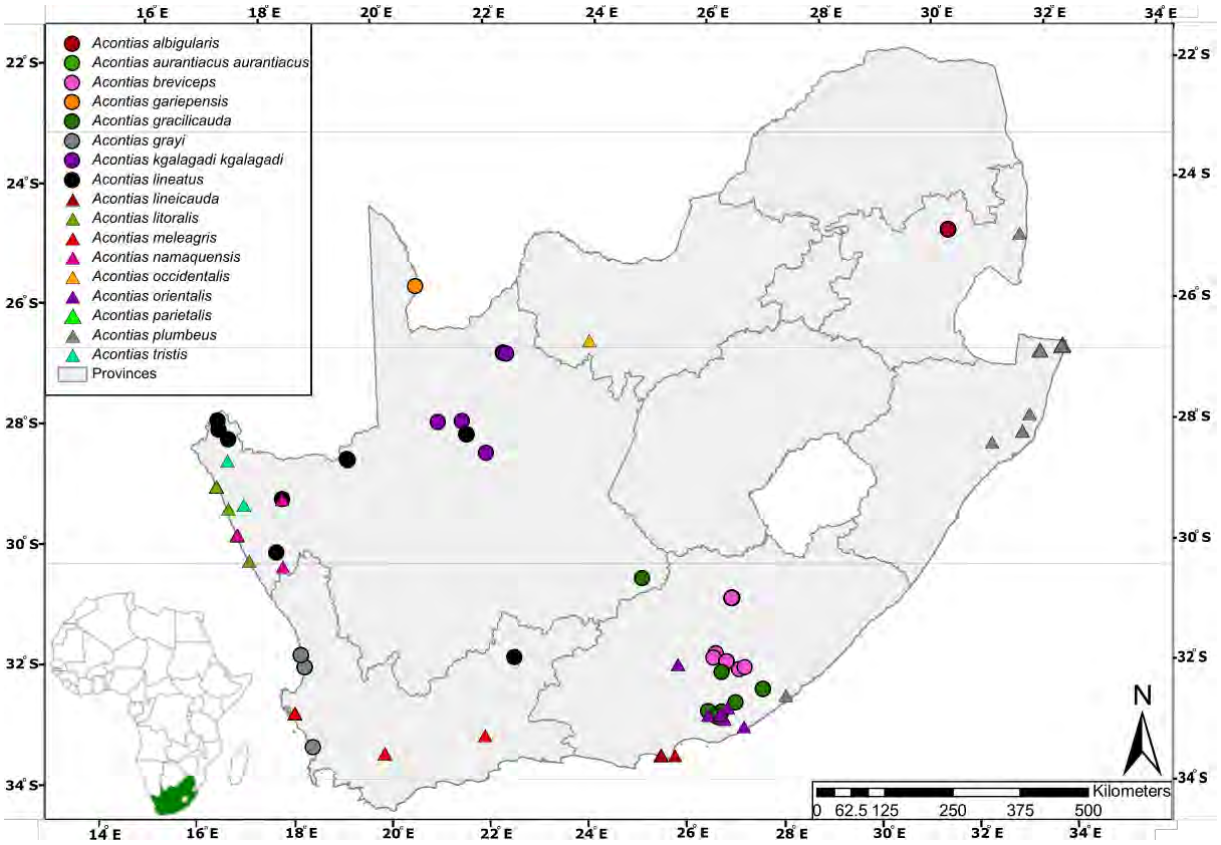


Figure 2.1: Sample localities of *Acontias* specimens used in this study. *Acontias bicolor* (three individuals; -18.2177, 32.7874) and *A. schmitzi* (one individual: -16.6486, 23.61389) are not indicated on this map as they are not found within South Africa"s borders.

Phylogenetic analyses

Tissue samples (tail or liver tissue) utilised in the phylogenetic analysis was obtained from previous collections (preserved in 95% ethanol). The majority of the sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov, Table A2). The number of individuals per species that were included in the generation of the phylogenetic tree ranged from one to six individuals.

Genomic DNA was isolated from tissues using a proteinase K digestion followed by a standard salt extraction method (Bruford *et al.* 1992) using lysis (Buffer ATL; Qiagen) and elution (Buffer AE; Qiagen) buffers. Standard PCR procedures as seen in Edwards *et al.* (2012) were utilised to amplify three mitochondrial genes (*16S* rRNA (*16S*), cytochrome b (*Cytb*) and cytochrome oxidase I (*COI*)) and one nuclear gene (Recombination activating gene subunit 1 (*RAG1*); Appendix Table A3). The prepared PCR products were sent to Macrogen Corp. in Amsterdam, Netherlands, for sequencing using only the forward primers in all cases. Sequence files were checked using BioEdit Sequence Alignment Editor V (Hall 1999) and aligned, along with the previously accessioned GenBank sequences, in MEGA v.6.0. (Tamura *et al.* 2013) using the ClustalW alignment method.

A concatenated phylogenetic tree was generated (genes used were *16S, Cytb, COI* and *RAG1*; Appendix Table A3). Outgroup taxa used were from the genus *Typhlosaurus* (*T. caecus*, *T. vermis*, *T. lomaie* and *T. meyeri*). jModelTest2 v2.1.6 (Darriba *et al.* 2012) was used to determine the nucleotide substitution models for each gene partition (models used: *16S*: HKY+I+G; *Cytb*: TPMuf+I+G; *COI*: HKY+G; *RAG1*: HKY+G). Bayesian Inference (BI) was done (using nucleotide substitution models for each gene partition) using MrBayes v.3.2 (Ronquist *et al.* 2012) in the CIPRES Science Portal (Miller *et al.* 2010), with 20 million generations run, with trees sampled every 1000 generations with a burn-in of 2000 generations; four chains were used (three hot and one cold). The phylogenetic tree was constructed using only the species that were used for the morphometric study in this paper. Thus, the entire *Acontias* genus is not represented in this phylogenetic tree nor are all the *Typhlosaurus* species. The species were grouped according to the clades produced in the phylogenetic tree.

Geometric morphometric analyses

In order to investigate differences in head shape between *Acontias* species, geometric morphometric analyses were conducted. Specimens were individually placed on grid paper comprised of 1 cm^2 squares. An external light source (Schott Mainz, KL30513) was used to provide additional light for photographs. The specimens were then photographed using a Canon digital camera (24MP Canon EOS 750D; set to F18, ISO 800, and a shutter speed of 1/200s) and Canon macro lens (EFS 55-250 mm macro-lens) from the dorsal and lateral view of the head region. The lateral view was taken from the right-hand side of the head. The specimens were all labelled according to their unique museum catalogue number (Appendix Table A2).

The program tpsUtil v1.76 (Rohlf 2018) was then used to build a **.tps* file from the images taken. The software program tpsDig2 v2.31 (Rohlf 2017) was then used to place landmarks on photographs of each of the specimens. Fourteen homologous landmarks were used for the dorsal view of the head (Fig. 2.2, Left) and six homologous landmarks were used for the lateral right side (Fig. 2.2, Right). For descriptions of the landmarks please refer to supporting information (Appendix Table A4). All the images were scaled. Further morphometric analysis was conducted using MorphoJ v1.07a (Klingenberg 2011). A preliminary Generalised Procrustes Analysis (GPA) was applied to the dataset. After which a covariant matrix was estimated, aligned by principal axes and the symmetric component selected for the dorsal dataset and the asymmetric component for the lateral dataset. A Principal Components Analysis (PCA) was then conducted on the components of the heads to identify which portions showed the most variation, and warped outline diagrams

(wireframes) were used to visualise the differences in the head shape. A second PCA was conducted on the *Acontias* individuals only (excluding *Typhlosaurus*).

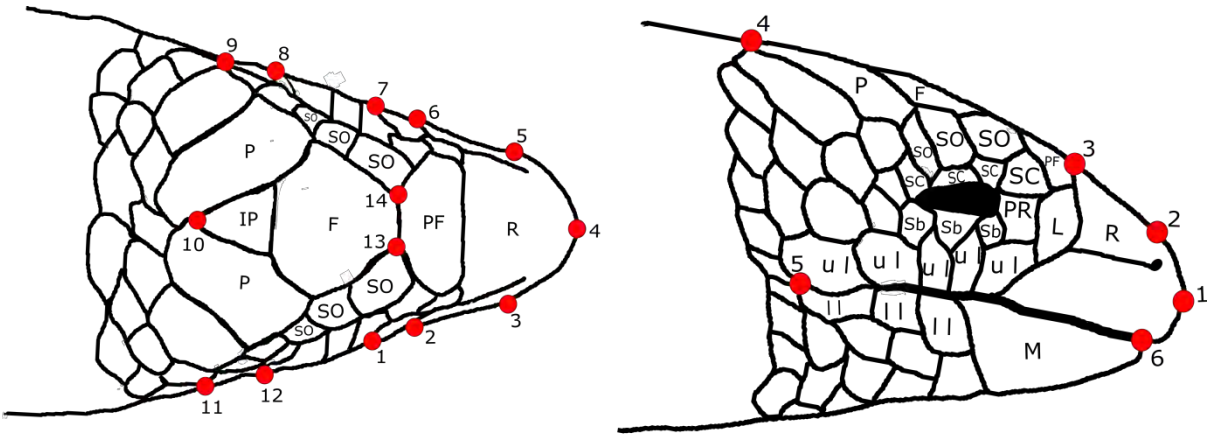


Figure 2.2: Line drawing of the placement of landmarks on photographs of dorsal (left) and lateral (right) aspects of the head for geometric morphometric analysis, where R = Rostral, M = Mental, PF = Prefrontal, F = Frontal, IP = Interparietal, P = Parietal, SO = Supraocular, SC = Supraciliary, Sb = Subocular, L = Loreal, PR = Preocular, UL = Upper labial and LL = Lower labial. Specimen used to generate diagram: *Acontias gracilicauda* (PEM R04141).

All phylogenetic analyses were conducted in R version 4.0.0 (R Core Team 2020) and R Studio version 1.3.959 (RStudio Team 2020). A phylogenetic tree produced from the methods mentioned previously was used in the phylogenetic signal analysis. To determine the phylogenetic signal and whether the traits are distributed as expected under Brownian Motion (BM) a phylogenetic signal test was conducted (function: “phylosig”, package: “Phytools”, Method: “K”). The method uses Blomberg‟s K and p-values to determine the amount of phylogenetic signal and how similar the results are compared to what is expected from BM. P-values based on 1000 randomisations.

Environmental variables and other influencing factors

Species distribution shapefiles were obtained from the IUCN Red List website (https://www.iucnredlist.org/) and updated where needed by incorporating new records from the PEM database using ArcMap v10.5.1 (ESRI: ArcGIS 2017; Fig. 2.3 and Fig 2.4). Clade distribution map was generated by merging the distribution shape files of the required species using the same software as for the species distribution. The general biome for each species was identified (only the nine major biomes found in southern Africa were used here as there are many subcategories within each biome, Appendix Table A1); biome shapefiles were obtained from the BGIS SANBI website (http://bgis.sanbi.org/Projects/Detail/208; Fig. 1.1). Species were categorised into biomes based on literature (Branch 1998; Alexander and

Marais 2007) and by overlaying the interpreted distribution of each species with the biome shapefiles and running intercept analyses in R. The biome that has the highest proportional values based on the intercept analysis was the biome selected for the species. The soil shapefile was downloaded from the Soil and Terrain Database for Southern Africa (SOTERSAF; https://files.isric.org/public/soter/SAF-SOTER.zip). The soil was categorised into five major topsoil groups (humic, organic, orthic, vertic, and melanic; Appendix Fig. A1 and Appendix Table A5). *Acontias* were categorised into soil types based on the same methods used for the biomes. A table indicating which biome and soil type each species is linked to can be found in the Appendices (Appendix Table A5).

Each species was placed *a priori* into phylogenetic clades based on the phylogenetic work from this study and previous studies (Lamb *et al*. 2010; Wagner *et al*. 2012; Busschau *et al*. 2017; Zhao *et al.* 2019; Appendix Table A6). Additionally, each species was categorised into a general "microhabitat" category based on information from literature (Branch 1998) (Appendix Table A6), namely: burrow, leaf-litter, in between roots or under stones (Appendix Table A7). This indicates where individuals of each species are usually found. For example, a species that has been classified in the "burrow" microhabitat means that individuals are mainly found in burrows in the soil while "leaf-litter" indicates that individuals are mostly found among the leaf-litter and not in the soil. Non South African species (e.g. *A. bicolor*) were excluded from the biome and soil as the shapefiles were restricted to South Africa only.

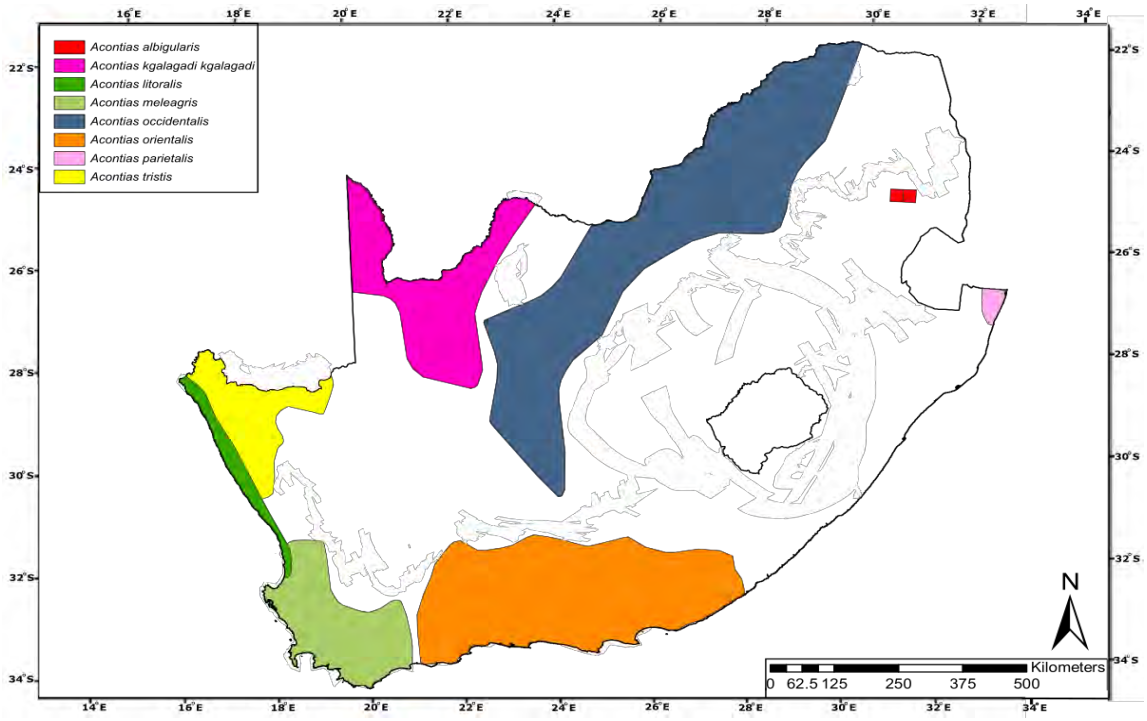


Figure 2.3: The distribution of eight (of the 19 species used in this study) *Acontias* species in South Africa. Two species that do not feature on this map are *A. schmitzi* (located in Zambia) and *A. bicolor* (located in Zimbabwe) because they are found too far outside of South Africa"s border.

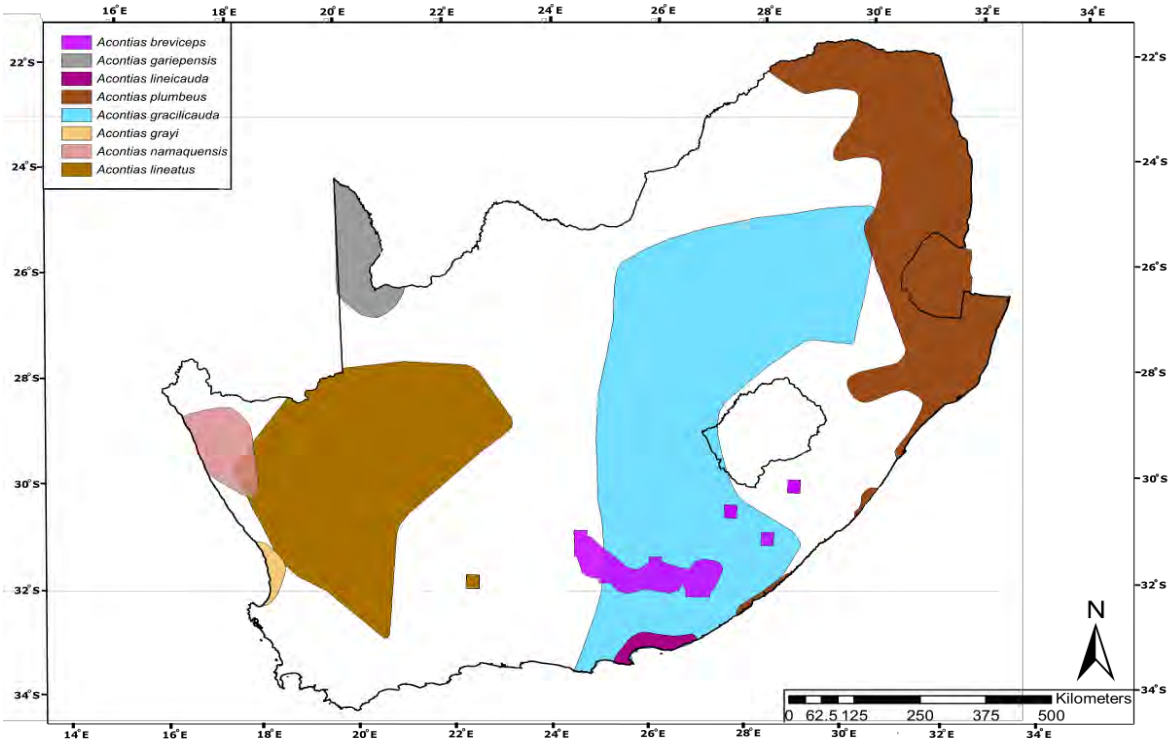


Figure 2.4: The distribution of eight (of the 19 species used in this study) *Acontias* species in South Africa. Two species that do not feature on this map are *A. schmitzi* (located in Zambia) and *A. bicolor* (located in Zimbabwe) because they are found too far outside of South Africa"s border.

Statistical Analyses

Statistical analyses were conducted in R version 4.0.0 (R Core Team 2020) and R Studio version 1.3.959 (RStudio Team 2020). An analysis of variance (ANOVA) was run for the Principal Component 1 (PC1) and Principal Component 2 (PC2) on both the dorsal and lateral view of the head for each category (soil, biome, clade, and microhabitat) to determine if there was any significant difference in head shape (package: „stats", functions: „aov", and „lm"). If significance was found, a Tukey's post-hoc test was conducted to determine which groups differed (package: „stats", function: „TukeyHSD").

To account for phylogenetic relationships, phylogenetic analysis of variance (phylANOVAs) was performed on the species means for the first two PC axes for the dorsal and lateral view of the head (packages: „ape" and „phytools", function: „phylANOVA"). This is to allow for evolutionary history to be taken into account and determine if there were any differences in morphometric data between the species and/or clades. Separate phylANOVAs were conducted for each category (soil, biome, clade, and microhabitat). The phylogeny for the genus produced in this study (see methods above) was imported into RStudio and plotted as a phylogenetic tree (package: „ape", function: „read.tree"). Simulation based phylANOVAs (Garland *et al.* 1993) were run with a "holm" correction, using 1000 simulations and branch lengths obtained from the genetic phylogeny (package: „phytools", function: „phyl.ANOVA", nsim: „1000", p.adj: „holm").

To visually depict how the phylogeny plots in the morphological space, for both dorsal and lateral view, a phylomorphospace plot was created (package: „phytools", function: „phylomorphospace"). To do this, an average of the PC1 and PC2 values were calculated for each species for each view and a phylogenetic tree with each species for each view was imported into R (see previous methods) to use in the function.

Results

Phylogenetic analyses

Six main phylogentic clades were identifeid in this study: Clade 1: *Acontias namaquensis*; Clade 2: *A. plumbeus*; Clade 3: *A. bicolor, A. parietalis* and *A. a. aurantiacus*; Clade 4: *A. gariepensis, A. schmitzi* and *A. a. kgalagadi*; Clade 5: *A. litoralis, A. tristis, A. grayi* and *A. lineatus*; Clade 6: *A. lineicauda, A. orientalis, A. meleagris, A. breviceps, A. occidentalis, A. albigularis* and *A. gracilicauda* (Fig. 2.5). Their distribution can be seen in Figure 2.6 (non South African species were not included in the distribution map). *Acontias namaquensis* has strong support (≥0.90) for it to be considered a separate clade (Fig. 2.5), although support

values to determine which species it is sister to is still uncertain (not well-supported; <0.90). *Acontias plumbeus* is considered a separate clade based on size (it is easily the largest species in the genus) and due to the strong support that it is sister to *A. bicolor, A. parietalis,* and *A. a. aurantiacus.* It is for this reason that the latter three species can be considered a clade on their own with strong support that *A. parietalis* is sister to *A. a. aurantiacus* and both are sister to *A. bicolor*. Clades 4 and 5 originate from a well-supported node with *A. schmitzi* and *A. k. kgalagadi* resolving as sister taxa, which are sister to *A. gariepensis* (well-supported). Within Clade 5, however, support for which species are more closely related to which is uncertain due to the low support value for *A. litoralis* being sister to *A. grayi* (0.61), thus forming a polytomy within the clade. The support is strong to indicate that all the speceis belong to the same clade. The species in Clade 6 all originate from the same node with stong support indicating that they do belong to the same monophyletic clade. Low support values within the clade (*A. occidentalis* and *A. albigularis* + *A. gracilicauda* = 0.73; Fig. 2.3) form polytomies and so the relationship of the species within Clade 6 is unclear with the exceptions of *A. orientalis* being sister to *A. meleagris* and *A. albigularis* being sister to *A. gracilicauda* (both are well supported). It is for these reasons that the chosen clades are seen as monophyletic and thus split accordingly. It must be noted, however, that low support was also found at the deeper nodes: split between Clades 2 + 3 and Clades 4 + 5 + 6 = 0.81, and Clade 6 and Clades 4 + 5 = 0.63 (Fig. 2.5). This indicates that the entire genus represented here is a polytomy made up of six monophyletic clades. Topology of the BI tree still reflects that of previous studies (Lamb *et al.* 2010; Busschau *et al.* 2017; Pietersen *et al.* 2018; Zhao *et al.* 2019) fairly well. The reason for having so many polytomies could be due to the lower number of individuals per species used for genetic analyisis and not including the full acontine ancestry.

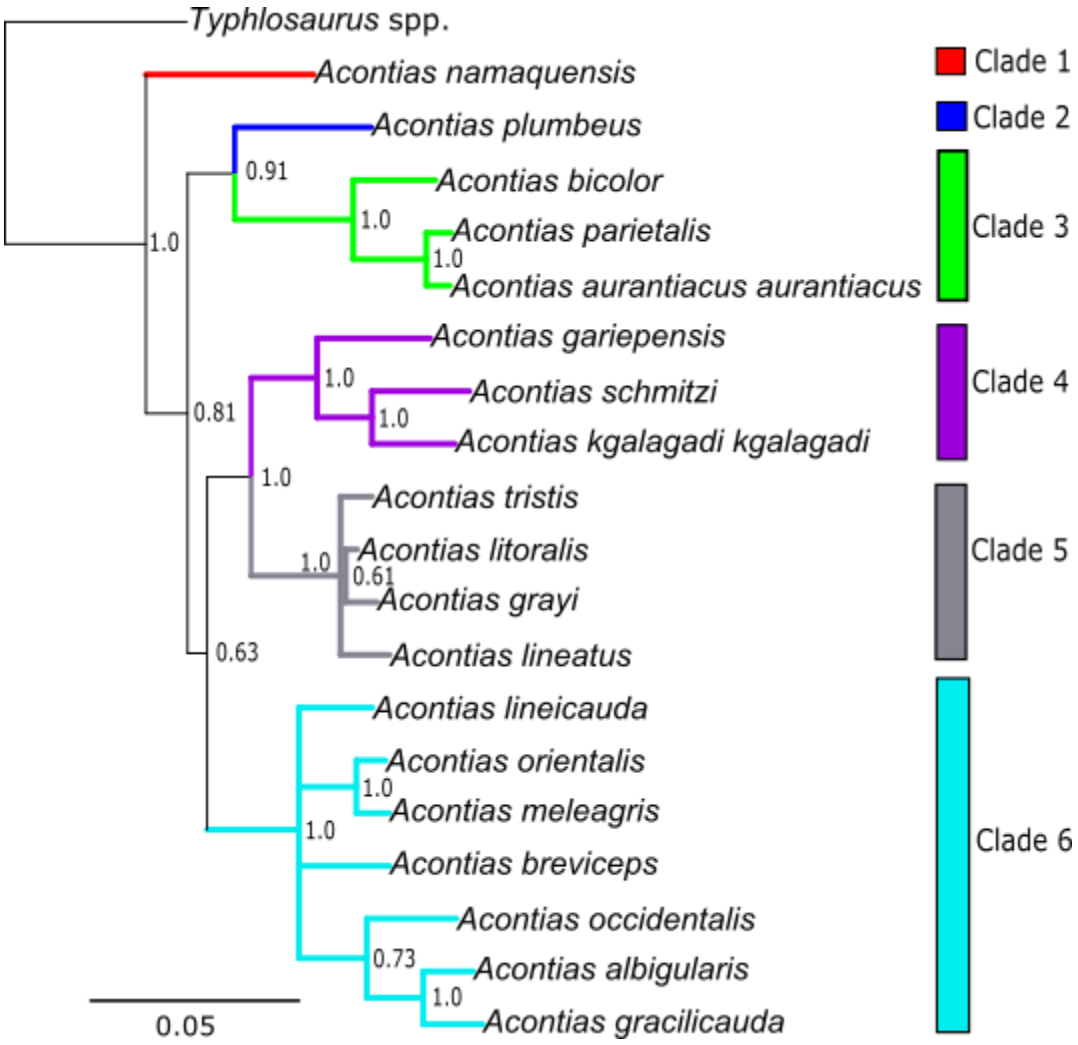


Figure 2.5: Simplified Acontinae Bayesian Inference phylogeny. The coloured branches correspond with the coloured blocks alongside indicating to which clade each species belongs. The numbers next to the nodes are the Bayesian posterior probability values. As the focus of this study is on *Acontias* and for visual ease, *Typhlosaurus caecus*, *T. lomaie* and *T. vermis* are labelled as *Typhlosaurus* spp. in this figure.

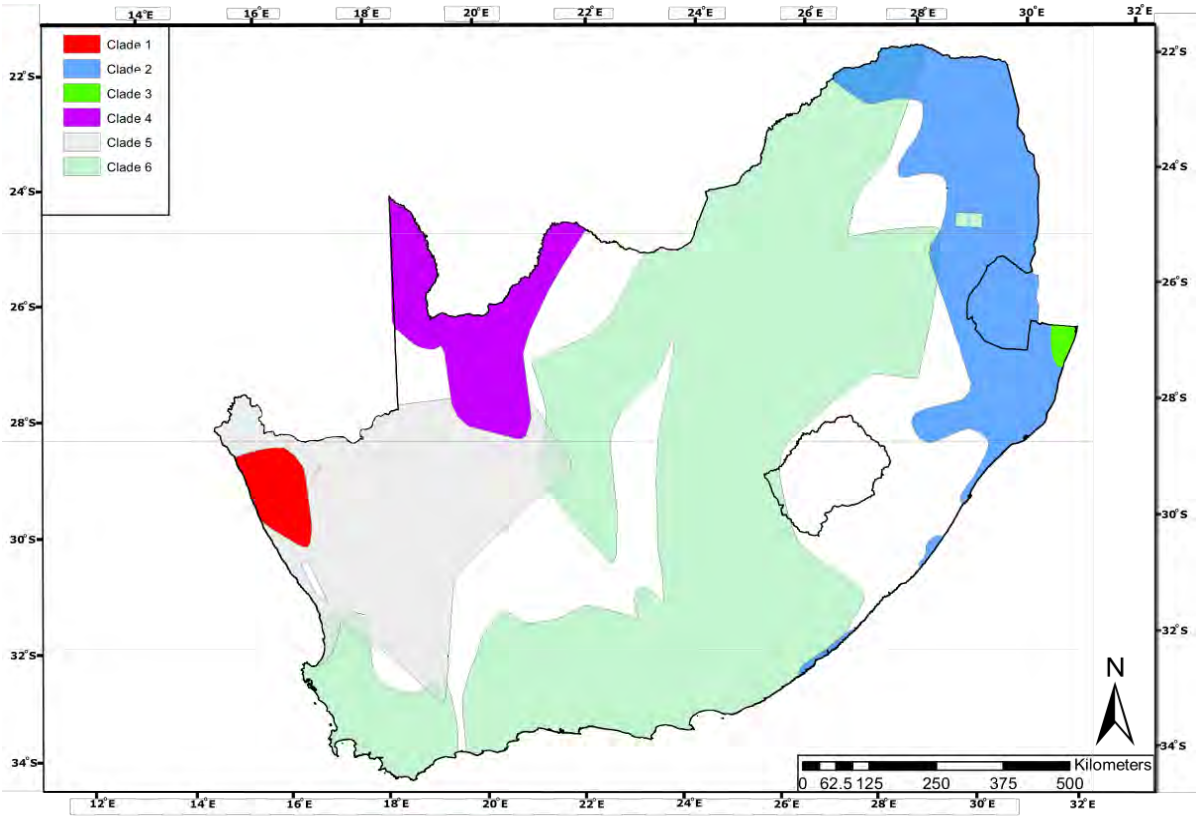


Figure 2.6: The distribution of the six phylogenetic *Acontias* clades from this study.

