A taxonomic review of the genus *Microacontias* (Scincidae: Acontiinae) based on DNA and morphological data

by

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Declaration:

I hereby declare that the work presented here is my own. It is submitted as the requirement for the degree of Master of Science at the University of the Free State, Bloemfontein, South Africa and has not been previously submitted for any other degree at this or any other university.

“Psalm 119:105”

“Thy word is a lamp unto my feet, and a light unto my path.”

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Abstract:

A taxonomic review of the genus *Microacontias* (Reptilia: Acontiinae) using DNA and morphological data

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The recent taxonomic review of the legless, fossorial skink genus *Acontias* by Daniels *et al.* (2006), using DNA sequence data, led to the erecting of the new genus *Microacontias* for all the small-bodied taxa. These are *Microacontias lineatus lineatus*, *Microacontias lineatus tristis*, *Microacontias lineatus grayi* and *Microacontias litoralis*. The latter species was, however, nested in the *lineatus* complex which raised questions about its true taxonomic status. Furthermore, the taxonomic status of the *lineatus* complex is questioned following the poor relationships between taxonomic diversity and morphological character states in these sub-terrestrial skinks (Daniels *et al*., 2002, 2005, and 2006). This study therefore addresses the following two questions: (i) should the taxonomic status of the *lineatus* complex be accepted as it stands and (ii) is *Microacontias litoralis* an independent species or does it form part of the *lineatus* complex. It is hypothesized that *M. l. lineatus* and *M. l. tristis* do not represent separate taxa (near identical morphology), while *M. l. grayi* and *M. litoralis* should be given independent status. The diagnostic methods utilized were (i) Mitochondrial DNA (mtDNA) sequence data (16S rRNA and Cytochrome b), (ii) morphometric data comprising head and body measurements, and (iii) meristic data comprised of various scale counts. The findings suggest that at least in terms of the gene fragments sequenced, the subspecies of the *lineatus* complex are very closely related. The phylogeny reports no distinct grouping of the taxa, nor could the morphological analyses separate the taxa as independent evolutionary units. The low genetic divergence but extensive color variation can be viewed as an example of morphological diversification despite genetic conservatism.

Die onlangse taksonomiese hersiening van die pootlose, sub-terrestriële skink genus, *Acontias*, deur Daniels *et al.* (2006) met behulp van DNS basispaaropeenvolgings data, het gelei tot die op-rigting van die nuwe genus *Microacontias* vir al die klein-liggaam taksa. Hierdie is *Microacontias lineatus lineatus, Microacontias lineatus tristis, Microacontias lineatus grayi* en *Microacontias litoralis*. Laasgenoemde takson was egter in die *lineatus* kompleks ingesluit wat vrae laat ontstaan het omtrent sy ware taksonomiese status. Afgesien daarvan is die taksonomiese status van die drie subspesies van die *lineatus* kompleks ook bevraagteken as gevolg van die swak korrelasie wat gevind was tussen die taksonomiese diversiteit en morfologiese eienskappe in hierdie sub-terrestriële skinke (Daniels *et al.,* 2002, 2005 en 2006). Hierdie studie ondersoek dus die volgende twee vrae: (i) moet die taksonomiese status van die *lineatus* kompleks aanvaar word soos dit huidiglik is, en (ii) is *M. litoralis* ‘n onafhanklike spesie of vorm dit deel van die *lineatus* kompleks. Ons stel voor dat *M. l. lineatus* en *M. l. tristis* nie afsonderlike taxa verteenwoordig nie (bykans identiese morfologie), terwyl *M. l. grayi* en *M. litoralis* onafhanklike status gegee moet word. Die diagnostiese metodes waarvan gebruik gemaak is sluit in (i) Mitochondriale DNS (mtDNS) basispaaropeenvolgingsdata (16S rRNS en Cytochroom b), (ii) morfometriese data, bestaande uit liniêre kop en liggaam afmetings, en (iii) meristiese data, wat verskeie skubtellings insluit. Die bevindinge stel voor dat, ten minste in terme van die geen basispaaropeenvolgingsfragmente, die subspesies van die *lineatus* kompleks baie nou verwant is. Die filogenetiese, asook die morfologiese analises, was

onsuksesfol om die taxa as aparte evolusionêre eenhede to onderskei. Die lae genetiese divergensie maar groot kleur variasie kan beskou word as ‘n voorbeeld van morfologiese diversifikasie ten spyte van genetiese konservatisme.

Contents:

Title page…………………………………………………………………………...…1

Declaration…………………………………………………………………….......2

Acknowledgements…………………………………………………………….....3

Dissertation abstract..……………………………………………….………..…4

Table of contents………………………………………………………….………9

List of tables…………………………………..………………………………..10

List of figures…………………………………………………………….……...…12

Chapter 1: Introduction……………………………...………………………...….14

Chapter 2: Materials and methods……………………………………..…………….25

Chapter 3: Results……….…………………………………………………………40

Chapter 4: Discussion………………………..……………………………………77

References……………………………………………………………………..90

List of tables:

Table 2.1 Number of specimens used for the morphometric and meristic analyses for each *Microacontias* taxon…..……………………..……..26

Table 2.2 List of *Microacontias* taxa sampled per locality. *N* represents the sample size per locality…………..……………………………………32

Table 3.1 Variation in head and body scale counts in the *Microacontias* taxa…………………………………………………………………..….41

Table 3.2 Variation in absolute head and body measurements expressed as ratios of SVL in the *Microacontias* taxa……………………………..43

Table 3.3 Results of a one-way multivariate analysis with the meristic data of all the taxa, $p < 0.001$ is indicative of significant differentiation…..44

Table 3.4 Results of a one-way multivariate analysis with the morphometric data of all the taxa, $p < 0.001$ is indicative of significant differentiation……………………………………………………….44

Table 3.5 Results of the PCA using the meristic data. See Table 3.1 for abbreviations…………………………………………………….…..…45

Table 3.6 Results of the PCA using the morphometric data. See Table 3.1 for abbreviations……………………………………………………………..48

Table 3.7 Results of the DFA based on the meristic data. See Table 3.1 for abbreviations……………………………………………………………...50

Table 3.8 Results of the DFA based on the morphometric. See Table 3.1 for abbreviations, all measurements are noted as ratios…………..….51

Table 3.9 The DFA classification matrix based on the meristic data……..…52

Table 3.10 The DFA classification based on the morphometric data…………55

Table 3.11 Euclidean distances of the meristic and morphometric data. The top right values represent the distances for the meristic data and the bottom left the morphometric data…………………………………...62

Table 3.12 List of the haplotype (combined 16S and cyt *b*) frequencies for each sampled locality for the *Microacontias* lineatus species complex………………………………………...…………….….68

Table 3.13 The nested contingency results based on 10000 permutations in GeoDIS. Significant support values for the used clades are $p < 0.05$. Inferences were made by using Templeton's key (2004)…..72

Table 3.14 Diversity measures for the *M. lineatus* subspecies sampled. N represents the sample size, Nh the number of haplotypes, Np the number of polymorphic sites, h the haplotype diversity and π_n the nucleotide diversity. ………………………………………..………74

Table 3.15 Pair-wise sequence divergences. The highlighted values indicate the significant pair-wise difference between haplotypes. Values are relative as they indicate haplotype distances, regardless of sample quantity and distribution congruency ($p < 0.05$)………..…….……75

List of figures:

Figure 1.1 The striped morphology of *M. l. lineatus* (top) and *M. l tristis* (bottom)..22

Figure 1.2 The morphology of *M. l. grayi*……………………………………..23

Figure 1.3 The two color morphs of *M. litoralis* (banded, top; orange, bottom)……………………………………………………………….24

Figure 2.1 Dorsal, ventral and lateral views of an Acontine skink, where R=Rostral, M=Mental, PF=Prefrontal, F=Frontal, IP=Interperietal, P=Parietal, SO=Supraocular, SC=Supraciliary, Sb=Subocular, L=Loreal, Pr=Preocular, ul=Upper labial, ll=Lower labial, CS=Anterior chin shields. Figure taken from Broadley & Greer (1969)…………………………………………………………...………28

Figure 2.2 Illustration of SVL and Tail length in lizards. Figure taken from Branch (1998)……………………………………………………….....29

Figure 2.3 Map showing locations where specimens of the *M. lineatus* species were complex collected during the present study..………………...33

Figure 3.1 PCA scatterplot based on the meristic data to show the spatial position of the taxa relative to each other at 95% confidence intervals …………………………………………………………...……46

Figure 3.2 PCA scatterplot based on the morphometric data to show the spatial position of the taxa relative to each other at 95% confidence intervals …………………………………………………….…………..49

Figure 3.3 DFA scatterplot based on the meristic data to show the spatial position of the taxa relative to each other at 95% confidence intervals ……………….……………………………………………..53

Figure 3.4 DFA scatterplot based on the morphometric data to show the spatial position of the taxa relative to each other, using roots one and two at 95% confidence intervals ……………………..…..…56

Figure 3.5 DFA scatterplot based on the morphometric data to show the spatial position of the taxa relative to each other, using roots one and three at 95% confidence intervals ……………………………..57

Figure 3.6 DFA scatterplot based on the morphometric data to show the spatial position of the taxa relative to each other, using roots two and three at 95% confidence intervals …………………………...…58

Figure 3.7 Results of the cluster analysis based on the meristic data….……60

Figure 3.8 Results of the cluster analysis based on the morphometric data for both the head and body measurements…………………...……..61

Figure 3.9 Maximum Parsimony phylogram derived from the two mtDNA loci (16S rRNA + cyt *b*) for the *Microacontias* taxa. The first value for each indicated node represents the bootstrap values for the parsimony analyses (%), while the last value for each indicated node represent the posterior probability (*p*P) value derived form the Bayesian inference analyses. Asterices indicate nodes that are unsupported with bootstrap values < 75% and posterior probabilities < 0.95 *p*P……………………………………..……….65

Figure 3.10 Minimum spanning network of the 53 haplotypes derived from the combined analyses of the two mtDNA loci for the *M. lineatus* species complex…………………………………...……………………..67

Chapter 1

Introduction

South Africa has three biodiversity hotspots, the Cape Floristic Region (CFR), the Succulent Karoo (Mittermeier *et al.,* 2004) and Maputaland-Pondoland Albany (Victor & Dold, 2003). Among these three biodiversity hotspots, the CFR is best known for its floral diversity, and contains in excess of 9000 plant species of which 70% are endemic (Goldblatt, 2002; Linder, 2003, 2005). Faunistically these areas are relatively depauperate, with reptiles the notable exception. For example, there are about 180 species of lizard in the CFR (nearly 40% of the total found in South Africa) and >20% of these are endemics (Mittermeier *et al.,* 2004), with skinks having the highest diversity (McGinley, 2008) with over 1300 species (Crottini *et al.*, 2009). Southern African herpetological endemicity currently stands at 52.5% in comparison with 12.7% of the more mobile avifauna (Branch, 1988) and the region contains 23 families, 115 genera and no less than 301 reptile and 95 amphibian species (Branch, 1988, 1998) that supports the ranking of the region as a herpetological hotspot (Tolley *et al.,* 2008).

Most of the estimations of species diversity and endemism are directly derived from traditional morphological alpha taxonomic studies (FitzSimons, 1943; de Witte & Laurent, 1943; Broadley, 1968a; Broadley & Greer, 1969; Rieppel, 1982; Welch, 1982; Haacke, 1986; Greer, 2002). Considering that a large number of species have been poorly described and that their taxonomic status therefore remains questionable, it is highly likely that additional taxa are nested among

these morphologically variable reptile species. Recent molecular based studies have further revealed a substantial increase in taxonomic diversity, suggesting that diversity at both the genus and species level, has been greatly underestimated using conventional alpha taxonomic features (Bauer & Lamb, 2003; Daniels *et al.,* 2005, 2006, 2007, 2009; Tolley *et al.,* 2006, 2008).

Fossorial, limbless skink taxa have received comparatively limited systematic attention, and taxonomic affinities remain dubious, hampering effective conservation of undescribed endemic evolutionary lineages (Daniels *et al.,* 2009). Where taxonomic revisions of fossorial taxa have been conducted recently, the results demonstrated that convergent morphological features traditionally used in the classification of the group have severely impeded taxonomic differentiation, leading to gross underestimation of alpha taxonomic diversity (Daniels *et al.,* 2002, 2005, 2006, 2007, 2009; Burbrink *et al.,* 2000). Consequently, a research project was initiated towards the end of the previous century to investigate the evolutionary relationships within the southern African fossorial skink subfamily, the Acontiinae. The Acontiinae together with the Scincinae, Lygosomatiinae and the Feyleniinae are the four subfamilies that constitute the family Scincidae (Greer, 1970).

The Acontiinae comprises completely limbless fossorial skinks that occupy varying microhabitats ranging from loose coastal sand to grassland and mountainous areas (Branch, 1998). The subfamily is confined to southern Africa

with 88% of the taxa endemic to South Africa (Branch, 1998), the exception being, *Acontias percivali percivali* which occurs in Kenya and Tanzania (Broadley & Greer, 1969). The Acontiinae traditionally consisted of three genera: *Acontias*, *Acontophiops*, and *Typhlosaurus* (FitzSimons, 1943; Broadley & Greer, 1969). Although the monophyly of the Acontiinae is well supported (Greer, 1970), phylogenetic relationships among its three genera have been the subject of considerable debate (FitzSimons, 1943; Broadley, 1968a; Broadley & Greer, 1969; Rieppel, 1982; Branch, 1998). *Acontias* (Cuvier, 1817) was originally subdivided into three species complexes (the *A. lineatus* complex, *A. plumbeus* complex, and the *A. meleagris* complex) by FitzSimons (1943). Broadley & Greer (1969) later recognized four species complexes (the *A. lineatus complex*, *A. percivali* complex, *A. meleagris* complex, and the *A. gracilicauda* complex) and three species (*A. plumbeus*, *A. breviceps*, and *A. litoralis*). Phylogenetically, the monotypic genus *Acontophiops* has been described as intermediate to *Acontias* and *Typhlosaurus* (Rieppel, 1982), *Acontias* having movable and transparent eyelids (pleisiomorphic state), *Acontophiops* (Sternfeld, 1919) transparent but immovable eyelids (apomorphic, intermediate state), and *Typhlosaurus* rudimentary eyes that are covered with a head shield (apomorphic state). *Typhlosaurus* (Wiegman, 1834) has been subjected to many revisions (FitzSimons, 1943; FitzSimons & Brain, 1958; Brain, 1959; Broadley, 1968a, 1990; Broadley & Greer, 1969; Haacke, 1975b; Bates *et al.,* 1992, 1998; Bauer *et al.,* 1997; Bauer, 2000; Bauer & Branch, 2003; Whiting *et al.,* 2004; Greer & Wadsworth, 2003; Michels & Bauer, 2004) and currently consists of three

species complexes (the *T. aurantiacus* species complex, *T. cregoi* species complex, and the *T. lineatus* species complex) and six species (*T. braini*, *T. caecus*, *T. gariepensis*, *T. lomiae*, *T. meyeri*, and *T. vermis*).

The first molecular phylogenetic analysis of the subfamily was conducted by Daniels *et al.* (2002). The authors demonstrated that *Acontophiops* is intermediate to *Acontias* and *Typhlosaurus*, a result that is congruent with earlier morphological findings. Daniels *et al.* (2006), using nDNA (genomic DNA) and mtDNA, demonstrated that two reciprocally monophyletic clades were present within *Acontias*. The genus was subsequently divided into two genera, *Acontias* and *Microacontias nov. gen.*, where *Acontias* was retained for the large to medium bodied taxa. The latter genus currently consists of one species complex, the *A. meleagris* complex (Daniels *et al.,* 2006), and seven acontine taxa. The complex is comprised of *A. m. meleagris* (Linnaeus, 1758)*, A. m. orientalis* (Hewitt, 1937), *A. percivali tasmani* (Hewitt, 1937), and *A. m. orientalis* lineicauda morph (Hewitt, 1937). The seven acontine taxa are: *A. plumbeus* (Bianconi, 1849), *A. p. percivali* (Loveridge, 1923), *A. p. occidentalis* (FitzSimons, 1935), *A. breviceps* (Essex, 1925), *A. gracilicauda gracilicauda* (Essex, 1925), *A. g. namaquensis* (Hewitt, 1937), and *A. poecilus* (Bourquin & Lambiris, 1996).

The new genus, *Microacontias*, was erected for the small bodied taxa that are biogeographically, morphologically and phylogenetically distinct from the rest of the *Acontias* taxa. The former genus currently consists of the *M. lineatus* (Peters,

1879a) species complex, which includes *M. litoralis* (Broadley & Greer, 1969) nested among its three subspecies, *M. l. lineatus* (Peters, 1879a), *M. l. tristis* (Werner, 1911) *and M. l. grayi* (Boulenger, 1887), suggesting that the species boundaries within this complex is in need of revision. The species complex forms the focal group of the present study.

The taxa in the *M. lineatus* species complex exhibit widespread overlap in morphological features as well as distribution, which question their taxonomic status. The first of these is the diagnostic morphological feature of body color variation, for example, longitudinal dorsal stripes of which *M. l. lineatus* has four to ten, while *M. l. tristis* has four to six, whereas *M. litoralis* and *M. l. grayi* are distinct with regard to body color. The second is the overlapping scale patterns, where *M. litoralis* and *M. l. tristis*, for example, each have four upper labials, while *M. l. lineatus* has five, and *M. l. grayi* has four (in 36% of taxa examined) or five (in 64% of taxa examined), as well as constant scale counts among all four taxa (for example, all the taxa have two suboculars, but *M. l. lineatus* can have two or three). Thirdly there are the overlapping distribution ranges. All four taxa inhabit the southern African west coast region. *Microacontias l. lineatus* and *M. l. tristis* occur inland throughout the Northern Cape Province as well as parts of the Western Cape Province, sharing distribution ranges. *Microacontias l. grayi* and *M. litoralis* inhabit a narrow coastal strip within the Western and Northern Cape Provinces, and also have overlapping ranges. The distribution range of *M. l. grayi* and *M. litoralis* overlaps with that of *M. l. lineatus* and *M. l. tristis*. Their current

distributions could have varied in geological time because of marine transgressions, glaciations, aridification as well as mobile sand dune formations (Siesser & Dingle, 1981; Rogers, 1987; Deacon *et al.,* 1992; Dale & McMillian, 1999), possibly causing population extinctions or bottlenecks, which negatively impacted genetic diversity. *Microacontias litoralis* is however genetically closely related to the three subspecies, further questioning the value of the traditional alpha taxonomic features and the species boundaries in the complex. In addition Daniels *et al*. (2006) demonstrated that *M. litoralis* is nested among the three *M. lineatus* subspecies, further casting doubt on the evolutionary affinities among the four taxa. Limited geographical sampling precluded these authors from resolving the taxonomic status of the species complex.