Geometric morphometric analyses

The PCAs that were conducted on the lateral views of *Acontias* and *Typhlosaurus,* and *Acontias* only, showed no clear separation between the genera (Appendix Figs A2 and A3). Principal component one (PC1) represented 61.34% of the variation and PC2 represented 25.51% of the variation. Variation in PC1 is mainly found in the „sharpness" and length of the rostral scale. Variation in PC2 is mainy found in the heat of the head and the length of the rostral scale. There was no significant difference in head shape between the two principal components (p-value > 0.05; Table 2.1).

Acontias and *Typhlosaurus* are clearly separated from each other in the dorsal view with the *Acontias* species forming distinct groups in the PCA (Fig. 2.7). The majority of the variation in the dataset was found in the PC1 axis (90.22%), which separated the *Acontias* and *Typhlosaurus* species. *Typhlosaurus* individuals have larger rostral scales (landmarks 2 to 6), narrower head and shorter back of the head (landmarks 8 to 12) (positive wire frame Fig. 2.7, bottom right). *Acontias* individuals, then, have smaller rostral scales (landmarks two to six), broader head widths, and longer posterior portions of the head (landmarks eight to 12) (negative wire frame Fig. 2.7, bottom left). The second largest proportion of the variation

(dorsal PC2, 9.78%) was found in the size of the rostral scale and the curvature of the head from the snout to the posterior region of the head (Fig. 2.7; dorsal PC2).

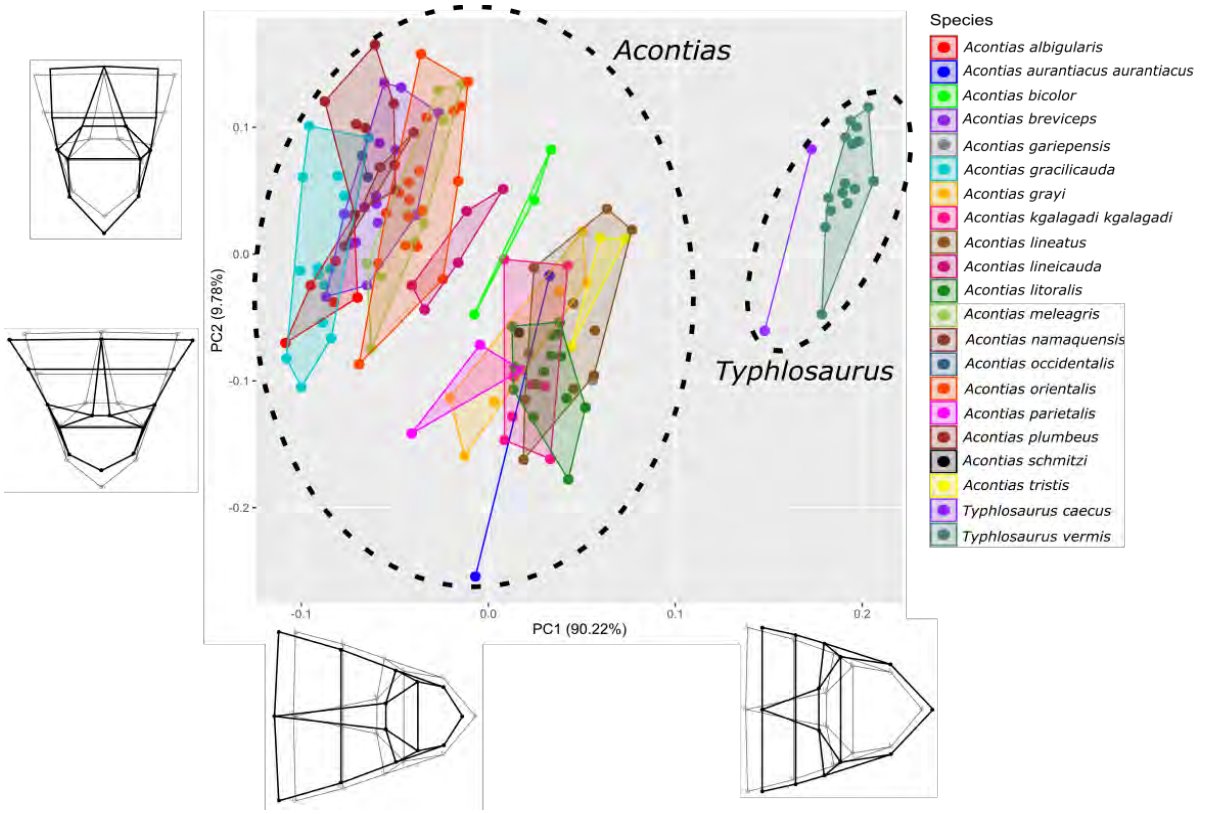


Figure 2.7: PCA plot of the first two principal components from the dorsal view of *Acontias* and *Typhlosaurus* species. Dots represent individuals and polygons represent the area the species fills in the morphospace. There is only one specimen representing *A. schmitzi* (black dot) and *A. gariepensis* (grey dot). Wireframe graphs (PC2 on the left and PC1 below) of representatives of the heads showing the deviation from the mean shape (shown in grey) on the positive and negative extremes (shown in black) of the dorsal PC1 and PC2 scores. Black dashed ovals represent main groupings.

When *Typhlosaurus* are removed from the analysis the remaining *Acontias* species are well grouped, forming two distinct clusters (Cluster A and Cluster B, Fig. 2.8). The majority of the variation was in PC1 making up 80.52% of the total variation while PC2 accounts for 19.48% of the variation. The majority of Cluster A fall on the negative PC1 and positive PC2 side of the plot (Fig. 2.8). This means that the majority of individuals in Cluster A exhibit a larger and more pointed rostral scale (landmarks 2 to 6), and a shorter (landmarks 8 to 12), more curved posterior region of the head (landmarks one, and seven to 12). The majority of the individuals in Cluster B fall on the positive PC1 and negative PC2 side of the plot. This indicates that most of the individuals in Cluster B exhibit a smaller and rounder rostral scale (landmarks 2 to 6), and a longer (landmarks 8 to 12), less curved posterior region of the head (landmarks 1, and 7 to 12). As PC1 accounts for around four times the variation of PC2, it is acceptable to state that PC1 has a greater influence on the head shape of *Acontias* species, i.e., the head shape will resemble that from PC1 more than

from PC2 in this case. The descriptions are a general trend in the head shape for the two clusters as there are individuals that do not fall into the aforementioned areas and some species have a wider spread in the morphospace.

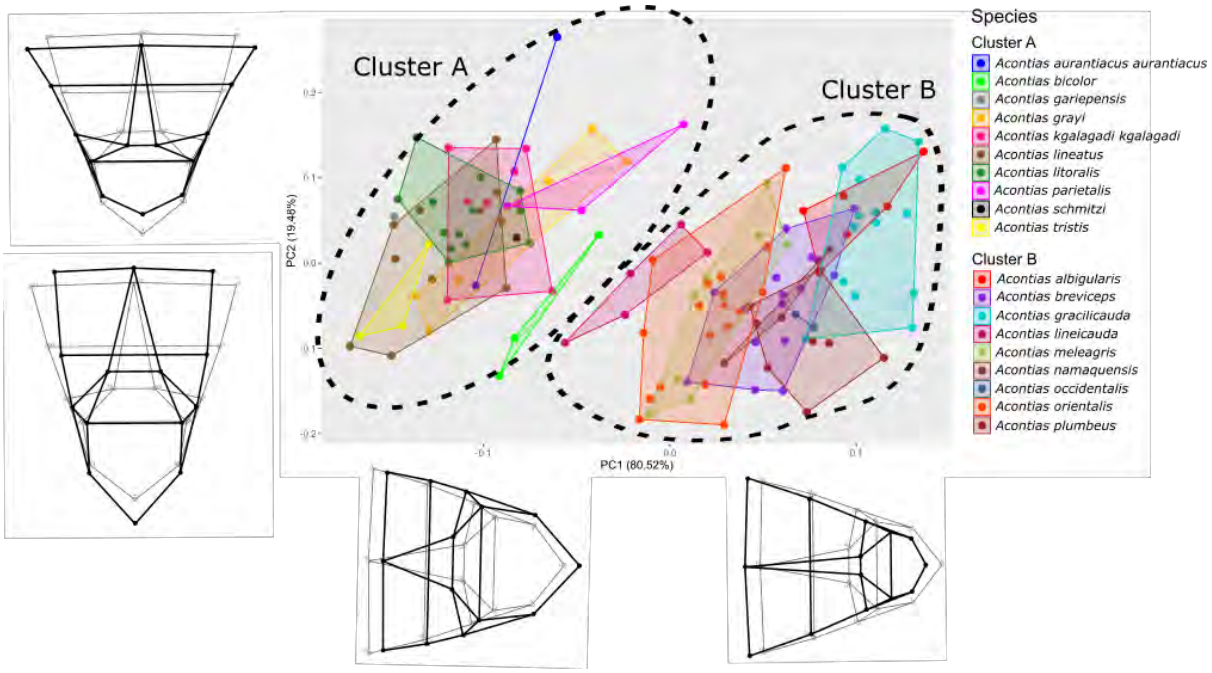


Figure 2.8: PCA plot of the first two principal components from the dorsal view of *Acontias* species only. Dots represent individuals and polygons represent the area the species fills in the morphospace. There is only one representative of *A. schmitzi* (black dot) and *A. gariepensis* (grey dot). Wireframe graphs (PC2 on the left and PC1 below) of representatives of the heads showing the deviation from the mean shape (shown in grey) on the positive and negative extremes (shown in black) of the dorsal PC1 and PC2 scores. Black dashed ovals represent the two main clusters observed.

Phylogenetic signal

For principal component one (PC1) on the dorsal side the trait (head shape) was a lot more similar than expected under BM and selection for particular shapes occurred (K = 2.87 and p-value = 0.001**). PC 2 dorsal view indicated more phylogenetic independence in the head shape and what one would expect from random trait occurrence (K = 0.13 and p-value = 0.84). The test on lateral view PC1 also indicated more phylogenetic independence (K = 0.27 and p-value = 0.16). The test on PC2 lateral view indicated close to phylogenetic independence with no significant difference (K = 0.12 and p-value = 0.86).

Link of morphometrics with ancestry

When species in the two clusters of *Acontias* are assigned to their representative phylogenetic clades a clear pattern starts to emerge (Fig. 2.9). The phylogenetic clades are grouped as follows: Cluster A comprises Clades 3 + 4 + 5 and Cluster B comprises Clades 1+ 2 + 6. Cluster A mostly falls on the negative PC1 and positive PC2 side of the plot, as indicated by the previous results. Individuals in Cluster B mainly fall on the positive PC1 and negative PC2 side of the plot. Clades may indicate along with the phylogenetic tree if species have similar head shapes because they are closely related. As the clades in each cluster are not sisters on the phylogenetic tree (Fig. 2.5), this indicates that similar head shape may not be due to ancestry, but rather due to convergent evolution of shape in response to another factor (possibly environmental in nature).

The “Phylogenetic Clade” categories were significantly different in dorsal PC1 (ANOVA: F-value = 72.5 df = 5, p-value < 0.0001****, Table 2.1). The post-hoc results indicate that the significant difference is found in 10 out of the 15 comparisons (Appendix Table A8). With ancestry taken into account there was still a significant difference between the clades (F-value = 68.7, p-value = 0.001; Table 2.1). When looking at PC2, there was significant difference in the dorsal view between the clades (F-value = 27.4, df = 5, p-value < 0.0001****; Table 2.1). Post-hoc results indicate significant differences in 10 of the 15 comparisons, with Clade 3 having a head shape significantly different from all other clades (Appendix Table A8).

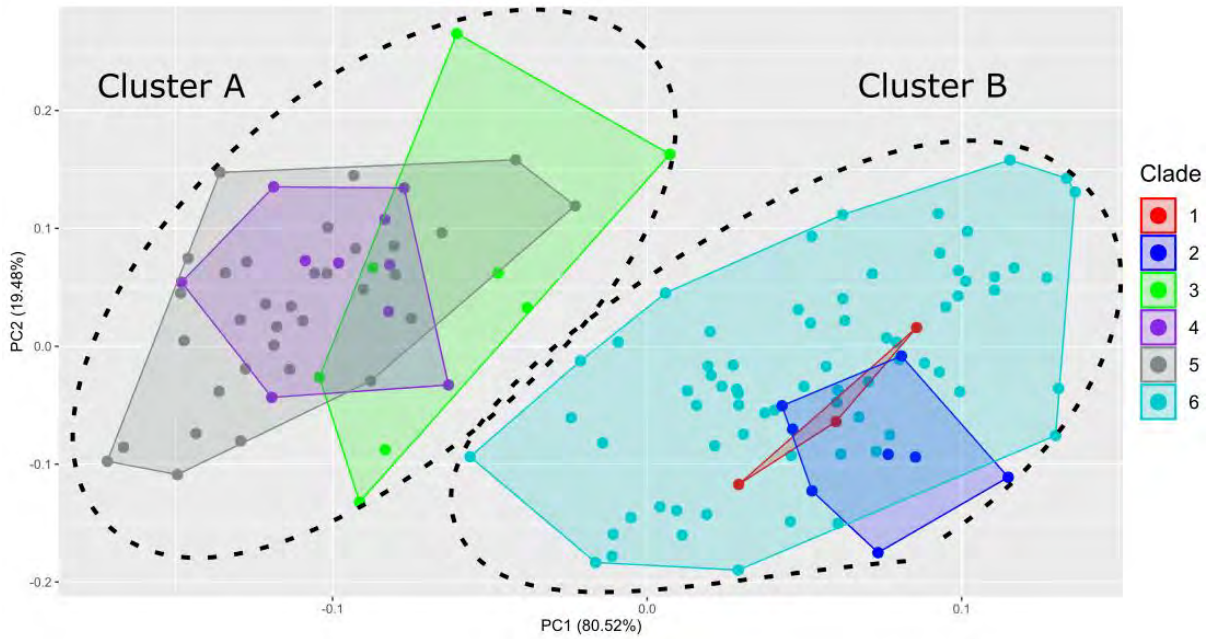


Figure 2.9: Plot of the first two principal components of dorsal view PCA, showing the *Acontias* phylogenetic clades. Clade colouration follows that of Figure 2.6 (Clade 1- *Acontias namaquensis*; Clade 2- *A. plumbeus*; Clade 3- *A. bicolor* and *A. a. aurantiacus*; Clade 4- *A. gariepensis, A. k. kgalagadi* and *A. schmitzi*; Clade 5- *A. grayi, A. lineatus, A. litoralis* and *A. tristis;* Clade 6- *A. albigularis, A. breviceps, A. gracilicauda, A. lineicauda, A. meleagris, A. occidentalis* and *A. orientalis*). Dashed black ovals represent the two main clusters identified based on overall head shape.

The lateral view indicated no separation between the phylogenetic clades (Appendix Fig. A4). There was no significant difference in lateral head shape between the clades in either principal component (all p-values > 0.05; Table 2.1).

Phylomorphospaces

The two *Typhlosaurus* species are clearly separate from the *Acontias* species in the plot. When considering the two *Acontias* clusters (A and B) from the previous figures, there is no grouping according the clusters in the phylomorphospace of the dorsal view (Fig. 2.10). That is, the species in Cluster A do not group closer to each other than to the species in Cluster B. They are mixed throughout the morphospace. As far as phylogenetic relationship goes *Acontias tristis* and *A. lineatus* plot closely together and *A. schmitzi* and *A. gariepensis* plot closely together. Also *A. breviceps* plots next to *A. schmitzi* and *A. gariepensis* despite not being closely related phylogenetically (Fig. 2.10). Most of the species that are closely related phylogenetically are not plotted closely together in the morphospace, *A. gariepensis, A. occidentalis* and *A. albigularis* are good examples.

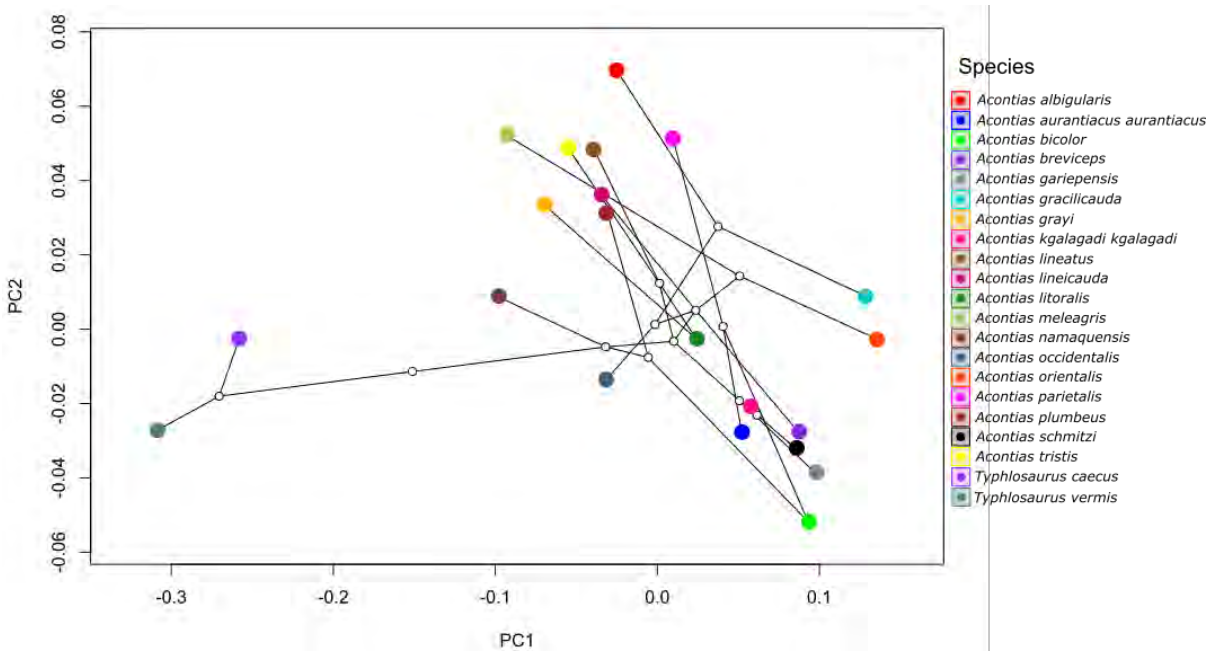


Figure 2.10: Phylomorphospace plot describing the evolution of dorsal head shape in *Acontias* and *Typhlosaurus*. Internal nodes are represented by hollow circles and species are represented by solid, colour dots.

There is no differentiation between *Acontias* and *Typhlosaurus* in lateral head shape. The species as a whole are more spread across the phylomorphospace in this lateral plot (Fig. 2.11) compared to the dorsal plot (Fig. 2.10). *Acontias albigularis, A. lineicauda,* and *T. caecus* are particularly noticeable as being separate along with one or two other species. Species that are phylogenetically closely related do not form tight groups based on their lateral head shape.

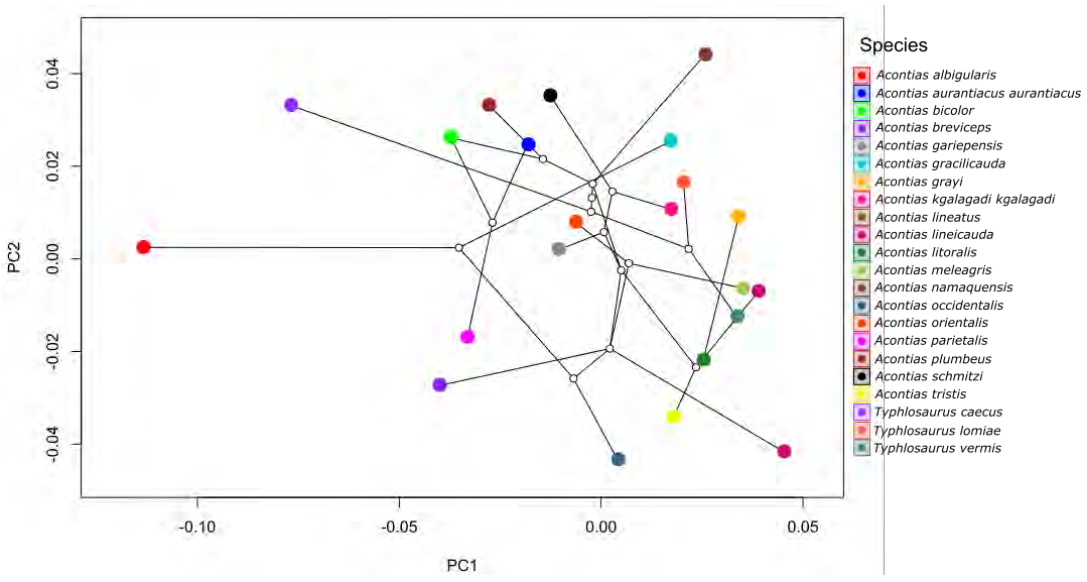


Figure 2.11: Phylomorphospace plot describing the evolution of lateral head shape in *Acontias* and *Typhlosaurus*. Internal nodes are represented by hollow circles and species are represented by solid, colour dots.

Soil types

Species were assigned and grouped according to the main soil types in which they occur (Fig. 2.12, Appendix Table A9). No species were found to be predominantly in the vertic soil category and so the vertic soil-type is excluded from the statistics. The organic soil comprised of *Acontias a. aurantiacus* (distribution is close enough to SA border that soil type can be implied) only and fell well within Cluster A indicating that they exhibit the larger and more pointed rostral scale (landmarks 2 to 6; Fig. 2.2), and a shorter (landmarks eight to 12; Fig. 2.2), more curved posterior region of the head (landmarks 1, and 7 to 12; Fig 2.2). Humic and orthic soil groups were split between the two clusters with the majority of species found in orthic soils in Cluster A and the majority found in humic soils in Cluster B thus exhibiting both types of head shape. Cluster B consists of all the individuals from the melanic soil group. The majority of individuals that fall into these two soil groups exhibit a smaller and rounder rostral scale (landmarks 2 to 6), and a longer (landmarks 8 to 12), less curved posterior region of the head (landmarks 1, and 7 to 12). It was expected to see the orthic soil type split between the clusters as it has the most species within it. As mentioned in the methods, soil was classified as orthic if it did not fall into any of the other four groups, thus leaving much of the country as orthic. What was not expected was that there is also a split between head shape in the humic soil category.

The head shape differed significantly (dorsal view) between the soil types in PC1 (ANOVA PC1: F-value = 12.5, df = 3; p-value < 0.0001****; Table 2.1). Differences were found between orthic-melanic, and humic-organic soils for PC1 (Appendix Table A10). With the inclusion of ancestry, there was still a significant difference in head shape between the soil types in PC1 (F-value = 13.4, p-value = 0.008**, Table 2.1). PC2 for the soil category of the dorsal view also showed significant differences in the head shape (ANOVA PC2: F-value = 13.4, df = 3, p-value < 0.0001****; Table 2.1). Ancestry indicated no significant difference in head shape between species found in different soils in PC2. The greatest differences occurred between melanic and all other soils for PC2 (Appendix Table A10). There was no significant difference in the lateral view in PC1 or PC2 for soil type (Table 2.1).

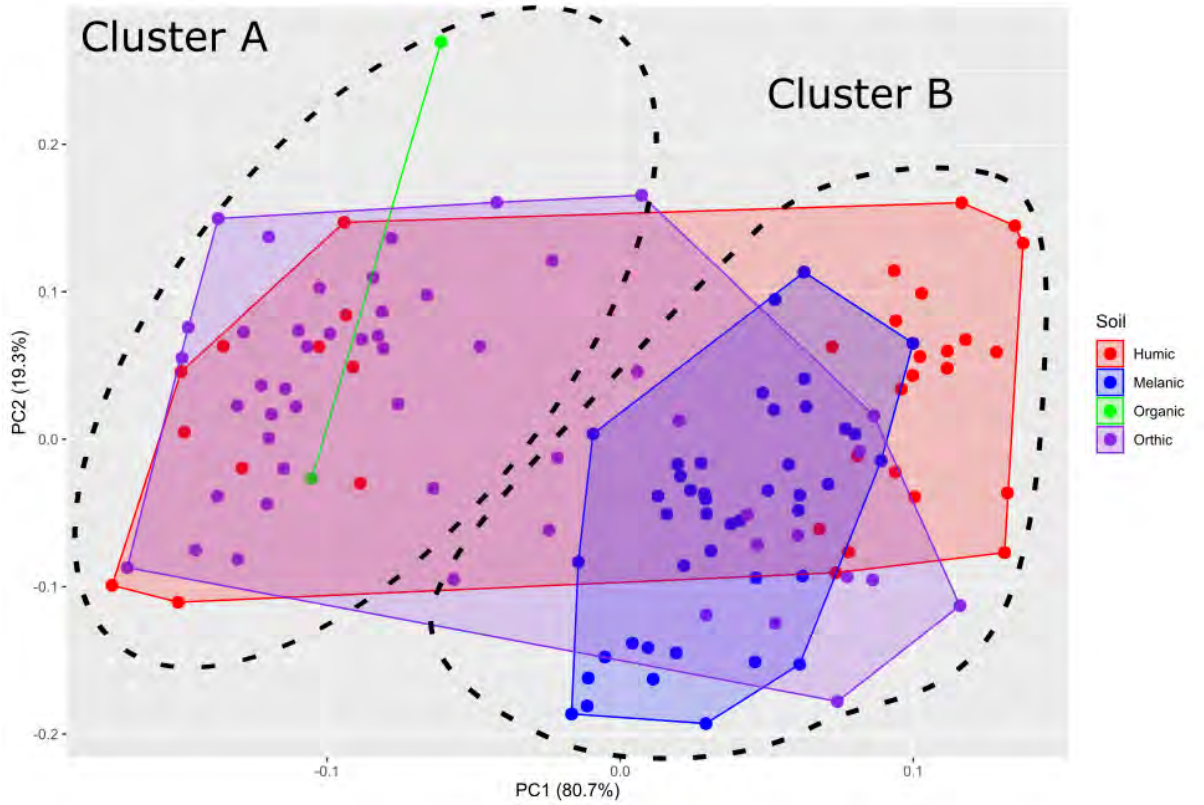


Figure 2.12: Plot of the first two principal components of dorsal view PCA, showing how *Acontias* individuals fall into the different soil categories. *Acontias* species found in each soil type are as follows: Humic: *A. albigularis, A. gracilicauda, A. lineatus* and *A. occidentalis*; Melanic: *A. breviceps, A. meleagris* and *A. orientalis*; Organic: *A. a. aurantiacus*; Orthic: *A. gariepensis, A. grayi, A. lineicauda, A. litoralis, A. k. kgalagadi, A. namaquensis*, *A. parietalis, A. plumbeus* and *A. tristis* (Appendix Table A6). Dashed black ovals represent the two main clusters identified based on overall head shape.

In the lateral view there was no distinction in head shape based on the soil type a species is found in (Appendix Fig. A5). Both the traditional and phylogenetic ANOVA results indicate that there is no significant difference in head shape in *Acontias* based on soil type (p-value > 0.05; Table 2.1).

Biomes

Species were assigned and grouped according to the main biomes they are associated with (Fig. 2.13). Each biome falls within one of the two clusters with the exceptions of the Fynbos and Succulent Karoo Biomes (Fig. 2.13). The Fynbos biome forms two distinct groups, one in each of the clusters while only three individuals from the Succulent Karoo Biome do not fall in Cluster A with the other individuals; these three individuals are all *A. namaquensis.* There is quite a lot of overlap between the head shapes of species found in the different biomes within each of the clusters. No biome is completely separated from the other biomes.

The ANOVA results showed that there was a significant difference in head shape (dorsal view) between biome types for PC1 (F-value = 33.3, df = 6, p-value < 0.0001****; Table 2.1). Post-hoc results indicate that 12 of the 21 comparisons indicate significant difference in head shape with the Grassland Biome being significantly different from all other biomes (Appendix Table A11). Dorsal view PC2 for biome types was also indicated significant difference in head shape (F-value = 7.1, df = 6, p-value < 0.0001****; Table 2.1). Post-hoc analysis revealed that significant differences in head shape was found in seven of the comparisons, with the Indian Ocean Coastal Belt representing four of them (Appendix Table A10). When accounting for ancestry, significant difference in head shape was found in species between the biomes for both principal components.

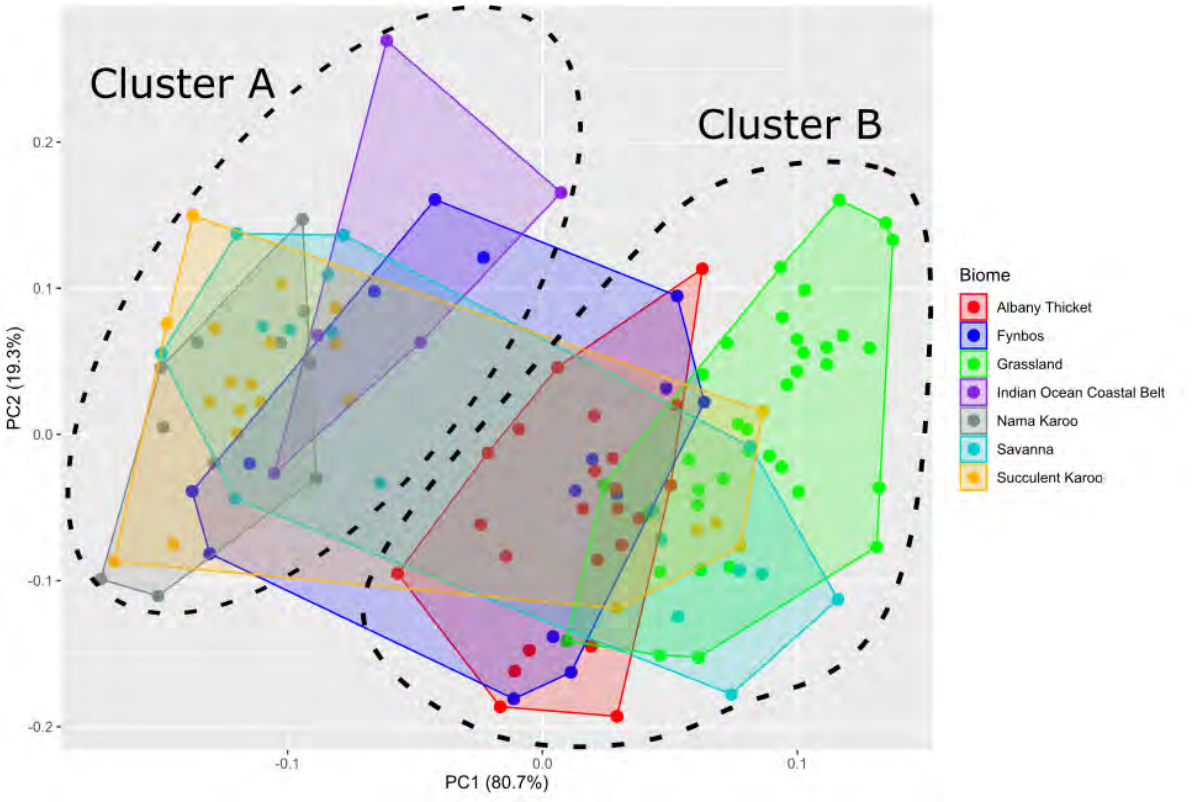


Figure 2.13: Plot of the first two principal components of dorsal view PCA, showing how *Acontias* individuals fall into the different biome categories. *Acontias* species found in each biome are as follows: Albany Thicket: *A. lineicauda, A. orientalis;* Fynbos: *A. grayi* and *A. meleagris;* Grassland: *A. albigularis, A. gracilicauda* and *A. breviceps;* Indian Ocean Coastal Belt: *A. a. aurantiacus* and *A. parietalis;* Nama Karoo: *A. lineatus;* Savanna: *A. gariepensis, A. k. kgalagadi,* and *A. plumbeus;* Succulent Karoo: *A. litoralis, A. namaquensis, A. occidentalis* and *A. tristis* (Appendix Table A6). Dashed black ovals represent the two main clusters identified based on overall head shape.

There was no separation in the lateral view PCA based on the biome in which the *Acontias* are found (Appendix Fig. A6). All of the biome polygons overlapped with each other. Both

traditional and phylogenetic ANOVA results indicated that there is no significant difference in head shape based on biome (all p-values > 0.05; Table 2.1).

Microhabitat

The “Microhabitat” category indicated a significant difference in head shape in dorsal PC1 (F-value = 5.6, df = 3, p-value = 0.001**; Table 2.1). The post-hoc analysis thus indicated significant differences between head shapes of individuals found in between roots and those found in the other microhabitats (Appendix Table A12). Taking ancestry into account, PC1 indicated a significant difference in head shape between “Microhabitat” categories in the dorsal view (p-value = 0.001**; Table 2.1). In the second principal component of the dorsal view, the “Microhabitat” category showed a significant difference in the ANOVA (F-value = 12.8, df = 3, p-value < 0.0001***, Table 2.1). Post-hoc analyses indicated that the significant difference in head shape is between individuals found in between roots and the other microhabitats, and between individuals found in leaf-litter and those that are found in burrows (Appendix Table A12). Phylogenetic ANOVA (F-value = 0.61, p-value = 0.007**; Table 2.1) indicated that there is a significant difference in head shape between “Microhabitat” categories.

The lateral view showed no significant difference between “Microhabitat” categories in PC1 for the ANOVA or phylogenetic ANOVA with both p-values > 0.05 (Table 2.1). In the second principal component of the lateral view, there was no significant difference found between the “Microhabitat” categories in the ANOVA results or phylogenetic ANOVA (Table 2.1).

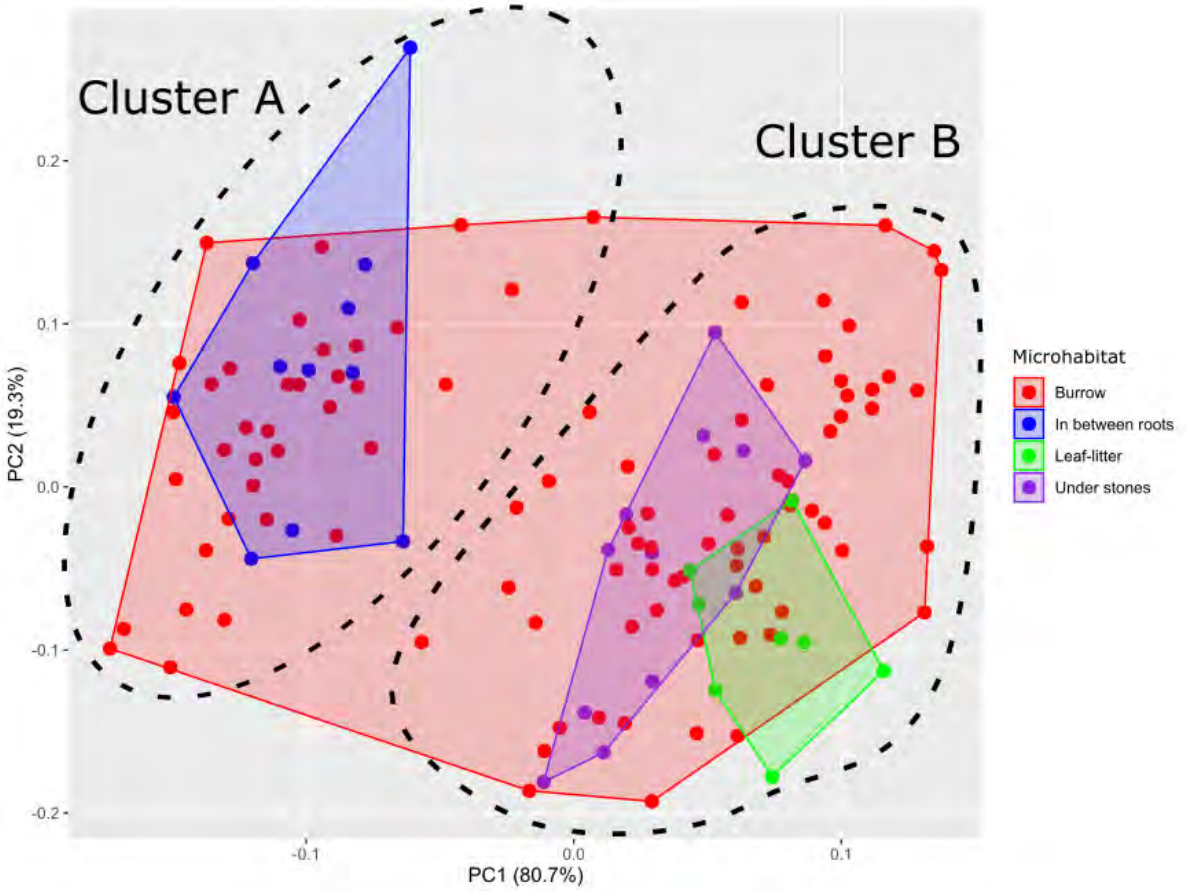


Figure 2.14: Plot of the first two principal components of dorsal view PCA, showing how *Acontias* individuals fall into the different microhabitat categories. *Acontias* species found in each microhabitat are as follows: Burrow: *A. albigularis*, *A. breviceps*, *A. gracilicauda*, *A. grayi*, *A. lineatus*, *A. lineicauda*, *A. litoralis*, *A. occidentalis*, *A. orientalis* and *A. tristis;* In between roots: *A. a. aurantiacus*, *A. gariepensis* and *A. k. kgalagadi*; Leaf-litter: *A. plumbeus*; Under stones: *A. meleagris* and *A. namaquensis*.

Table 2.1: Results of the traditional ANOVA and phylogenetic ANOVA for both principal components using soil, biome, clade, and microhabitat groupings of *Acontias*. The traditional p-value is that obtained from a standard ANOVA without the inclusion of ancestry. The phylogenetic p-value is that obtained by conducting a phylogenetic ANOVA where ancestry is taken into account. Values that represent significance are indicated in bold as well as with an asterisk for p-values less than or equal to 0.05 (*) or double asterisk (**) for significance p-value ≤ 0.01 or triple asterisk (***) for significance p-value ≤ 0.001 or quadruple asterisk (****) for p-value ≤ 0.0001.

PC #	Category	Traditional F-value	df	Traditional p-value	Phylogenetic p-value
PC1 Dorsal	**Soil**	12.5	3	**< 0.0001****	**0.008****
	Biome	33.3	6	**< 0.0001****	**0.001*****
	Clade	72.5	5	**< 0.0001****	**0.001****
	Microhabitat	5.6	3	**0.001****	**0.001****
PC2 Dorsal	**Soil**	13.4	3	**< 0.0001****	0.1
	Biome	7.1	6	**< 0.0001****	**0.01****
	Clade	27.4	5	**< 0.0001****	0.50
	Microhabitat	12.8	3	**< 0.0001****	**0.007****
PC1 Lateral	**Soil**	1.9	3	0.14	0.90
	Biome	0.9	6	0.50	0.59
	Clade	1.5	5	0.21	0.84
	Microhabitat	0.3	3	0.85	0.60
PC2 Lateral	**Soil**	0.66	3	0.58	0.50
	Biome	0.6	6	0.76	0.17
	Clade	0.8	5	0.55	0.14
	Microhabitat	0.7	3	0.55	0.17

Discussion

Overall head shape

Typhlosaurus and *Acontias* were clearly separated from each other based on dorsal head shape. *Typhlosaurus* have large rostral scales and a shorter posterior head region. The chosen landmarks were useful in delineating genera and the dorsal side of the head can be used, along with various scale sizes, to distinguish the two genera from one another. In the lateral view, however, *Acontias* and *Typhlosaurus* cannot be distinguished from each other.

The dorsal view without *Typhlosaurus* allowed for a better visualisation of the head shape differences among *Acontias*. Two clear clusters were formed (Cluster A and Cluster B). The species in Cluster A exhibit a large, pointed rostral scale, and a shorter more curved posterior region of the head. There are a few individuals in this cluster that do not conform exactly to this head shape in that they have a less curved and narrower posterior head region. These individuals fall within negative PC1 and PC2 on the PCA. No species falls completely in that section of the PCA and the individuals that do are the minority. The larger and more pointed rostral scale of Cluster A most likely indicates that the individuals dig through the soil by swinging their heads side to side thus „excavating" and moving the soil out from in front of them.

There is also considerable variation in the shape and arrangement of rostral elements and the nasofrontal region of the cranium in fossorial species (Wu *et al.* 2015; Cyriac and Kodandaramaiah 2020). This was seen in our study with the larger rostral scales and shorter posterior head region in *Typhlosaurus* and Cluster A, with Cluster B exhibiting a smaller rostral scale and longer posterior head region. The angle of the rostrum was not looked at in this study as the lateral PCA showed no grouping between species but it might be worth looking into for future studies.

The three distinct groups on the dorsal PCA indicate that *Acontias* and *Typhlosaurus* group very closely according to their body size (and thus head size) and environment, with mainly large-bodied *Acontias* forming a group and mainly small-bodied *Acontias* forming another group. This links to the idea that narrower/smaller bodied fossorial animals require less effort to burrow (produce higher push forces relative to their size) and thus tend to burrow deeper into the sandier soils, than thicker/larger bodied species in the more compact soils (Navas *et al.* 2004; Wu *et al.* 2015; Le Guilloux *et al.* 2020).The type of movement through soil can vary depending on the soil and/or the species (Herrel and Measey 2010). Larger bodied legless skink species (e.g. *A. plumbeus* and *A. meleagris*) with longer, taller and broader heads produced higher push forces than the smaller bodied species (e.g. *A. lineatus* and *A. litoralis*) with narrower heads in absolute terms, but once body mass was

accounted for it was no longer the case with regards to relative force (Le Guilloux *et al.* 2020).

Phylogenetic relationship

The species that comprise Cluster A are as follows: *A. a. aurantiacus, A. bicolor,* and *A. parietalis* (Clade 3)*, A. gariepensis, A. k. kgalagadi,* and *A. schmitzi* (Clade 4)*, A. grayi, A. lineatus, A. litoralis,* and *A. tristis* (Clade 5)*.* Clade 3 is sister to Clade 2, which does not fall within Cluster A. *Acontias parietalis* is most closely related to *A. a. aurantiacus.* Clade 4 is sister to Clade 5 and both fall within Cluster A. Clade 4 shows *A. schmitzi* most closely related to *A. k. kgalagadi* with them then sister to *A. gariepensis* (keep in mind that *A. schmitzi* and *A. gariepensis* are only represented by a single individual each). Clade 5 consists of a polytomy and thus it is uncertain which species are most closely related to each other in the clade. It is unclear where *A. namaquensis* groups in the phylogeny due to a polytomy at the basal node of the genus. *Acontias plumbeus* is also the only member in its Clade and is sister to Clade 3; however, it does not group with the taxa in this Clade when it comes to head shape, *A. plumbeus* exhibits the head shape of Cluster B while its closest relatives exhibit that of Cluster A. Clade 6 makes up the majority of Cluster B and so differences found within this clade may be more informative. The basal node of this cluster is also a polytomy with the only certain relationships being that *A. orientalis* and *A. meleagris* are sister taxa and that *A. albigularis* and *A. gracilicauda* are sister taxa.

What is clear is that the clades split into two distinct groups based on head shape. The groups do not strictly follow the phylogenetic clade grouping as the three clades that are most closely related are not the three grouped into the same cluster. This could mean that there are perhaps convergent evolutionary forces driving head shape. Perhaps looking at the environmental variables will give a better indication. The phylogenetic clades will be worth further investigating as significant difference was found in both the traditional and phylogenetic ANOVAs in the PC1 dorsal view.

Phylogenetic signal

Principal component one of the dorsal view indicated phylogenetic independence with a low Blomberg"s K-value and the significant p-value indicated that the resulting head shape is likely due to selection and not what one would expect from random. This could mean that the head shape could be selected for due to the type of soil, biome, or microhabitat the species are found in and not retained from ancestry. Phylogenetic signal for PC2 was high with K-value close to zero and a non-significant p-value. This means that changes in head shape in

the second principal component in the dorsal view is not from selection and rather due to phylogenetic links.

The first principal component in the lateral view has a fairly strong phylogenetic signal with K-value closer to zero than one and a non-significant p-value. This indicates that the head shape is what one would expect from Brownian motion evolution. The same could be said for PC2 of the lateral view with a low K-value and non-significant p-value.

Phylomorphospace

Acontias and *Typhlosaurus* are clearly separated in the dorsal phylomorphospace plot indicating that their head shape was retained from the respective ancestors. Within the *Acontias* genus there are very few closely related species that occur in a similar morphospace. This leads to the thought that the evolution of *Acontias* head shape is not from retaining the ancestral head shape but due to another factor. Such a factor could be environmental such as soil type, biome type, or microhabitat.

The lateral phylomorphospace does not have *Acontias* and *Typhlosaurus* separated on the plot. This could mean that the evolution of the lateral head shape in these genera is not retained from their respective ancestor but rather due to another factor. This is further supported in that within the *Acontias* species there are not many phylogenetically closely related species with similar lateral head shapes. They are spread out on the plot.

Biomes

Acontias parietalis and *A. a. aurantiacus* (distribution is close enough to SA border that the biome type can be implied) are found within the Indian Ocean Coastal Belt Biome (IOCB). *Acontias gariepensis* and *A. k. kgalagadi* are found in the same biome (savanna) and exhibit similar head shapes. *Acontias tristis* and *A. litoralis* are both found in the Succulent Karoo Biome and their head shapes are fairly similar, grouping quite closely in the PCA. *Acontias namaquensis* also occurs in the Succulent Karoo Biome but the head shape differs greatly from the other two species sharing the biome. *Acontias grayi* is the only species within Cluster A and *A. meleagris* is the only species from Cluster B to occur within the Fynbos Biome. This split causes the Fynbos biome to spread across much of the PCA when in fact there are two clear groupings within the biome in each cluster. There may be other factors influencing the head shape of these species since biome type does not seem to have as great of an influence as expected. A possible reason for the Fynbos biome forming two clusters is that this biome varies in the type of vegetation cover and density. Cluster A contains mostly biomes with little vegetation at lower densities while Cluster B mainly

contains biomes with more vegetation at higher densities. This aspect can be looked at more closely in future research. *Acontias lineatus* is the only species to be found in the Nama-Karoo Biome but the head shapes are variable within the species. This variation is mainly in PC2 but might not be as evident, due to variation in PC1 when looking at the specimens with the naked eye, as PC1 accounts for the majority of the variance in head shape.

Soil

In this study, soil type is used as a proxy for microhabitat in that *Acontias* are fossorial and therefore spend the majority of their lives in the soil. *Acontias meleagris* and *A. grayi* occur in different soil types even though they are in the same biome; this could be part of the reason that their head shapes are so different. It could be that soil is one of the driving factors in their head shape evolution. *Acontias lineatus* is the only species from Cluster A that is found in humic soil, but as mentioned previously, shares a biome with other species from Cluster A, so soil is more than likely not the driver in this case (Janse van Vuuren 2009). Head shape in the different soil types was found to be significantly different in both the traditional and phylogenetic ANOVAs for PC1 and in the traditional ANOVA for PC2. Soil should then be investigated further as a driving factor in *Acontias* head shape as it seems to show potential, especially since the phylogenetic signal in dorsal PC1 indicated phylogenetic independence. It is also known that *Typhlosaurus* inhabit the much sandier regions of the country along the West Coast compared to most *Acontias. Acontias* in Cluster A, fall closest to *Typhlosaurus* in morphospace and could indicate that they are found in soil conditions similar to that which *Typhlosaurus* are found in.

Microhabitat

The difference in head shape between *Acontias* species found in different microhabitats was only significant in the dorsal side of the head. Traditional ANOVAs indicated a significant difference between the head shapes. The individuals found in between roots were found to have head shapes most different from all other microhabitat groups. A significant difference was found in the phylogenetic ANOVA as well. It will be worth investigating microhabitat more closely and perhaps refine the categories used as well, to improve results. There was no significant difference in head shape with regard to microhabitat for the lateral view.

Conclusion

The results from the traditional ANOVAs indicate that for PC1 there was a significant difference in head shape for all the categories. This indicates that environmental factors such as soil, biome and microhabitat types can play a role in determining the head shape of *Acontias*. This could be expected as *Acontias* use their heads to burrow and that is the structure that is interacting with the environmental factors. For the phylogenetic p-values for PC1 all categories indicated significant differences in head shape within them. PC1 for the dorsal head shape showed to be phylogenetically independent which further supports the idea that dorsal head shape evolution is linked to environmental factors. The “Microhabitat” category along with the soil-type tries to give a better idea of the microhabitat in which the species are located, by stating where they are mostly found. This gives an idea of how the different species use the different environments, which can provide some insight into their head shape. There was significant difference in head shape between the microhabitat categories thus indicating that the microhabitat has an influence on the evolution of *Acontias* head shape. In the case of PC2 all of the categories were significant for traditional p-value, while the phylogenetic p-value showed significant difference in two of the categories. This indicates that the head shape in PC2 is most likely due to ancestry and not environmental factors. This is supported with PC2 having high phylogenetic signal in dorsal head shape.

Pitfalls and improvements

This study grouped the species used into broad categories, especially in terms of the biome and soil categories. Only the main biome and soil category in which the species are found were used as their grouping, when in fact some are found in more than one soil or biome category. The soil and biome categories in themselves are broad groupings, and as mentioned previously, many soil types and subcategories within each biome occur. Even with this in mind, the head shape of *Acontias* does seem to be influenced by environmental factors such as soil, biome and microhabitat, as two distinct groups were formed, however, looking at it at a finer scale may be even more insightful.

The lateral head view results indicated no significant difference in head shape for either principal component. The reason for this could be because many of the landmarks used in the lateral analyses were not based on definite positions, such as where scales meet, and had more arbitrary positions, such as midway along the rostral (landmark 2), which increases the chance of variation due to inconsistent placement. Landmarks one and six are also included in the arbitrary landmarks, raising the total to three, thus, half of the landmarks used to describe the lateral shape of the head could be inconsistent. The majority of the landmarks used in the dorsal view could be placed with greater consistency and thus

could be the reason for finding significant difference in head shape. If the lateral landmarks were placed with greater consistency or describe the shape in more detail, there may be significant differences in lateral head shape. To improve on the lateral analyses the use of sliding semi-landmarks with main landmarks should be used when defining the shape of the snout/rostrum. This will provide a better description of the shape of the snout as the landmarks in this study alone may not be sufficient.

Future research

Future research can include a more in-depth analysis at a finer scale to see whether similar results are obtained. It is worth noting to increase the sample size in the species that were lacking individuals, include more *Acontias* species, as well as to try and incorporate the full *Acontias* genus would be suitable for further research. In addition to this, it will be worthwhile using the same individuals for all analyses as far as possible. This study did manage to do that for almost all aspects. This will allow for links to be made more easily as well as to improve visualisation of the data. The diet of the different *Acontias* was not taken into account for this study and it has been shown that diet can affect the head shape of lizards (Herrel *et al.* 1999; Herrel *et al.* 2008). It might be worth looking into for further research even if the diet of the species is fairly similar.

This study provided a good stepping stone to build on for future research, as mentioned above. Furthermore, incorporating three-dimensional analyses of the skull could give a better idea of what shape changes are occurring in the bones and not just the scales on the surface. Comparing the results from the two analyses will be very interesting to see. The reason 3D geometric morphometric analyses were not used in this study, even though the skulls were included in the CT-scans (next chapter), is because the resolution of the skull in the scan was not high enough to provide the necessary detail we needed for the analyses. If the CT-scans were conducted only on the skulls of the *Acontias* and *Typhlosaurus* and not the entire specimen the resolution would have been of a better quality.

Chapter 3: Put your back into it: using vertebral counts as a possible species delineation method for *Acontias*

Image showing *Acontias orientalis* moving above ground (top; photographer: Chad Keates) and a CT-scan of how the vertebrae were counted (bottom); each white dot represents ten vertebrae counted.

Chapter 3

Put your back into it: using vertebral counts as a possible species delineation method for *Acontias*

Introduction

Fossorial squamates share many morphological characteristics, such as reduced eyes and limbs, and elongation of the body. This elongation can result from trunk (body) elongation, tail elongation or both and it can be due to increasing the number or length of the vertebrae in that region. This can be achieved because the high degree of stabilising selection on vertebral number is more relaxed in lizards and snakes than in other taxa, such as mammals (Greer 1991; Bergmann and Irschick 2012). Some snake species possess over 300 precaudal vertebrae (Head and Polly 2007; Muller *et al.* 2010) and some smaller species (in length) can still possess close to 150 total vertebrae (Weinell *et al.* 2020). In salamanders, body elongation is achieved through either addition or lengthening of the vertebrae in the trunk and tail (Wiens *et al.* 2006; Brandley *et al.* 2008; Bergmann and Morinaga 2019; Bergmann *et al.* 2020). Body elongation in lizards and snakes takes place through the addition of vertebrae in the body or both the body and tail (Wiens *et al.* 2006; Brandley *et al.* 2008; Bergmann and Irschick 2012; Bergmann and Morinaga 2019; Bergmann *et al.* 2020).

Somatic growth and *Hox* gene expression are primary factors driving the formation of the vertebrate body axis (Richardson *et al.* 1998; Muller *et al.* 2010). The vertebrae are derived from the embryonic somites through resegmentation indicating that any addition to the vertebral number occurs in the developmental stages. The number of somites formed during embryogenesis determines the number of vertebrae an organism will have (with the exception of frogs that reabsorb most of the tail somites during metamorphosis) (Handrigan and Wassersug 2007; Woltering 2012). The size of vertebrae relative to the number of vertebrae can provide information about the rate of somatic growth. As mentioned, body elongation can occur in either the trunk or tail region and the region that is affected depends on which *Hox* genes are expressed (Burke *et al.* 1995; Muller *et al.* 2010). It was also found that, in general, terrestrial species of snakes and lizards went through tail elongation, while fossorial species went through trunk elongation, i.e. terrestrial species have longer tails than fossorial species (Polly *et al.* 2001; Brandley *et al.* 2008).

The evolution of the elongate body form is also related to various ecological variables such as soil texture, substrate coverage, and plant primary productivity (Grizante *et al.* 2012). In gymnopthalmid lizards, for example, species that inhabit looser soil are more elongate than those found in more compact soil (Barros *et al.* 2011; Bergmann *et al.* 2020).

The genus *Chalcides* exhibit higher numbers of presacral vertebrae and are associated with sandier soil zones along the coast of Italy (Caputo *et al.* 1995). Other studies on functional morphology suggest that elongate, limbless bodies enhance burrowing performance during subterranean locomotion (Lee 1998).

Vertebral counts have been shown to be a successful delineation method in many species, including the snake-eels (Böhlke 1997; McCosker 2010). A study conducted by Watanabe *et al.* (2011) used total vertebral number of three species of tropical eels to determine differences in their population structure. It was found that *Anguilla megastoma* could be split into an eastern and western population (Watanabe *et al.* 2011). Vertebral counts of species were not only used for delineation, but they were also used to determine manoeuvrability in studies conducted on snakes (Kelley *et al.* 1997) and fossorial lizards (Gans and Fusari 1994). Papenfuss and Parham (2013) used vertebral numbers as part of their species delineation method when describing four new species of legless lizards (*Anniella*) in California. *Anniella pulchra* originally thought to be a single lineage was discovered to have a total of five genetic lineages within it (Papenfuss and Parham 2013). Three of these lineages can be differentiated from *Anniella pulchra* through a combination of colouration, scalation, and skeletal characters (number of trunk vertebrae), while the fourth is differentiated by its karyotype (Papenfuss and Parham 2013). The species in that study could not be differentiated by vertebral counts alone but the counts did greatly aid in the delineation process. Some of these studies used x-ray or computed tomography to count the vertebrae as opposed to dissecting the specimen and then doing the count.

The conserved body shape of *Acontias* makes it difficult to easily tell species apart, especially when none have any easily identifiable external features at a quick glance. The use of colouration alone is not viable, as differences in colouration could be due to environmental plasticity (Rosenblum 2006; Busschau *et al.* 2017). More often than not, further information is needed on the specimen in order to determine the species identification, such as locality, number of ventral scales, head scalation patterns, etc. Traditional identifying features in lizards include snout-vent length, leg development, diet, reproductive mode, scale counts and locality data (Meiri 2018). Working with features such as scale counts can be tedious as many *Acontias* are not very large, and have small scales, making identifying and counting the scales difficult. This makes delineating species within this genus rather difficult (Daniels *et al.* 2006; Daniels *et al.* 2009; Engelbrecht *et al.* 2013). A number of cryptic species have been discovered within this subfamily in recent years with the aid of DNA sequencing techniques (Daniels *et al.* 2009; Lamb *et al.* 2010; Wagner *et al.* 2012; Busschau *et al.* 2017; Conradie *et al.* 2018; Pietersen *et al.* 2018; Zhao *et al.* 2019) thus supporting the idea that morphological identification might not be enough in this case.

This is not to say that morphological studies should be ignored completely but rather used in combination with genomic data. Vertebral counts in *Acontias* as a means of species delineation was briefly looked into by Wagner *et al.* (2012) and Conradie *et al.* (2018). Lamb *et al.* (2010) used caudal vertebrae number as a means to differentiate between the *Acontias* and *Typhlosaurus* genera. Wagner *et al.* (2012) used vertebral number to determine whether an undescribed Zambian fossorial skink species belonged in *Acontias* or *Typhlosaurus* genus. They found that it belonged in *Acontias* and it was described as a new species, e.g. *Acontias schmitzi.* This information means that the use of vertebral number can be used to describe new species and should be further investigated. Conradie *et al.* (2018) used vertebral counts as a descriptive statistic to aid in the description of two new *Acontias* species (*A. albigularis* and *A. wakkerstroomensis).* Conradie *et al* (2018) also found that *A. meleagris* had a higher trunk vertebral count than both *A. breviceps* and *A. gracilicauda.* This leads to the thought that there could be other *Acontias* species or groups that can be differentiated on vertebral number. The same authors also noted that *A. meleagris* is usually associated with well-drained sandier soils along the coast (Branch 1998) and has a higher vertebral count than other the other species investigated in the study that are found in more compact soils inland. This is interesting because *Typhlosaurus* species, which are found in similar soils and locations, also have a high number of vertebral counts. Could this indicate that there is a link between soil type and vertebral count in legless lizards?