As can be seen in Figures 1.1 to 1.3, the *Microacontias* taxa show great color variation. *Microacontias l. lineatus* and *M. l. tristis* share a striped morphology, being distinguished from each other on the basis of their respective head and body scale counts. Four color morphs thus make up the genus. *Microacontias l. lineatus* and *M. l. tristis* are both striped, the former having four to ten dorsal longitudinal stripes (Figure 1.1 top) while the latter has four to six (Figure 1.1 bottom). *Microacontias l. grayi* (Figure 1.2) has light orange body coloration with a narrow, dark, transverse marking on the posterior edge of the dorsal scales, providing a speckled appearance. *Microacontias litoralis* has two color morphs. One has an orange-brown body with a broad, dark, dorsal, longitudinal band of

purplish-brown coloration (Figure 1.3 top) while the other one has uniform light to an occasionally dark orange body coloration (Figure 1.3 bottom).

The delineation of species boundaries is critical for species conservation and management, and dubious taxonomic groups (such as subspecies and species complexes) can potentially severely hamper the recognition of species diversity. In this respect, it is critical to evaluate traditional alpha taxonomic groupings, in order to reject or affirm their status. In the present study both molecular and morphological analyses were used to address the following research questions:

(1) What are the correct taxonomic relationships within the *M. lineatus* species complex?

> (1.1) Are the taxa sufficiently divergent genetically to be recognized as independent evolutionary significant units?
>
> (1.2) Are the taxa as currently defined, morphometrically, and meristically distinct?

(2) Which factors are potentially responsible for driving the process of speciation or the lack thereof in this group?

Figure 1.1. The striped body coloration of *M. l. lineatus* (top) and *M. l tristis* (bottom). (Photographs taken by Prof Neil Heideman, Keetmanshoop (top), Pofadder-Kakamas (bottom), South Africa)

Figure 1.2. The orange speckled body coloration of *M. l. grayi.* (Photograph taken by Johan Marais, Lamberts Bay, South Africa)

South Africa)

Figure 1.3. The two color morphs of *M. litoralis* (purplish-brown banded, top; orange, bottom) (Photographs taken by Prof Neil Heideman, MacDougal Bay, South Africa)

Chapter 2

Materials and Methods

2.1 Morphological analyses

2.1.1 Sample Collection

Taxa comprising the *M. lineatus* species complex were hand-collected from under stones, logs and decaying plant material (leaf litter) from known (Broadley & Greer, 1969; FitzSimons, 1943, Branch, 1998) and new localities in the Western and Northern Cape provinces of South Africa. Freshly collected animals were killed by freezing at -20˚C. Additional specimens used in morphometric and meristic analyses were obtained from two museum collections (Northern Flag Ship Museum, Pretoria and IZIKO Museum, Cape Town). Refer to section 2.1.2 and 2.1.3, as well as Table 2.1 for sample sizes used per taxon for the meristic and morphometric analyses. Voucher specimens of newly collected samples were preserved in 70% ethanol and will be deposited in the collection of the National Museum in Bloemfontein, Free State Province, South Africa at the termination of the present study.

2.1.2 Meristic analyses

A meristic analysis was carried out on head and body scales of a selected 300 specimens (Table 2.1). The scale categories chosen for the analyses were those used by FitzSimons (1943) and Broadley & Greer (1969) to discriminate between the *Microacontias* taxa. A dissection microscope was used to perform the various counts.

Table 2.1. Number of specimens used for the morphometric and meristic analyses for each *Microacontias* taxon.

Taxa	Morphometric analysis	Meristic analysis
M. l. lineatus	11*	27*
M. l. tristis	35	94
M. l. grayi	54	97
M. litoralis	54	82

*The small sample size of *M. l. lineatus* specimens used in the analyses was due to the difficulty of finding specimens in the field as well as the unsuitability of museum specimens that were brittle due to long storage.

The scales counted (Figure 2.1) were: supraoculars (SO), suboculars (Sb), supracilliaries (SC), upper labials (UL), mid-body scale rows (MSR) (including ventral rows), subcaudals (SD), and ventral counts (VC).

2.1.3 Morphometric analyses

A morphometric analysis was carried out on head and body measurements of a selected 154 specimens (Table 2.1). Juveniles were excluded by selecting adult specimens (specimens within the 80% range of the max SVL; Broadley & Greer, 1969). The measurements used in the analyses were those used by FitzSimons (1943) and Broadley & Greer (1969) to discriminate between the *Microacontias* taxa.

Voucher specimens which were stored in ethanol were dried with a paper towel prior to processing. A vernier caliper was used to take the various measurements (Figure 2.1 and 2.2) to the nearest 0.1mm. The following measurements were taken: snout to vent length (SVL), tail length (TL), head width (HW), head length (HL), head height (HH), rostral scale length (RL), and mental scale length (ML). Measurements were log-transformed prior to analyses in order to normalize the distribution of the data. In order to remove the effect of physical size when comparing measurements among taxa they were standardized by expressing them as ratios of logSVL.

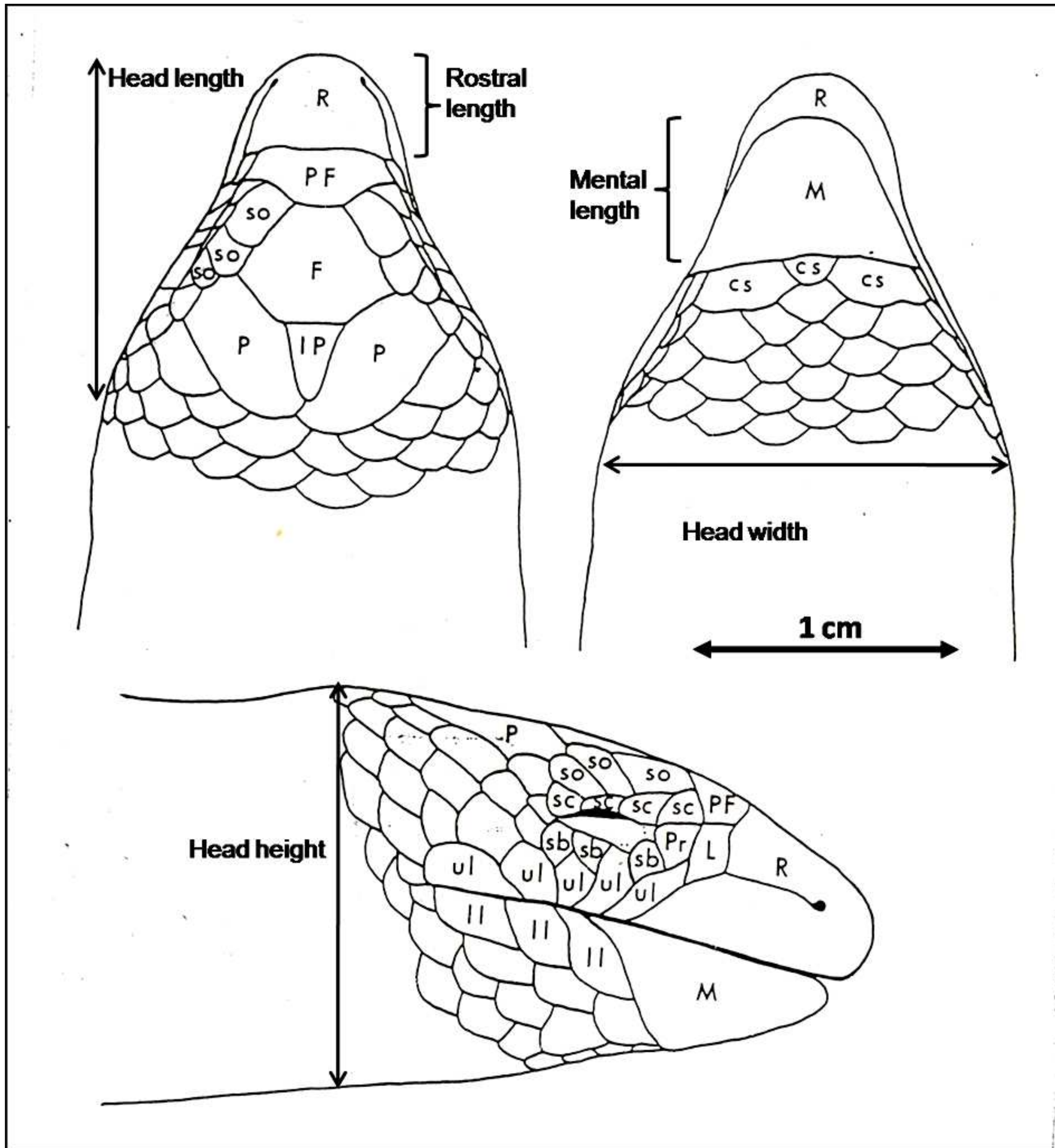

Figure 2.1. Dorsal, ventral and lateral views of an Acontine skink, where R=Rostral, M=Mental, PF=Prefrontal, F=Frontal, IP=Interperietal, P=Parietal, SO=Supraocular, SC=Supraciliary, Sb=Subocular, L=Loreal, Pr=Preocular, ul=Upper labial, ll=Lower labial, CS=Anterior chin shields. Figure taken from Broadley & Greer (1969).

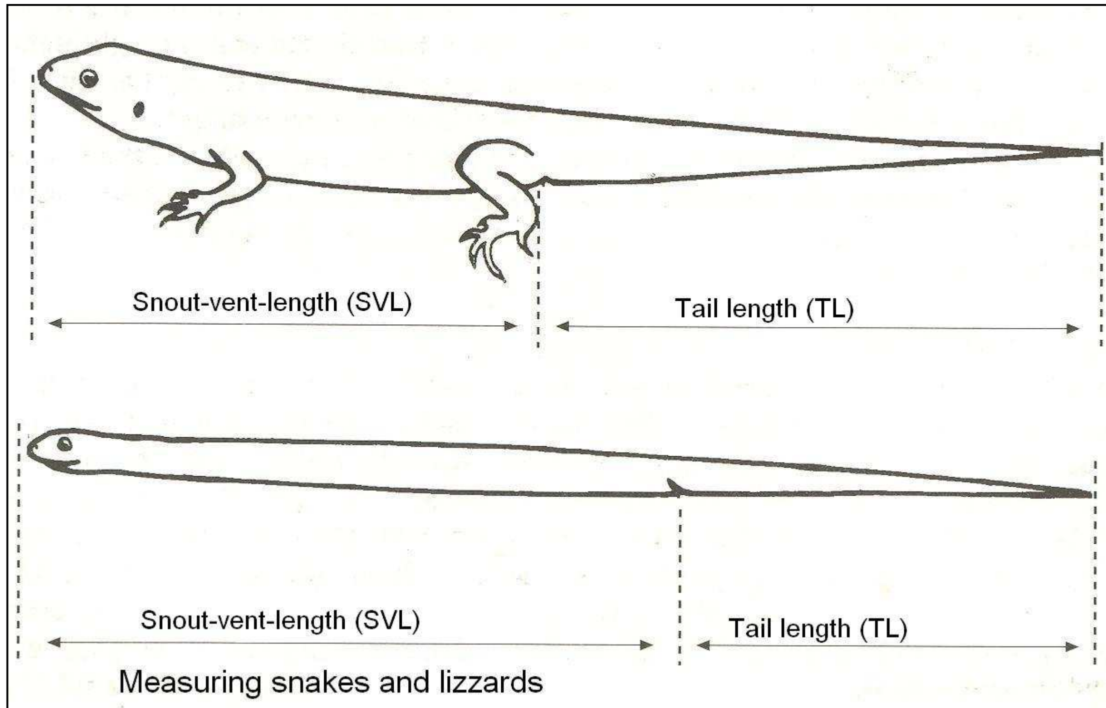

Figure 2.2. Illustration of SVL and Tail length in lizards. Figure taken from Branch (1998).

2.1.4 Statistical analyses

Univariate and multivariate statistical procedures were carried out on both the meristic and morphometric data in order to explore their usefulness in discriminating taxonomically between the taxa. The univariate analyses involved summarizing the data into means (±1 SD) and ranges. The multivariate procedures involved Discriminant Function Analysis (DFA), Principle Component Analysis (PCA) and Cluster Analysis which test existing classification of taxa as well as generate classification hypotheses. The DFA is an *a priori* procedure whereas the PCA is an *a posteriori* procedure. Cluster analysis, generates dendograms, which allows the visualization of morphometric relationships between taxa based on linear distances in morphometric space. All these procedures were carried out with using the program STATISTICA ver.6 (Statsoft, Inc., 2001). Differences between data sets were considered significant at $p < 0.05$.

2.2 DNA Analysis

2.2.1 Sample Collection

A total of 70 samples (Table 2.2, Figure 2.3) of the four taxa that comprise the *M. lineatus* species complex were hand-collected from under stones, logs and decaying plant material (leaf litter) in the Western and Northern Cape provinces of South Africa. Of the 70 specimens, three were collected from neighboring countries, one specimen from Botswana and two from Namibia. The sample from Botswana represents the first distribution record from the country. Co-ordinates of each sample site were recorded using a hand-held GPS (Garmin, *e-trex* model). Animals were killed by freezing at -20˚C. Carcasses were biopsied and liver or muscle tissue samples taken from each specimen and preserved in 95% ethanol. Voucher specimens were preserved in 70% ethanol and will be deposited in the collection of the National Museum in Bloemfontein, Free State Province, South Africa at the termination of the present study.

2.2.2 DNA extraction, PCR and Sequencing

Tissue samples were washed in sterile water prior to DNA extraction. DNA was extracted (from either muscle or liver samples) using a DNeasy® Blood and Tissue kit (Qiagen), following the instructions of the manufacturer. Extracted DNA was stored in a fridge at 4˚C until required for PCR. Two genes were sequenced during the present study; these were the two mtDNA loci 16S rRNA and cytochrome b (cyt *b*).

Table 2.2: List of *Microacontias* taxa sampled per locality. *N* represents the sample size per locality.

Taxon	Locality	*N*	GPS Co-ordinates	
M. l. lineatus	Pofadder road to Kakamas	5	29°05' 463" S	19°30' 5 27" E
	McDougall's Bay	4	29°11' 160" S	16°52' 480" E
	Upington	2	28°26' 024" S	21°18' 323" E
	Leseding Research station, Botswana*	1	18°25' 832" S	21°53' 765" E
	Ariamsvlei, Namibia	1	28°05' 600" S	19°36' 380" E
	Keetmanshoop, Namibia	1	26°36' 324" S	18°07' 951" E
	Van Rhynsdorp	5	31°39' 160" S	18°34' 369" E
	Kuboes	1	28°27' 289" S	16°59' 751" E
M. l. tristis	Anenous Pass	4	29°13' 093" S	17°36' 943" E
	Springbok road to Steinkopf	4	29°31' 017" S	17°51' 524" E
	Kammieskroon	2	30°12' 041" S	17°56' 679" E
M. l. grayi	Doring Bay*	2	31°49' 090" S	18°14' 280" E
	Albani Farm, near Lamberts Bay*	5	32°09' 501' S	18°18' 902" E
	Steenboksfontein Farm, near Lamberts Bay*	5	32°09' 966" S	18°19' 902" E
	Graafwater	5	32°09' 372" S	18°36' 467" E
	Elands Bay*	4	32°18' 557" S	18°20' 760" E
M. litoralis	Doring Bay*	4	31°49' 090" S	18°14' 280" E
	Graafwater*	5	32°09' 372" S	18°36' 467" E
	Omega Farm, near Lutzville*	3	31°30' 934' S	18°20' 760" E
	Vredendal road to Van Rhynsdorp*	3	31°39' 157" S	18°34' 371" E
	Skaapvlei Farm, near Lutzville*	4	31°29' 212" S	18°04' 789" E

* Indicates new locality records.

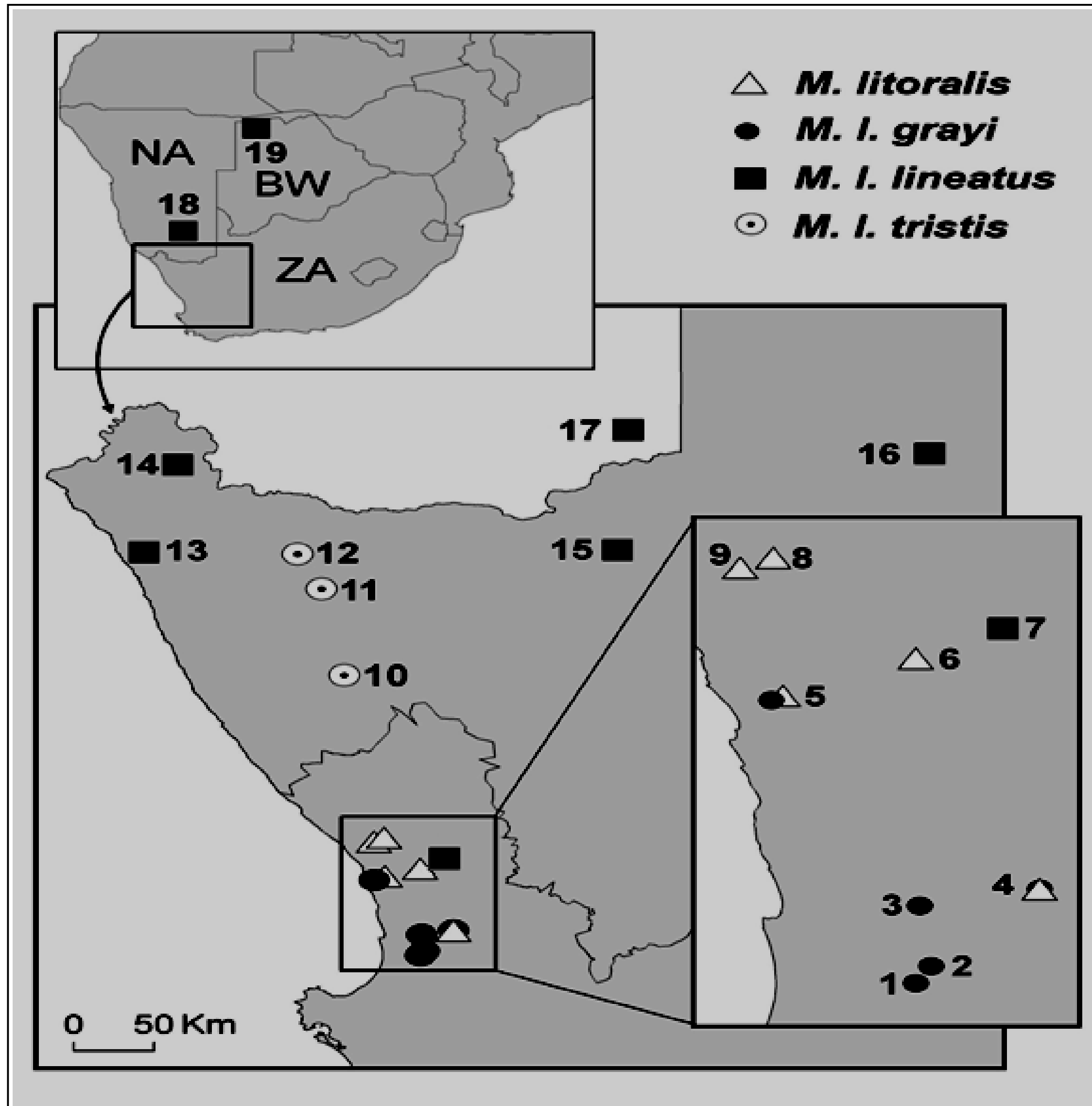

Figure 2.3: Map showing specimens of the *Microacontias lineatus* species complex collected during the present study. (1) Elands Bay, (2) Steenboksfontein, (3) Albani, (4) Graafwater, (5) Doring Bay, (6) Vanrhynsdorp road to Vredendal, (7) Vanrhynsdorp, (8) Omega, (9) Skaapvlei farm, (10) Kamieskroon, (11) Springbok road to Steinkopf, (12) Anenous Pass, (13) McDougalls bay, (14) Kuboes, (15) Pofadder, (16) Upington, (17) Ariamsvlei, (18) Keetmanshoop, and (19) Leseding Research Station, Botswana.