Aims and Objectives

Aim: The aim of this study is to investigate whether vertebral number has taxonomic utility in differentiating between and/or grouping of *Acontias* species and whether the vertebral number is linked to environmental characters or retained through ancestry.

Objective: 1) Determining the strength of the phylogenetic signal between *Acontias* species regarding vertebral number 2) To run traditional and phylogenetic ANOVAs to determine whether or not vertebral number is an ancestral trait or whether it is influenced by environmental factors.

Question: 1) “Can vertebral number have taxonomic utility?” 2) “Is the number of vertebrae found in *Acontias* species a trait retained through ancestry or is it influenced by environmental factors?”

Hypotheses: 1) *Acontias* species that exhibit similar vertebral counts will be more closely related phylogenetically. 2) *Acontias* with higher vertebral counts are found in looser soils while *Acontias* species with lower vertebral counts are found in more compact soils.

Methodology

Sampling

Specimens were obtained from the Port Elizabeth Museum"s (PEM) herpetology collection, Eastern Cape, South Africa (Appendix Table A13). Eighteen species of *Acontias* (*A. gariepensis* was excluded because the specimen had a truncated tail) were used in the clade and species analyses section in this chapter. For the environmental analyses, only 16 of the 18 species were used. *Acontias bicolor* and *A. schmitzi* were excluded as they occur outside the area for which we had soil and biome layers. The number of individuals from each species varies according to their availability in the museum collection (Appendix Table A13).

Vertebral counts

In order to count the vertebrae without damaging the specimens, all specimens were sent to the Central Analytical Facility (CAF) at Stellenbosch University for scanning (du Plessis *et al.* 2016). The General Electric Phoenix VTomeX L240 microCT scanner was used to produce computer tomography (CT) scans. A 245 kV X-ray tube, with no copper filter, was set at 80 V, 150 µA, with a two second detector time. X-ray exposure was 250 ms per image during a full 360-degree rotation of the sample. Each scan contained two *Acontias* specimens where possible. A total of 103 individuals were used for this chapter. This included data from Conradie *et al.* (2018) to supplement the final dataset. Optimal parameters were selected according the guidelines set out in du Plessis *et al* (2017). Raw X-ray data files were processed using VGStudio Max version 3.3 (Volume Graphics) to produce a series of tomogram images. The resulting sectioned images were of a resolution of 1000 times smaller than the width of the sample as advised in the guidelines (du Plessis *et al.* 2017). These volumes were then rendered in Dragonfly software (Object Research Systems (ORS) Inc 2018) and used for the vertebral counts of the specimens. Trunk vertebrae were counted from the first vertebrae after the neck vertebrae until the vertebrae at the cloaca. The vertebra at the cloaca was identified by the drastic reduction in size or absence of ribs connected to it (Fig. 3.1). The cloaca could also be seen in the same image as the vertebrae when the scan is angled in the right way and the density threshold adjusted (both of these factors vary with each individual in each scan). Boxplots for the absolute counts were produced (package:"stats", function: „boxplot"; R Core Team 2020).

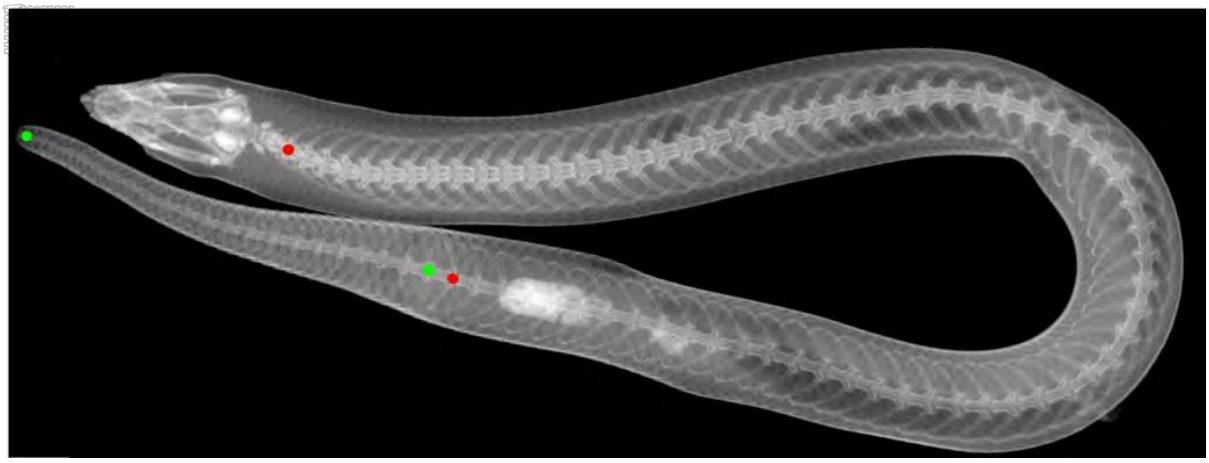


Figure 3.1: X-ray taken form Conradie *et al.* (2018) of *Acontias gracilicauda* (PEM R20214). Red dot closest to skull represents the first trunk vertebrae and the red dot furthest from the skull represents the last trunk vertebrae counted. The green dot furthest from the tail tip represents the first tail vertebrae and the green dot closest to the tail tip represents the last tail vertebrae counted.

Ancestry influence analysis

Each species was placed *a priori* into a phylogenetic clade based on, but not identical to, the work from Chapter 2 and previous studies (Lamb *et al*. 2010; Busschau *et al*. 2017; Zhao *et al.* 2019). The difference between the clade in this chapter and Chapter 2 is that there are only four clades in this chapter. We have merged Clades 2 and 3, and 4 and 5 from Chapter 2 based on each forming a single phylogenetic group. This was done to increase the sample size of the clades for this chapter for statistical purposes. This reduced the number of single species clades in the dataset. It is this grouping that was used for the "Clade" analyses. The Clades are now as follows: Clade 1: *A. namaquensis*; Clade 2: *A. plumbeus*, *A. bicolor, A. parietalis* and *A. a. aurantiacus*; Clade 3: *A. schmitzi, A. k. kgalagadi, A. tristis, A. litoralis, A. grayi* and *A. lineatus*; Clade 4: *A. lineicauda, A. orientalis, A. meleagris, A. breviceps, A. occidentalis, A. albigularis* and *A. gracilicauda* (Fig. 2.3). *Acontias plumbeus,* although clearly much larger, is merged with the *A. a. aurantiacus* clade as the number of vertebrae are similar and it is sister to this clade. The *A. k. kgalagadi* clade is merged with the *A. litoralis* clade as they are sister clades. *Acontias namaquensis* remains a clade on its own as it does not fall within any of the other clades on the phylogenetic tree.

Phylogenetic signal analyses were conducted in R studio version 4.0.0 (R Core Team 2020) to test how strong of an influence phylogeny has on the number of trunk and tail vertebrae in *Acontias* (package: "phytools", function: "phylosig"). Blomberg"s K-statistic is used for phylogenetic signal in this case and a logical hypothesis test was conducted as part of the function with 1000 simulations.

Environmental factors

To determine whether vertebral counts were linked to their environment that they inhabit, each species was categorised into a biome category (Biome), a soil-type category (Soil), and a microhabitat category (Microhabitat). Species distribution shapefiles were obtained from the IUCN Red List website (https://www.iucnredlist.org/) and updated where needed by incorporating new records from the PEM database using ArcMap v10.5.1 (ESRI: ArcGIS 2017; Fig. 2.3 and Fig. 2.4). Biome shapefiles were obtained from the BGIS SANBI website (http://bgis.sanbi.org/Projects/Detail/208; Fig. 1.1). Only the nine major biomes found in southern Africa were used here as there are many subcategories within each biome, Appendix Table A1. The soil shapefile was downloaded from the Soil and Terrain Database for Southern Africa (SOTERSAF; https://files.isric.org/public/soter/SAF-SOTER.zip). The soil was categorised into five major topsoil groups (humic, melanic, organic, orthic, and vertic; Appendix Fig. A1; Table A5). Biome and soil layers were overlaid with the interpreted species distribution ranges and an intersect analysis was conducted in R. The biome and soil type that had the highest proportion (based on the intersect analysis) was used for each species. The "Microhabitat" category was based on information from literature (Branch 1998) to give a better idea of the microhabitat (Appendix Table A6) they can be found in, namely: "burrower", "leaf-litter dweller", "in between roots" or "under stones" (Appendix Table A7) for the South African species.

Statistical analysis

Statistical analyses were conducted in R version 4.0.0 (R Core Team 2020) and R Studio version 1.3.959 (RStudio Team 2020). Boxplots and descriptive statistics for the absolute counts were produced. In order to determine if there is any significant difference between the vertebral counts of the *Acontias* species and the vertebral counts of the clades, Kruskal-Wallis tests were conducted (package: „stats", function: „kruskal.test", R Core Team 2020) for each test. If significance was found in the Kruskal-Wallis test, a Dunn"s post-hoc test was conducted to determine where the differences occurred (package: „dunn.test", function: „"dunn.test"; R Core Team, 2020).

A Kruskal-Wallis test was run for the four vertebral count regions (absolute counts for total, trunk, tail, and ratio) for each category (Soil, Biome, and Microhabitat) to determine if there was any significant difference in the vertebral number (package: „stats", function: „Kruskal.test). If significance was found, a Dunn"s post-hoc test was conducted to determine which groups differed (package: „dunn.test", function: „dunn.test").

The phylogeny for the genus produced in this thesis (see Chapter 2) was imported into RStudio and plotted as a phylogenetic tree (package: „ape", function: „read.tree", R Core Team 2020). Simulation based phylANOVAs (Garland *et al*. 1993), on the four categories (Clade, Microhabitat, Soil and Biome) were run with a "holm" correction, using 1000 simulations and branch lengths obtained from the genetic phylogeny (package: „phytools", function: „phyl.ANOVA", nsim: „1000", p.adj: „holm"; R Core Team 2020). Phylogenetic analyses of variance (phylANOVAs) were performed to account for phylogenetic relationships (ancestry) of the absolute vertebral counts (packages: „ape" and „phytools", function: „phylANOVA", R Core Team, 2020).

Results

Vertebral counts

The results of the descriptive statistics vary with each species (Table 3.1). The species that had the highest and lowest values per body region with regards to range and variance were as follows: *Total vertebrae*: *Acontias lineicauda* had the lowest variance (0.92) and shared the lowest range (2) with *A. a. aurantiacus,* and *A. occidentalis.* The highest variance was in *A. tristis* (27.00) and the largest range was found in *A. meleagris, A. k. kgalagadi* and *A. plumbeus* with a range of 11 vertebrae. *Trunk vertebrae*: The lowest range and variance in this body region were found in *A. a. aurantiacus* (both zero). This is because there are only two specimens for this species and they both have 69 vertebrae. *Acontias gracilicauda* has the largest range (13) and *A. tristis* has the highest variance of 19.00. *Tail vertebrae*: *Acontias namaquensis* has the smallest range (1) and lowest variance (0.33). *Acontias breviceps* has the largest range (10) and highest variance (8.84).

Table 3.1: Descriptive statistics (Mean ± standard deviation (sd), Range (min-max), and Variance of each category per *Acontias* species). *Acontias schmitzi, A. gariepensis* and *A. parietalis* were excluded from this table as there was only one individual from each species with vertebral count data.

Species	Descriptive Statistic	Total vertebrae	Trunk vertebrae	Tail vertebrae	Ratio Trunk:Tail vert
Acontias albigularis	Mean ± sd	94.80 ± 2.30	71.80 ± 2.62	23.00 ± 0.94	3.13 ± 0.21
	Range	92-100	69-78	22-25	2.84-3.55
	Variance	2.29	6.84	0.89	0.04
Acontias a. aurantiacus	Mean ± sd	90.00 ± 1.41	69.00 ± 0.00	21.00 ± 1.41	3.30 ± 0.22
	Range	89-91	69-69	20-22	3.14-3.45
	Variance	2.00	0.00	2.00	0.05
Acontias bicolor	Mean ± sd	103.67 ± 3.51	80.66 ± 1.53	23.00 ± 2.00	3.66 ± 0.35
	Range	100-107	79-82	21-25	3.28-3.95
	Variance	12.30	2.30	4.00	0.12
Acontias breviceps	Mean ± sd	97.30 ± 4.52	75.30 ± 2.75	22.20 ± 2.97	3.43 ± 0.42
	Range	89-104	71-80	19-29	2.52-4.05
	Variance	20.46	2.57	8.84	0.17
Acontias gracilicauda	Mean ± sd	94.30 ± 3.65	71.70 ± 3.80	22.60 ± 2.12	3.20 ± 0.39
	Range	90-102	66-79	20-26	2.64-3.65
	Variance	13.34	14.46	4.49	0.15
Acontias grayi	Mean ± sd	96.50 ± 1.22	74.17 ± 0.98	22.33 ± 0.82	3.32 ± 0.14
	Range	95-98	73-75	21-23	3.17-3.57
	Variance	1.50	0.97	0.67	0.02
Acontias k. kgalagadi	Mean ± sd	101.29 ± 4.46	82.57 ± 3.21	20.14 ± 0.90	4.11 ± 0.19
	Range	95-106	78-86	19-21	3.76-4.30
	Variance	19.90	10.29	0.81	0.04
Acontias lineatus	Mean ± sd	102.44 ± 2.60	77.11 ± 2.57	25.33 ± 0.87	3.05 ± 0.16
	Range	98-106	73-80	24-27	2.81-3.33
	Variance	6.78	6.61	0.75	0.02
Acontias lineicauda	Mean ± sd	96.75 ± 0.96	76.75 ± 1.71	20.00 ± 0.82	3.85 ± 0.24
	Range	96-98	75-79	19-21	3.57-4.16
	Variance	0.92	2.92	0.67	0.06
Acontias litoralis	Mean ± sd	90.91 ± 1.92	68.36 ± 2.66	22.55 ± 1.04	3.04 ± 0.25
	Range	89-96	66-75	21-24	2.75-3.57
	Variance	3.69	7.05	1.07	0.06
Acontias meleagris	Mean ± sd	102.8 ± 3.36	80.20 ± 2.04	22.30 ± 1.42	3.57 ± 0.28
	Range	99-110	77-83	20-25	3.07-3.95
	Variance	11.29	4.18	2.01	0.08
Acontias namaquensis	Mean ± sd	97.33 ± 3.51	74.67 ± 3.06	22.67 ± 0.58	3.29 ± 0.09
	Range	94-101	72-78	22-23	3.22-3.39
	Variance	12.33	9.33	0.33	0.01
Acontias occidentalis	Mean ± sd	101.00 ± 1.41	79.50 ± 0.71	21.50 ± 2.12	3.72 ± 0.40
	Range	100-102	79-80	20-23	3.43-4.00
	Variance	2.00	0.50	4.50	0.16
Acontias orientalis	Mean ± sd	100.33 ± 3.27	78.33 ± 3.83	22.00 ± 0.89	3.57 ± 0.29
	Range	94-103	71-82	21-23	3.09-3.90
	Variance	10.67	14.67	0.80	0.08
Acontias plumbeus	Mean ± sd	92.50 ± 4.72	74.00 ± 3.79	18.50 ± 1.64	4.02 ± 0.33
	Range	87-98	70-79	16-21	3.67-4.44
	Variance	22.30	14.4	2.7	0.11
Acontias tristis	Mean ± sd	99.00 ± 5.20	75.00 ± 4.36	24.00 ± 1.00	3.12 ± 0.12
	Range	96-105	72-80	23-25	3.00-3.20
	Variance	27.00	19.00	1.00	0.01

The absolute vertebral numbers per species has a fair amount of variation. *Acontias meleagris* has the individual with the most vertebrae (110, Table 3.1 and Fig. 3.2A) and *A. plumbeus* has the individual with the fewest vertebrae (87, Table 3.1 and Fig. 3.1A). With the number of trunk vertebrae (Fig. 3.2B), *A. litoralis* has the fewest vertebrae (68.36 ± 2.66) and *A. k. kgalagadi* has the most (82.57 ± 3.21). When looking at the number of tail vertebrae (Fig. 3.2C), the species group more closely together with most of them between 20 and 24 vertebrae. *Acontias lineatus* has a higher mean (25.33 ± 0.87) and *A. plumbeus* has a lower mean (18.50 ± 1.64) number of vertebrae compared to the mentioned range (Table 3.1). *Acontias breviceps* has the largest range in tail vertebrae number, ranging from 20 to 29 vertebrae, although the majority of individuals still exhibited 25 vertebrae or less. When looking at the ratio between the trunk vertebral number and the tail vertebral number, *A. k. kgalagadi* has the highest ratio and *A. litoralis* has the lowest ratio (Fig. 3.2D).

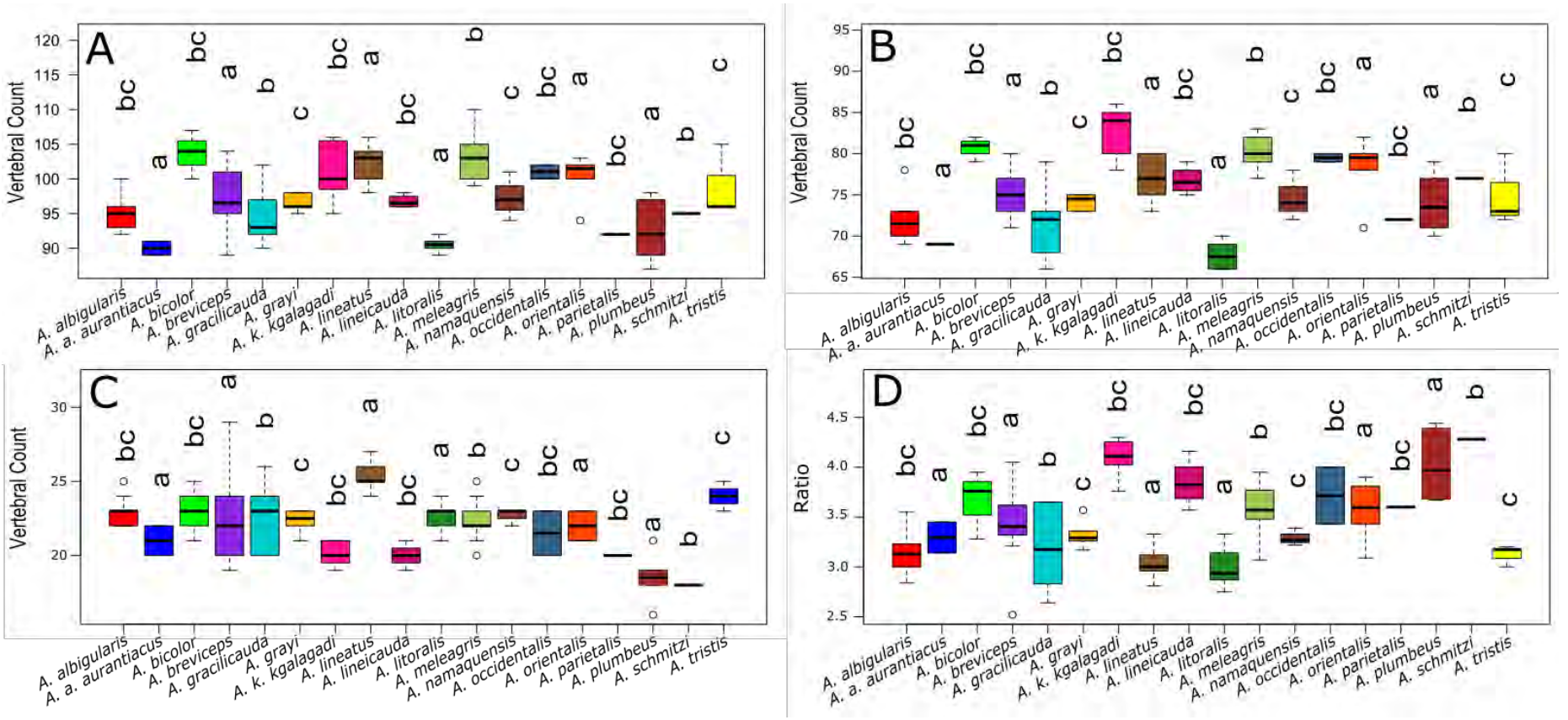


Figure 3.2: Boxplots of the absolute vertebral counts per species: A) Total vertebral count, B) Trunk vertebral count, C) Tail vertebral count, and D) Ratio of trunk vertebral number to tail vertebral number. Lowercase letters above the plots indicate significant difference. Species sharing a letter indicates no significant difference between them.

The absolute vertebral number per phylogenetic clade still has a large amount of variation (Fig. 3.3). It should be noted that Clade 1 is represented by *A. namaquensis* only. When looking at the total numbers of vertebrae (Fig. 3.3A), Clades 1, 3, and 4 all have similar mean values (Clade 1: 97.3 ± 3.51; Clade 3: 97.45 ± 5.64; Clade 4: 97.6 ± 4.55). Clade 2 has the lowest mean (94.8 ± 6.46; Fig. 3.3B) shows a similar trend to the number of total vertebrae with the mean values close together. Clade 1 and 2 have the lowest means and Clade 4 has the highest mean. Clade 2 has the lowest mean for the number of tail vertebrae and Clades 1 and 3 have the highest mean values (Fig. 3.3C). The ratio of number of trunk vertebrae to number of tail vertebrae (Fig. 3.3D) indicates that Clade 3 has the lowest mean ratio and Clade 2 has the highest mean ratio.

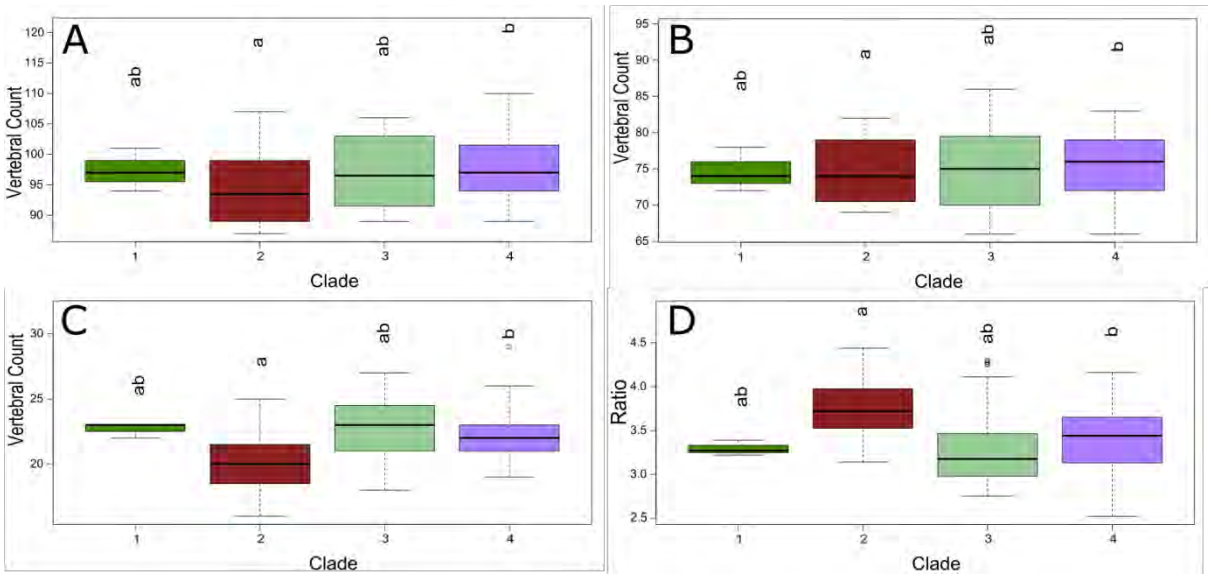


Figure 3.3: Boxplots of the absolute vertebral counts per clade: A) Total vertebral count, B) Trunk vertebral count, C) Tail vertebral count, and D) Ratio of trunk vertebral number to tail vertebral number. Lowercase letters above the plots indicate significant difference. Species sharing a letter indicates no significant difference between them.

Phylogenetic signal

When looking at whether there is a strong phylogenetic signal in the number of trunk vertebrae in *Acontias,* we found that it was weak (K-value = 0.18). This indicates that there is phylogenetic independence with regards to this trait. The non-significant p-value (p-value = 0.83) supports this as it indicates that the trait has developed close to what one would expect from random when tested against 1000 simulations. The number of tail vertebrae are quite strongly linked to what one would expect under Brownian motion (K-value = 0.62). A significant p-value was found when the K-value was tested (p-value = 0.02*). The total number of vertebrae is also phylogenetically independent (K-value = 0.17) and is close to

matching the results from simulation (p-value = 0.85). The ratio between trunk and tail vertebrae is partly independent of phylogeny (K-value = 0.59) but the result compared to 1000 simulations is not similar to what can be expected from random (p-value = 0.01*).

Statistical analyses

All four categories of vertebral counts (total, trunk, tail, and ratio between trunk and tail) showed significant difference between species using the Kruskal-Wallis test (Table 3.2). The difference in total vertebral number was highly significant between species with *A. litoralis* being significantly different to most other species. This was followed by *A. lineatus,* with regard to total vertebrae according to the Dunn"s post hoc analysis (Appendix Table A14). This means that those species are most likely able to be differentiated from other species based on the total vertebral number. Ancestry indicated that there was no significant difference in total vertebral count between species. The number of trunk vertebrae was also found to be significantly different between species according to the Kruskal-Wallis test (Table 3.2). Post hoc analysis indicated that *A. litoralis* is significantly different from most other species with regards to the number of trunk vertebrae (Appendix Table A15). There is no significant difference in trunk vertebral number between the *Acontias* species due to ancestry. Tail vertebral numbers are highly significant between species (Table 3.2). *Acontias lineatus* is significantly different from most species based on tail vertebrae number (Appendix Table A16). There is no significant difference found between species based on the number of tail vertebrae with regards to ancestry. The ratio between trunk vertebral number and tail vertebral number is also shown to have a significant difference between species (Table 3.2), with *A. k. kgalagadi* being significantly different from the most species it was compared to (Appendix Table A17). When ancestry was taken into account, no significant difference is found between the species.

Table 3.2: Results of the Kruskal-Wallis test and phylogenetic ANOVA for the different vertebral count categories of *Acontias* species. The traditional p-value is that obtained from a standard ANOVA without the inclusion of phylogeny. The phylogenetic p-value is that obtained by conducting a phylogenetic ANOVA where ancestry is taken into account. Values that represent significance are indicated in bold as well as with an asterisk for p-values less than or equal to 0.05 (*) or double asterisk (**) for significance p ≤ 0.01 or triple asterisk (***) for significance p ≤ 0.001 or quadruple asterisk (****) for significance ≤ 0.0001.

Category	df	chi-squared (H stat)	Traditional p-value	Phylogenetic F-value	Phylogenetic p-value
Total vertebrae	17	67.87	**< 0.0001****	9.59	0.82
Trunk Vertebrae	17	73.29	**< 0.0001****	5.92	0.91
Tail vertebrae	17	58.28	**< 0.0001*****	4.46	0.97
Ratio (Trunk:Tail)	17	66.34	**< 0.0001****	21.12	0.51

The *Acontias* species were placed *a priori* into phylogenetic clades as described in this chapter. Total vertebral number of the clades indicates no significant difference between them (Table 3.3) (Appendix Table A18). The Kruskal-Wallis test indicates that there is no significant difference in the number of trunk vertebrae between clades (Table 3.3; Appendix Table A18). There is no significant difference found between clades when looking at ancestry. The number of tail vertebrae is significantly different between the clades based on the Kruskal-Wallis test (Table 3.3). Clade 2 is significantly different from Clades 3 and 4 (Appendix Table A19). When accounting for ancestry, there is no significant difference in the number of tail vertebrae between the clades. When looking at the ratio between the number of trunk vertebrae to the number of tail vertebrae in the clades, a significant difference was found between the clades (Table 3.3). Clade 2 is significantly different from Clade 3. Ancestry reveals there is no significant difference between clades with regards to vertebral ratio.

Table 3.3: Results of the Kruskal-Wallis test and phylogenetic ANOVA for the different vertebral count categories of *Acontias* clades. The traditional p-value is that obtained from a standard ANOVA without the inclusion of ancestry. The phylogenetic p-value is that obtained by conducting a phylogenetic ANOVA where ancestry is taken into account. Values that represent significance are indicated in bold as well as with an asterisk for p-values less than or equal to 0.05 (*) or double asterisk (**) for significance p ≤ 0.01 or triple asterisk (***) for significance p ≤ 0.001 or quadruple asterisk (****) for significance p ≤ 0.0001.