These loci have been widely used in reconstructing evolutionary relationships among Squamates (Cunningham, 1992; Thomas *et al.,* 1995; Prychitko *et al.,* 1997; Schluenzen *et al.,* 2000; Huber *et al.,* 2002; Daniels *et al.,* 2002, 2005, 2006, 2009; Whiting *et al.,* 2004; Poulakakis *et al.,* 2007; Alfaro *et al.,* 2008; Dolman & Hugall, 2008; Kyriaz *et al.,* 2008) and have also been proven useful at understanding evolutionary relationships within species complexes within the above mentioned studies.

The PCR reaction consisted of a 25µl total volume, 14.9µl millipore water, 3µl $MgCl_2$ (25mM), 2.5µl 10x Mg^{2+} free buffer, 0.5µl dNTP mixture (10mM), 0.5µl forward primer and 0.5µl reverse primer, both (10mM), 0.1µl Taq, 3µl template whole genomic DNA, according to Daniels *et al*. (2002). The PCR temperatures for the run were as follows: 94˚C denaturation for four minutes; cycles of: 94˚C for 30s, 50˚C for 40s, 72˚C for 1 minute, for 32 cycles, and a final elongation period of 72˚C for 7 min, according to Daniels *et al*. (2006). The two sets of primers used were as follows: 16Sa (5'-CGC CTG TTT ACT AAA AAC AT-3') and 16Sb (5'-CCG GTC TGA ACT CAG ATC ACG T-3') primers were used to amplify the 16S gene (Cunningham, 1992) and the cyt *b* WWF (5'-AAA YCA YCG TTG TWA TTC AAC TAC-3') and cyt *b* R2 (5'-GGG TGR AAK GGR ATT TTA TC-3') primers were used to amplify the cyt *b* gene (Whiting *et al*., 2004). PCR products were visualized on a 1% agarose gel. PCR products were electrophoresed on a 1% agarose gel containing ethidium bromide for 45 minutes at 80V, and were visualized under UV light.

Those PCR products were gel purified using a PCR purification kit (Qiagen), and cycle sequenced using standard protocols (3µl purified PCR product, 1µl florescent-dye terminators with a ABI PRISM Dye terminator Cycle Sequencing kit (Perkin-Elmer), and 1µl of a 10µM primer pair mix, samples were only sequenced in the forward direction). Unincorporated dideoxynucleotides were removed by gel filtration using Sphadex G-25 (sigma). Sequencing was performed on an ABI 377 automated sequencer.

2.2.3 Phylogenic Analysis

Sequences were checked for base ambiguity in Sequence Navigator (Applied Biosystems). All sequences (16S rRNA and cyt *b*) were aligned in CLUSTAL X version 1.6 (Thompson *et al.,* 1997) using the default parameters of the program and further adjusted by eye where obvious mismatches were made by the computational alignment. As a result of ambiguity in the first 30 bases of these genes, the first 30 bases were trimmed and excluded from the analysis. In addition, ambiguity within the sequence gene regions that could not be aligned with confidence was excluded from the analysis. For the phylogenetic analyses I combined the two mtDNA loci since the mitochondria is maternally inherited and the mtDNA genes are all linked loci.

We preformed maximum parsimony (MP) and Bayesian analyses on the sequence data to reconstruct evolutionary relationships among taxa. Phylogenetic data was analyzed using MP was executed in PAUP*4 version beta

10 (Swofford, 2002). For the MP analysis, trees were generated using the heuristic search option with TBR branch swapping using 1000 random taxon additions. Phylogenetic confidence among nodes was estimated using bootstrapping (Felsenstein, 1985), analyzing 1000 pseudo replicates of data sets.

Bayesian inferences were used to investigate optimal tree space using the program MrBayes 3.0b4 (Ronquist & Huelsenbeck 2003). The best-fit substitution model was chosen using Akaike's information criterion (AIC) (Akaike, 1973), since this reduced the amount of unnecessary parameters that contribute little to describing the data by penalizing more complex models (Bernham & Anderson, 2002; Nylander *et al.*, 2004). A partitioned Bayesian analyses was used during the analyses of the two loci, using their respective substitution models as calculated in MODELTEST (Posada & Crandall, 1998). For each analysis, four Markov chains were run, with each chain starting from a random tree and five million generations generated. Sampling from the chain occurred every 5000th tree for each of the two partitions (two loci mtDNA, followed by the total evidence). A 50% majority rule consensus tree was generated from the trees retained (after the burn-in trees were discarded using likelihood plots), with posterior probabilities for each node estimated by the percentage of time the node was recovered. For the Bayesian analyses, data sets were run a minimum of four times to test that they converge on the same topology. Bootstrap values >75% and posterior probabilities >0.95 (*pP*) were regarded as indicating strong

nodal support. Uncorrected ('p') sequence divergence values were calculated between samples.

2.2.4 Outgroup selection

I used the three *Microacontias* sister genera, *Acontias, Acontophiops* and *Typholosaurus* as outgroups. These included three representative *Typholosaurus* species (*Typhlosaurus lomii*, *T. vermis*, and *T. caecus)*, the monotypic *Acontophiops lineatus* and two representative *Acontias* species (*Acontias percivali occidentalis*, and *Acontias breviceps*). Genbank accession numbers are as follows: *T. lomii,* (16S AY028894, Daniels *et al.,* 2002 & cyt *b* DQ249097, Daniels *et al.,* 2006)*; T. vermis*, (16S AY028895, Daniels *et al.,* 2002 & cyt *b* DQ249098, Daniels *et al.,* 2006); *T. caecus*, (16S DQ249033 & cyt *b* DQ249096, Daniels *et al.,* 2006); *Acontophiops lineatus*, (16S AY649142, Brandley *et al.,* 2005 & cyt *b* DQ249084, Daniels *et al.,* 2006); *Acontias percivali occidentalis*, (16S DQ249032 & cyt *b* DQ249095, Daniels *et al.,* 2006) and *Acontias breviceps*, (16S DQ249031 & cyt *b* DQ249094, Daniels *et al.,* 2006).

2.2.5 Population genetic analyses

A haplotype network was constructed using TCS 1.21 (Clement *et al*., 2000) Haplotypes were connected into a single network with a 95% parsimony probability. The haplotype network was converted into a nested statistical design as outlined in Templeton *et al.* (1992); and Crandall & Templeton (1996) for each of the two partial gene fragments (16S rRNA and cyt *b*). Geographic and genetic

associations were tested using the software GeoDIS version 2.0 (Posada *et al.*, 2000) under a null hypothesis of no geographic association among haplotypes using 10,000 permutations. I estimated the clade distance (D_c), which measures the geographic spread of a clade, and the nested distance (D_N), which measures how a clade is geographically distributed relative to other clades in the same higher-level nesting category (Posada *et al.,* 2006). Templeton's (2004) inference key was then used to interpret patterns of population structure. The conclusions of NCA have recently been debated (Knowles & Maddison, 2002; Templeton, 2004) and are not definite, and I am mindful of this fact.

In addition to the NCA, I performed an analysis of molecular variation on the data (AMOVA) using ARLEQUIN version 3.0 (Excoffier *et al.*, 2005) to calculate gene and nucleotide diversity among haplotypes. Permutation procedures used 10,000 randomizations to test for significance. Deviations in allele frequencies were investigated with Fu's F statistic (Fu, 1997) using ARLEQUIN version 3.0. I also assessed the demographic history of groups by performing a mismatch distribution. This method explores the signature of population fluctuation in DNA sequence data. In this regard, DnaSP (Rozas *et al.,* 2006) was used to obtain an estimate of the population expansions.

2.3 Species concepts and criteria

Criteria for species designation have to be well defined beforehand in order to correctly evaluate the groups of interest, instead of using a reductionistic approach (Sites & Crandall, 1997; de Queiroz, 2005; Mulcahy, 2007). de Queiroz (1998, 2005, 2007) states that current definitions of species are actually delineation criteria, that speciation is gradual and continuous, and as diverging lineages undergo this transition, they pass through various stages exhibiting some of these characteristics that are used as criteria. de Queiroz (2007) also argues that the definitions of what species essentially entail should remain separate from the criteria used to define them. The morphological species concept (Simpson, 1961) and phylogenetic species concept (Hennig, 1966; Ridley, 1989; de Queiroz, 1998, 1999; Meier & Willmann, 2000) may in unison explain the taxonomic confusion within this group. Therefore, I followed these definitions to interpret species boundaries. With the aid of the meristic data, linear head and body measurements, phylogeny, and haplotype analysis I hope to clarify the status of the taxa under consideration.

With regard to morphological characters, species designation would be considered if at least one character state occurs constantly within a taxon to the exclusion of the rest of the taxa in the complex. With regard to molecular characters, monophyletic groups will be considered as species, but as working hypotheses, requiring further investigation.

Results

Part 1: Morphology

3.1 Univariate analyses

Preliminary statistical analyses (data not shown) revealed limited sexual size and shape dimorphisms within *M. litoralis*, however, with no consequence on the current dataset (no changes in graphical presentation of results), and no sexual size and shape dimorphisms were noted within the rest of the *M. lineatus* complex (preliminary DFA, results not shown). This allowed samples to be pooled in order to increase sample size. However, age related dimorphisms within the morphometric data were observed, thus juveniles were excluded from the analyses.

3.1.1 Meristic data

A summary of the descriptive analyses of the scale counts is given in Table 3.1, which demonstrates their general overlap. For example, all of the taxa have a MSR count of 14 with the exception of a single *M. l. grayi* specimen which had 15. Similarly all the taxa also have an SD scale count which overlapped within a range of 34 to 45. The only clear discriminators between the two striped forms, namely, *M. l. lineatus* and *M. l. tristis,* are the SO and UL counts, while *M. litoralis* is the only taxon with a non overlapping ventral count range.

Table 3.1. Variation in head and body scale counts in the *Microacontias* taxa. MSR = Mid-body Scale Row count, SD = Subcaudals, SO = Supraoculars, SC = Supraciliaries, Sb = Suboculars, UL = Upper labials, and VC = ventral scale counts. N refers to sample size.

Taxa	N	MSR	SD	SO (%)		SC (%)		Sb (%)		UL (%)		VC
				1	2	2	3	2	3	4	5	
M. l. lineatus	27	14	36 – 45	0	100	78	22	15	85	0	100	161 – 181
M. l. tristis	94	14	34 – 40	100	0	18	82	100	0	100	0	164 – 181
M. l. grayi	97	14(15)*	34 – 39	69	31	0	100	100	0	36	64	161 – 172
M. litoralis	82	14	34 – 40	100	0	23	77	100	0	100	0	147 – 160

*A single specimen was found with an MSR count of 15.

3.1.2 Morphometric data

The absolute measurements were standardized by expressing them as ratios of SVL (Table 3.2). These relative head and body measurements also showed no clear discrimination between the taxa, due to their range overlap.

3.2 Multivariate analyses

A one-way multivariate analysis indicated significant group separation ($p < 0.01$) for both the meristic (Table 3.3) and morphometric (Table 3.4) data sets. Discriminant Function Analyses (DFA) and Principle Component Analyses (PCA) followed in suit.

3.2.1 Principle Component Analysis (PCA)

3.2.1.1 Meristic data

The first two factors accounted for 47.75% of the total variance within the dataset with SO, Sb, and UL being the greatest contributors, as shown in Table 3.5. The weights of the first component (Factor 1) were mostly negative with varying magnitude. The PCA scatter plot (Figure 3.1) revealed *M. l. tristis* and *M. litoralis* to be overlapping, with *M. l. lineatus* and *M. l. grayi* independent of the group as well as each other.

3.2.1.2 Morphometric data

The first two factors accounted for 59.71% of the total variance within the dataset where HW ratio, HL ratio, and HH ratio were the most discriminative, as shown in

Table 3.2. Variation in absolute head and body measurements expressed as ratios of SVL in the *Microacontias* taxa. SVL = Snout-Vent length, TL = Tail length, HW = Head width, HL = Head length, HH = Head height, ML = Mental scale length, and RL = Rostral scale length.

Taxa		TL/SVL	HW/SVL	HL/SVL	HH/SVL	ML/SVL	RL/SVL
M. l. lineatus	Min	0.231	0.029	0.021	0.021	0.020	0.013
	Max	0.257	0.053	0.049	0.049	0.046	0.017
	Average	0.243	0.034	0.034	0.032	0.014	0.020
M. l. tristis	Min	0.065	0.026	0.022	0.022	0.017	0.011
	Max	0.281	0.039	0.045	0.045	0.045	0.019
	Average	0.213	0.031	0.035	0.027	0.014	0.020
M. l. grayi	Min	0.081	0.016	0.021	0.022	0.017	0.010
	Max	0.255	0.039	0.074	0.074	0.050	0.037
	Average	0.211	0.032	0.039	0.033	0.017	0.022
M. litoralis	Min	0.071	0.020	0.016	0.016	0.020	0.008
	Max	0.264	0.036	0.046	0.046	0.044	0.019
	Average	0.226	0.030	0.0289	0.034	0.014	0.021

Table 3.3. Results of a one-way multivariate analysis with the meristic data of all the taxa, $p < 0.001$ is indicative of significant differentiation.

	Test	Value	F	Effect	Error	*P*
Intercept	Wilks lambda	0.000	1912515	7	290.000	0.000
Species	Wilks lambda	0.045	77	21	833.274	0.000

Table 3.4. Results of a one-way multivariate analysis with the morphometric data of all the taxa, $p < 0.001$ is indicative of significant differentiation.

	Test	Value	F	Effect	Error	*P*
Intercept	Wilks lambda	0.000	66762.16	7	144.000	0.00
Species	Wilks lambda	0.075	28.87	21	414.040	0.00

Table 3.5. Results of the PCA using the meristic data. See Table 3.1 for abbreviations.

Factor	Eigen value	% Total variance	Cumulative Eigen value	Cumulative %	Eigenvectors of Factor weights						
					MSR	SD	SO	SC	Sb	UL	VC
1	2.2276	31.82	2.2276	31.82	-0.003	-0.076	-0.374	0.217	-0.383	-0.324	-0.072
2	1.115	15.93	3.3426	47.75	-0.476	0.462	-0.119	-0.478	0.098	-0.334	-0.305
3	1.0108	14.44	4.3534	62.19	0.516	-0.216	-0.002	-0.241	0.021	0.038	-0.785
4	0.9679	13.83	5.3213	76.02	0.639	0.741	-0.166	-0.006	-0.017	-0.019	0.217
5	0.8636	12.34	6.1849	88.36	-0.275	0.435	0.181	0.680	-0.103	0.378	-0.494
6	0.4544	6.49	6.6393	94.85	0.191	-0.016	0.605	0.550	0.623	-1.049	-0.074
7	0.3607	5.15	7	100	-0.072	-0.133	1.119	0.353	1.149	0.217	-0.075

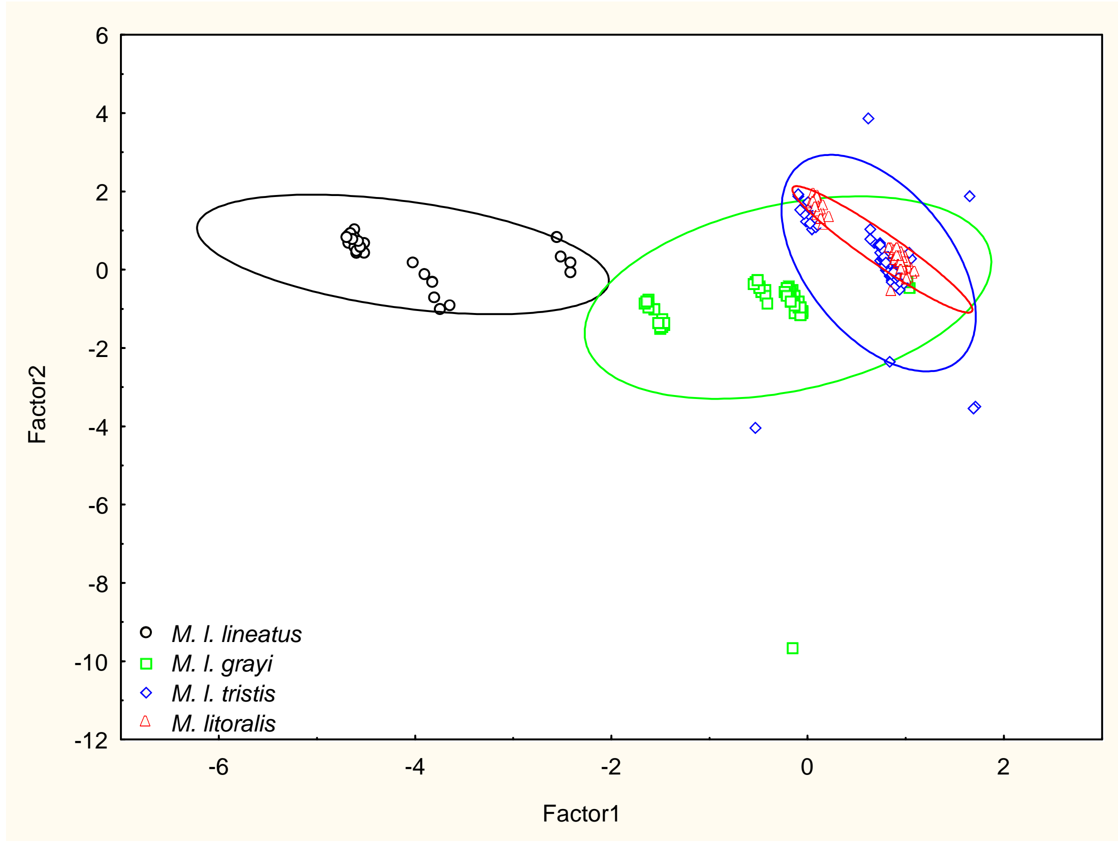

Figure 3.1. PCA scatterplot based on the meristic data to show the spatial position of the taxa relative to each other at 95% confidence intervals.

Table 3.6. The weights of the the first component (Factor 1) were mostly negative with varying magnitude, suggesting variation along this component to be more related to shape, rather than size. The PCA scatter plot (Figure 3.2) shows that all of the taxa are morphometrically inseparable, and form no distinct groups.

3.2.2 Discriminant function analysis (DFA)

In the analyses, the test statistic (*F*) showed that the models all had highly significant discriminatory power (Table 3.7, meristic data; Table 3.8, morphometric data).

3.2.2.1 Meristic data

In the case of the meristic data all the variables were included in the model with Sb being the best discriminator (lowest partial Wilks' lambda), and SD being the poorest discriminator (highest partial Wilks' lambda) between the taxa. Table 3.9 shows that the percentage correctly classified specimens in each taxon varied from 75% to 96%. Four (15%) specimens of *M. l. lineatus* were classified as *M. l. tristis*, 24 (25%) specimens of *M. l. tristis* were classified as *M. l. grayi*, five (5%) specimens of *M. l. grayi* were classified as *M. litoralis*, and 14 (17%) specimens of *M. litoralis* were classified as *M. l. grayi* (Table 3.9). When represented graphically in two-dimentional space (Figure 3.3), *M. l lineatus* and *M. l. grayi* are clearly positioned independently, while *M. l. tristis* and *M. litoralis* overlap. The apparent classification error rate is given as 47/300 or 15.7%.

Table 3.6. Results of the PCA using the morphometric data. See Table 3.1 for abbreviations.

Factor	Eigen value	% Total variance	Cumulative Eigen value	Cumulative %	Eigenvectors of Factor weights						
					SVL	TL/ SVL	HW/ SVL	HL/ SVL	HH/ SVL	ML/ SVL	RL/ SVL
1	2.593	37.050	2.593	37.050	-0.488	-0.007	-0.473	0.161	-0.338	-0.484	-0.406
2	1.600	22.858	4.195	59.908	-0.189	0.132	-0.289	-0.699	0.597	-0.094	-0.102
3	0.995	14.220	5.189	74.128	-0.085	-0.986	-0.042	-0.115	0.049	0.017	0.061
4	0.711	10.15	5.900	84.282	0.033	-0.049	0.236	-0.091	-0.045	0.452	-0.852
5	0.465	6.65	6.365	90.929	-0.709	0.073	-0.099	0.185	0.068	0.619	0.246
6	0.404	5.768	6.769	96.700	-0.447	0.033	0.748	-0.319	-0.196	-0.307	0.069
7	0.231	3.304	7.000	100.000	0.125	0.048	-0.258	-0.573	-0.694	0.274	0.174

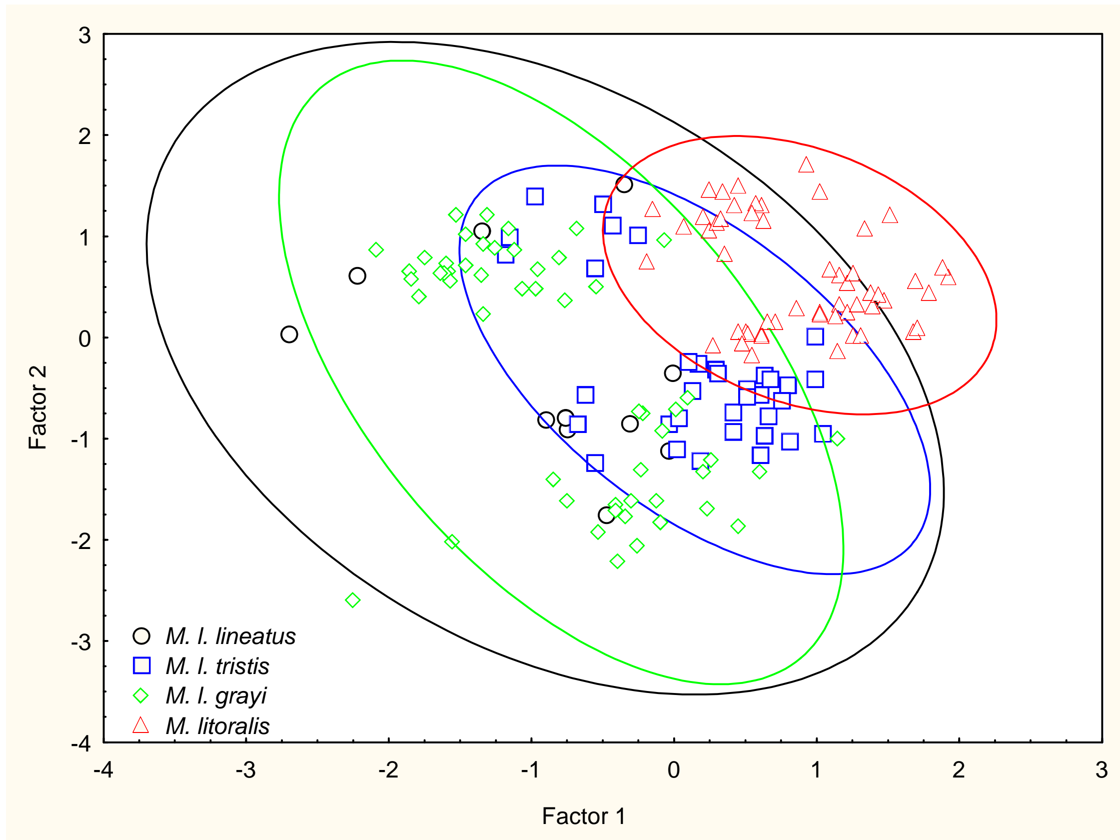

Figure 3.2. PCA scatterplot based on the morphometric data to show the spatial position of the taxa relative to each other at 95% confidence intervals.

Table 3.7. Results of the DFA based on the meristic data. See Table 3.1 for abbreviations. DFA summary: approximate Wilks' lambda: 0.04543; $F_{(21,833)} = 76.778$; $p < 0.001$.

Scales	Wilks' lambda	Partial lambda	F-remove 3,290	*p*	Tolerance	R^2
Sb	0.152	0.299	226.683	0.000	0.993	0.007
UL	0.085	0.536	83.671	0.000	0.994	0.006
SO	0.056	0.816	21.817	0.000	0.992	0.008
SC	0.052	0.869	14.600	0.000	0.988	0.012
VC	0.050	0.905	10.096	0.000	0.995	0.005
SD	0.046	0.995	0.486	0.692	0.996	0.011
MSR	0.045	0.999	0.059	0.981	0.989	0.0113

Table 3.8. Results of the DFA based on the morphometric. See Table 3.1 for abbreviations, all measurements are given as ratios of SVL. DFA summary: approximate Wilks' lambda: 0.07504; $F_{(21,414)} = 28.870$; $p < 0.001$.

Measurements	Wilks' lambda	Partial lambda	F-remove 3,290	*p*	Tolerance	R^2
HW	0.250	0.300	111.934	0.000	0.845276	0.155
HH	0.113	0.663	24.385	0.000	0.307288	0.693
HL	0.096	0.779	13.617	0.000	0.381382	0.619
ML	0.092	0.816	10.803	0.000	0.855538	0.144
TL	0.077	0.970	1.465	0.227	0.994852	0.005
RL	0.076	0.990	0.455	0.715	0.869352	0.131

Table 3.9. The DFA classification matrix based on the meristic data.

	Taxa	Percent correctly classified	1	2	3	4
1	*M. l. lineatus*	85%	23	4	0	0
2	*M. l. tristis*	75%	0	73	24	0
3	*M. l. grayi*	94%	0	0	89	5
4	*M. litoralis*	82%	0	0	14	68

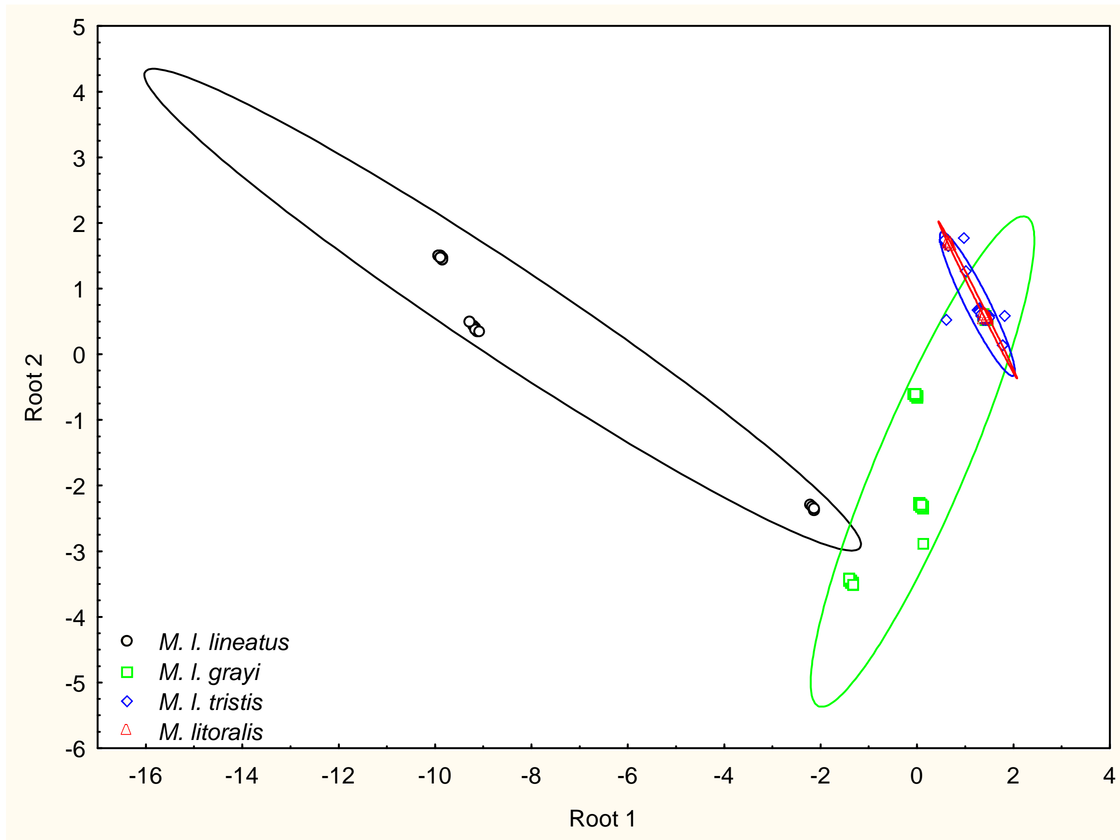

Figure 3.3. DFA scatterplot based on the meristic data to show the spatial position of the taxa relative to each other at 95% confidence intervals.

3.2.2.2 Morphometric data

In the case of the morphometric data all the variables were also included in the model with HW being the best discriminator (lowest partial Wilks' lambda) and RL being the lowest discriminator (highest partial Wilks' lambda) (Table 3.8). Table 3.10 shows that the percentage correctly classified specimens in each taxon ranged from 45% to 100%. When represented graphically in two-dimentional space (Figure 3.4, Root 1 vs. Root 2) *M. l. tristis* and *M. l. grayi* show substantial overlap, while *M. l. lineatus* is positioned where the two former taxa overlap. *Microacontias litoralis* is clearly independant from the group. Separation of the taxa becomes increasingly poorer with the interaction of Root 1 vs. Root 3 (Figure 3.5) and Root 2 vs. Root 3 (Figure 3.6). The apparent classification error rate was 18/154 or 11.69%.

3.2.3 Cluster analyses

Figures 3.7 (meristic data) and 3.8 (morphometric data) show dendograms of the cluster analysis using Euclidian distances (Table 3.11). This method shows a "statistical phylogenetic tree" that is based on correlations between variances among the taxa. The meristic data, varyingly, supports the earlier morphological studies with the closer relationship of *M. l. lineatus* to *M. l. tristis*, and *M. l. grayi,* but with *M. l. lineatus* and *M. l. grayi* being closer related than *M. l. tristis*, but again with *M. litoralis* being the most differentiated on the basis of the scale counts from the rest of the group.

Table 3.10: The DFA classification based on the morphometric data.

	Taxa	Percent correctly classified	1	2	3	4
1	*M. l. lineatus*	45%	5	2	4	0
2	*M. l. tristis*	80%	3	28	4	0
3	*M. l. grayi*	91%	0	5	49	0
4	*M. litoralis*	100%	0	0	0	54

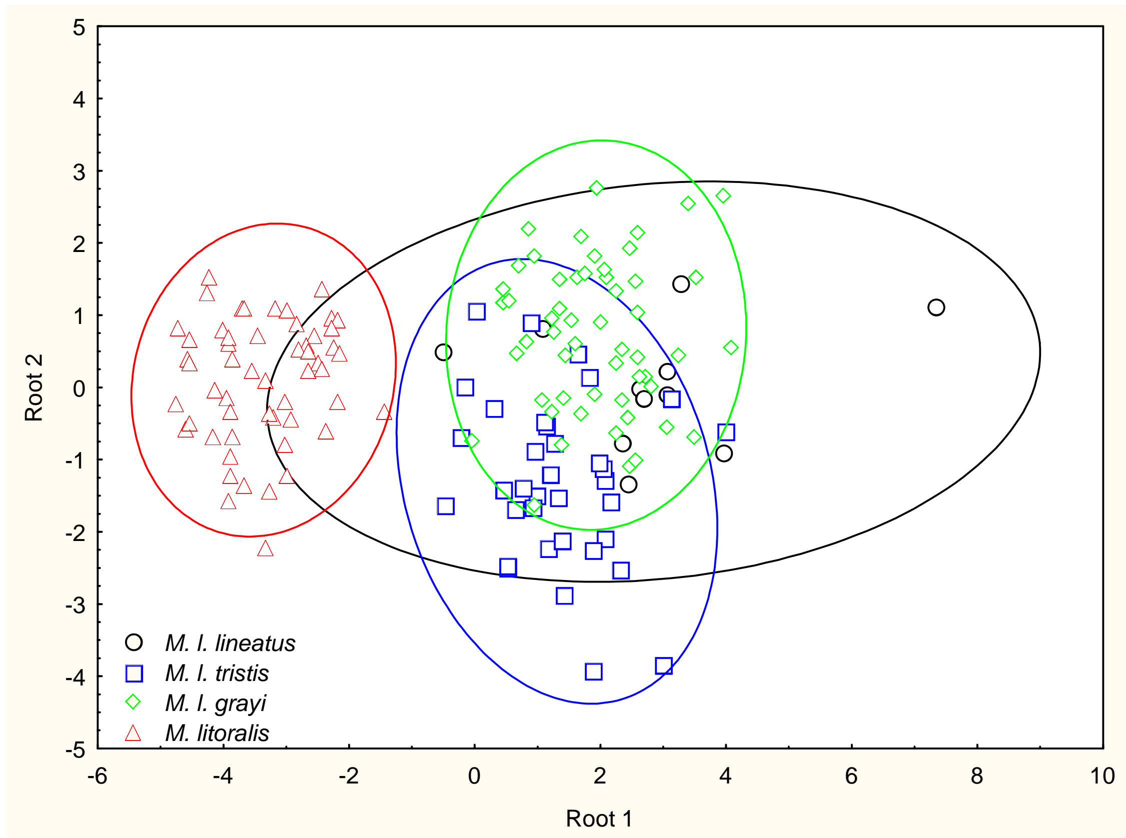

Figure 3.4. DFA scatterplot based on the morphometric data to show the spatial position of the taxa relative to each other, using roots one and two at 95% confidence intervals.

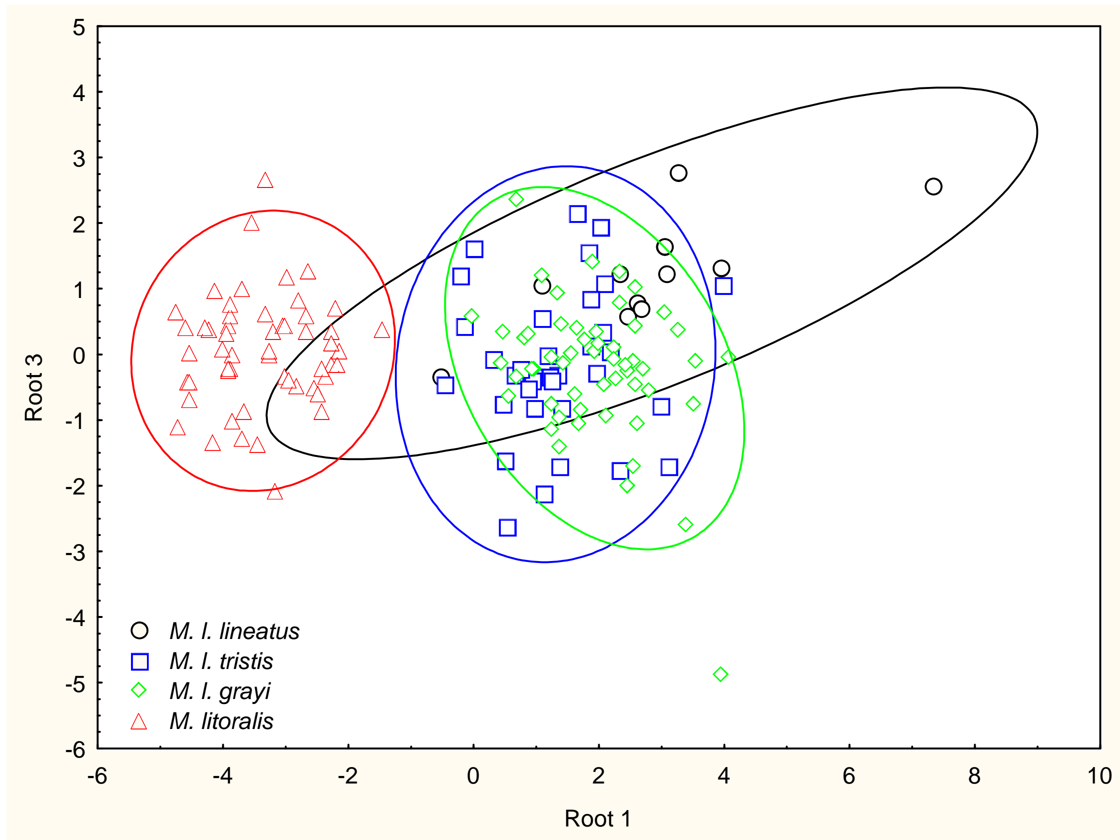

Figure 3.5. DFA scatterplot based on the morphometric data to show the spatial position of the taxa relative to each other, using roots one and three at 95% confidence intervals.