Category	df	chi-squared (H-statistic)	Traditional p-value	Phylogenetic F-value	Phylogenetic p-value
Total vertebrae	3	2.92	0.40	15.17	0.22
Trunk Vertebrae	3	0.66	0.88	6.34	0.52
Tail vertebrae	3	11.78	**0.008****	3.77	0.63
Ratio (Trunk:Tail)	3	11.51	**0.009****	0.02	0.99

Kruskal-Wallis test results indicate that the “Soil” category was significant for all regions (Total: chi-square = 17.38, df = 3, p-value = 0.0006***Trunk: chi-square = 16.61, df = 3, p-value = 0.009**; Tail: chi-square = 18.65, df = 3, p-value = 0.0002***) and ratio (chi-square = 16.35, df = 3, p-value < 0.0001****; Table 3.4). The total number of vertebrae for individuals found in melanic soil differed significantly from both organic and orthic-soil inhabiting individuals. Individuals found in melanic soil have a significantly different number of trunk vertebrae from individuals found in the other soil types (Appendix Table A20). Humic-Orthic and Melanic-Humic were significantly different with regards to the number of tail vertebrae in *Acontias* species (Table A21), while organic and melanic soils were both significantly different from humic soils when looking at the ratio trunk:tail vertebrae (Appendix Table A21). Phylogenetic ANOVA results indicate no significant difference in the number of vertebrae with regards to soil types; Table 3.4).

Kruskal-Wallis test results indicate that there is a significant difference in the number of vertebrae between species found in different biome categories in all three regions (all p-values < 0.001***; Table 3.7) and in the ratio (p-value < 0.001***; Table 3.4). In the total vertebrae the highest significance was found between the Nama Karoo and Succulent Karoo biomes (Appendix Table A22). In the trunk region the highest significance was found between the Succulent Karoo and Fynbos biome and the Succulent Karoo and Savanna Biome (Appendix Table A22). The tail vertebrae have the highest significance in the Nama-Karoo and Savanna biomes, each showing significant difference from four biomes (Appendix Table A23). Significant difference in ratio of trunk to tail vertebrae was found between the Savanna and Succulent Karoo with most other biomes (Appendix Table A23).

Microhabitat is significant according to the Kruskal-Wallis test in all vertebral count regions (Total: chi-square = 15.40, df = 3, p-value = 0.002**; Trunk: chi-square = 18.91, df = 3, p-value = 0.0003***, Tail: chi-square = 26.58, df = 3, p-value < 0.0001****; Table 3.7) and ratio (chi-square = 28.78, df = 3, p-value < 0.0001****; Table 3.4). Post hoc results indicate significant differences for the total vertebrae count in “under stones” - “leaf-litter" and “leaf-litter”-“burrow” (Appendix Table A24). The trunk vertebrae number was significantly different between “burrow”-“in between roots” and “burrow”-“under stones”. The tail vertebrae count indicated significant difference in vertebral count in four of the six microhabitat category comparisons (Appendix Table A25). The ratio between trunk vertebral number and tail vertebral number indicated significant differences between “burrow”, “in between roots” and “burrow” those found under stones (Appendix Table A25). When ancestry was considered there is a significant difference in the ratio between the microhabitat categories (df = 3, p-value = 0.001**; Table 3.4).

Table 3.4: Results of the Kruskal-Wallis and phylogenetic ANOVA for the four absolute vertebral count regions using soil, biome, and microhabitat groupings of *Acontias*. The traditional p-value is that obtained from a standard ANOVA without the inclusion of ancestry. The phylogenetic p-value is that obtained by conducting a phylogenetic ANOVA where ancestry is taken into account. Values that represent significance are indicated in bold as well as with an asterisk for p-values less than or equal to 0.05 (*) or double asterisk (**) for significance p ≤ 0.01 or triple asterisk (***) for significance p ≤ 0.001 or quadruple asterisk (****) for significance p ≤ 0.0001.

Vertebral region	Category	df	Chi-square (H-statistic)	Traditional p-value	Phylogenetic p-value
Total	Soil	3	17.38	**0.0006*** **	0.066
	Biome	6	36.54	**< 0.0001****	0.62
	Microhabitat	3	15.40	**0.002** **	0.18
Trunk	Soil	3	16.61	**0.0009*** **	0.09
	Biome	6	42.30	**< 0.0001****	0.86
	Microhabitat	3	18.91	**0.0003*** **	0.31
Tail	Soil	3	18.65	**0.0002*** **	0.19
	Biome	6	47.91	**< 0.0001****	0.93
	Microhabitat	3	26.58	**< 0.0001****	0.60
Ratio	Soil	3	16.35	**< 0.001** **	0.10
	Biome	6	53.22	**< 0.0001****	0.12
	Microhabitat	3	28.78	**< 0.0001****	**0.001** **

Discussion

The concept of “species” has been highly debated for centuries and so attempting to produce methods to aid in delineating species from each other is often useful. In this chapter, the use of vertebral counts and various morphological measurements were tested

as possible species and clade delineation methods for *Acontias.* In the past, using vertebral counts has been useful in delineating species with similar body plans to *Acontias* (Watanabe *et al.* 2011; Papenfuss and Parham 2013). It is for this reason that the technique was thought to be useful for *Acontias* (Conradie *et al.* 2018). Environmental factors, such as soil and biome, were also looked at to determine if there is a link between environmental factors and vertebral counts and body length.

The absolute vertebral counts indicated that there was a significant difference between *Acontias* species and clades with regards to vertebral number with the exception of the number of trunk vertebrae between clades. This indicated that species and clades should be able to be differentiated based on vertebral numbers in particular regions. However, the post hoc tests revealed that these significant differences in vertebral numbers were only between some species and clades and not all, and in some cases it was between very few species. In addition to this the phylogenetic ANOVA revealed that once ancestry was taken into account there was no significant difference in any of the vertebral numbers between the clades. This indicates that vertebral count is most likely not a factor that can be used in species or clade delineation. Keep in mind that to increase the sample size, the clades were different in this chapter from Chapter 2. If more species were to be included and the clades remained the same as in Chapter 2, the results may differ. It is worthwhile to take a more in-depth look at vertebral count as a species delineation option for *Acontias* clades. Given that vertebral count did indicate strong evidence with significant difference between some species and clades, it could be used in conjunction with other common identifying characters in the delineation method. Examples of such characters can be seen in Table 3.5.

Phylogenetic independence was found in the number of trunk and total vertebrae and the numbers are what one would expect from BM. The number of tail vertebrae and the ratio between trunk and tail vertebrae were not phylogenetically independent indicating a phylogenetic link for those traits.

One method that already aids in the delineation of *Acontias* into different groups is based on SVL, and incorporating vertebral counts with this method could prove to be helpful. It was found in previous studies that body elongation in squamates occurs through the addition of vertebrae and the lengthening of the body or both the body and tail (Wiens *et al.* 2006; Brandley *et al.* 2008; Bergmann and Irschick 2012; Bergmann and Morinaga 2019; Bergmann *et al.* 2020). Variation in the number of tail vertebrae is much lower than in the trunk vertebrae, which support the idea that fossorial species most likely underwent body elongation and not tail elongation.

In this study, all *Acontias* species looked at showed similar results to Wagner *et al.* (2012) except not all of the individuals from *A. bicolor* and *A. meleagris* possessed 25 or more tail vertebrae, at least one individual from each of these species did. *Acontias lineatus* was the only species where eight out of nine individuals possessed 25 or more tail vertebrae, with *Acontias tristis* close behind (25, 23, 24). Lamb *et al.* (2010) also noted that the *Acontias* genus should possess a minimum of 21 tail vertebrae. The majority of individuals in this study met this prerequisite, but there were a few individuals that possessed 18 to 20 tail vertebrae. This could have been due to a number of factors. When looking at the tail vertebrae it is sometimes difficult to observe them clearly due to their small size on the images. Some of the images also had scales on the tail that were of a higher density and could not clearly be excluded in the image, thus obscuring the view of some of the vertebrae making them difficult to count. The counts were not far off from the minimum 21 vertebrae mentioned in Lamb *et al.* (2010) and so they were still acceptable for use in this study (possibly due to truncated tails). Data from this chapter (before being supplemented from other sources) was also compared to that of Conradie *et al.* (2018) and counts fall within or just outside the ranges presented there. This helps confirm that the counts in this study fall within the correct range.

Table 3.5: Identifying characters that are often used in *Acontias* species delineation (used in this study) with the inclusion of vertebral counts. Values in the table were obtained from the literature as well as from this study. Sb = subocular, SO = supraoccular and SC = supracilliary scales.

Species	SVL range (mm)	Mean midbody scale	Ventral scale	Subcaudal scale	Sb	SO	SC	Chin shields	Total vertebrae	Trunk vertebrae	Tail vertebrae	Vert. count Clade
Acontias albigularis	70–195	14	146–159	33–39	3	3	4	3	89–101	68–73	21–28	4
Acontias a. aurantiacus	150–200	12	141–168	26–33	2	2	2	5	89–91	69	20–22	2
Acontias bicolor	170–192	16–20	170–192	31–37	2	3	2	5	100–107	79–82	21–25+	2
Acontias breviceps	130–170	16	147–183	27–38	3	3	4	3	73–97	68–80	18–24	4
Acontias gariepensis	100–120	12	170–189	32–38	0	0	1	3	-	-	-	3
Acontias gracilicauda	200–230	18	146–177	25–45	3	3	4	3	89–105	68–81	21–24	4
Acontias grayi	130–145	14–15	160–170	34-39	1–2	1	3	3	95–98	73–75	22–23	3
Acontias k. kgalagadi	130–145	14	165–195	26–35	1	0	2	4	97–106	78–86	19–21	3
Acontias lineatus	130–145	14	160–182	35–45	2–3	1–2	3–4	3	103–106	76–82	22–27	3
Acontias lineicauda	123–171	14	178–186	28–42	2	2	4	3	96–98	75–79	19–21	4
Acontias litoralis	110–115	12–14	145–160	34–40	2	1	3	3	89–91	66–70	21–24	3
Acontias meleagris	200–230	14–18	160–193	32–43	3	2–3	3–4	3	99–110	77–83	20–25	4
Acontias namaquensis	200–230	16–18	158–179	35–46	3	3	4	3	94–101	72–78	22–23	1
Acontias occidentalis	180–220	14–18	165–181	27–42	3	3	3–4	3	100–103	79–82	20–23	4
Acontias orientalis	200–	14–16	161–	28–42	3	2–	4	3	94–103	71–82	21–23	4

Species	SVL range (mm)	Mean midbody scale	Ventral scale	Subcaudal scale	Sb	SO	SC	Chin shields	Total vertebrae	Trunk vertebrae	Tail vertebrae	Vert. count Clade
	230		186			3						
Acontias parietalis	150–200	12	157–168	28–34	2	2	2	6	92	72	20	2
Acontias plumbeus (Bianconi, 1849)	250– > 300	16–20	146–165	25–36	2–3	3	4	3–5	89–97	70–79	16–19	2
Acontias schmitzi	~ 190	14	173	~ 26	1	1	0	4	95–104	77–81	18–23	3
Acontias tristis	~ 130	12–14	160–181	36–45	2	1	3	3	96–105	72–80	23–25	3

Environmental factors were used to determine if there is any link between the environment and vertebral number. *Acontias* use their body and head to burrow and move through the substrate, so you can expect to find different vertebral numbers (body lengths) to allow for different flexibilities to move through the different substrates. For all the absolute vertebral counts a significant difference between species was found for all environmental variables except in the total vertebrae with regards to soil category and tail vertebrae with regard to microhabitat category. This indicates that the number of vertebrae a species has, whether it is total or just in specific regions, could be linked to the environment in which that species inhabits. The phylogenetic ANOVA indicated that there is a significant difference between the ratio of trunk to tail vertebral number with regards to the microhabitat category. This indicates that the microhabitat category should perhaps be investigated further and be refined.

Combining the results from the environmental analyses in this chapter with the distribution data, biome maps, and soil maps, a pattern begins to emerge. The smaller bodied *Acontias* tend to group together and are found in similar environments close to the West Coast of South Africa. This is close to the *Typhlosaurus* distributions and thus the environments where these *Acontias* are found share similar traits to those where *Typhlosaurus* are found. *Typhlosaurus* are also small and thin-bodied like some of the *Acontias* species. The larger bodied *Acontias* are found in other regions of the country that have different environmental pressures. The soils found in the more inland regions of the country are known to be coarser than the sandier soils of the coast. Perhaps *Acontias* have developed larger, thicker bodies to better traverse through the substrates.

As mentioned previously the development of vertebrae is controlled by *Hox* gene expressions or rather groups of *Hox* gene expressions with each group relating to different regions of the vertebral axis. In mammals, for example, there will be a group that controls each of the cervical (neck), thoracic, lumber regions, etc. In squamates, such as snakes and legless lizards, however, these regions are not clear due to the suppression of certain *Hox* genes leaving most of the vertebrae to have a similar design. The vertebral axis in these cases is referred to as being “deregionalised”. The only two clear regions are those of the tail vertebrae and the rest of the trunk vertebrae (Burke *et al.* 1995; Richardson *et al.* 1998). It is not easy to tell the cervical vertebrae from the rest but it is possible. Taking a more in-depth look at the neck vertebrae of head-first burrowing species, such as *Acontias* and *Typhlosaurus,* can also prove to be interesting. Are the neck vertebrae denser and/or more compact than other vertebrae in the body, as a result of being exposed to higher compression forces, due to head-first burrowing?

Future research

Future studies can include using more *Acontias* species in the sample set in an attempt to represent the complete phylogeny. To include *Typhlosaurus* in the vertebral count work can also be interesting as they will form a good outgroup for the study. One is then able to compare the results obtained from the *Acontias* to *Typhlosaurus* and determine where the similarities and differences lie, for example, would *Acontias* species that live in similar environments to *Typhlosaurus* species have similar numbers of vertebrae in their trunk and tail relative to *Typhlosaurus*? Van Damme and Vanhooydonck (2002) showed that lacertid lizards that inhabit different microhabitats have significantly different numbers of presacral vertebrae (Van Damme and Vanhooydonck 2002; Conradie *et al.* 2018). This can also then be linked to the work done by Kelley *et al.* (1997) where the effect of vertebral number on manoeuvrability in a species of garter snake (*Thamnophis elegans*) was looked at. It suggested that higher numbers of vertebrae increases manoeuvrability while lower numbers of vertebrae aid in increasing speed and acceleration capabilities (Kelley *et al.* 1997). *Acontias* that occur in microhabitats that require a higher manoeuvrability may have a higher number of vertebrae than *Acontias* species that do not need this requirement.

Chapter 4: Conclusion

Acontias breviceps Essex, 1925 (Short headed legless skink; photographer: Chad Keates)

Chapter 4: Conclusion

In this study, we looked to give better insight into the scincid genus *Acontias.* We aimed to achieve this by trying to determine what is (are) the driving factor(s) behind head shape evolution in the genus - is it retained from ancestry or due to environmental factors? This is interesting to look into due to the simplistic, highly conserved body plan of the genus. We also looked into a possible novel method to delineate *Acontias* clades, as new methods to assist in identifying different species that look alike are always welcomed. In addition to this, we attempted to determine whether there is a link between vertebral number and environmental factors. This could pave the way for further studies to look into whether certain body types are adapted to burrowing in certain substrates for *Acontias,* and to look more closely at body and tail elongation.

The head morphometrics chapter (Chapter 2) looked to answer the question of what is driving the evolution of head morphology in *Acontias* by looking at “Soil”, “Biome”, and “Microhabitat” as factors. We found good support that the environment plays a role in the evolution of dorsal head shape of *Acontias.* This is because a significant difference in head shape between species was found for all factors, except in the microhabitat category, before accounting for ancestry. Once ancestry was accounted for there were still significant differences between the dorsal head shapes in most of the factors in the first principal component. The phylogenetic signal in the PC1 dorsal view also indicated that the head shape could be a result of selection and not random. This was expected as species are known to exhibit adaptations that best suit the environment in which they are found. The *Acontias* species exhibit two main dorsal head shapes: the first consists of a large, more pointed rostral scale and a shorter more curved posterior region of the head and the second a smaller, more rounded rostral scale and a longer less curved posterior head region. These two head shapes linked to the different soil and biome types that the species are found in. Species found in the sandier soils closer to the coast exhibited the larger rostral scale head shape than those found more inland in the less sandy soils.

The vertebral analysis (Chapter 3) looked to answer the question of whether vertebral number can be used as a clade delineation method for the *Acontias* genus. Previous studies done on species with similar body plans to *Acontias* have been successful in delineating species based on the vertebral number, thus we were hopeful that it will be successful in *Acontias* as well. What we discovered was that this method has potential to be a delineation method for clades, however further research is required. Significant differences were found between some species without accounting for ancestry, but once ancestry was considered, there was no significant difference in most cases. Clades are more promising to look into because both Kruskal-Wallis and phylogenetic ANOVAs indicated a significant

difference with regard to vertebral number. The number of tail vertebrae had a strong phylogenetic signal and so could potentially be used to delineate clades of *Acontias* or the genus itself from other genera. Although we were not able to say for sure whether vertebral counts can be used as a delineation method, it is a step in the right direction and these methods can still be used for some species (as a confirmation of sorts) in combination with other delineation methods, such as the identifying characters mentioned in Table 3.5.

The second part of Chapter 3 looked at whether there is a link between the environment and vertebral number in *Acontias.* We found that there were links between these factors and *Acontias* vertebral counts, particularly in the “Soil” and “Biome” categories. It will still be worth investigating deeper into this, as not all *Acontias* species are included in this study and results may change. What we did discover was that the *Acontias* grouped once again according to the soil and biome in which they are found. Smaller bodied species grouped together and are found in sandier soils closer to the coast of South Africa and the larger, thicker bodied species are found more inland. This same pattern was mentioned in Chapter 2, where the *Acontias* grouped according to their head shapes. Looking at the two patterns together we can deduce that the smaller bodied *Acontias* with larger rostral scales and more pointed snouts are found in the sandier, looser soils close to the cost and the larger bodied, more round snouted *Acontias* species are found in the more inland regions of the country. This is a great find and opens the door to determine if the other *Acontias* species not used in this study follow the same pattern. We expect that they will, but further studies to test this will be needed to confirm our theory.

In general, this study showed that *Acontias* is a difficult group to delineate morphologically due to its conserved body plan across its species, however there is still potential to use morphological delineation methods in combination with other methods to aid this process. We say this because it was found that *Acontias* head shape and size can vary with the environment in which they are found. Genetic analyses are able to delineate species and discover cryptic species within the genus; and while some aspects of these types of analyses are expensive now, they are becoming more affordable with the evolution of technology and may be used even more frequently in the future. Finally, it was discovered that *Acontias* evolution may not only be affected by ancestry and the environment, but also from convergent evolution due to their lifestyle and microhabitat. This is because none of the analyses showed a 100% clear conclusion as none resulted in a clear pattern between all the species.

References

AERTS, P., VAN DAMME, R., VANHOOYDONCK, B., ZAAF, A. & HERREL, A. 2000. Lizard Locomotion: How Morphology Meets Ecology. *Netherlands Journal of Ecology* **50** (2), 261-277.

ALBERT, E.M., ZARDOYA, R. & GARCIA-PARIS, M. 2007. Phylogeographical and speciation patterns in subterranean worm lizards of the genus *Blanus* (Amphisbaenia: Blanidae). *Molecular Ecology* **16** (7), 1519-1531.

ALEXANDER, G. & MARAIS, J., 2007. A guide to the reptiles of southern Africa. Struik Publishers

ALIABADIAN, M., KABOLI, M., NIJMAN, V. & VENCES, M. 2009. Molecular identification of birds: performance of distance-based DNA barcoding in three genes to delimit parapatric species. *Public Library of Science One* **4** (1), e4119.

BARRACLOUGH, T.G., 2019. The Evolutionary Biology of Species. (Charles, H., Godfray, J., Dunne, J., Sheldon, B.C., eds). pp 271. Oxford University Press, Great Clarendon Street, Oxford, United Kingdom

BARROS, F.C., HERREL, A. & KOHLSDORF, T. 2011. Head shape evolution in Gymnophthalmidae: does habitat use constrain the evolution of cranial design in fossorial lizards? *Journal of Evolutionary Biology* **24** (11), 2423-2433.

BATES, M.F., BRANCH, W.R., BAUER, A.M., BURGER, M., MARAIS, J., ALEXANDER, G. & DE VILLIERS, M.S., (eds). 2014 (reprint 2014). Atlas and Red List of the Reptiles of South Africa, Lesotho and Swaziland. eds). pp *Suricata* 1, South African National Biodiversity Institute, Pretoria

BAUER, A.M., CHILDERS, J.L., BROECKHOVEN, C. & MOUTON, P.L.N. 2019. A new *Nucras* Gray, 1838 (Squamata: Lacertidae) from the Strandveld of the Western Cape, South Africa. *Zootaxa* **4560** (1), 149-163.

BERGMANN, P. & IRSCHICK, D.J. 2012. Vertebral Evolution and the Diversification of Squamate Reptiles. *Evolution* **66** (4), 1044-1058.

BERGMANN, P.J. & MORINAGA, G. 2019. The convergent evolution of snake-like forms by divergent evolutionary pathways in squamate reptiles. *Evolution* **73** (3), 481-496.

BERGMANN, P.J., MANN, S.D.W., MORINAGA, G., FREITAS, E.S. & SILER, C.D. 2020. Convergent Evolution of Elongate Forms in Craniates and of Locomotion in Elongate Squamate Reptiles. *Integrative and Comparative Biology* **60** (1), 190-201.

BICKFORD, D., LOHMAN, D.J., SODHI, N.S., NG, P.K., MEIER, R., WINKER, K., INGRAM, K.K. & DAS, I. 2007. Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution* **22** (3), 148-155.

BÖHLKE, E.B. 1997. Notes on the identity of elongate unpatterned Indo-Pacific morays, with description of new species (Muraenidae, Subfamily Muraninae). *Proceedings of the Academy of Natural Sciences of Philadelphia* **147** 89-109.

BRANCH, W.R., 1998. Field guide to snakes and other reptiles of southern Africa. (Parker, P., Reid, H., eds). pp 397. Struik Publishers

BRANDLEY, M.C., HUELSENBECK, J.P. & WIENS, J.J. 2008. Rates and patterns in the evolution of snake-like body form in squamate reptiles: evidence for repeated re-evolution of lost digits and long-term persistence of intermediate body forms. *Evolution* **62** (8), 2042-2064.

BROADLEY, D.G. & GREER, A.E. 1969. A revision of the genus *Acontias* Cuvier (Sauria: Scincidae). *Arnoldia Zimbabwe* **4** (26), 1-29.

BROECKHOVEN, C. & DU PLESSIS, A. 2018. X-ray microtomography in herpetological research: a review. *Amphibia-Reptilia* **39** (4), 377-401.

BRUFORD, M.W., HANOTTE, M., BROOKFIELD, J.F.Y. & BURKE, T., 1992. Molecular genetic analysis of populations: A practical approach. (Hoelzel, A.R., eds). pp 225-270. Information Retrieval Limited Press, Oxford, United Kingdom

BURBRINK, F.T., GRAZZIOTIN, F.G., PYRON, R.A., CUNDALL, D., DONNELLAN, S., IRISH, F., KEOGH, J.S., KRAUS, F., MURPHY, R.W., NOONAN, B., RAXWORTHY, C.J., RUANE, S., LEMMON, A.R., LEMMON, E.M. & ZAHER, H. 2020. Interrogating Genomic-Scale Data for Squamata (Lizards, Snakes, and Amphisbaenians) Shows no Support for Key Traditional Morphological Relationships. *Systematic Biology* **69** (3), 502-520.

BURKE, A.C., NELSON, C.E., MORGAN, B.A. & TABIN, C. 1995. Hox genes and the evolution of vertebrate axial morphology. *Development* **121** 333-346.

BUSSCHAU, T., CONRADIE, W., JORDAAN, A. & DANIELS, S.R. 2017. Unmasking evolutionary diversity among two closely related South African legless skink species (Acontinae: *Acontias*) using molecular data. *Zoology (Jena)* **121** 72-82.

BUSSCHAU, T., CONRADIE, W. & DANIELS, S.R. 2019. Evidence for cryptic diversification in a rupicolous forest-dwelling gecko (Gekkonidae: *Afroedura pondolia*) from a biodiversity hotspot. *Molecular Phylogenetics and Evolution* **139** 1-52.

BUSSCHAU, T., CONRADIE, W. & DANIELS, S.R. 2020. One species hides many: Molecular and morphological evidence for cryptic speciation in a thread snake

(Leptotyphlopidae: *Leptotyphlops sylvicolus* Broadley & Wallach, 1997). *Journal of Zoological Systematics and Evolutionary Research* **59** (1), 195-221.

CAPUTO, V., LANZA, B. & PALMIERI, R. 1995. Body elongation and limb reduction in the genus *Chalcides* Laurenti 1768 (Squamata Scincidae): a comparative study. *Tropical Zoology* **8** (1), 95-152.

CERÍACO, L.M.P., HEINICKE, M.P., PARKER, K.L., MARQUES, M.P. & BAUER, A.M. 2020. A review of the African snake-eyed skinks (Scincidae: *Panaspis*) from Angola, with the description of a new species. *Zootaxa* **4747** (1), 77-112.

CONRADIE, W., BUSSCHAU, T. & EDWARDS, S. 2018. Two new species of *Acontias* (Acontinae, Scincidae) from the Mpumalanga Highveld escarpment of South Africa. *Zootaxa* **4429** (1), 89-106.

CONRADIE, W., DEEPAK, V., KEATES, C. & GOWER, D.J. 2020. Kissing cousins: a review of the African genus *Limnophis* Günther, 1865 (Colubridae: Natricinae), with the description of a new species from north-eastern Angola. *African Journal of Herpetology* **69** (1), 79-107.

COOK, L.G., EDWARDS, R.D., CRISP, M.D. & HARDY, N.B. 2010. Need morphology always be required for new species descriptions? *Invertebrate Systematics* **24** 322-326.

COYNE, J. 1992. Genetics and Speciation. *Nature* **335** 511-515.

CRONQUIST, A. 1978. Once again, what is a species? *Biosystematics in Agriculture* **2** 3-20.

CYRIAC, V. & KODANDARAMAIAH, U. 2020. Warning signals promote morphological diversification in the fossorial uropeltid snakes (Squamata: Uropeltidae). *Zoological Journal of the Linnean Society* **20** 1-14.

DA SILVA, F.O., FABRE, A.C., SAVRIAMA, Y., OLLONEN, J., MAHLOW, K., HERREL, A., MULLER, J. & DI-POI, N. 2018. The ecological origins of snakes as revealed by skull evolution. *Nature Communication* **9** (1), 376.

DANIELS, S.R., HEIDEMAN, N.J., HENDRICKS, M.G. & WILSON, B.A. 2002. A molecular phylogeny for the South African limbless lizard taxa of the subfamily Acontinae (Sauria: Scincidae) with special emphasis on relationships within *Acontias*. *Molecular Phylogenetics and Evolution* **24** 315-323.

DANIELS, S.R., HEIDEMAN, N.J., HENDRICKS, M.G., MOKONE, M.E. & CRANDALL, K.A. 2005. Unraveling evolutionary lineages in the limbless fossorial skink genus *Acontias* (Sauria: Scincidae): are subspecies equivalent systematic units? *Molecular Phylogenetics and Evolution* **34** (3), 645-654.

DANIELS, S.R., HEIDEMAN, N.J.L., HENDRICKS, M.G.J. & CRANDALL, K.A. 2006. Taxonomic subdivisions within the fossorial skink subfamily Acontinae (Squamata: Scincidae) reconsidered: a multilocus perspective. *Zoologica Scripta* **35** (4), 353-362.

DANIELS, S.R., HEIDEMAN, N.J.L. & HENDRICKS, M.G.J. 2009. Examination of evolutionary relationships in the Cape fossorial skink species complex (Acontinae: *Acontias meleagris meleagris*) reveals the presence of five cryptic lineages. *Zoologica Scripta* **38** (5), 449-463.

DARRIBA, D., TABOADA, G.L., DOALLO, R. & POSADA, D. 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* **9** (8), 772.

DE QUEIROZ, K. 2005. A Unified Concept of Species and Consequences for the Future of Taxonomy. *Proceedings of the California Academy of Science* **56** (18), 196-215.

DE QUEIROZ, K. 2007. Species concepts and species delimitation. *Systematic Biology* **56** (6), 879-886.

DE SCHEPPER, N., ADRIAENS, D. & DE KEGEL, B. 2005. Moringua edwardsi (Moringuidae: Anguilliformes): cranial specialization for head-first burrowing? *Journal of Morphology* **266** (3), 356-368.

DESALLE, R., EGAN, M.G. & SIDDALL, M. 2005. The unholy trinity: taxonomy, species delimitation and DNA barcoding. *Philosophical Transactions of the Royal Society of London. Series B. Biological Sciences* **360** (1462), 1905-1916.

DU PLESSIS, A., LE ROUX, S.G. & GUELPA, A. 2016. The CT Scanner Facility at Stellenbosch University: An open access X-ray computed tomography laboratory. *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms* **384** 42-49.

DU PLESSIS, A., BROECKHOVEN, C., GUELPA, A. & LE ROUX, S.G. 2017. Laboratory x-ray micro-computed tomography: a user guideline for biological samples. *GigaScience* **6** (6), 1-11.