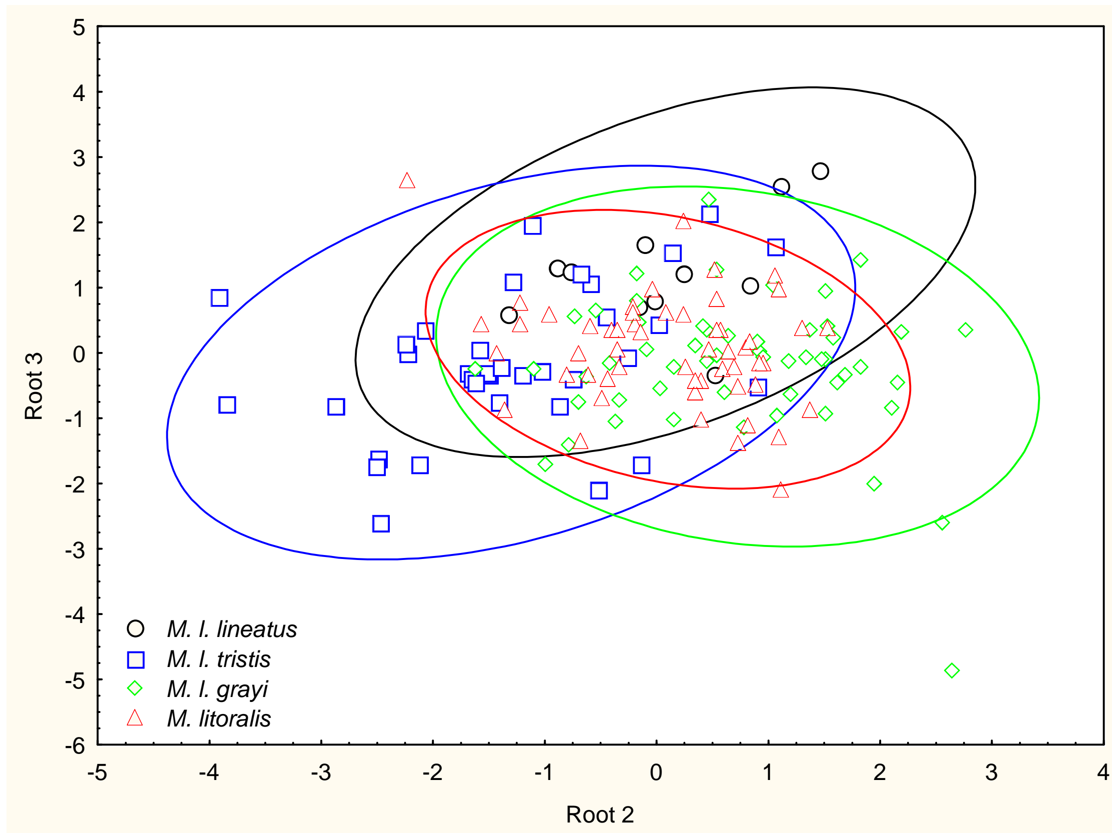

Figure 3.6. DFA scatterplot based on the morphometric data to show the spatial position of the taxa relative to each other, using roots two and three at 95% confidence intervals.

The morphometric data supports the earlier morphological studies with the closer relationship of *M. l. lineatus* to *M. l. tristis*, and *M. l. grayi* closer to the former two than to *M. litoralis*. *Microacontias litoralis* was again the most differentiated from the rest of the group on the basis of size and shape. Mahalanobis distances could not be calculated for the morphometric data as the data entries consisted of non-real numbers (values containing decimals which could not be rounded-off as it would have over-standardized the data).

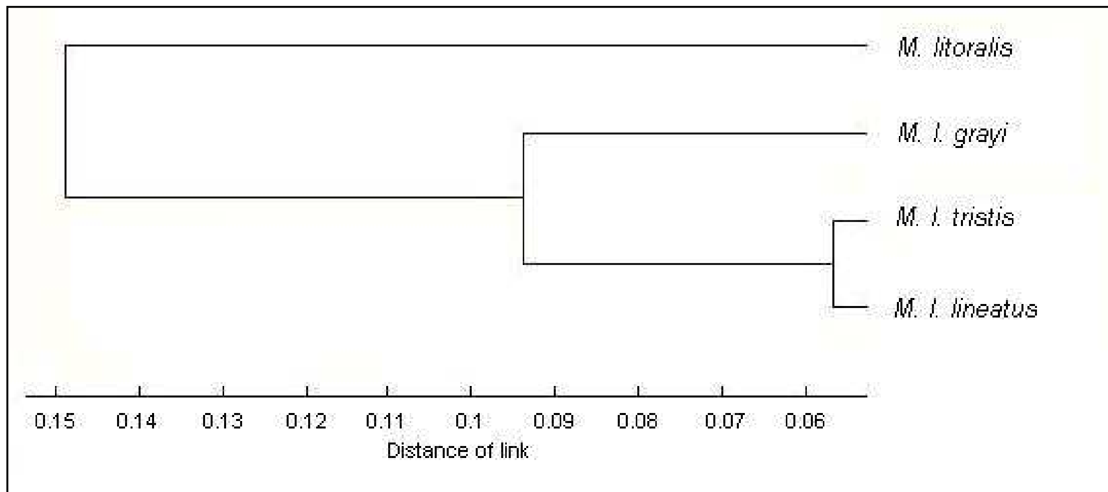

Figure 3.7. Results of the cluster analysis based on the meristic data.

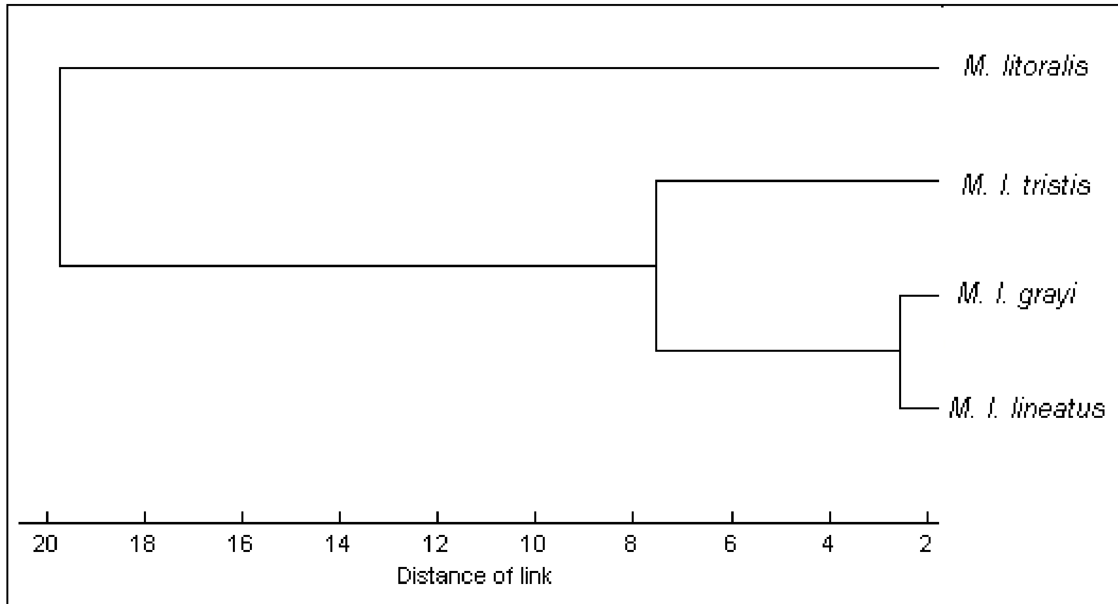

Figure 3.8. Results of the cluster analysis based on the morphometric data for both the head and body measurements.

Table 3.11. Euclidean distances of the meristic and morphometric data. The top right values represent the distances for the meristic data and the bottom left for the morphometric data.

		Euclidean distances			
		1	2	3	4
1	*M. l. lineatus*	0	7.5295	2.5673	19.7228
2	*M. l. grayi*	0.0938	0	6.3895	12.5374
3	*M. l. tristis*	0.0567	0.0747	0	18.8351
4	*M. litoralis*	0.1488	0.0944	0.1310	0

Part 2: Molecular Phylogeny

3.3.1 Combined mtDNA analyses (16S rRNA & cyt *b*)

The combined data for the two mtDNA loci yielded a total of 1082 base pairs for the 70 ingroup specimens. Sequences have been deposited in Genbank (Accession numbers are: GQ259670 – GQ259734 + GQ292543 for the 16S rRNA, 66 amplicons; and GQ259604 – GQ259669 + GQ292544 for the cyt *b*, 67 amplicons). For the 16S rRNA locus the substitution model was TVM+G (-lnL = 1962.87; AIC = 3941.74), the base frequencies were A = 35.70%, C = 24.45%, G = 19.02% and T = 20.83%, the rate matrix was R(a) [A-C] = R(c) [A-T] = 2.09, R(b) [A-G] = R(e) [C-T] = 5.16, R(d) [C-G] = 0.97, R(f) [G-T] = 1.00 and the gamma shape (G) was 0.25. For cyt *b* the substitution model was K81uf+I+G (Kimura, 1981; -lnL = 2648.60, AIC = 5311.19), the base frequencies were A = 32.84%, C = 31.84%, G = 13.81%, T = 21.51%, the rate matrix was R(a) [A-C] = R(f) [G-T] = 1.00, R(b) [A-G] = R(e) [C-T] = 6.80, R(c) [A-T] = R(d) [C-G] = 0.21, the proportion of invariable sites (I) was 0.563 and G was 1.230. These two models were used during the partitioned BI analyses. The MP analyses retrieved a single tree with a tree length of 742 steps (Consistency Index = CI = 0.354, Retention Index = RI = 0.6361) derived from 222 parsimony informative characters. Since I observed topological congruence between the BI and MP analyses, I will only discuss the MP topology. Both topologies derived from the BI and MP were highly congruent (Figure 3.9) and characterized by short internal branches and tip branches.

Figure 3.9. Maximum Parsimony phylogram derived from the two mtDNA loci (16S rRNA + cyt *b*) among the *Microacontias*. The first value for each indicated node represents the bootstrap values for the parsimony analyses (%), while the last value for each indicated node represent the posterior probability (*p*P) value derived form the Bayesian inference analyses. Asterices indicate nodes that are unsupported with bootstrap values < 75% and posterior probabilities < 0.95 *p*P.

Furthermore, no evidence was observed for any distinct phylogenetic groupings among the four taxa in the species complex. The phylogeny supports the monophyly of the *Microacontias* with high support (100%/1.00*p*P). There were no statistically well supported clades within the ingroups and the high level of interdigitation among the subspecies is indicative of a general lack of support for the current taxonomic designations. The Botswana sample was basal, and sister to the remaining ingroup taxa.

3.3.2 Sequence divergence:

For both 16S rRNA and cyt *b* the uncorrected sequence divergence values among the taxa were: < 2.0% for *M. l. tristis* and *M. litoralis*; < 2.0% for *M. l. lineatus* and *M. litoralis*; < 0.6% for *M. l. grayi* and *M. litoralis*; < 1.8% for *M. l. tristis* and *M. l. lineatus*; < 4.0% for *M. l. tristis* and *M. l. grayi*; and < 2.5% for *M. l. lineatus* and *M. l. grayi*. The maximum of sequence divergences within the complex was < 4.0% for cyt *b*. Among the large-bodied *Acontias* and *Typhlosaurus* species the divergence between the genera ranged from 0.5% to 10.6% for the 16S rRNA, while it was up to 15.2% for cyt *b*. The outgroups were separated from the *Microacontias* by divergence values of 9.8% to 15.1% for 16S rRNA and 8.0% to 15.3% for cyt *b*, while the Botswana sample was separated from the *Microacontias* by 7.9% to 11% for 16S rRNA and 8.8% to 11.4% for cyt *b*, while it was separated from the outgroups by 11.76% to 13.7% for 16S rRNA and 11.8% to 14.3% for cyt *b*.

3.4 Population genetic analyses derived from combined (16S rRNA & cyt *b*) data

I retrieved 53 haplotypes for the 70 samples sequenced. Table 3.12 gives the haplotype distribution. The minimum spanning network supported the phylogenetic results and revealed a general absence of taxonomic differentiation among the *Microacontias* taxa. In a number of instances I observed shared haplotypes between subspecies. For example, haplotypes one (*M. l. grayi* and *M. litoralis*), eight (*M. l. lineatus* and *M. litoralis*), 24 (*M. l. grayi* and *M. l. tristis*), 38 (*M. l. grayi* and *M. litoralis*), and 40 (*M. l. grayi* and *M. litoralis*) are shared among different taxonomic units. Haplotype 51 represents a specimen from Springbok (*M. l. tristis*) and haplotypes 52 and 53 (both *M. l. lineatus*) represent specimens from Kuboes and Botswana respectively.

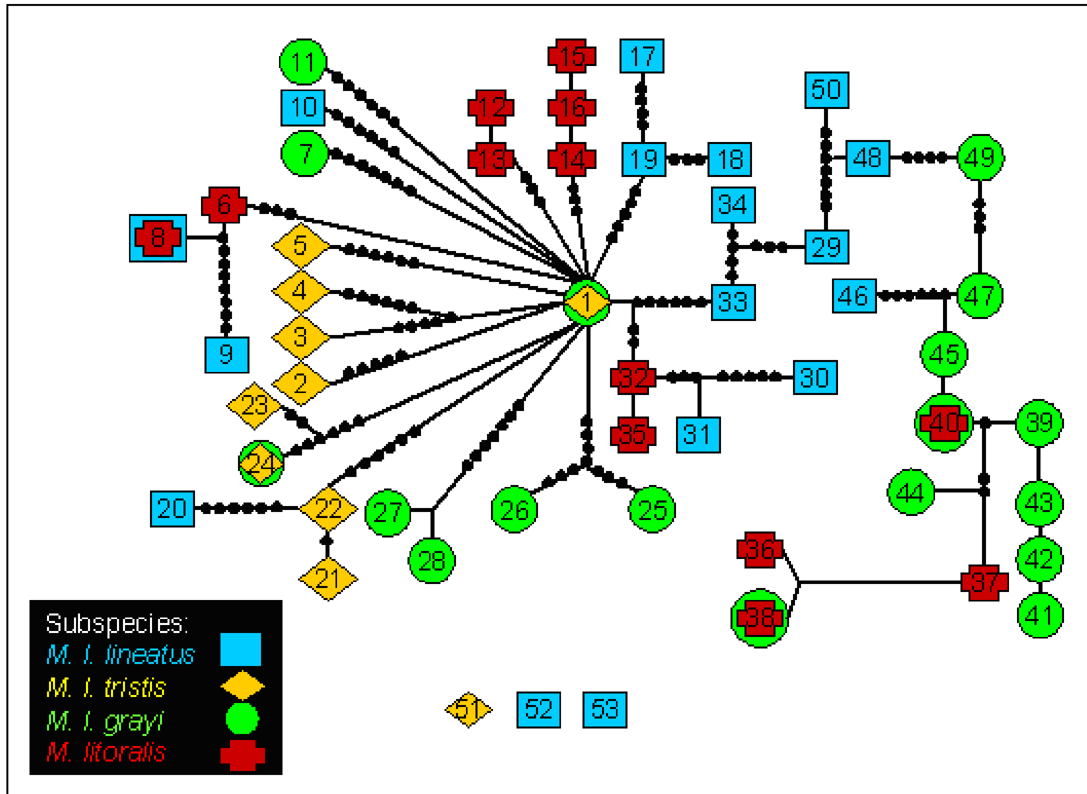

Figure 3.10. Minimum spanning network of the 53 haplotypes derived from the combined analyses of the two mtDNA loci for the *M. lineatus* species complex.

Table 3.12: List of the haplotype (combined 16S and cyt *b*) frequencies for each sampled locality for the *Microacontias lineatus* species complex.

Haplotype		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Localities	Subspecies																				
Anenous	*M. l. tristis*	1	1	1	1																
VanRhynsdorp	*M. l. lineatus*	1																	1	1	1
Keetmanshoop	*M. l. lineatus*	1																			
Springbok	*M. l. tristis*					1															
Skaapvlei farm	*M. litoralis*						1						1	2							
Steenboksfontein	*M. l. grayi*							1				1									
Upington	*M. l. lineatus*								1	1											
Doring Bay	*M. l. grayi*																				
	M. litoralis								1												
McDougall's Bay	*M. l. lineatus*										1										
Omega farm	*M. litoralis*												1								
Kamieskroon	*M. l. tristis*														1	1	1				
Ariamsvlei	*M. l. lineatus*																	1			

Table 3.12 Continued.

Haplotype		21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
Localities	Subspecies																			
Springbok	*M. l. tristis*	1	1																	
Kamieskroon	*M. l. tristis*			1	1															
VanRhynsdorp	*M. l. lineatus*				1															
Steenboksfontein	*M. l. grayi*					3														
Albani farm	*M. l. grayi*						1													
Graafwater	*M. l. grayi*							1	1										1	
	M. litoralis															1	1		1	
McDougall's Bay	*M. l. lineatus*									1				1	1					
Pofadder	*M. l. lineatus*										1	1								
Omega farm	*M. litoralis*												1			1				
Doring Bay	*M. l. grayi*																	2		
	M. litoralis																			
Elands Bay	*M. l. grayi*																			1

Table 3.12 Continued.

Haplotype		40	41	42	43	44	45	46	47	48	49	50	51	52	53
Localities	Subspecies														
Albani farm	*M. l. grayi*	2				1	1								
Graafwater	*M. l. grayi*								1		1				
	M. litoralis	2													
Doring Bay	*M. l. grayi*							1			1				
	M. litoralis	1													
Elands Bay	*M. l. grayi*		1	1	1										
Pofadder	*M. l. lineatus*							1		1		1			
Springbok	*M. l. tristis*												1		
Kuboes	*M. l. lineatus*													1	
Leseding, Botswana	*M. l. lineatus*														1

A statistical parsimony network constructed, using TCS, demonstrated that there are no discrete groupings among the haplotypes (figure 3.10). The Nested Clade Analysis (NCA) retrieved 53 haplotypes which were partitioned into 41 one-step clades, 35 two-step clades, 30 three-step clades, 20 four-step clades, and four five-step clades (nesting not shown). The clade distances (D_c) for clades 3-6, 4-9, 5-3 and the total cladogram (figure not shown), were significantly small ($p < 0.05$), reflecting an under-dispersed geographic spread of haplotypes within these clades (results not shown). The nested clade distances (D_N) show reversals from this pattern, with one or two D_N values being significantly large ($p < 0.05$). The haplotype cladogram revealed no divergent groups. Contingency analysis (Table 3.13) indicated no significant association between nested clades and geography ($p < 0.05$). I observed a pattern consistent with gene flow and isolation by distance for one three-step clade (3-6), and a pattern consistent with restricted gene flow and isolation by dispersal, but long distance dispersal was

Table 3.13: The nested contingency results based on 10000 permutations in GeoDIS. Significant support values for the used clades are $p < 0.05$. Inferences were made by using Templeton's key (2004).

Clade	χ^2	Probability	Inference chain	Inferred chain
3-6	20.77	0.002	1-2-3-4	Restricted gene-flow with isolation by distance
4-9	64.87	0.002	1-2-3-5-6-7	Restricted gene-flow with dispersal, but long distance dispersal
5-3	14.60	0.016	1-2-3-5-6-7-8	Sampling design inadequate to describe isolation by distance vs. long distance dispersal
Total	112.66	0.023	1-2-3-5-6-7-8	Sampling design inadequate to describe isolation by distance vs. long distance dispersal

observed for one four-step clade (4-9). In both the five-step clade (5-3) and the total cladogram, inadequate geographic sampling precluded any conclusive outcome.

AMOVA results over all populations ($\Phi ST=0.51078$) revealed that 51.08% of the variation occurs among populations (df = 18; Va = 5.62; $p < 0.001$), and 48.92% of the variation within populations (df = 51; Vb = 5.38; $p < 0.001$). For genetic diversity measures, see table 3.14. The table illustrates the range of haplotypic and nuclear diversity. Almost every haplotype is unique, with a low number of polymorphic sites resulting in a high frequency of haplotypes; congruently the nuclear diversity (base pair assortment) is low with few base pair substitutions giving rise to private haplotypes. The significant pair-wise Fst values are highlighted in table 3.15, indicating significant population structuring.

Table 3.14: Diversity measures for the *M. lineatus* subspecies sampled. *N* represents the sample size, *Nh* the number of haplotypes, *Np* the number of polymorphic sites, *h* the haplotype diversity and π_n the nucleotide diversity.

Locality	Subspecies	*N*	*Nh*	*Np*	*h*	π_n
Elands Bay	*M. l. grayi*	4	4	4	1.0000 +/- 0.1768	0.002008 +/- 0.001659
Steenboksfontein	*M. l. grayi*	5	3	36	0.7000 +/- 0.2184	0.016960 +/- 0.010646
Albani farm	*M. l. grayi*	5	4	28	0.9000 +/- 0.1610	0.010751 +/- 0.006882
Graafwater	*M. l. grayi / M. litoralis*	10	8	42	0.9556 +/- 0.0594	0.013655 +/- 0.007562
Doring Bay	*M. l. grayi / M. litoralis*	6	5	22	0.9333 +/- 0.1217	0.007600 +/- 0.004747
Omega farm	*M. litoralis*	3	3	11	1.0000 +/- 0.2711	0.006796 +/- 0.005465
Skaapvlei farm	*M. litoralis*	4	3	9	0.8333 +/- 0.2224	0.004171 +/- 0.003091
Vredendal	*M. litoralis*	3	3	2	1.0000 +/- 0.2722	0.001236 +/- 0.001270
Kamieskroon	*M. l. tristis*	2	2	6	1.0000 +/- 0.5000	0.005556 +/- 0.006001
Springbok	*M. l. tristis*	4	4	35	1.0000 +/- 0.1768	0.016204 +/- 0.010962
Anenous Pass	*M. l. tristis*	4	4	11	1.0000 +/- 0.4768	0.010721 +/- 0.007783
McDougall's Bay	*M. l. lineatus*	4	4	26	1.0000 +/- 0.1768	0.012203 +/- 0.008349
VanRhynsdorp	*M. l. lineatus*	5	3	13	1.0000 +/- 0.1265	0.011696 +/- 0.007846
Pofadder	*M. l. lineatus*	5	5	20	1.0000 +/- 0.1265	0.009453 +/- 0.006095
Upington	*M. l. lineatus*	2	2	9	1.0000 +/- 0.5000	0.008341 +/- 0.008792
Ariamsvlei	*M. l. lineatus*	1	1	N/A	N/A	N/A
Keetmanshoop	*M. l. lineatus*	1	1	N/A	N/A	N/A
Leseding, Botswana	*M. l. lineatus*	1	1	N/A	N/A	N/A
Kuboes	*M. l. lineatus*	1	1	N/A	N/A	N/A

Table 3.15: Pair-wise sequence divergences. The highlighted values indicate the significant pair-wise difference between haplotypes. Values are relative as they indicate haplotype distances, regardless of sample quantity and distribution congruency ($p < 0.05$).

	1	2	3	4	5	6	7	8	9	10	11
1	0.00000										
2	-0.71930	0.00000									
3	0.22185	-0.74242	0.00000								
4	**0.32009**	-0.52174	**0.19097**	0.00000							
5	0.65057	-0.05882	**0.55145**	**0.50562**	0.00000						
6	**0.60208**	-0.12500	0.50226	0.42624	**0.50345**	0.00000					
7	**0.58982**	-0.29474	**0.52250**	**0.49601**	**0.41802**	0.33628	0.00000				
8	**0.61826**	-0.06250	**0.56807**	**0.54334**	**0.56160**	**0.40159**	**0.15525**	0.00000			
9	**0.49402**	-1.28750	**0.47683**	**0.41478**	**0.57498**	0.45950	**0.51801**	**0.56019**	0.00000		
10	**0.51377**	-0.81609	**0.50000**	**0.48023**	0.55274	0.43316	**0.35718**	**0.27729**	**0.48014**	0.00000	
11	**0.56782**	-0.69231	**0.46323**	0.40757	0.18848	0.45244	0.07565	0.30951	**0.50259**	**0.36118**	0.00000
12	0.54630	1.00000	0.02542	0.14634	0.79310	0.61702	0.64088	0.60769	0.44207	0.48867	0.66154
13	0.70644	0.71429	**0.59111**	**0.53658**	**0.70805**	0.75806	**0.58369**	**0.64115**	0.55646	**0.61826**	0.62500
14	**0.42664**	-1.37634	**0.39015**	**0.37760**	0.29936	0.26754	-0.02198	0.19878	**0.37885**	**0.26318**	0.03309
15	**0.55277**	-0.52632	**0.48712**	**0.44369**	**0.46026**	0.38485	0.06988	**0.29949**	**0.30787**	**0.31950**	**0.20357**
16	0.89727	1.00000	0.86597	0.82968	0.95373	0.90863	0.91399	0.89613	0.81056	0.86356	0.92414
17	**0.74564**	0.69048	**0.64539**	**0.58377**	**0.72222**	**0.71527**	**0.20530**	**0.43190**	**0.52443**	**0.48675**	**0.48110**
18	0.46315	0.14286	0.21569	0.06118	0.71917	0.63415	**0.59599**	0.63300	**0.50923**	0.57551	0.60426
19	0.76156	1.00000	0.69895	0.59302	0.90955	0.82857	0.84078	0.81915	0.68448	0.76276	0.85235

Table 3.15 Continued:

	12	13	14	15	16	17	18	19
11								
12	0.00000							
13	0.93651	0.00000						
14	0.41765	0.38693	0.00000					
15	0.54688	**0.55826**	-0.02956	0.00000				
16	1.00000	0.98611	0.84889	0.87815	0.00000			
17	0.90877	**0.87606**	0.07048	0.07323	0.97676	0.00000		
18	0.64706	0.84585	**0.41201**	0.54180	0.93970	0.83779	0.00000	
19	1.00000	0.97279	0.72358	0.78439	1.00000	0.95813	0.85714	0.00000

Chapter 4

Discussion

The morphological and molecular datasets were unable to unambiguously differentiate between the *Microacontias* taxa due to extensive character state overlap. Morphological characters in these skinks are prone to homoplasy (Hedges & Maxon, 1996; Wiens *et al*., 2003; Crottini *et al*., 2009) which is presumably a result of their fossorial habitat, limiting the reliability of their morphology in species diagnoses. The molecular data did not recover the taxa as distinct units as is evident from their interdigitization and the absence of any distinctly monophyletic subclades. This suggests that the complex is a single, polymorphic species represented by a diversity of color morphs (two striped forms, *M. l. lineatus* and *M. l. tristis*; an orange speckled form, *M. l. grayi*; as well as an orange and melanistic form, *M. litoralis*). These color morphs have distribution ranges which overlap to various degrees, but they show no color mixing in their areas of sympatry. This is incongruent with the uncovering of shared haplotypes between the taxa, which suggests extensive gene flow. A possible reason for this anomaly may be that they represent shared ancient ancestral polymorphisms. On the other hand the shared haplotypes may be indicative of ongoing gene flow, with the maintenance of color integrity in each one being under the control of complex genetic systems. Low sequence divergence distances between the ingroup taxa (< 4.0%) compared to the outgroup taxa (as high as 15.3% between the taxa of *Acontias,* and *Typhlosaurus*, respectively) suggest that the taxa have only just started to diverge from each other, indicating incomplete lineage sorting (Funk & Omland, 2003).

Morphology

Both uni- and multivariate analyses of the meristic and morphometric data show that the diagnoses based on scale counts, and linear head and body measurements are generally unable to unambiguously discriminate between the four *Microacontias* taxa. Both data sets (meristic and morphometric) show range overlaps which the multivariate techniques were unable to disentangle. There were nevertheless a few notable exceptions where the diagnostic features showed clear discrimination between certain taxa. For example, the two striped forms, *M. l. lineatus* and *M. l. tristis,* consistently differ from each other in terms of the SO and UL counts. *Microacontias litoralis*, on the other hand, displays a non-overlapping VC with the rest of the taxa, supporting its taxonomic distinction. Some scale counts show low numerical variability, but their ranges still overlap, reducing their utility for discriminating between the taxa. For example, SC count tends to be one in *M. l. lineatus* and two in *M. l. tristis*, but there are specimens in which these counts are reversed.

The results of both the PCA and DFA, with regard to the meristic data show some concordance with the findings of the univariate analyses by identifying SO and UL as the main discriminators between the *Microacontias* taxa. As previously mentioned the two scale counts are the only clear discriminators between *M. l. lineatus* and *M. l. tristis*. This is also reflected in the scatter plot in which these two taxa are clearly separate in meristic space. Notably, both the PCA and DFA show agreement in the spatial arrangement of the taxa with respect to each other, where all the *M. lineatus* taxa are spatially separated, while *M. litoralis* completely overlaps with *M. l. tristis*. The inability of

the DFA to unambiguously discriminate between the taxa is further reflected by the fairly high classification error range of 6.0% - 25.0%. The topology of the dendogram derived from the cluster analysis clearly show *M. litoralis* as standing separate from the rest of the group, with *M. l. tristis* as its closest taxon in terms of Euclidean distance.

Both the PCA and DFA results, with regard to the morphometric data, confirmed the univariate findings as the *M. lineatus* taxa were also not clearly separable on the basis of linear head and body dimensions (in morphometric space), because of the high degree of data overlap. *Microacontias litoralis*, however, is completely separated from the group within the DFA scatter plot, supporting its current taxonomic designation as an independent taxon. The analyses ranked the HH, HW, and HL as the best discriminators and also confirmed the importance of head shape, rather than head size as taxonomic discriminators, as stated by Broadley & Greer (1969). The inability of the DFA to unambiguously discriminate between the taxa is further reflected by the high classification error of up to 65%. The topology of the dendogram derived from the cluster analysis again clearly shows *M. litoralis* as standing separately from the rest of the group, but this time with *M. l. grayi* as its closest taxon in terms of Euclidean distance.

In conclusion, the meristic data appeared to be more useful than the morphometric data as it was better able to discriminate between the *Microacontias* taxa. The high classification error of the morphometric data is noteworthy, which may be indicative of the low taxonomic utility of such data within this group. Both types of analyses of the

data (PCA and DFA) supported each other, as well as earlier classifications. The sole reliance on external morphological characters such as body scales in designating evolutionary lineages in fossorial reptiles has been shown to be fundamentally flawed due to suspected convergence of the character states in the sub-terrestrial habitat as demonstrated in several studies e.g. Broadley & Greer (1969) (*Acontias*), Daniels *et al.,* (2002, 2005, 2006) (*Acontias*) and Kearney & Stuart (2004) (Amphisbaenians). However, variable meristic features have been proven useful for species differentiation (Brandley *et al.,* 2005), if well defined (species designation would be considered if at least one character state occurs constantly within a taxon to the exclusion of the rest of the taxa in the complex). Thus, the obvious shortage of well-defined synapomorphies, as well as deciding which of these are useful, is clearly an obstacle in determining the taxonomic relationships of fossorial skinks (Daniels *et al.,* 2006) when using scale counts and body measurements.

Phylogeny

The monophyly of the *Microacontias* complex (previously comprising the *Acontias lineatus* complex and *Acontias litoralis*) is confirmed with strong bootstrap and posterior probability support (100% / 1.00 *p*P) which is corroborated with similar findings in Daniels *et al.* (2006). The previous phylogenetic placement of the genera *Acontias*, *Acontophiops* and *Typhlosaurus* relative to each other is also corroborated with strong Bootstrap support (100%). The phylogenetic placement of the *Microacontias* taxa, however, differs from that of previous studies (Daniels *et al.,* 2006) where it is nested within *Acontias,* possibly due to the use of different, additional genes (the 16S rRNA

mtDNA gene is conjointly used). Here *Microacontias* is shown as a monophyletic clade which is more closely related to *Typhlosaurus* than *Acontias*.

Within the *Microacontias* complex taxonomic relationships are problematic, with the topology recovered showing low statistical support and inconsistent grouping of taxa. All trees were characterized by short internal nodes and similarly short tip branches suggesting early stages of recent diversification. The Botswana specimen was recovered as a sister clade to the monophyletic clade comprising the rest of the *Microacontias* taxa, with strong (100%) bootstrap support, suggesting that it may be a new lineage.

Sequence divergence

Sequence divergence values are applied here with caution and are not regarded as absolute values for inferring species relationships as it indicates relatedness of sequence data, rather than being benchmarks to speciation (Daniels *et al*., 2002). Collectively the data derived from the present and previous studies (Daniels *et al*., 2002, 2005, 2006) at least partially support the subspecific status of the *Microacontias* taxa. Sequence divergences (among the four acontine genera) as derived from the 16S rRNA and cyt *b* mtDNA genes varied from 3.4% to 15.1% (16S rRNA) and 7.9% to 15.5% (cyt *b*). The former result compares favorably with results of Crottini *et al*., (2009) who found congeneric scincine distances of 5.5% to 12.6% for the 16S rRNA gene, and the latter with both Johns & Avise (1998) and Harris (2002) who suggested a general average of congeneric reptile cyt *b* divergences at 13.6%. The placement of *Microacontias* is

unexpected as the BI and MP analyses place it closer to *Typhlosaurus* than *Acontias* which is in contrast to current taxonomy. Sequence divergences indicate the *Microacontias* taxa to differ from *Acontias* by 9.8% to 14.3% (16S rRNA) and 12.8% to 15.3% (cyt *b*) whereas it differs from *Typhlosaurus* by 12.3% to 15.1% (16S rRNA) and 9.2% to 12.3% (cyt *b*); a miniscule margin that thus places *Microacontias* closer to *Typhlosaurus*. In a previous study (Daniels *et al.,* 2006) *Microacontias* is more closely related to *Acontias* (11% maximum sequence divergence, cyt *b*) than *Typhlosaurus* (14% maximum sequence divergence, cyt *b*), but this could be a result of their limited *Microacontias* sample sizes. Sequence divergences between the *Microacontias* taxa ranged between 0% and 4%. The lowest sequence divergences found between taxa supports closer relationships of *M. l. lineatus* to *M. l. tristis* (0.2% to 1.8%), and *M. l. grayi* to *M. litoralis* (0% to 0.6%). These low intraspecific genetic distances suggest that differentiation within the *Microacontias* is a relatively recent event. Mott & Vieites (2009) similarly report that lack of resolution within South American amphisbaenids could be due to recent diversification. The Botswana specimen (identified morphologically as *M. l. lineatus* by Dr. Mike Bates, Bloemfontein National Museum, South Africa; five UL, two SO, three Sb, three SC, head shield counts and form match those of *Acontias*/*Microacontias*; rostral, prefrontal, frontal and interparietal) is clearly a lineage on its own as it stands as intermediate between *Microacontias* and *Typhlosaurus* (100% Bootstrap support). Sequence divergences place it closer to the former (7.9% to 11%, 16S rRNA and 8.8% to 11.4%, cyt *b*) than the latter (13.1% to 13.7%, 16S rRNA and 14.3%, cyt *b*). These distances compare favorably to congeneric distances within the subfamily (10% to 14%), and are higher than the differences of known *Acontias* taxa, for

example, *Acontias meleagris meleagris* and *Acontias gracilicauda gracilicauda* that differ by 6.1% (Daniels *et al*., 2006) calling for attention to resolve this anomaly.

Distribution analyses

Microacontias l. lineatus and *M. l. tristis* are co-distributed within the Northern Cape province, westwards to and down the western coastal region of South Africa including the west coast of the Western Cape Province, with scattered localities in Namibia (Broadley & Greer, 1969; Branch, 1998). In addition a *M. l. lineatus* specimen was found (chance sampling) in Botswana. This sympatric distribution could likely be the cause of confusion with regard to discriminating between these two taxa (near identical morphology), as subspecies or independent species are expected to occur allopatric. They enclose the distribution of the coastal occurring *M. litoralis* (confined to the narrow coastal strip of western South Africa), that includes a pocket-sized niche for *M. l. grayi* to the west-southwestern coastal regions of South Africa around the Graafwater - Doring Bay area (Broadley & Greer, 1969; Branch, 1998). Relationships between taxa remain troublesome as the phylogenetic, and phylogeographic analyses also revealed the general absence of structure within or between the populations and taxa.

The haplotype network also displayed no subdivision of taxa and localities with no distinct groupings further confounding the taxonomic status of this group. Low genetic variation (haplotypic and nucleotype diversity) is observed within the sequence data, which is suggestive of little or no genetic divergence, continuous gene flow (within lineages), or possibly low sample sizes, as explained by the low genetic distances

between ingroup taxa. The Nested clade analysis was also unable to confirm any distinct groupings, other than the sharing of haplotypes between *M. litoralis* and both *M. l. lineatus* and *M. l. grayi*, while *M. l. tristis* shared haplotypes with *M. l. grayi*. AMOVA results indicate little distinction of where most variation lies, with marginal difference, as 51.08% of the variation lies within populations and 48.92% variation between populations. Templeton's key (2004) suggests that long distance dispersal and/or isolation by distance may be the cause of clustering groups (nested phylogeny and shared haplotypes per locality/region) among *Microacontias* taxa.