DUNN, C.P. 2003. Keeping taxonomy based in morphology. *TRENDS in Ecology and Evolution* **18** (6), 270-271.

EDWARDS, S., VANHOOYDONCK, B., HERREL, A., MEASEY, G.J. & TOLLEY, K.A. 2012. Convergent Evolution Associated with Habitat Decouples Phenotype from Phylogeny in a Clade of Lizards. *Public Library of Science One* **7** (12), e51636.

EDWARDS, S., 2014. Chapter 5: Convergence in Morphology is Preceded by Convergence in Performance in Lizards. In: Lizards: Thermal Ecology, Genetic Diversity and Functional Role in Ecosystems, First edition (Kiernan, M.P., eds). pp 99-120. Nova Science Publishers, Inc.

,
ENGELBRECHT, H.M., 2012. Systematics of the Cape legless skink *Acontias meleagris* species complex, Unpublished PhD Dissertation. University of Stellenbosch, p. 116.

ENGELBRECHT, H.M., VAN NIEKERK, A., HEIDEMAN, N.J.L., DANIELS, S.R. & PARMAKELIS, A. 2013. Tracking the impact of Pliocene/Pleistocene sea level and climatic oscillations on the cladogenesis of the Cape legless skink, *Acontias meleagris* species complex, in South Africa. *Journal of Biogeography* **40** (3), 492-506.

ERIKSSON, C.E., RUPRECHT, J. & LEVI, T. 2020. More affordable and effective noninvasive single nucleotide polymorphism genotyping using high-throughput amplicon sequencing. *Molecular Ecology Resources* **20** (6), 1505-1516.

FABRE, A., BICKFORD, D., SEGALL, M. & HERREL, A. 2016. Impact of diet, habitat use and behaviour on head shape evolution in homalopsid snakes. *Biological Journal of the Linnean Society* **118** 634-647.

FABRE, A.C., CORNETTE, R., HUYGHE, K., ANDRADE, D.V. & HERREL, A. 2014. Linear versus geometric morphometric approaches for the analysis of head shape dimorphism in lizards. *Journal of Morphology* **275** (9), 1016-1026.

FEY, M., 2010. Soils of South Africa. (Hanks, K., eds). pp 16-147. Cambridge University Press,
FIŠER, C., ROBINSON, C.T. & MALARD, F. 2018. Cryptic species as a window into the paradigm shift of the species concept. *Molecular Ecology* **27** (3), 613-635.

FITZSIMONS, V.F. 1943. The lizards of South Africa: Systematic account of the lizards (Sauria) of Southern Africa. *Transvaal Museum Memoirs* **1** (1), 1-6.

GANS, C. 1960. Studies on amphisbaenids (Amphisbaenia, Reptilia). A taxonomic revision of the Trogonophinae, and a functional interpretation of the amphisbaenid adaptive pattern. *Bulletin of the American Museum of Natural History* **119** 129-204.

GANS, C. 1969. Amphisbaenians-reptiles specialized for a burrowing existence. *Nature* **99** 146-151.

GANS, C., 1974. Biomechanics: an Approach to Vertebrate Biology. (May, C., Burke, K., eds). pp 141-160. Lippincott Williams & Wilkins, Ann Arbor, Michigan

GANS, C. & FUSARI, M. 1994. Locomotor analysis of surface propulsion by three species of reduced-limb fossorial lizards (*Lerista:* Scincidae) from Western Australia. *Journal of Morphology* **222** 309-326.

GANS, C. & MONTERO, R. 2008. An atlas of amphisbaenian skull anatomy. *Biology of the Reptilia* **21** 621-738.

GARLAND, T., DICKERMAN, A., JANIS, C. & JONES, J. 1993. Phylogenetic analysis of covariance by computer simulation. *Systematic Biology* **42** (3), 265-292.

GIL, M.J., GUERRERO, F. & PEREZ-MELLADO, V. 1993. Observations on Morphometrics and Ecology in *Blanus cinereus* (Reptilia: Amphisbaenia). *Journal of Herpetology* **27** (2), 205.

GRAB, S. & KNIGHT, J., 2015. Landscapes and Landforms of South Africa (Grab, S., Knight, J., eds). pp 187. Springer International Publishing, Switzerland

GRAY, J.A., SHERRATT, E., HUTCHINSON, M.N. & JONES, M.E.H. 2019. Changes in ontogenetic patterns facilitate diversification in skull shape of Australian agamid lizards. *BMC Evolutionary Biology* **19** (1), 7.

GREER, A.E. 1991. Limb Reduction in Squamates: Identification of Lineages and Discussion of the Trends. *Journal of Herpetology* **25** (2), 166-173.

GRIZANTE, M.B., BRANDT, R. & KOHLSDORF, T. 2012. Evolution of body elongation in gymnophthalmid lizards: relationships with climate. *Public Library of Science* **7** (11), e49772.

GROVES, C. 2017. Phylogenetic Species Concept. *The International Encyclopedia of Primatology* **2** 1-2.

HALL, T. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41** 95-98.

HALLERMANN, J., CERÍACO, L.M.P., SCHMITZ, A., ERNST, R., CONRADIE, W., VERBURGT, L., MARQUES, M.P. & BAUER, A.M. 2020. A review of the Angolan House snakes, genus *Boaedon* Duméril, Bibron and Duméril (1854) (Serpentes: Lamprophiidae), with description of three new species in the *Boaedon fuliginosus* (Boie, 1827) species complex. *African Journal of Herpetology* **69** (1), 29-78.

HANDRIGAN, G.R. & WASSERSUG, R.J. 2007. The anuran *Bauplan*: a review of the adaptive, developmental, and genetic underpinnings of frog and tadpole morphology. *Biological Reviews of the Cambridge Philosophical Society* **82** (1), 1-25.

HEAD, J. & POLLY, D. 2007. Dissociation of somatic growth from segmentation drives gigantism in snakes. *Biology Letters* **3** (3), 296-298.

HEIDEMAN, N.J. 1989. The adaptive significance of vertebral form in the pelvic regions of *Mabuya capensis* and *Acontias plumbeus* (Reptilia: Scincidae). *South African Journal of Zoology* **25** (2), 109-113.

HEIDEMAN, N.J.L., DANIELS, S.R., MASHININI, P.L., MOKONE, M.E., THIBEDI, M.L., HENDRICKS, M.G.J., WILSON, B.A. & DOUGLAS, R.M. 2008. Sexual dimorphism in the African legless skink subfamily Acontinae (Reptilia: Scincidae). *African Zoology* **43** (2), 192-201.

HERREL, A., SPITHOVEN, L., VAN DAMME, R. & DE VREE, F. 1999. Sexual Dimorphism in head shape of *Galliotia galloti*: Testing the niche divergence hypothesis by functional analysis. *Functional Ecology* **13** 289-297.

HERREL, A., MEYERS, J.J. & VANHOOYDONCK, B. 2001. Correlations between habitat use and body shape in a phrynosomatid lizard (*Urosaurus ornatus*): A population-level analysis. *Biological Journal of the Linnean Society* **74** 305-314.

HERREL, A., SCHAERLAEKEN, V., MEYERS, J.J., METZGER, K.A. & ROSS, C.F. 2007. The evolution of cranial design and performance in squamates: Consequences of skull-bone reduction on feeding behavior. *Integrative and Comparative Biology* **47** (1), 107-117.

HERREL, A., HUYGHE, K., VANHOOYDONCK, B., BACKELJAU, T., BREUGELMANS, K., GRBAC, I., VAN DAMME, R. & IRSCHICK, D.J. 2008. Rapid large-scale evolutionary divergence in morphology and performance associated with exploitation of a different dietary resource. *Proceedings of the National Academy of Sciences of the United States of America* **105** (12), 4792-4795.

HERREL, A. & MEASEY, G.J. 2010. The kinematics of locomotion in caecilians: effects of substrate and body shape. *Journal of Experimental Zoology. Part A, Ecological Genetics and Physiology* **313** (5), 301-309.

HEWITT, J. 1938. Descriptions of new forms of the genus acontias Lin. *Transactions of the Royal Society of South Africa* **26** (1), 39-48.

HIPSLEY, C.A., RENTINCK, M.N., RODEL, M.O. & MULLER, J. 2016. Ontogenetic allometry constrains cranial shape of the head-first burrowing worm lizard *Cynisca leucura* (Squamata: Amphisbaenidae). *Journal of Morphology* **277** (9), 1159-1167.

HOHL, L.d.S.L., DE CASTRO LOGUERCIO, M.F., SICURO, F.L., DE BARROS-FILHO, J.D. & ROCHA-BARBOSA, O. 2017. Body and skull morphometric variations between two shovel-headed species of Amphisbaenia (Reptilia: Squamata) with morphofunctional inferences on burrowing. *PeerJ* **5** e3581.

HULL, D.L., 1997. The ideal species concept-and why we can't get it. In: In. *Species: The Units of Biodiversity*, (Claridge, M.F., Dawah, H.A., Wilson, M.R., eds). pp 357-380. Chapman & Hall, London

HULL, D.L., 1999. On the plurality of species: questioning the party line. In: *Species: New Interdisciplinary Essays*, (Wilson, R.A., eds). pp 23–48. The MIT Press, Cambridge, Massachusetts

JANSE VAN VUUREN, L.C., 2009. A taxonomic review of the genus *Microacontias* (Scincidae: Acontinae) based on DNA and morphological data, Unpublished Master thesis. University of the Free State, p. 145.

JÖRGER, K.M. & SCHRÖDL, M. 2013. How to describe a cryptic species? Practical challenges of molecular taxonomy. *Frontiers in Zoology* **10** (1), 1-27.

KALIONTZOPOULOU, A., CARRETERO, M.A. & LLORENTE, G. 2008. Head shape allometry and proximate causes of head sexual dimophism in *Podarcis* lizards: joining linear and geometric morphometrics. *Biological Journal of the Linnean Society* **93** 111-124.

KALIONTZOPOULOU, A. 2011. Geometric morphometrics in herpetology: modern tools for enhancing the study of morphological variation in amphibians and reptiles. *Basic and Applied Herpetology* **25** 5-32.

KAZI, S. & HIPSLEY, C. 2018. Conserved evolution of skull shape in Caribbean head-first burrowing worm lizards (Squamata: Amphisbaenia). *Biological Journal of the Linnean Society* **125** (1), 14-29.

KEATES, C., CONRADIE, W., GREENBAUM, E. & EDWARDS, S. 2019. A snake in the grass: Genetic structuring of the widespread African grass snake (*Psammophylax* Fitzinger 1843), with the description of a new genus and a new species. *Journal of Zoological Systematics and Evolutionary Research* **57** (4), 1039-1066.

KELLEY, K.C., ARNOLD, S.J. & GLADSTONE, J. 1997. The effects of substrate and vertebral number on locomotion in the garter snake *Thamnophis elegans*. *Functional Ecology* **11** 189-198.

KLINGENBERG, C.P. 2011. MorphoJ: an integrated software package for geometric morphometrics. *Molecular Ecology Resources* **11** 353-357.

KREHENWINKEL, H., POMERANTZ, A., HENDERSON, J.B., KENNEDY, S.R., LIM, J.Y., SWAMY, V., SHOOBRIDGE, J.D., GRAHAM, N., PATEL, N.H., GILLESPIE, R.G. & PROST, S. 2019. Nanopore sequencing of long ribosomal DNA amplicons enables portable and simple biodiversity assessments with high phylogenetic resolution across broad taxonomic scale. *Gigascience* **8** (5), 1-16.

KULLENKAMPFF, K., VAN ZYL, F., KLAUS, S. & DANIELS, S.R. 2019. Molecular evidence for cryptic species in the common slug eating snake *Duberria lutrix lutrix* (Squamata, Lamprophiidae) from South Africa. *ZooKeys* **838** 133-154.

LAMB, T., BISWAS, S. & BAUER, A.M. 2010. A phylogenetic reassessment of African fossorial skinks in the subfamily Acontinae (Squamata: Scincidae): Evidence for parallelism and polyphyly. *Zootaxa* **2657** 33-46.

LE GUILLOUX, M., MIRALLES, A., MEASEY, G.J., VANHOOYDONCK, B., O'REILLY, J., LOWIE, A. & HERREL, A. 2020. Trade-offs between burrowing and biting force in fossorial scincid lizards? *Biological Journal of the Linnean Society* **130** 310-319.

LEE, M. 1998. Convergent evolution and character correlation in burrowing reptiles: towards a resolution of squamate relationships. *Biological Journal of the Linnean Society* **65** 369-453.

LEE, M.S.Y., SKINNER, A., CAMACHO, A. & PATTEN, M. 2013. The relationship between limb reduction, body elongation and geographical range in lizards (*Lerista*, Scincidae). *Journal of Biogeography* **40** (7), 1290-1297.

MAIN, D.C., JANSENVAN VUUREN, B. & TOLLEY, K.A. 2018. Cryptic diversity in the common flap-necked chameleon *Chamaeleo dilepis* in South Africa. *African Zoology* **53** (1), 11-16.

MAYER, W. & PAVLICEV, M. 2007. The phylogeny of the family Lacertidae (Reptilia) based on nuclear DNA sequences: convergent adaptations to arid habitats within the subfamily Eremiainae. *Molecular Phlogenetics and Evolution* **44** (3), 1155-1163.

MAYR, E., 1942. Systematics and the Origin of Species (Dunn, L.C., Clarke, H.T., Detwiler, S.R., Schrader, F., eds). pp 1-334. Columbia University Press, New York

MCBRAYER, L.B. & CORBIN, C., 2007. *Patterns of head shape variation in lizards: morphological correlates of foraging mode*. (Reilly, S.M., McBrayer, L.B., Miles, D.B., eds). pp 271-301. Cambridge University Press, Cambridge, United Kingdom

MCCOSKER, J.E. 2010. Deepwater Indo-Pacific species of the snake-eel genus *Ophichthus* (Anguilliformes: Ophichthidae), with descrition of nine new species. *Zootaxa* **2505** 1-39.

MEDINA, M.F., BAUER, A.M., BRANCH, W.R., SCHMITZ, A., CONRADIE, W., NAGY, Z.T., HIBBITTS, T.J., ERNST, R., PORTIK, D.M., NIELSEN, S.V., COLSTON, T.J., KUSAMBA, C., BEHANGANA, M., RODEL, M.O. & GREENBAUM, E. 2016. Molecular phylogeny of *Panaspis* and *Afroablepharus* skinks (Squamata: Scincidae) in the savannas of sub-Saharan Africa. *Molecular Phylogenetics and Evololution* **100** 409-423.

MEIER, R. & WILLMANN, R., 2000. *The Biological Species Concept*. In: Species Concepts and Phylogenetic Theory: A Debate, (Wheeler, Q., Meier, R., eds). pp 17-29. Columbia University Press, New York

MEIRI, S. 2018. Traits of lizards of the world: Variation around a successful evolutionary design. *Global Ecology and Biogeography* **27** (10), 1168-1172.

MENDES, R.G., 2017. Phylogeny and evolution of head shape in the Amphisbaenia (Reptilia: Squamata). Universidade de São Paulo.

MILLER, M.A., PFEIFFER, W. & SCHWARTZ, T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. *Proceedings of the Gateway Computing Environments Workshop (GCE), 14 Nov. 2010, New Orleans, LA* 1-8.

MILLER, S.E., HAUSMANN, A., HALLWACHS, W. & JANZEN, D.H. 2016. Advancing taxonomy and bioinventories with DNA barcodes. *Philosophical Transactions of the Royal Society of London. Series B. Biological Sciences* **371** (1702).

MISHLER, B.D. & WILKINS, J.S. 2018. The Hunting of the SNaRC: A Snarky Solution to the Species Problem. *Philosophy, Theory, and Practice in Biology* **10** (20200323).

MUCINA, L., RUTHERFORD, M.C. & POWRIE, L.W., 2006. *Vegetation Atlas of South Africa, Lesotho and Swaziland*. eds). pp 30-583 and 748-790. Strelitzia 19, Stellenbosch, South Africa

MULLER, J., SCHEYER, T.M., HEAD, J.J., BARRETT, P.M., WERNEBURG, I., ERICSON, P.G., POL, D. & SANCHEZ-VILLAGRA, M.R. 2010. Homeotic effects, somitogenesis and the evolution of vertebral numbers in recent and fossil amniotes. *Proceedings of the National Academy of Sciences of the United States of America* **107** (5), 2118-2123.

MULLER, J., HIPSLEY, C.A. & MAISANO, J.A. 2016. Skull osteology of the Eocene amphisbaenian *Spathorhynchus fossorium* (Reptilia, Squamata) suggests convergent evolution and reversals of fossorial adaptations in worm lizards. *Journal of Anatomy* **229** (5), 615-630.

NAVAS, C.A., ANTONIAZZI, M.M., CARVALHO, J.E., CHAUI-BERLINK, J.G., JAMES, R.S., JARED, C., KOHLSDORF, T., PAI-SILVA, M.D. & WILSON, R.S. 2004. Morphological and physiological specialization for digging in amphisbaenians, an ancient lineage of fossorial vertebrates. *Journal of Experimental Biology* **207** (Pt 14), 2433-2441.

OBJECT RESEARCH SYSTEMS (ORS) INC, 2018. Dragonfly 4.1, Montreal, Canada.

OPENSHAW, G.H. & KEOGH, J.S. 2014. Head shape evolution in monitor lizards (*Varanus*): interactions between extreme size disparity, phylogeny and ecology. *Journal of Evolutionary Biology* **27** (2), 363-373.

OXFORD NANOPORE TECHNOLOGIES, 2020, https://nanoporetech.com/products/minion, 23 December, 2020

PADIAL, J., MIRALLES, A., DE LA RIVA, I. & VENCES, M. 2010. The integrative future of taxonomy. *Frontiers in Zoology* **7** (16), 2-14.

PALUMBI, S., 1996. *Nucleic acids II: the polymerase chain reaction*. In: Molecular Systematics, 2nd edition (Hillis, D.M., Moritz, C., Mable, B.K., eds). pp 205-247. Sinauer Associates, Sunderland, MA.

PAPENFUSS, T.J. & PARHAM, J.F. 2013. Four New Species of California Legless Lizards (*Anniella*). *Breviora* **536** 1-17.

PIETERSEN, D.W., SCHOLTZ, C.H. & BASTOS, A.D.S. 2018. Multi-locus phylogeny of southern African *Acontias aurantiacus* (Peters) subspecies (Scincidae: Acontinae) confirms the presence of three genetically, geographically and morphologically discrete taxa. *Zootaxa* **4442** (3), 427-440.

POLLY, D., HEAD, J. & COHN, M. 2001. Testing modularity and dissociation: the evolution of regional proportions in snakes. *Beyond Heterochrony: The Evolution of Development* 307-335.

PORTILLO, F., BRANCH, W.R., TILBURY, C.R., NAGY, Z.T., HUGHES, D.F., KUSAMBA, C., MUNINGA, W.M., ARISTOTE, M.M., BEHANGANA, M. & GREENBAUM, E. 2019. A Cryptic New Species of *Polemon* (Squamata: Lamprophiidae, Aparallactinae) from the Miombo Woodlands of Central and East Africa. *Copeia* **107** (1), 22.

POUGH, F.H., ANDREWS, R.M., CADLE, J.E., CRUMP, M.L., SAVITZKY, A.H. & D., W.K., 2004. Herpetology. Third edition (Pough, F.H., eds). pp 726. Prentice-Hall, Upper Saddle River, NJ

PRÖTZEL, D., VENCES, M., SCHERZ, M.D., VIEITES, D. & GLAW, F. 2017. Splitting and lumping: An intergrative taxonomic assessment of Malagasy chameleons in the *Calumma guibei* complex results in the new species *C. gehringi* sp. nov. *Vertebrate Zoology* **67** (2), 231-249.

R CORE TEAM, 2020. R: A language and environment for statistical computing, *R Foundation for Statistical Computing*, Vienna, Austria.

REGAN, C.T. 1925. Organic Evolution. *Nature* **116** (2915), 398-401.

RICHARDSON, M.K., ALLEN, S.P., WRIGHT, G.M., RAYNAUD, A. & HANKEN, J. 1998. Somite number and vertebrate evolution. *Development* **125** 151-160.

ROHLF, F. 2017. TpsDig2©, v 2.31. *Free software. Ecology & Evolution, SUNY, Stony Brook University*.

ROHLF, F. 2018. TpsUtil©, v 1.76. *Free software. Ecology & Evolution, SUNY, Stony Brook University*.

RONQUIST, F., TESLENKO, M., VAN DER MARK, P., AYRES, D.L., DARLING, A., HOHNA, S., LARGET, B., LIU, L., SUCHARD, M.A. & HUELSENBECK, J.P. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61** (3), 539-542.

ROSENBLUM, E. 2006. Convergent Evolution and Divergent Selection:Lizards at the White Sands Ecotone. *The American Naturalist* **167** (1), 1-15.

RSTUDIO TEAM, 2020. RStudio: Integrated Development Environment for R. RStudio, PBC, Boston, MA, http://www.rstudio.com/.

SHERRATT, E., GOWER, D.J., KLINGENBERG, C.P. & WILKINSON, M. 2014. Evolution of Cranial Shape in Caecilians (Amphibia: Gymnophiona). *Evolutionary Biology* **41** (4), 528-545.

SHINE, R., BRANCH, W.R., HARLOW, P.S., WEBB, J.K., SHINE, T. & LANNOO, M.J. 2006. Biology of Burrowing Asps (Atractaspididae) from Southern Africa. *Copeia* **2006** (1), 103-115.

ŠMÍD, J., ENGELBRECHT, H., TAFT, J.M., TELFORD, N.S., MAKHUBO, B.G., BAUER, A.M. & TOLLEY, K.A. 2018. A contribution to the phylogeny and taxonomy of the *Pachydactylus weberi* group (Squamata: Gekkonidae): a case of intraspecific colour polymorphism confounding taxonomy. *African Journal of Herpetology* **67** (2), 113-126.

SOUTH AFRICAN NATIONAL BIODIVERSITY INSTITUTE (2006-2018). The Vegetation Map of South Africa Lesotho and Swaziland, Mucina, L., Rutherford, M.C., Powrie, L.W., (eds.), http://bgis.sanbi.org/Projects/Detail/186, Version 2018.

SPAWLS, S., HOWELL, K., HINKEL, H. & MENEGON, M., 2018. Field guide to East African reptiles. Second edition (Spawls, S., eds). pp 544. Bloomsbury Publishing, London, United Kingdom

STEPANOVA, N. & BAUER, A.M. 2021. Phylogenetic history influences convergence for a specialized ecology: comparative skull morphology of African burrowing skinks (Squamata; Scincidae). *BMC Ecology and Evolution* **21** (1), 86.

STRUCK, T.H., FEDER, J.L., BENDIKSBY, M., BIRKELAND, S., CERCA, J., GUSAROV, V.I., KISTENICH, S., LARSSON, K., LIOW, L.H., NOWAK, M.D., STEDJE, B., BACHMANN, L. & DIMITROV, D. 2018. Finding evolutionary processes hidden in cryptic species. *Trends in Ecology & Evolution,* **33** (3), 153-163.

TAFT, J.M., 2018. Phylogenetic assessment and historical biogeography of *Psammophis leightoni* complex, Unpublished Master Thesis. University of the Western Cape, p. 64.

TAMURA, K., STECHER, G., PETERSON, D., FILIPSKI, A. & KUMAR, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* **30** (12), 2725-2729.

TEMPLETON, A. 2001. Using pylogeographic analyses of gene trees to test species status and processes. *Molecular Ecology* **10** 779-791.

TRONTELJ, P. & FIŠER, C. 2009. Perspectives: Cryptic species diversity should not be trivialised. *Systematics and Biodiversity* **7** (1), 1-3.

UETZ, P., FREED, P. & HOŠEK, J., 2020, The Reptile Database, http://www.reptile-database.org, 5 February, 2021

VAN DAMME, R. & VANHOOYDONCK, B. 2002. Speed versus manoeuvrability: assocuation between vertebral number and habitat structure in lacertid lizards. *Journal of Zoology London* **258** 327-334.

VANHOOYDONCK, B., VAN DAMME, R. & AERTS, P. 2000. Ecomorphological correlates of habitat partitioning in Corsican lizards. *Functional Ecology* **14** 358-368.

VANHOOYDONCK, B., BOISTEL, R., FERNANDEZ, V. & HERREL, A. 2011. Push and bite: trade-offs between burrowing and biting in a burrowing skink (*Acontias percivali*). *Biological Journal of the Linnean Society* **102** 91-99.

VASCONCELOS, R., MONTERO-MENDIETA, S., SIMO-RIUDALBAS, M., SINDACO, R., SANTOS, X., FASOLA, M., LLORENTE, G., RAZZETTI, E. & CARRANZA, S. 2016. Unexpectedly High Levels of Cryptic Diversity Uncovered by a Complete DNA Barcoding of Reptiles of the Socotra Archipelago. *Public Library of Science* **11** (3), e0149985.

VOLUME GRAPHICS, https://www.volumegraphics.com/, May, 2019

WAGNER, P., BROADLEY, D.G. & BAUER, A.M. 2012. A New Acontine Skink from Zambia (Scincidae: *Acontias* Cuvier, 1817). *Journal of Herpetology* **46** (4), 494-502.

WATANABE, S., MILLER, M.J., AOYAMA, J. & TSUKAMOTO, K. 2011. Analysis of vertebral counts of the tropical anguillids, *Anguilla megastoma*, *A. obscura*, and *A. reinhardtii*, in the western South Pacific in relation to their possible population structure and phylogeny. *Environmental Biology of Fishes* **91** (3), 353-360.

WEINELL, J.L., PALUH, D.J., SILER, C.D. & BROWN, R.M. 2020. A New, Miniaturized Genus and Species of Snake (Cyclocoridae) from the Philippines. *Copeia* **108** (4), 907-923.

WIENS, J.J. & SLINGLUFF, J.L. 2001. How Lizards Turn into Snakes: A Phylogenetic Analysis of Body-Form Evolution in Anguid Lizards. *Evolution* **55** (11), 2303.

WIENS, J.J., BRANDLEY, M.C. & REEDER, T.W. 2006. Why Does a Trait Evolve Multiple Times within a Clade? Repeated Evolution of Snakelike Body Form in Squamate Reptiles. *Evolution* **60** (1), 123.

WOLLENBERG VALERO, K.C., MARSHALL, J.C., BASTIAANS, E., CACCONE, A., CAMARGO, A., MORANDO, M., NIEMILLER, M.L., PABIJAN, M., RUSSELLO, M.A., SINERVO, B., WERNECK, F.P., SITES, J.W., JR, WIENS, J.J. & STEINFARTZ, S. 2019. Patterns, Mechanisms and Genetics of Speciation in Reptiles and Amphibians. *Genes (Basel)* **10** (9).

WOLTERING, J. 2012. From Lizard to Snake; Behind the Evolution of an Extreme Body Plan. *Current Genomics* **13** 289-299.

WU, N.C., ALTON, L.A., CLEMENTE, C.J., KEARNEY, M.R. & WHITE, C.R. 2015. Morphology and burrowing energetics of semi-fossorial skinks (*Liopholis* spp.). *Journal of Experimental Biology* **218** (Pt 15), 2416-2426.

YANG, Z., 2014. Molecular Evolution: A Statistical Approach. (Yang, Z., eds). pp 366-377. Oxford University Press, Oxford, United Kingdom

ZACHOS, F., 2016. Species Concepts in Biology: Historical Development, Theoretical Foundations and Practical Relevance. eds). pp Springer Nature, Springer International Publishing AG Switzerland

ZACHOS, F. 2018. (New) Species concepts, species delimitation and the inherent limitations of taxonomy. *Journal of Genetics* **97** 811-815.

ZELDITCH, M.L., SWIDERSKI, D.L., SHEETS, H.D. & FINK, W.L., 2004. Geometric Morphometrics for Biologists: A Primer. (Zelditch, M.L., eds). pp 432

Elsevier Academic Press, London, United Kingdom

ZHAO, Z., VERDÚ-RICOY, J., MOHLAKOANA, S., JORDAAN, A., CONRADIE, W. & HEIDEMAN, N. 2019. Unexpected phylogenetic relationships within the world's largest limbless skink species (*Acontias plumbeus*) highlight the need for a review of the taxonomic status of *Acontias poecilus*. *Journal of Zoological Systematics and Evolutionary Research* **57** (2), 445-460.