Biogeography

Calibrating rates of molecular evolution for a cladogenic event is difficult in the absence of fossil data, or distinct biogeographic events. Nevertheless when looking at a generalized molecular clock calibration of 2% per million years for the cyt *b* mtDNA gene (Johns & Avise, 1998), our cyt *b* sequence divergence data (0% - 4%) suggests ongoing differentiation from about two million years ago till the present. This calibration places the differentiation within the Quaternary (2.6 mya - present). The principal events of the Quaternary include the formation of cemented and mobile dunes. These dunes advanced the movement of coastal forelands, alluvial sands and acid sand plains and the exposure of the Plio-Pleistocene Bredasdorp Formation (Siesser and Dingle, 1981; Rogers, 1987; Decon *et al.,* 1992; Dale and McMillian, 1999). These processes are the consequence of the icehouse-hothouse cycles of the Pleistocene (Tyson and Partridge, 2000) and their associated progressive and regressive stages. During these transgressive stages, strong Pleistocene wind regimes would have developed

aeolianites (Hesp *et al.,* 1989) which gave rise to the large areas of calcareous sandy substrata along the west coast (Cowling *et al.,* 2008), thus providing the current habitat for the taxa.

When the taxa were mapped on the geography of South Africa, two distinct biogeographic areas could be defined. These include a narrowly distributed West Coast Cape Floristic Region (CFR) distribution of *M. litoralis* and *M. l. grayi*, with a more widely dispersed Succulent Karoo distribution for *M. l. lineatus* and *M. l. tristis*, with one exceptional record of *M. l. lineatus* within the CFR distribution. Both these areas historically consisted of alluvial sand belts (Cowling *et al.,* 2008). The formation of these vegetation belts and mobile sand formations is closely linked to the climatic and geological histories of the areas (Cowling *et al.,* 2008). With sub-terranean taxa, the movement of sand dunes could have resulted in populations being separated and again reunited periodically. In such a dynamic situation, secondary contact between populations (Mulcahy, 2007; Parham & Papenfuss, 2009) could be a possible explanation for the observed inconsistent phylogeny. New transgressions and regressions could have hampered genetic isolation to such an extent that genetic diversification was stopped or slowed down, causing little further sequence divergence, and, therefore, little morphological evolution. Substratum preference, different route of dispersal, mountain ranges, coastal dune belt dispersion, climatic shifts, marine transgressions, refugal areas such as inland habitats and niches, and tectonic upheavals have been explanations in many phylogenetic studies (Matthee & Flemming, 2002; Daniels *et al*., 2004; DeMenocal, 2004; Tolley *et al*., 2006; Daniels *et al*., 2007;

Cowling *et al.*, 2008). Another hypothesis is the sand dune dispersal (Siesser & Dingle, 1981; Rogers, 1987; Deacon *et al.,* 1992; Dale & McMillian, 1999). The climatic and geographic changes may have aided in speciation among the limbless lizard fauna of South Africa, but these coinciding with the secondary contact theory may have led to the lack thereof.

Color

It is difficult to assign functions to pigmentation as it can be the result of natural selection, sexual selection or neutral evolution under genetic drift. Color patterns are mostly thought to signal visual stimuli, thus their presence and function in subterranean taxa is unknown (Wollenberg & Measley, 2009). The handicap theory (Zahavi, 1975; Guilford & Dawkins, 1993), states that color patterns are most often interpreted as having an adaptive selective evolutionary advantage. Possible functions of color patterns may include physiological adaptations (thermoregulation), visual signals in the context of aposematism, mimicry or crypsis, or predation signals (Wollenberg & Measley, 2009).

Little conclusive work has been done on the mode of color inheritance and maintenance to explain its variation. Hoffman & Blouin (1999) argue that complex methods such as pigmentation, spread of pigments, pigment concentration, and mobilization of pigments/pigment clusters can cause varied phenotypes, and many factors/mechanisms control these phenotypes. For example, stripes are caused by concentration increases in pigment, rather than spread of pigment. Temperature, light,

humidity, and other factors such as warmth and cold conditions contribute to these variable origins of pigmentation. Gray & McKinnon (2006) state that color polymorphisms may be inherited or transferred by a host of factors, such as genetic drift and gene flow, divergent selection and gene flow, sensory bias, disruptive selection and frequency dependant selection. In addition, they state that the scale of the environmental variation is important, as divergent selection can be expected in a population that experiences a single, variable environment. According to Mouton and Van Wyk (1990, 1995), cooler paleo-climatic conditions are thought to be the cause or origin of many melanistic features of lizards occurring along the southern African west coast, possibly explaining the convergent morphology of fossorial skinks. This is, however, an inappropriate designation for the *Microacontias* taxa, as *M. litoralis* displays both its color patterns in sympatry, both forms were found within a single brood.

The *Microacontias* taxa exhibit diverse morphological patterns that conflict with their current taxonomic status, derived from morphological and molecular studies (Broadley & Greer, 1969; Daniels *et al.,* 2006). *Microacontias l. lineatus* shares a striped morphology with *M. l. tristis* (four to ten longitudinal stripes for the former, eight or more are rarely observed, and four to six stripes for the latter). *Microacontias l. grayi* has an orange colored body with transverse markings on the posterior edge of the dorsal and lateral scales, providing a speckled appearance, and *M. litoralis* has two color forms, based on the presence or absence of a dark dorsal longitudinal band of a purplish-brown color. In addition to color variation, low genetic divergence can be viewed as an example of morphological diversification with genetic conservatism. Color pattern is,

however, an unreliable indicator of species boundaries as demonstrated with the inverse situation found in *A. meleagris* complex in which Daniels *et al.*, (2006) showed morphological conservatism with genetic diversification. Similarly, Burbrink *et al.,* (2000) in the case of the American rat snake, *Elaphe obsolete*, in North America, and Sinclair *et al.,* (2004) in the case of the night lizard genus, *Xantusia*, found morphological conservatism with genetic diversification. Mulcahy (2007), on the other hand, found morphological variation coinciding with molecular diversification, as well as morphological variation and molecular conservatism in Nightsnake (*Hypsiglena torquata*) populations of North America. Color as well as the absence or presence of stripes are thus labile characters. Deheyn & Jangoux (1998) reported that progeny of *Amphipholis squamata* display color patterns of the brooding parent, no intermediate varieties were found during rearing that could indicate cross breeding; whether this may also be the underlying cause for the apparent lack of hybridization within the *Microacontias* complex is something that may be worth investigation in future studies. Multigenerational breeding studies are necessary to prove a mode of inheritance because complex modes of inheritance, such as quantitative threshold traits (Wright, 1934) can mimic simple Mendelian ratios in the F1 generation. The underlying cause or control of color within this complex thus merits further investigation.

Conclusions:

According to the criteria defined earlier under the morphological and phylogenetic species concepts, the *Microacontias* taxa do not merit elevation to species status based on the findings obtained in this study. Their subspecific status is reinforced, as well as

the placement of *M. litoralis* to within the complex. The redesignation of *Microacontias litoralis* as *Microacontias lineatus litoralis* would on the basis thereof be a logical step. It shows total meristic overlap with *M. l. tristis* for the body scale characters analyzed, apart from being phylogenetically nested within the complex. Only the Botswana specimen (*M. l. lineatus*) shows sufficient DNA sequence divergence to be tentatively recognized as a new species, but this needs further investigation. Follow-up studies using genetic markers with finer resolution (e.g. microsatellites) may provide a clearer picture of the interrelationships among the taxa and the gene flow dynamics among them.

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Figure 1.1. The striped body coloration of *M. l. lineatus* (top) and *M. l tristis* (bottom). (Photographs taken by Prof Neil Heideman, Keetmanshoop (top), Pofadder-Kakamas (bottom), South Africa)

Figure 1.2. The orange speckled body coloration of *M. l. grayi.* (Photograph taken by Johan Marais, Lamberts Bay, South Africa)

Figure 1.3. The two color morphs of *M. litoralis* (purplish-brown banded, top; orange, bottom) (Photographs taken by Prof Neil Heideman, MacDougal Bay, South Africa)

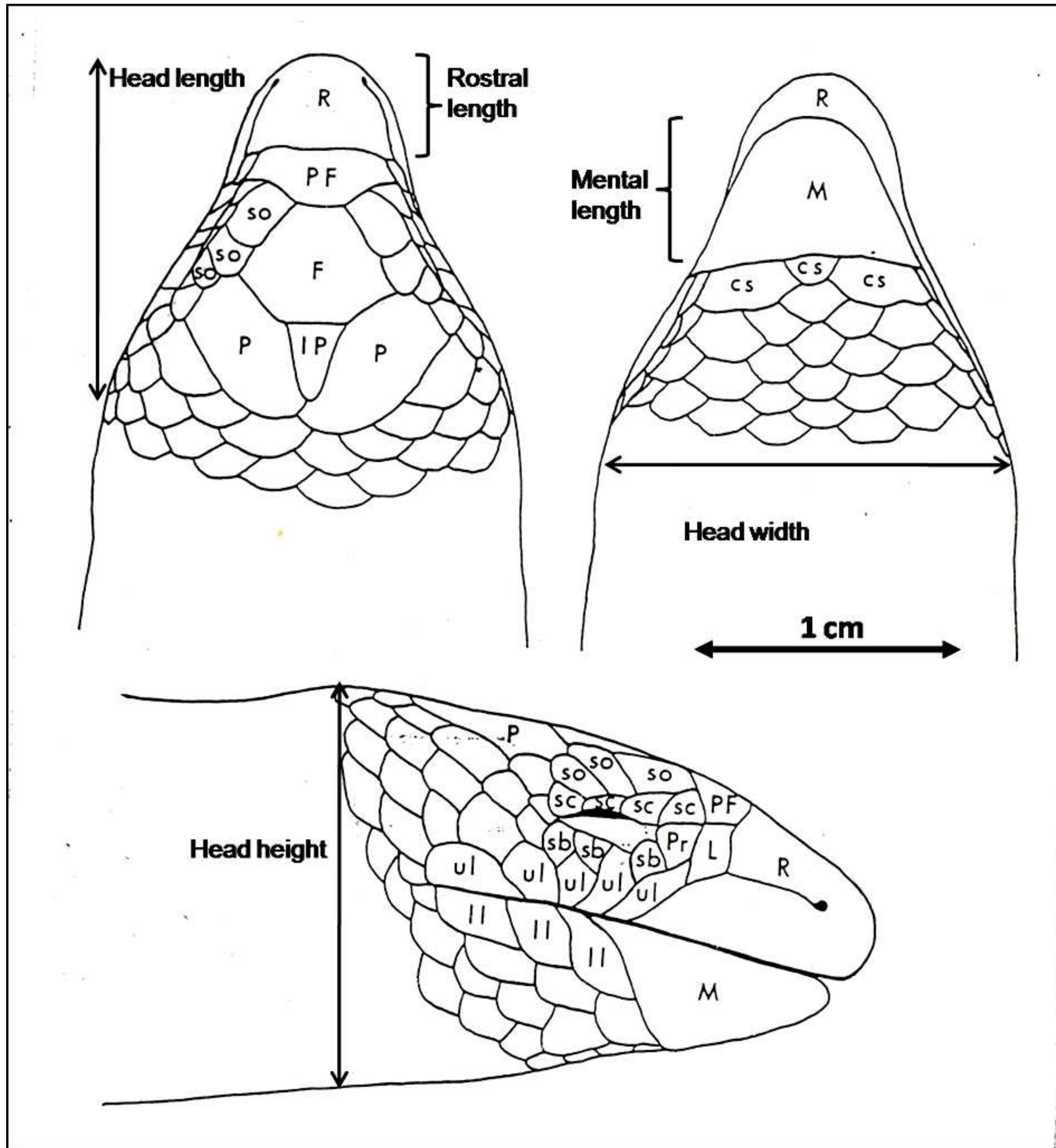

Figure 2.1. Dorsal, ventral and lateral views of an Acontine skink, where R=Rostral, M=Mental, PF=Prefrontal, F=Frontal, IP=Interperietal, P=Parietal, SO=Supraocular, SC=Supraciliary, Sb=Subocular, L=Loreal, Pr=Preocular, ul=Upper labial, ll=Lower labial, CS=Anterior chin shields. Figure taken from Broadley & Greer (1969).

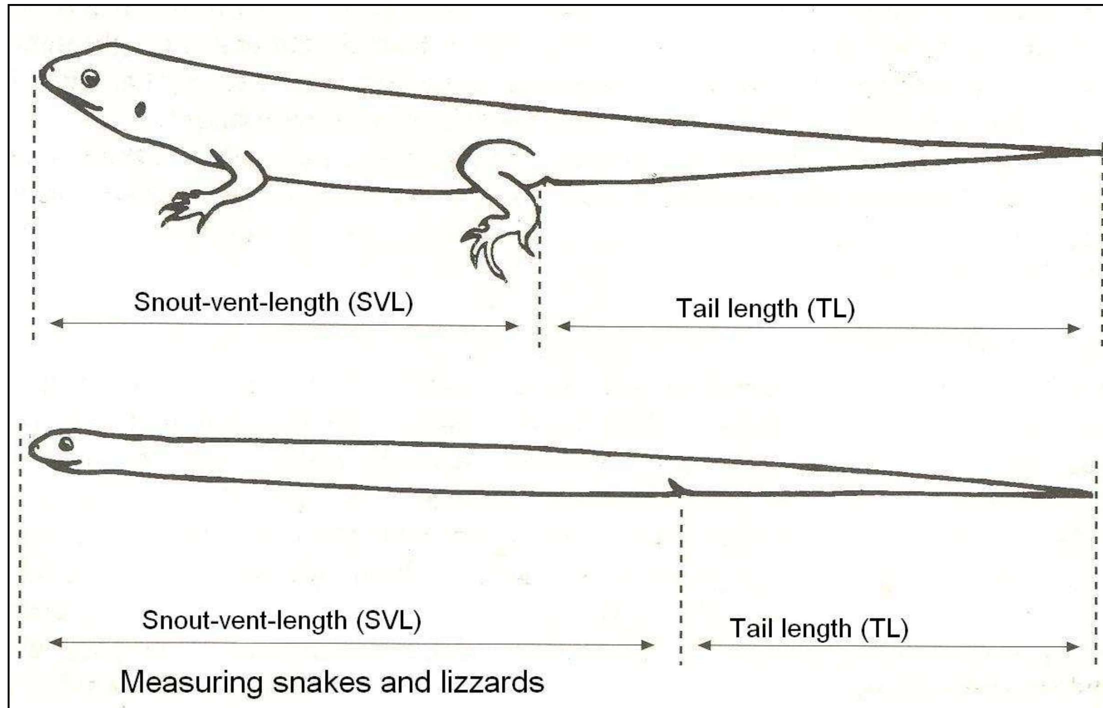

Figure 2.2. Illustration of SVL and Tail length in lizards. Figure taken from Branch (1998).

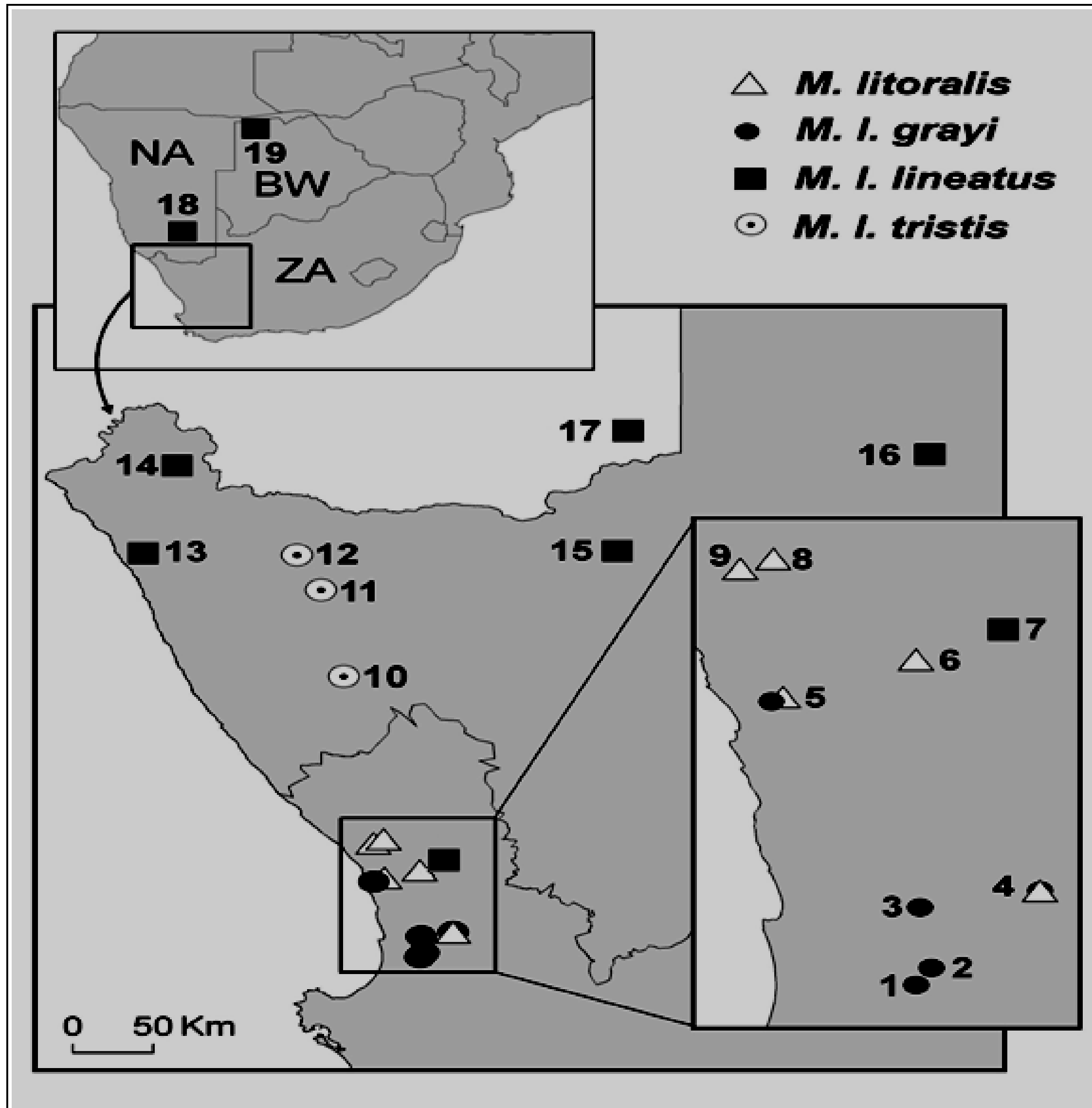

Figure 2.3: Map showing specimens of the *Microacontias lineatus* species complex collected during the present study. (1) Elands Bay, (2) Steenboksfontein, (3) Albani, (4) Graafwater, (5) Doring Bay, (6) Vanrhynsdorp road to Vredendal, (7) Vanrhynsdorp, (8) Omega, (9) Skaapvlei farm, (10) Kamieskroon, (11) Springbok road to Steinkopf, (12) Anenous Pass, (13) McDougalls bay, (14) Kuboes, (15) Pofadder, (16) Upington, (17) Ariamsvlei, (18) Keetmanshoop, and (19) Leseding Research Station, Botswana.