ZINK, R.M. & KLICKA, J., 2018. *Species Concepts and Speciation Analysis*. In: Ornithology: Foundation, Analysis and Application, (Morrison, M.L., Rodewald, A.D., Voelker, G., Colón, M.R., Prather, J., eds). pp 39-60. Johns Hopkins University Press, Baltamore

Appendices

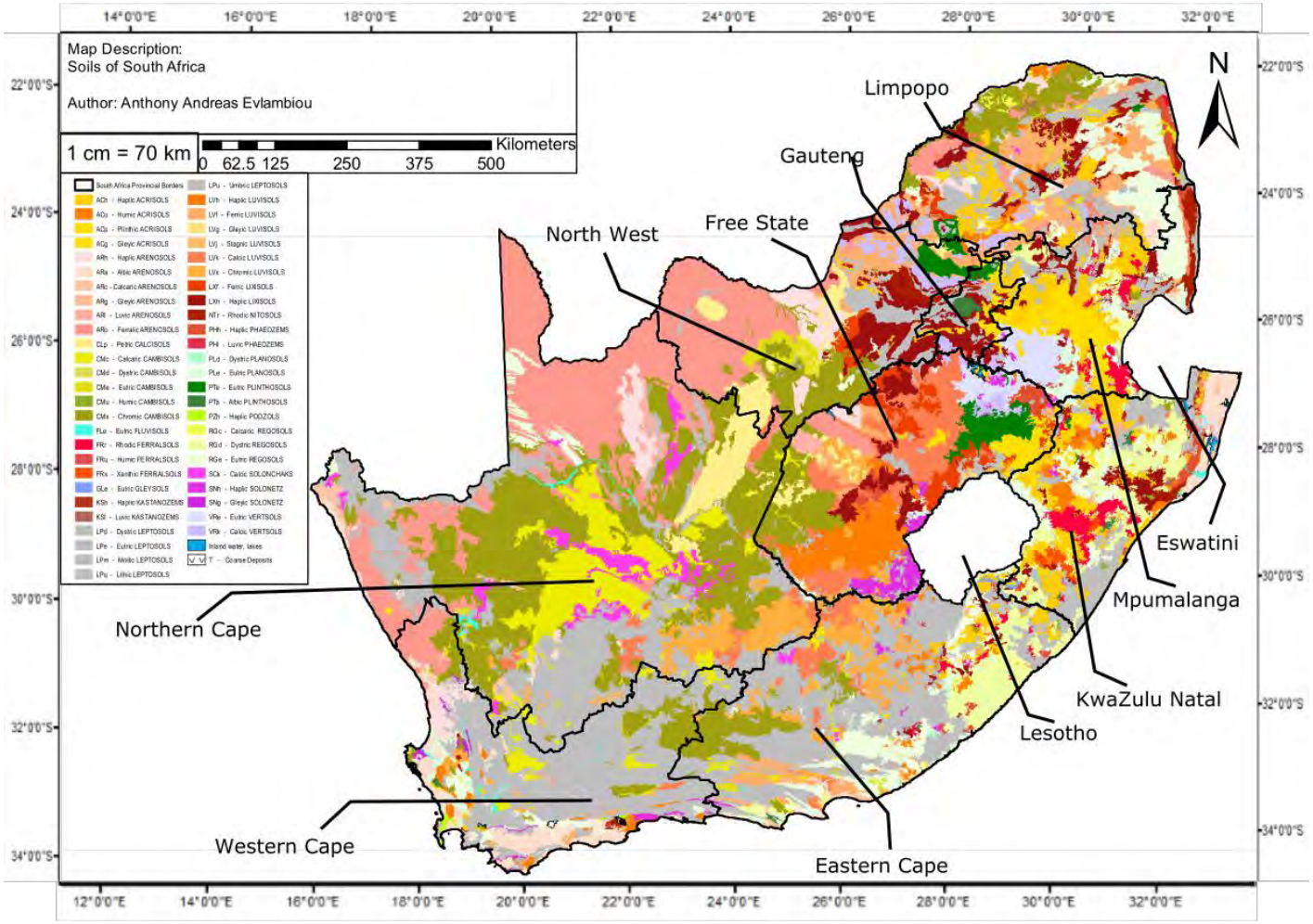


Figure A1: Map of the dominant soils found in South Africa. This study grouped the soils based on their top soil characteristics (Organic, Humic, Vertic, Melanic and Orthic; Fey *et al.* 2010). The soil abbreviations from this map are grouped into the before mentioned categories as follows: Organic- Gle; Humic- ACh, ACu, ACp, ACg, CMc, CMd, CMe, CMu, CMx, FRr, Fru, FRx, LVh, LVf, LVg, LVj, LVk, LVx, LXf, and LXh; Vertic- VRe, VRk, PHh, and PHl; Melanic – KSh, KSl, CLp, LPd, Lpe, LPm, LPq, LPu, and FLe; Orthic- ARa, ARc, ARg, ARh, ARi, ARo, NTr, PLd, PLe, PTe, PTa, PZh, RGc, RGd, RGe, SCk, SNh, and SNg.

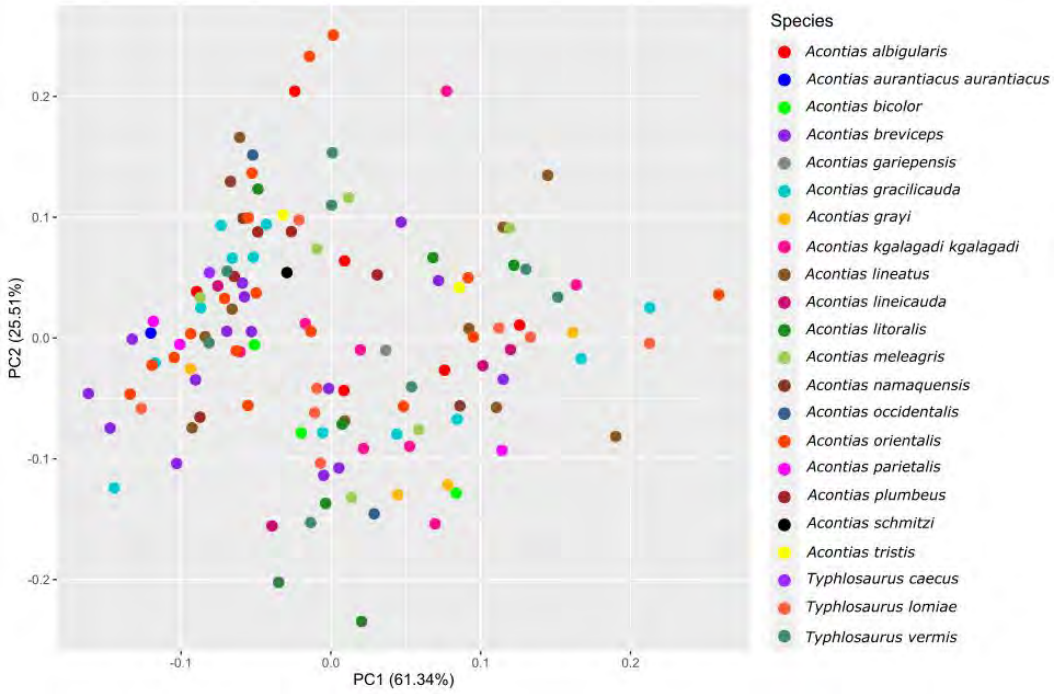


Figure A2: Plot of the first two principal components of *Acontias* and *Typhlosaurus* species from the lateral view. Note that *A. gariepensis* and *A. schmitzi* are represented by only one individual each. The polygons for the species are left out for this figure because there is too much overlap between them.

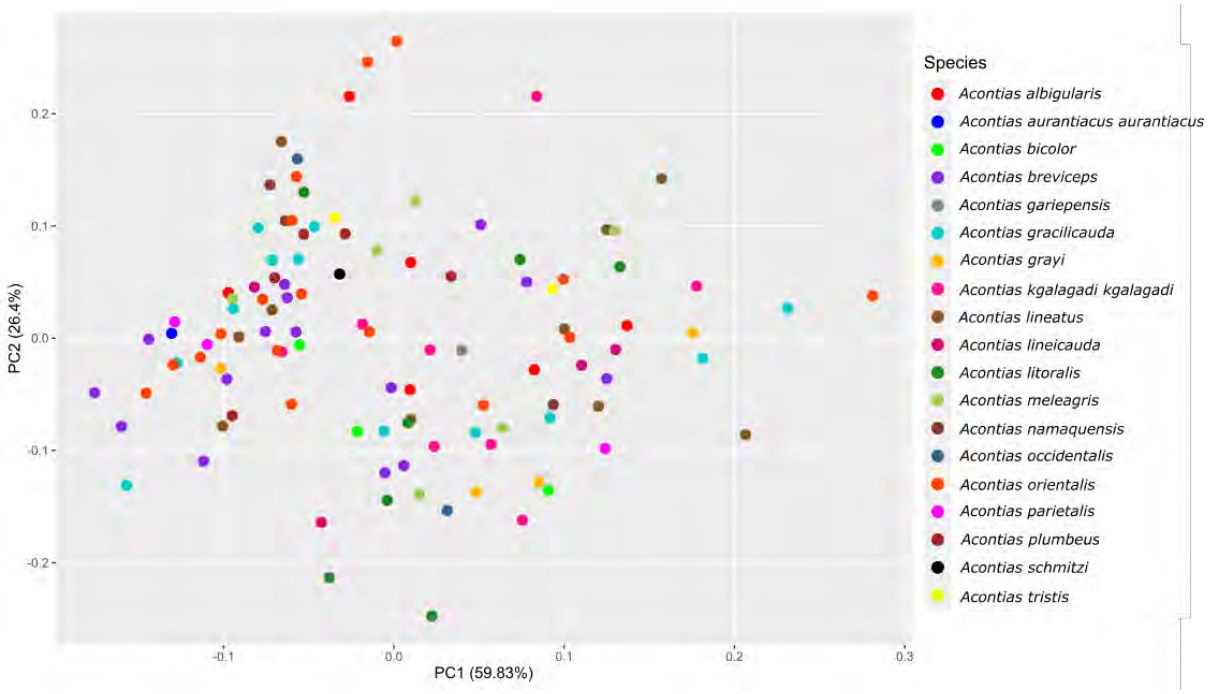


Figure A3: Plot of the first two principal components of *Acontias* species from the lateral view. Note that *A. gariepensis* and *A. schmitzi* are represented by only one individual each. The polygons for the species are left out for this figure because there is too much overlap between them.

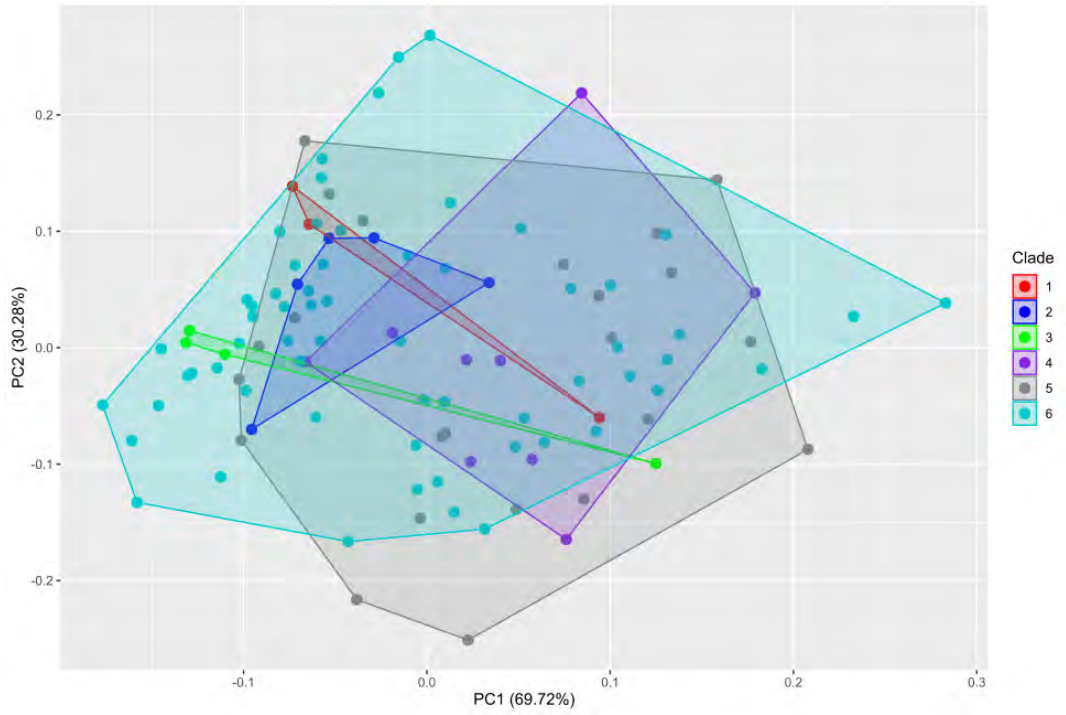


Figure A4: Plot of the first two principal components of *Acontias* phylogenetic clades from the lateral view. Clade colouration follows that of Figure 2.6 (Clade 1- *Acontias namaquensis*; Clade 2- *A. plumbeus*; Clade 3- *A. bicolor* and *A. a. aurantiacus*; Clade 4- *A. gariepensis, A. k. kgalagadi* and *A. schmitzi*; Clade 5- *A. grayi, A. lineatus, A. litoralis* and *A. tristis;* Clade 6- *A. albigularis, A. breviceps, A. gracilicauda, A. lineicauda, A. meleagris, A. occidentalis* and *A. orientalis*).

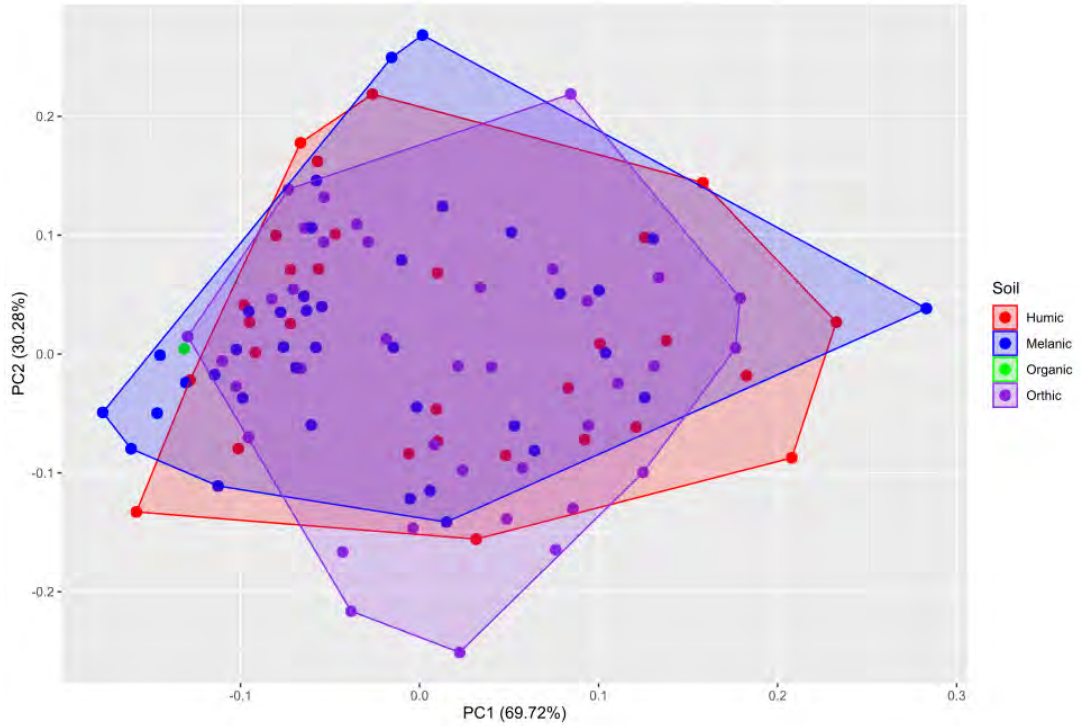


Figure A5: Plot of the first two principal components of lateral view PCA, showing how *Acontias* individuals fall into the different soil categories. *Acontias* species found in each soil type are as follows: Humic: *A. albigularis, A. gracilicauda, A. lineatus* and *A. occidentalis*; Melanic: *A. breviceps, A. meleagris* and *A. orientalis*; Organic: *A. a. aurantiacus*; Orthic: *A. gariepensis, A. grayi, A. lineicauda, A. litoralis, A. k. kgalagadi, A. namaquensis*, *A. parietalis, A. plumbeus* and *A. tristis* (Appendix Table A6).

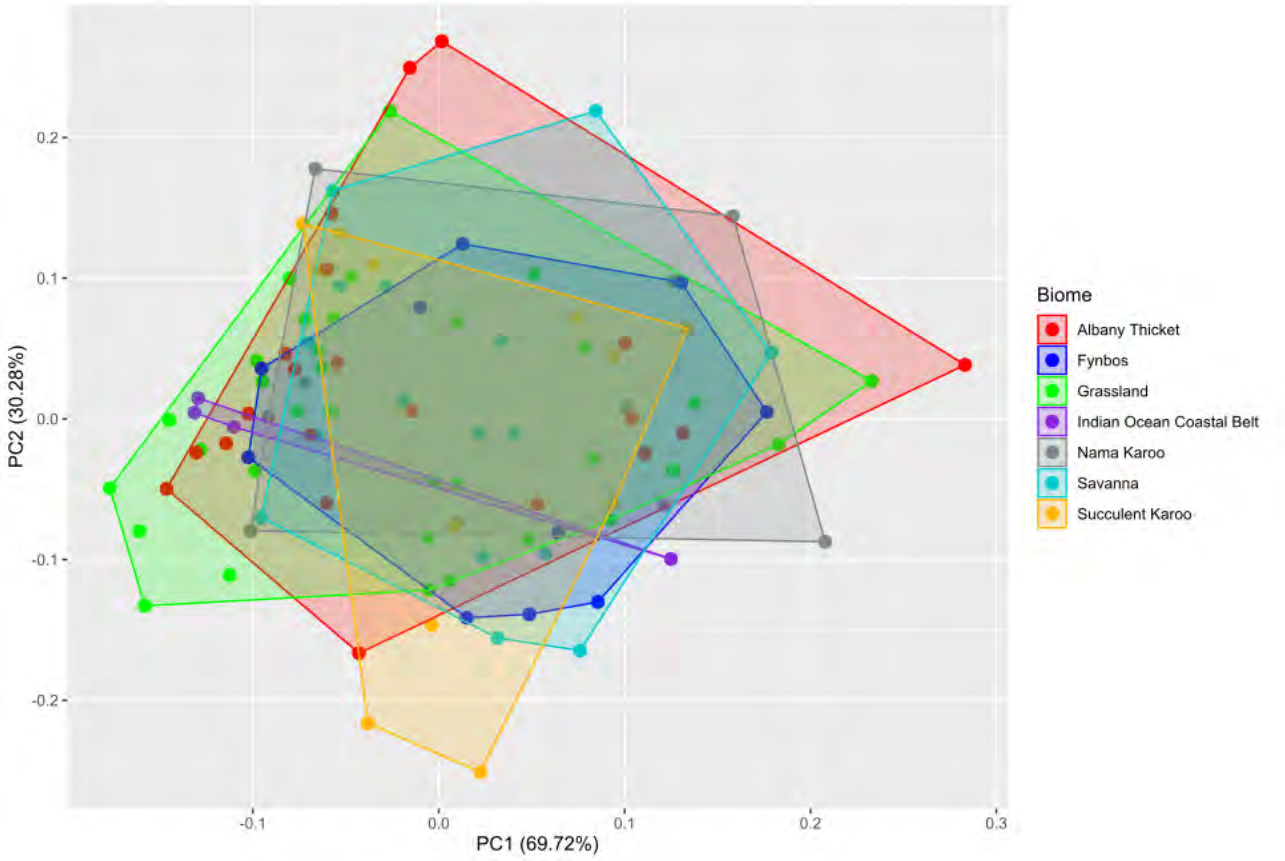


Figure A6: Plot of the first two principal components of lateral view PCA, showing how *Acontias* individuals fall into the different biome categories. *Acontias* species found in each biome are as follows: Albany Thicket: *A. lineicauda, A. orientalis;* Fynbos: *A. grayi* and *A. meleagris;* Grassland: *A. albigularis, A. gracilicauda* and *A. breviceps;* Indian Ocean Coastal Belt: *A. a. aurantiacus* and *A. parietalis;* Nama Karoo: *A. lineatus;* Savanna: *A. gariepensis, A. k. kgalagadi,* and *A. plumbeus;* Succulent Karoo: *A. litoralis, A. namaquensis, A. occidentalis* and *A. tristis* (Appendix Table A6).

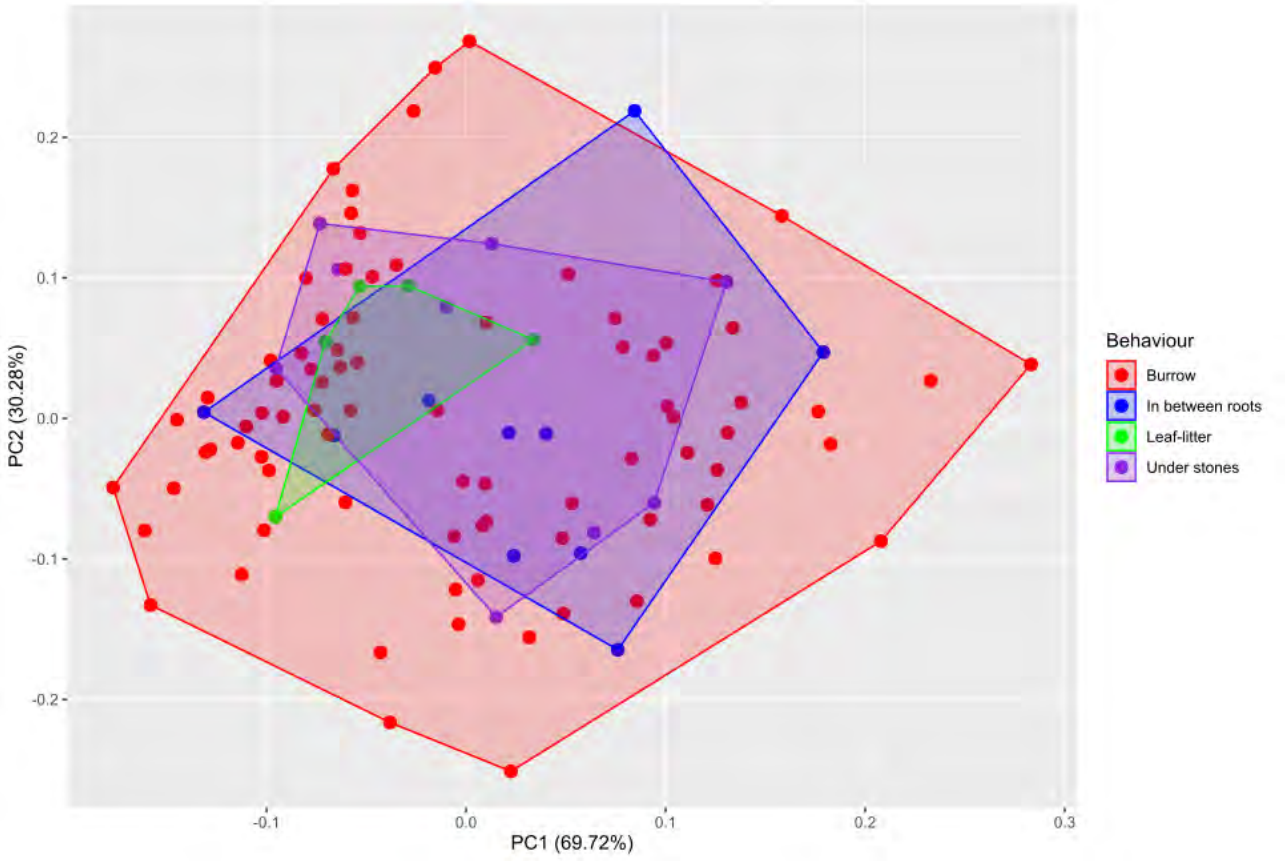


Figure A7: Plot of the first two principal components of lateral view PCA, showing how *Acontias* individuals fall into the different microhabitat categories. *Acontias* species found in each microhabitat are as follows: Burrow: *A. albigularis*, *A. breviceps*, *A. gracilicauda*, *A. grayi*, *A. lineatus*, *A. lineicauda*, *A. litoralis*, *A. occidentalis*, *A. orientalis* and *A. tristis;* In between roots: *A. a. aurantiacus*, *A. gariepensis* and *A. k. kgalagadi*; Leaf-litter: *A. plumbeus*; Under stones: *A. meleagris* and *A. namaquensis*.

Table A1: Short description of Biomes in South Africa

Biome	Precipitation	Vegetation	Soil
Desert	<70-80 mm annually	Sparse perennial vegetation	Very rocky substrate with little to no soil. Alluvial soil found close to the coast
Fynbos	±480 mm annually	Fynbos, Renosterveld and Strandveld	Heavy-textured with large fine-sand and silt factions
Nama-Karoo	Often unreliable 70-500 mm annually	Low shrubs intermixed with grasses, succulents, geophytes and annual forbes.	Range from red and yellow sands to non-swelling clays. Varies from deep, uniform, coarse-textured to shallow, sandy loam.
Succulent Karoo	Typically unimodal winter rainfall ±100-200 mm annually	Predominantly consists of succulents	Usually some sort of sandy soil variation with some more calcareous based. Largely fine-grained.
Forest	>525 mm with strong winter rain. >725 mm with strong summer rain.	Evergreen stands ranging from three to 30 m.	Varies is depth, water holding capacity and nutrient status. High clay soils often become waterlogged.
Albany Thicket	±200-900 mm annually	Often clumped and varies with landscape and local climate.	Vary from deep, well-structured to shallow, rocky, and unstructured
Grassland	±400-2500 mm annually	Graminoids dominate. Small to medium shrubs are rare or confined.	Usually deep, fertile soils but a wide range can occur.
Savanna	<200- ±1300 mm depending on the season. On average 500-750 mm outside of the extremes.	Grasses with sparsely spaced trees and shrubs.	Red-yellow well-drained soils lacking a strong texture and contrast. High clay content and swelling properties.
Indian Ocean Coastal Belt	±800-1300 mm	Mixture of trees, epiphytes and grasses which are dominant in different zones	Very heterogeneous; shallow, sandy, highly leached acidic soils, well-drained, acidic sands, and humic gleysols.

Table A2: List of the specimens used in the study

Species	Geometric morphometrics	Genetic material used
Acontias albigularis	n=6 PEM R16668, PEM R20660 PEM R16674, PEM R20662 PEM R05139, PEM R20657	n=3 PEM R20654 (16S; KT592575, Cytb; KT592629), PEM R20656 (16S; KT592577, Cytb; KT592631), PEM R20662 (16S; KT592585, Cytb; KT592639)
Acontias a. aurantiacus	n=2 PEM R21743, PEM R21742	n=2 PEM R21743 (16S; MG897347.1, Cytb; MG897318.1), PEM R21744 (16S; MG897348.1, Cytb; MG897319.1)
Acontias bicolor	n=3 PEM R05583, PEM R05582 PEM R05581	n=1 CAS209628 (16S; HQ180045.1, Cytb; HQ180099.1, RAG1; HQ180126.1)
Acontias breviceps	n=16 PEM R03050, PEM R21855 PEM R21854, PEM R18999 PEM R21856, PEM R21847 PEM R04232, PEM R06503 PEM R20253, PEM R21262 PEM R21852, PEM R22131 PEM R19000, PEM R03904 PEM R21848, PEM R18993	n=6 PEM R18999 (16S; KT592559, Cytb; KT592613), PEM R19001 (16S; KT592556, Cytb; KT592610), PEM R19010 (16S;KT592557, Cytb; KT592611), PEM R21848 (16S; LT999774.1, Cytb; LT999784.1), PEM R21855 (16S; LT999778.1, Cytb; LT999787.1), PEM R21856 (16S; LT999779.1, Cytb; LT999788.1)
Acontias gariepensis	n=1 PEM R09111	n=1 CAS214467 (16S; HQ180029.1, COI; HQ180056.1, Cytb; HQ180083.1, RAG1; HQ180110.1)
Acontias gracilicauda	n=14 PEM R01420, PEM R04129 PEM R04141, PEM R06413 PEM R08245, PEM R20214 PEM R22551, PEM R06512 PEM R17110, PEM R10202 PEM R07268, PEM R03660 PEM R02982, PEM R03815	n=3 ag5 (16S; KT592563, Cytb; KT592617), ag6 (16S; KT592572, Cytb KT592626), ag7 (16S; KT592573, Cytb; KT592627)

Species	Geometric morphometrics	Genetic material used
Acontias grayi	n=6 PEM R02034, PEM R02033 PEM R17803, PEM R18313 PEM R12936, PEM R17838	n=2 *Acontias grayi* 1 (16S; DQ249029.1, COI; DQ249074.1, Cytb; DQ249086.1), *Acontias grayi* 2 (16S; DQ249030.1, COI; DQ249075.1, Cytb; DQ249087.1)
Acontias k. kgalagadi	n=8 PEM R09105, PEM R17807 PEM R20474, PEM R17795 PEM R18574, PEM R16731 PEM R18632, PEM R18575	n=2 CAS206986 (16S; HQ180026.1, COI; HQ180053.1, Cytb; HQ180080.1, RAG1; HQ180107.1), KF263 (16S; this study, COI; this study, Cytb; this study)
Acontias lineatus	n=11 PEM R07649, PEM R16917 PEM R16722, PEM R12476 PEM R07668, PEM R11917 PEM R11921, PEM R12477 PEM R09292, PEM R04684 PEM R16916	n=3 CAS201850 (16S; HQ180032.1, COI; HQ180059.1 , Cytb; HQ180086.1, RAG1; HQ180113.1), *Acontias lineatus* 1 (16S; AY028874.1 , COI; AY028852.1, Cytb; DQ249088.1), *Acontias lineatus* 2 (16S; AY028875.1, COI; AY028853.1, Cytb; DQ249089.1)
Acontias lineicauda	n=5 PEM R24298 PEM R23203 PEM R23206 PEM R23204 PEM R23205	n=1 Aconline01 (16S, Cytb; HQ180091.1, RAG1; HQ180118.1)
Acontias litoralis	n=13 PEM R09422, PEM R16758 PEM R16459, PEM R18222 PEM R16724, PEM R15805 PEM R16729, PEM R16733 PEM R17822, PEM R18221 PEM R07172, PEM R16425 PEM R15804	n=3 CAS206800 (16S; HQ180030.1, COI; HQ180057.1 , Cytb; HQ180084.1, RAG1; HQ180111.1), *Acontias litoralis* A1 (16S; AY217945.1, Cytb; AY217791.1), *Acontias litoralis* 1 (Cytb; Q249092.1)
Acontias meleagris	n=9 PEM R08717, PEM R02008 PEM R02006, PEM R02007 PEM R01278, PEM R02013 PEM R01279, PEM R02014	n=3 Moss1 (16S; AY683721, COI; AY683774, Cytb; AY683799), Moss2 (16S; AY683722,

Species	Geometric morphometrics	Genetic material used
	PEM R02507	COI; AY683775, Cytb; AY683795), Moss3 (16S; AY683723, COI; AY683776, Cytb; AY683798)
Acontias namaquensis	n=3 PEM R18207, PEM R17806 PEM R16753	n=3 PEM R18218 (16S; KT592550.1, Cytb; KT592604.1), PEM R18207 (16S; KT592549.1, Cytb; KT592604.1), PEM R16753 (16S; HQ180033, COI; HQ180060, Cytb; HQ180087, RAG1; HQ180114)
Acontias occidentalis	n=2 PEM R12408, PEM R21034	n=3 *Acontias occidentalis* isolate Ao1 (16S; MH464264.1 , Cytb; MH488726.1), CAS209634 (16S; HQ180034.1, Cytb; HQ180088.1), *Acontias occidentalis* LV006 (16S; KT592590.1, Cytb; KT592644.1)
Acontias orientalis	n=18 PEM R06523, PEM R07286 PEM R10192, PEM R06569 PEM R09049, PEM R09050 PEM R06474, PEM R09045 PEM R09099, PEM R09043 PEM R09056, PEM R09046 PEM R09072, PEM R09055 PEM R09071, PEM R22224 PEM R22225, PEM R09057	n=2 AOR1 (16S; AY028881, COI; AY028857), AOR3 (16S; AY028883, COI; AY028859)
Acontias parietalis	n=3 PEM R16793, PEM R08389 PEM R08514	n=1 PEM R16794 (16S; HQ180043.1, Cytb; HQ180097.1, RAG1; HQ180124.1)
Acontias plumbeus	n=8 PEM R05915, PEM R17848 PEM R16472, PEM R17849 PEM R12076, PEM R09811 PEM R07139, PEM R07292	n=3 Aconplum05 (16S; HQ180040.1, Cytb; HQ180094.1, RAG1; HQ180121.1), Aconplum06 (16S; HQ180041.1, Cytb; HQ180095.1 , RAG1; HQ180122.), PEMR Field PJ2 (16S; MG897377.1, Cytb; MG897343.1)
Acontias schmitzi	n=1 PEM R22015	n=1 WC3680 (16S; TBA, COI; TBA, Cytb; TBA)

Species	Geometric morphometrics	Genetic material used
Acontias tristis	n=3 PEM R20054, PEM R02178 PEM R02179	n=3 *Acontias tristis* 1 (16S; AY028876.1, COI; AY028866.1, Cytb; DQ249090.1), *Acontias tristis* 2 (16S; AY028877.1, COI; AY028867.1, Cytb; DQ249091.1), NMBF36 (16S; HQ180031.1 , COI; HQ180058.1, Cytb; HQ180085.1 , RAG1; HQ180112.1)
Typhlosaurus caecus	n=2 PEM R17794, PEM R06402	n=2 CAS207015 (16S; HQ180023.1 , COI; HQ180050.1 , Cytb; HQ180077.1, RAG1; HQ180104.1), CAS207011 (16S; HQ180129.1 , COI; HQ180132.1, Cytb; HQ180135.1 , RAG1; HQ180138.1), *Typhlosaurus caecus* A3 (16S; AY217947.1 , Cytb; AY217793.1)
Typhlosaurus lomiae	n=8 PEM R17890, PEM R17892 PEM R17893, PEM R17895 PEM R17896, PEM R17897 PEM R17899, PEM R18229	n=1 CAS206870 (16S; HQ180022.1, COI; HQ180049.1 Cytb; HQ180076.1, RAG1; HQ180103.1)
Typhlosaurus vermis	n=15 PEM R18216, PEM R06128 PEM R16725, PEM R17836 PEM R17832, PEM R16449 PEM R18215, PEM R17799 PEM R03485, PEM R15803 PEM R02377, PEM R15809 PEM R18227, PEM R09416 PEM R16446	n=1 CAS206853 (16S; HQ180021.1, COI, HQ180048.1, Cytb; HQ180075.1, Rag1; HQ180102.1)

Table A3: List of primer details used in this study. Key to column headings: T_{AN} = Annealing temperature used in the PCRs

Gene region name	Primer name	Forward (Fwd)/ Reverse (Rev)	Primer sequence	T_{AN} (^{o}C)	Primer source
16S	16Sa:L2510	Fwd	5"-CGCCTGTTTATCAAAAACAT-3"	50	(Palumbi 1996)
	16Sb:H3080	Rev	5"CCGGTCTGAACTCAGATCACGT-3"	50	
Cytb	WWF	Fwd	5"-AAAYCAYCGTTGTWATTCAACTAC−3"	48	(Whiting, Bauer, &
	Cytb-R2	Rev	5"-GGGTGRAAKGGRATTTTATC-3"	48	Sites, 2003)
CO1	LBF	Fwd	5"-CTGCAGGAGGAGGAGATCAACA-3"	56	(Lamb *et al.*
	LBR	Rev	5"-GTCTGGGTAGTCTGATCGTCGTGGTAT-3"	56	2010)
RAG1	RAG1-f0	Fwd	5"-AAAGGGCTACATCCTGG-3"	49	(Mayer and
	RAG1-R1	Rev	5"-AAAATCTGCCTTCCTGTTATTG-3"	49	Pavlicev 2007)

Table A4: Explanation of landmarks used in the dorsal and lateral morphometric analysis

Landmark #	Description
	Dorsal view
1	Where the upper labial next to the rostrum meets the neighbouring upper labial.
2	Where the rostral scale meets the neighbouring upper labial.
3	Midway along the edge of the rostral scale
4	Most forward point of the snout
5	Midway along the edge of the rostral scale, opposite landmark 3
6	Where the rostral scale meets the neighbouring upper labial.
7	Where the upper labial next to the rostrum meets the neighbouring upper labial. Mirror of landmark 1
8	Where the supraoccular and supracilliary meet; moved to the outer edge of the image.
9	Where the rear of the upper labial meets the outline of the specimen.
10	Where the point of the interparietal meets both parietals.
11	Where the rear of the upper labial meets the outline of the specimen. Mirrors landmark nine.
12	Where the supraoccular and supracilliary meet; moved to the outer edge of the image. Mirrors landmark 8.
13 & 14	Where the prefrontal, supraoccular and frontal scales join.
	Lateral view
1	The most front part of the snout
2	Midway along the rostral scale
3	Where the rostral scale meets the prefrontal scale
4	Where the most rear of the parietal scale meets the outline of the image.
5	Where the most rear upper labial and lower labial meet.
6	Where the rostral scale and mental touch when the mouth is closed.

Table A5: Basic key indicating identifying features of the topsoil categories according to Fey *et al.* (2010)

Topsoil groups	Concepts	Diagnostic horizon or material for identification
Organic	Wetland or montane peat	Organic O
Humic	Rich in humus; drains freely; low base status	Humic A
Vertic	Swelling, cracking clay	Vertic A
Melanic	Black, structured clay; high base status	Melanic A
Orthic	Not classified as the other four topsoils	Orthic A

Table A6: Biome, soil and microhabitat category allocation for *Acontias*

Species	Biome cat.	Soil cat.	Microhabitat cat.	Clade #
Acontias albigularis	Grassland	Humic	Burrow	6
A. a. aurantiacus	Indian Ocean Coastal Belt	Organic	In between roots	3
A. breviceps	Grassland	Melanic	Burrow	6
A. gariepensis	Savanna	Orthic	In between roots	4
A. gracilicauda	Grassland	Humic	Burrow	6
A. grayi	Fynbos	Orthic	Burrow	5
A. k. kgalagadi	Savanna	Orthic	In between roots	4
A. lineatus	Nama Karoo	Humic	Burrow	5
A. lineicauda	Albany Thicket	Orthic	Burrow	6
A. litoralis	Succulent Karoo	Orthic	Burrow	5
A. meleagris	Fynbos	Melanic	Under stones	6
A. namaquensis	Succulent Karoo	Orthic	Under stones	1
A. occidentalis	Savanna	Humic	Burrow	6
A. orientalis	Albany Thicket	Melanic	Burrow	6
A. parietalis	Indian Ocean Coastal Belt	Orthic	Burrow	3
A. plumbeus	Savanna	Orthic	Leaf-litter	2
A tristis	Succulent Karoo	Orthic	Burrow	5

Table A7: A brief description of the microhabitat categories for *Acontias.*

Microhabitat category	Description
Burrow	Species that are often found in burrows in the substrate
Leaf-litter	Species that are often found in the leaf-litter and not in the soil
In between roots	Species that are often found burrowing in between the roots of plants and not in the open soil
Under stones	Species that are often found when turning over objects such as stones

Table A8: Post hoc results Tukey"s test: PC1 (left) and PC2 (right) for phylogenetic "Clade" category for *Acontias* for dorsal view.

	Clade #	1	2	3	4	5	6
1	Clade 1		NS	NS	NS	NS	NS
2	Clade 2	NS		NS	NS	NS	NS
3	Clade 3	*	***		NS	NS	NS
4	Clade 4	***	***	NS		NS	NS
5	Clade 5	***	***	*	NS		NS
6	Clade 6	NS	NS	***	***	***	

	Clade #	1	2	3	4	5	6
1	Clade 1		NS	NS	NS	NS	NS
2	Clade 2	NS		NS	NS	NS	NS
3	Clade 3	**	***		NS	NS	NS
4	Clade 4	**	***	NS		NS	NS
5	Clade 5	**	***	NS	NS		NS
6	Clade 6	NS	*	***	***	***	

Table A9: Results of the intercept analysis for the soil and top three biome types of *Acontias* species. The bold text indicates the category used for the species.

Species	Soil	Biome
A. albigularis	**Humic 73.25%**	Forest 3.62%
	Melanic 5.92%	**Grassland 76.49**
	Orthic 20.84%	Savanna 19.89%
A. breviceps	Humic 13.57%	Albany Thicket 13.53%
	Melanic 80.21%	**Grassland 59.64%**
	Orthic 6.22%	Nama-Karoo 17.38%
A. gariepensis	**Orthic 96.89%**	Nama-Karoo 5.53%
		Savanna 87.83%
A. gracilicauda	**Humic 51.45 %**	Albany Thicket 7.24%
	Melanic 21.80%	**Grassland 71.24%**
	Orthic 18.75%	Savanna 10.61%
	Vertic 7.04%	
A. grayi	Humic 0.03%	**Fynbos 69.15%**
	Melanic 0.09%	Succulent Karoo 28.74%
	Orthic 96.24%	
A. k. kgalagadi	Humic 9.65%	Grassland 0.05%
	Melanic 1.91%	Nama-Karoo 9.63%
	Orthic 85.74%	**Savanna 86.06%**
A. lineatus	**Humic 56.72%**	**Nama-Karoo 58.50%**
	Melanic 19.90%	Savanna 5.56%
	Orthic 18.62%	Succulent Karoo 23.68%
A. lineicuada	Humic 30.73%	**Albany Thicket 70.41%**
	Melanic 3.32%	Fynbos 6.83%
	Orthic 57.73%	Savanna 11.18%
A. litoralis	Humic 1.49%	Desert 1.78%
	Melanic 1.16%	Fynbos 19.07%
	Orthic 95.21%	**Succulent Karoo 75.27%**
A. meleagris	Humic 10.67%	Forest 0.06%
	Melanic 43.12%	**Fynbos 84.59%**
	Orthic 35.57%	Succulent Karoo 8.08%
A. namaquensis	Humic 4.84%	Fynbos 3.62%
	Melanic 45.56%	Nama-Karoo 2.38%
	Orthic 49.49%	**Succulent Karoo 93.26%**
A. occidentalis	**Humic 50.77%**	Grassland 8.39%
	Melanic 22.47%	Nama-Karoo 11.15%
	Orthic 22.65%	**Savanna 78.91%**
A. orientalis	Humic 27.74%	**Albany Thicket 27.72%**
	Melanic 53.80%	
	Orthic 17.15%	Fynbos 19.91%
		Nama-Karoo 26.75%
A. parietalis	**Orthic 97.33%**	Forest 0.20%
		IOCB 48.18%
		Savanna 37.61%
A. plumbeus	Humic 31.80%	Grassland 20.42%
	Melanic 15.10%	IOCB 4.35%
	Orthic 38.29%	Savanna 71.89%
A. tristis	Humic 7.28%	Desert 13.42%
	Melanic 33.82%	Nama-Karoo 11.14%
	Orthic 43.66%	**Succulent Karoo 56.96%**

Table A10: Post hoc results Tukey"s test for PC 1 (left) and PC 2 (right) with regards to soil type for *Acontias* species dorsal view.

	Soil category	1	2	3	4
1	Humic		NS	NS	NS
2	Melanic	NS		NS	NS
3	Organic	NS	NS		NS
4	Orthic	***	***	NS	

	Soil category	1	2	3	4
1	Humic		NS	NS	NS
2	Melanic	***		NS	NS
3	Organic	NS	**		NS
4	Orthic	NS	***	NS	

Table A11: Post hoc results Tukey"s test for PC1 (left) and PC 2 (right) for "Biome" category for *Acontias* species for dorsal view.

Biome category	1	2	3	4	5	6	7
Albany Thicket		NS	NS	NS	NS	NS	NS
Fynbos	NS		NS	NS	NS	NS	NS
Grassland	***	***		NS	NS	NS	NS
IOCB	NS	NS	***		NS	NS	NS
Nama-Karoo	***	***	***	NS		NS	NS
Savanna	NS	NS	***	NS	***		NS
Succulent Karoo	***	**	***	NS	NS	***	

	Biome category	1	2	3	4	5	6	7
1	Albany Thicket		NS	NS	NS	NS	NS	NS
2	Fynbos	NS		NS	NS	NS	NS	NS
3	Grassland	NS	NS		NS	NS	NS	NS
4	IOCB	***	NS	**		NS	NS	NS
5	Nama-Karoo	**	NS	*	NS		NS	NS
6	Savanna	NS	NS	NS	*	NS		NS
7	Succulent Karoo	***	NS	**	NS	NS	NS	

Table A12: Post hoc results Tukey"s test: PC1 and PC2 for "Microhabitat" category for *Acontias* species for dorsal view.

	Microhabitat category	1	2	3	4
1	Burrow		NS	NS	NS
2	In between roots	*		NS	NS
3	Under stones	NS	**		NS
4	Leaf-litter	NS	**	NS	

	Microhabitat category	1	2	3	4
1	Burrow		NS	NS	NS
2	In between roots	**		NS	NS
3	Under stones	NS	***		NS
4	Leaf-litter	***	***	NS	

Table A13: List of individuals used in vertebral count and morphological measurements

Species	Vertebral Count
Acontias albigularis	n=10 PEM R05139, PEM R16674, PEM R20650, PEM R20654, PEM R20656, PEM R20657, PEM R20658, PEM R20660, PEM R20661, PEM R20662
Acontias a. aurantiacus	n=2 PEM R21742, PEM R21743
Acontias bicolor	n=3 PEM R05581, PEM R05582, PEM R05583
Acontias breviceps	n=10 PEM R03049, PEM R06558, PEM R18694, PEM R18912, PEM R18994, PEM R19385, PEM R20253, PEM R21262, PEM R21855, PEM R21856
Acontias gracilicauda	n=10 PEM R01420, PEM R02982, PEM R03815, PEM R06413, PEM R17110, PEM R20214, PEM R20939, PEM R20941, PEM R21295, PEM R21296
Acontias grayi	n=6 PEM R02033, PEM R02034, PEM R12936, PEM R17803, PEM R17838, PEM R18313
Acontias k. kgalagadi	n=7 PEM R16731, PEM R17795, PEM R17807, PEM R 18574, PEM R18575, PEM R18632, PEM R20474
Acontias lineatus	n=9 PEM R07649, PEM R 07668, PEM R11917, PEM R 11921, PEM R 12476, PEM R12477, PEM R 16722, PEM R16916, PEM R16917
Acontias lineicauda	n=4 PEM R23203, PEM R23204, PEM R23205, PEM R24298
Acontias litoralis	n=10 PEM R07172, PEM R09422, PEM R15804, PEM R15805, PEM R16425, PEM R16459, PEM R 16724, PEM R16729, PEM R16733, PEM R18221
Acontias meleagris	n=10 PEM R01260, PEM R01279, PEM R02006, PEM R02008, PEM R02013, PEM R02507, PEM R02508 PEM R03285, PEM R03588, PEM R8118
Acontias namaquensis	n=3 PEM R16753, PEM R17806, PEM R18207
Acontias occidentalis	n=2 PEM R12408, PEM R21034
Acontias orientalis	n=6 PEM R06474, PEM R06523, PEM R09043, PEM R09055, PEM R10192, PEM R22225
Acontias parietalis	n=1 PEM R08389
Acontias plumbeus	n=6 PEM R05915, PEM R07292, PEM R09811, PEM R12076, PEM R16472, PEM R17848
Acontias schmitzi	n=1 PEM R22015
Acontias tristis	n=3 PEM R02178, PEM R02179, PEM R20054

Table A14: Post hoc results for the different absolute total vertebral count values of *Acontias* species. The level of significance is measured as follows: a single asterisk (*) equals low significance. Double asterisk equals (**) moderately significant, and triple asterisk (***) equals highly significant.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*A. albigularis*		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
2	*A. a. aurantiacus*	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
3	*A. bicolor*	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
4	*A. breviceps*	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
5	*A. gracilicauda*	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
6	*A. grayi*	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
7	*A. k. kgalagadi*	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
8	*A. lineatus*	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
9	*A. lineicauda*	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS
10	*A. litoralis*	NS	NS	*	NS	NS	NS	**	***	NS		NS	NS	NS	NS	NS	NS	NS	NS
11	*A. meleagris*	NS	NS	NS	NS	*	NS	NS	NS	NS	***		NS	NS	NS	NS	NS	NS	NS
12	*A. namaquensis*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS
13	*A. occidentalis*	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	NS		NS	NS	NS	NS	NS
14	*A. orientalis*	NS	NS	NS	NS	NS	NS	NS	*	NS	***	NS	NS	NS		NS	NS	NS	NS
15	*A. parietalis*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS
16	*A. plumbeus*	NS	NS	NS	NS	NS	NS	*	**	NS	NS	*	NS	NS	NS	NS		NS	NS
17	*A.schmitzi*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS
18	*A. tristis*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	

Table A15: Post hoc results for the different absolute trunk vertebral count values of *Acontias* species. The level of significance is measured as follows: a single asterisk (*) equals low significance. Double asterisk equals (**) moderately significant, and triple asterisk (***) equals highly significant.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*A. albigularis*		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
2	*A. a. aurantiacus*	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
3	*A. bicolor*	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
4	*A. breviceps*	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
5	*A. gracilicauda*	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
6	*A. grayi*	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
7	*A. k. kgalagadi*	**	NS	NS	NS	**	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
8	*A. lineatus*	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
9	*A. lineicauda*	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS
10	*A. litoralis*	NS	NS	**	NS	NS	NS	***	***	NS		NS	NS	NS	NS	NS	NS	NS	NS
11	*A. meleagris*	**	NS	NS	NS	**	NS	NS	NS	NS	***		NS	NS	NS	NS	NS	NS	NS
12	*A. namaquensis*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS
13	*A. occidentalis*	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS
14	*A. orientalis*	NS	NS	NS	NS	**	NS	NS	NS	NS	**	NS	NS	NS		NS	NS	NS	NS
15	*A. parietalis*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS
16	*A. plumbeus*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS
17	*A.schmitzi*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS
18	*A. tristis*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	

Table A16: Post hoc results for the different absolute tail vertebral count values of *Acontias* species. The level of significance is measured as follows: a single asterisk (*) equals low significance. Double asterisk equals (**) moderately significant, and triple asterisk (***) equals highly significant.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*A. albigularis*		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
2	*A. a. aurantiacus*	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
3	*A. bicolor*	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
4	*A. breviceps*	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
5	*A. gracilicauda*	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
6	*A. grayi*	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
7	*A. k. kgalagadi*	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
8	*A. lineatus*	NS	NS	NS	NS	NS	*	***		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
9	*A. lineicauda*	NS	NS	NS	NS	NS	NS	NS	**		NS	NS	NS	NS	NS	NS	NS	NS	NS
10	*A. litoralis*	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS
11	*A. meleagris*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS
12	*A. namaquensis*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS
13	*A. occidentalis*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS
14	*A. orientalis*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS
15	*A. parietalis*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS
16	*A. plumbeus*	*	NS	NS	NS	NS	NS	NS	***	NS	NS	NS	NS	NS	NS	NS		NS	NS
17	*A.schmitzi*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS
18	*A. tristis*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	

Table A17: Post hoc results for the different absolute value of the ratio between the number of trunk vertebrae and the number of tail vertebrae of *Acontias* species. The level of significance is measured as follows: a single asterisk (*) equals low significance. Double asterisk equals (**) moderately significant, and triple asterisk (***) equals highly significant.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*A. albigularis*		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
2	*A. a. aurantiacus*	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
3	*A. bicolor*	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
4	*A. breviceps*	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
5	*A. gracilicauda*	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
6	*A. grayi*	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
7	*A. k. kgalagadi*	**	NS	NS	NS	*	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
8	*A. lineatus*	NS	NS	NS	NS	NS	NS	***		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
9	*A. lineicauda*	**	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS
10	*A. litoralis*	NS	NS	NS	NS	NS	NS	***	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS
11	*A. meleagris*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS
12	*A. namaquensis*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS
13	*A. occidentalis*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS
14	*A. orientalis*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS
15	*A. parietalis*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS
16	*A. plumbeus*	**	NS	NS	NS	NS	NS	NS	**	NS	***	NS	NS	NS	NS	NS		NS	NS
17	*A.schmitzi*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS
18	*A. tristis*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	

Table A18: Post hoc results for the different absolute total (left) and trunk (right) vertebral count of *Acontias* Clades. The level of significance is measured as follows: a single asterisk (*) equals low significance. Double asterisk equals (**) moderately significant, and triple asterisk (***) equals highly significant.

	Clade #	1	2	3	4
1	Clade 1		NS	NS	NS
2	Clade 2	NS		NS	NS
3	Clade 3	NS	NS		NS
4	Clade 4	NS	NS	NS	

	Clade #	1	2	3	4
1	Clade 1		NS	NS	NS
2	Clade 2	NS		NS	NS
3	Clade 3	NS	NS		NS
4	Clade 4	NS	NS	NS	

Table A19: Post hoc results for the different absolute tail (left) and ratio between trunk and tail (right) vertebral count of *Acontias* Clades. The level of significance is measured as follows: a single asterisk (*) equals low significance. Double asterisk equals (**) moderately significant, and triple asterisk (***) equals highly significant.

	Clade #	1	2	3	4
1	Clade 1		NS	NS	NS
2	Clade 2	NS		NS	NS
3	Clade 3	NS	**		NS
4	Clade 4	NS	*	NS	

	Clade #	1	2	3	4
1	Clade 1		NS	NS	NS
2	Clade 2	NS		NS	NS
3	Clade 3	NS	**		NS
4	Clade 4	NS	NS	NS	

Table A20: Post hoc results of the absolute total vertebral number (left) and absolute trunk vertebral number (right) with regards to soil type. The level of significance is measured as follows: a single asterisk (*) equals low significance. Double asterisk equals (**) moderately significant, and triple asterisk (***) equals highly significant.

	Soil category	1	2	3	4
1	Humic		NS	NS	NS
2	Melanic	NS		NS	NS
3	Orthic	NS	*		NS
4	Organic	NS	***	NS	

	Soil category	1	2	3	4
1	Humic		NS	NS	NS
2	Melanic	**		NS	NS
3	Orthic	NS	*		NS
4	Organic	NS	**	NS	

Table A21: Post hoc results of the absolute tail vertebral number (left) and ratio of trunk:tail vertebral number (right) with regards to soil type. The level of significance is measured as follows: a single asterisk (*) equals low significance. Double asterisk equals (**) moderately significant, and triple asterisk (***) equals highly significant.

	Soil category	1	2	3	4
1	Humic		NS	NS	NS
2	Melanic	*		NS	NS
3	Orthic	***	NS		NS
4	Organic	NS	NS	NS	

	Soil category	1	2	3	4
1	Humic		NS	NS	NS
2	Melanic	**		NS	NS
3	Orthic	NS	NS		NS
4	Organic	**	NS	NS	

Table A22: Post hoc results of the absolute total vertebral number (left) and absolute trunk vertebral number (right) with regards to biome type. The level of significance is measured as follows: a single asterisk (*) equals low significance. Double asterisk equals (**) moderately significant, and triple asterisk (***) equals highly significant.

	Biome category	1	2	3	4	5	6	7
1	Albany Thicket		NS	NS	NS	NS	NS	NS
2	Fynbos	NS		NS	NS	NS	NS	NS
3	Grassland	NS	*		NS	NS	NS	NS
4	IOCB	NS	*	NS		NS	NS	NS
5	Nama-Karoo	NS	NS	**	**		NS	NS
6	Savanna	NS	NS	NS	NS	NS		NS
7	Succulent Karoo	NS	**	NS	NS	***	NS	

	Biome category	1	2	3	4	5	6	7
1	Albany Thicket		NS	NS	NS	NS	NS	NS
2	Fynbos	NS		NS	NS	NS	NS	NS
3	Grassland	NS	**		NS	NS	NS	NS
4	IOCB	NS	NS	NS		NS	NS	NS
5	Nama-Karoo	NS	NS	NS	NS		NS	NS
6	Savanna	NS	NS	**	NS	NS		NS
7	Succulent Karoo	**	***	NS	NS	**	***	

Table A23: Post hoc results of the absolute tail vertebral number (left) and ratio of trunk:tail vertebral number (right) with regards to biome type. The level of significance is measured as follows: a single asterisk (*) equals low significance. Double asterisk equals (**) moderately significant, and triple asterisk (***) equals highly significant.

	Biome category	1	2	3	4	5	6	7
1	Albany Thicket		NS	NS	NS	NS	NS	NS
2	Fynbos	NS		NS	NS	NS	NS	NS
3	Grassland	NS	NS		NS	NS	NS	NS
4	IOCB	NS	NS	NS		NS	NS	NS
5	Nama-Karoo	***	**	**	**		NS	NS
6	Savanna	NS	**	***	NS	***		NS
7	Succulent Karoo	NS	NS	NS	NS	NS	***	

	Biome category	1	2	3	4	5	6	7
1	Albany Thicket		NS	NS	NS	NS	NS	NS
2	Fynbos	NS		NS	NS	NS	NS	NS
3	Grassland	NS	NS		NS	NS	NS	NS
4	IOCB	NS	NS	NS		NS	NS	NS
5	Nama-Karoo	**	NS	NS	NS		NS	NS
6	Savanna	NS	NS	***	NS	***		NS
7	Succulent Karoo	**	*	NS	NS	NS	***	

Table A24: Post hoc results of the absolute total vertebral number (left) and absolute trunk vertebral number (right) with regards to microhabitat type. The level of significance is measured as follows: a single asterisk (*) equals low significance. Double asterisk equals (**) moderately significant, and triple asterisk (***) equals highly significant.

	Microhabitat category	1	2	3	4
1	Burrow		NS	NS	NS
2	In between roots	NS		NS	NS
3	Under stones	NS	NS		NS
4	Leaf-litter	**	NS	**	

	Microhabitat category	1	2	3	4
1	Burrow		NS	NS	NS
2	In between roots	*		NS	NS
3	Under stones	**	NS		NS
4	Leaf-litter	NS	NS	NS	

Table A25: Post hoc results of the absolute tail vertebral number (left) and ratio of trunk:tail vertebral number (right) with regards to microhabitat type. The level of significance is measured as follows: a single asterisk (*) equals low significance. Double asterisk equals (**) moderately significant, and triple asterisk (***) equals highly significant.

	Behaviour category	1	2	3	4
1	Burrow		NS	NS	NS
2	In between roots	**		NS	NS
3	Under stones	***	NS		NS
4	Leaf-litter	NS	*	**	

	Behaviour category	1	2	3	4
1	Burrow		NS	NS	NS
2	In between roots	***		NS	NS
3	Under stones	***	NS		NS
4	Leaf-litter	NS	NS	N	