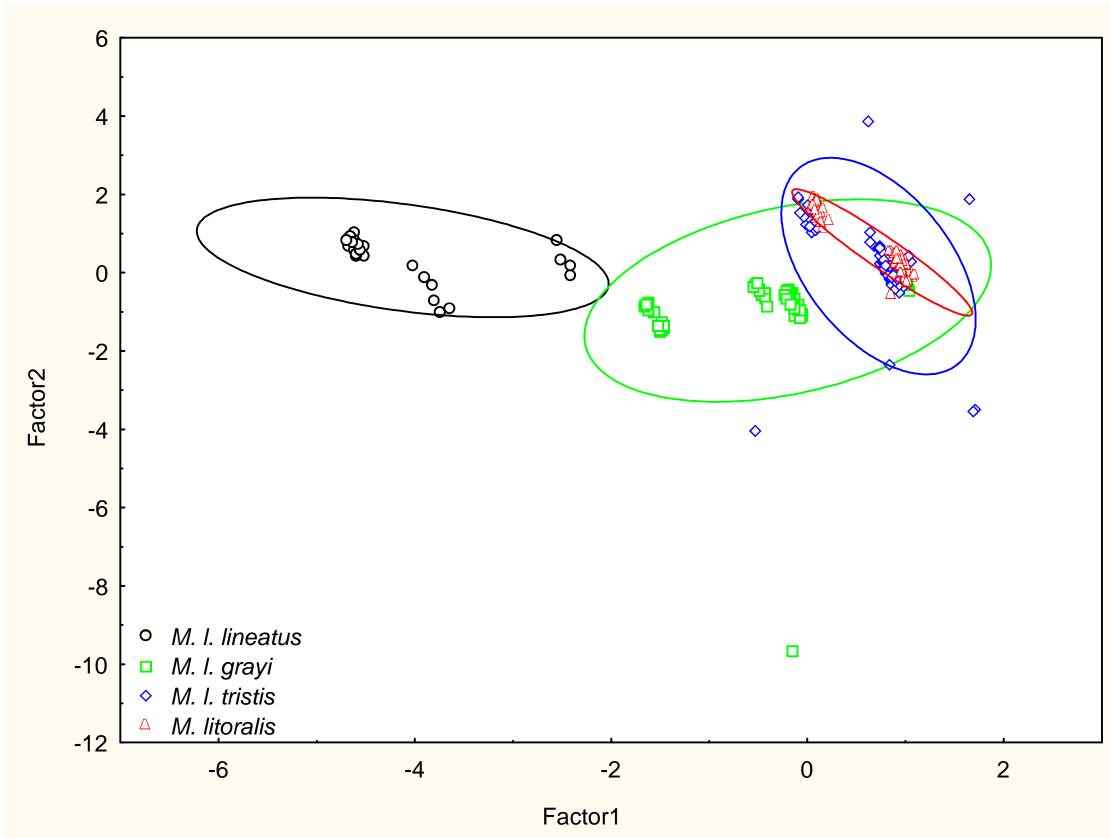

Figure 3.1. PCA scatterplot based on the meristic data to show the spatial position of the taxa relative to each other at 95% confidence intervals.

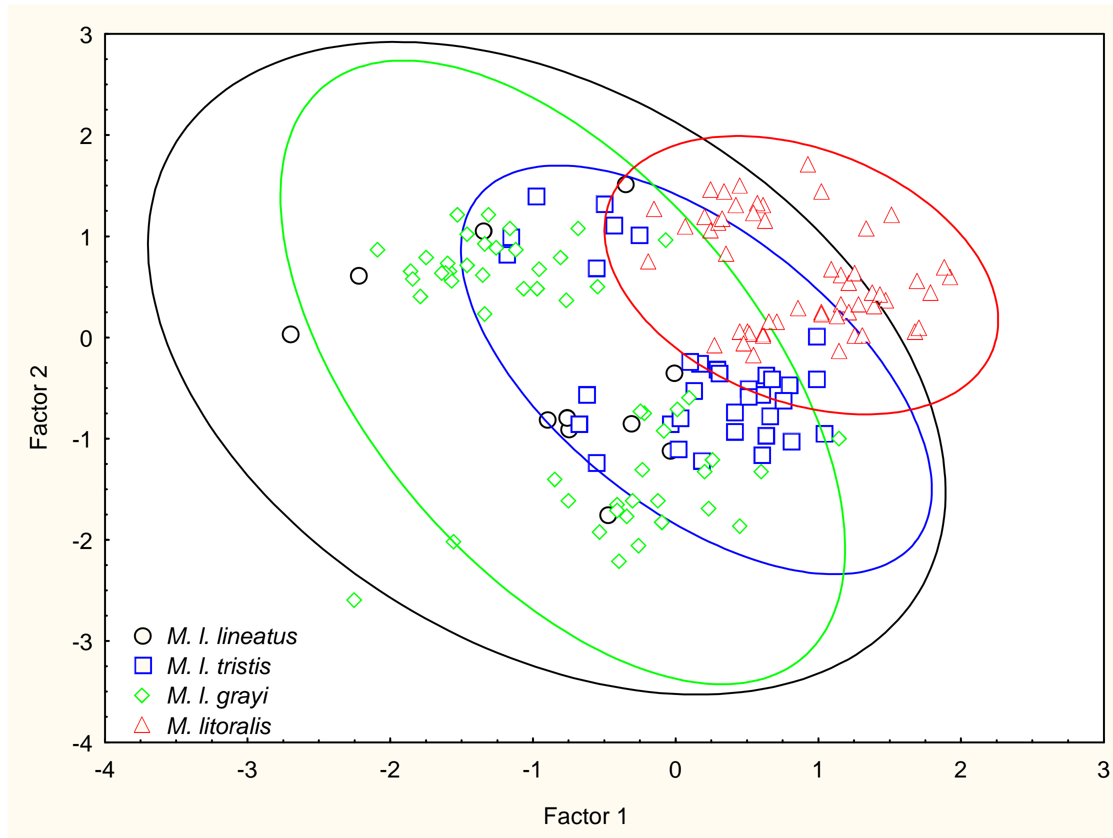

Figure 3.2. PCA scatterplot based on the morphometric data to show the spatial position of the taxa relative to each other at 95% confidence intervals.

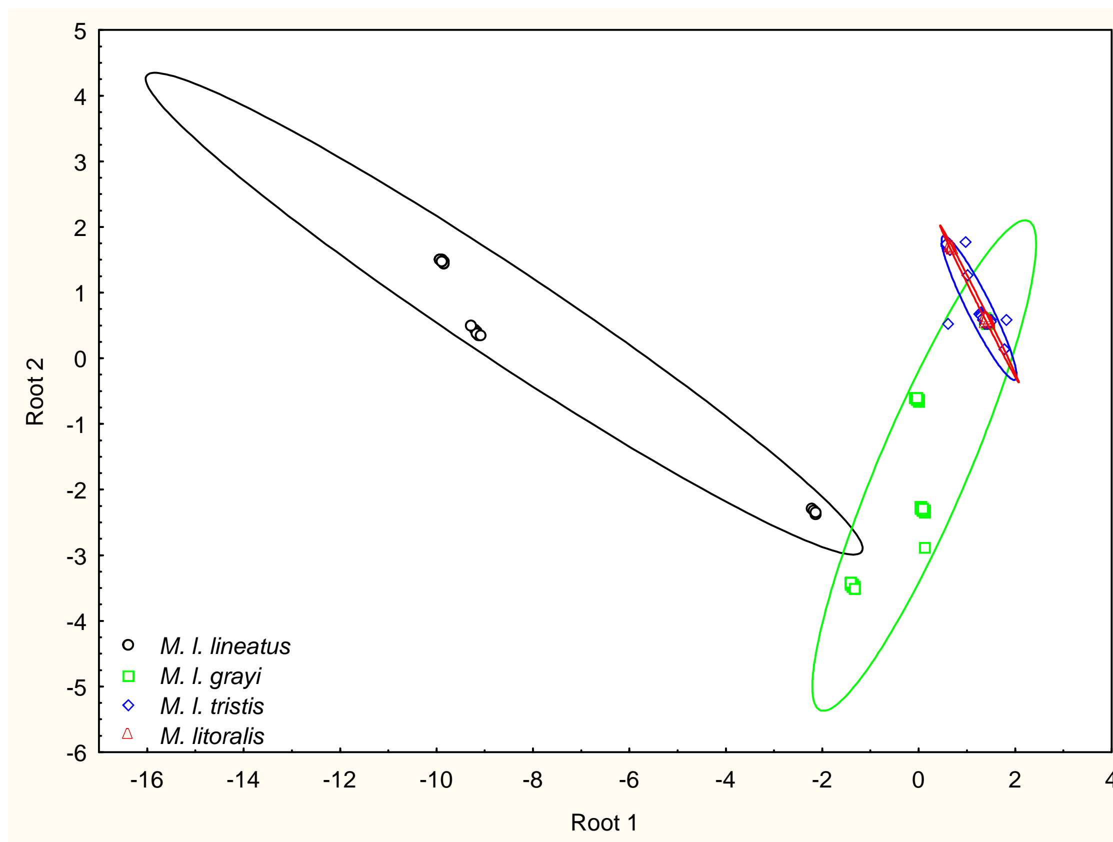

Figure 3.3. DFA scatterplot based on the meristic data to show the spatial position of the taxa relative to each other at 95% confidence intervals.

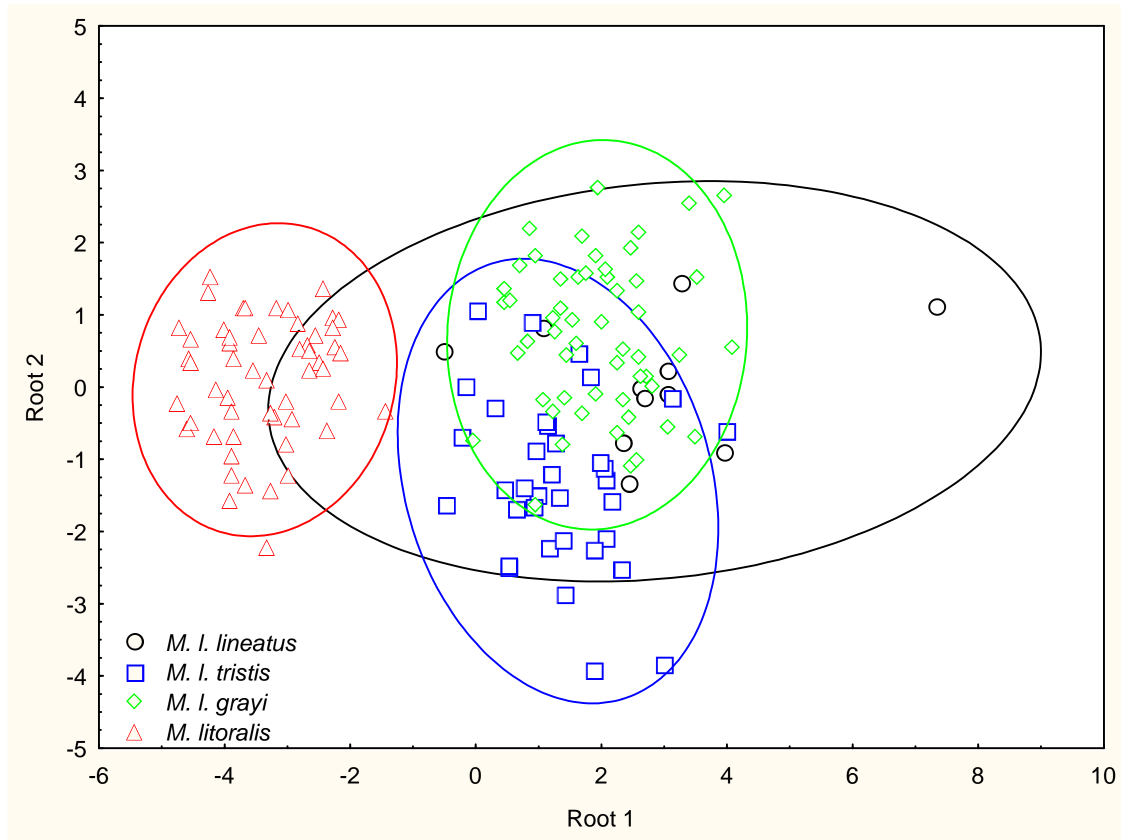

Figure 3.4. DFA scatterplot based on the morphometric data to show the spatial position of the taxa relative to each other, using roots one and two at 95% confidence intervals.

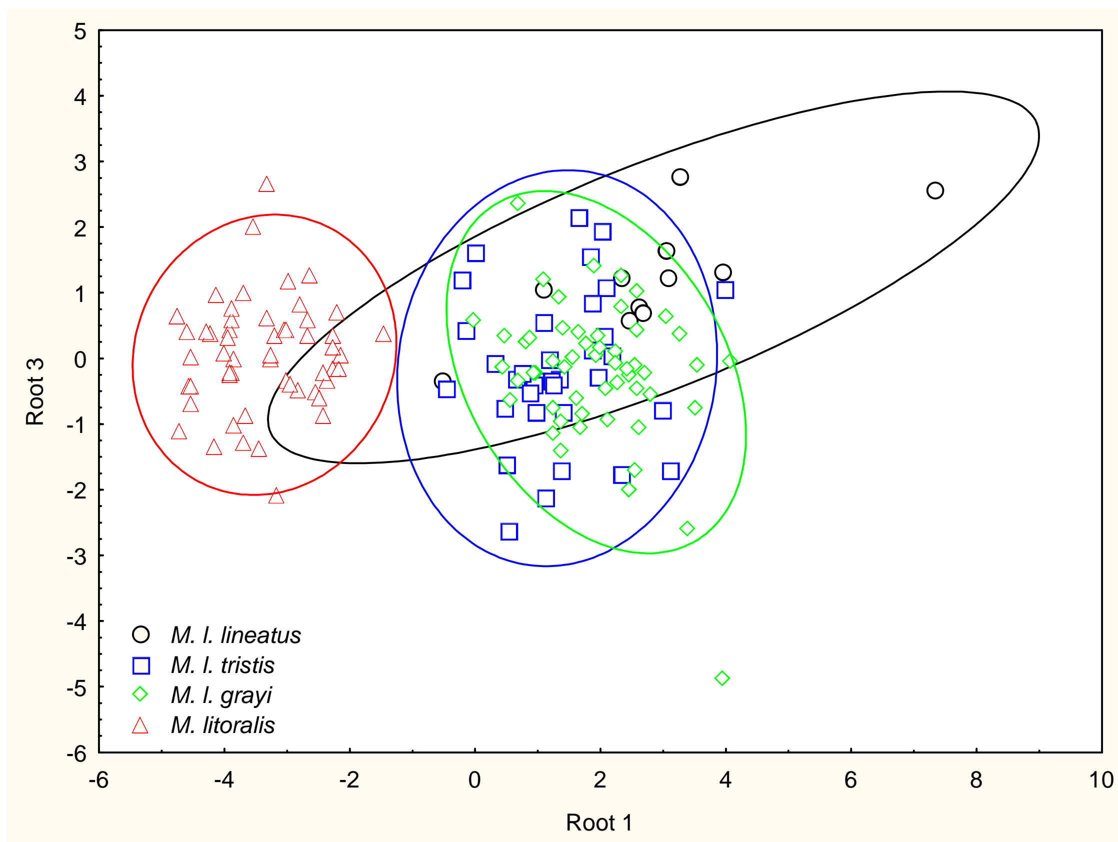

Figure 3.5. DFA scatterplot based on the morphometric data to show the spatial position of the taxa relative to each other, using roots one and three at 95% confidence intervals.

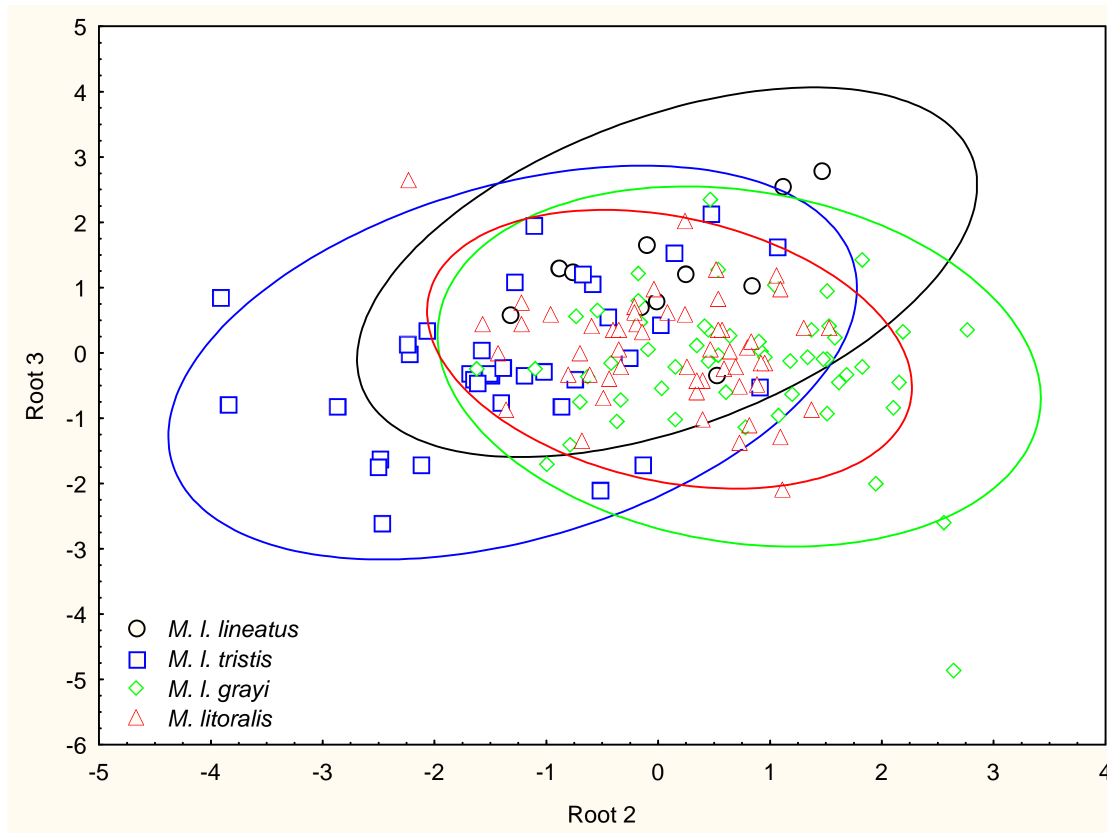

Figure 3.6. DFA scatterplot based on the morphometric data to show the spatial position of the taxa relative to each other, using roots two and three at 95% confidence intervals.

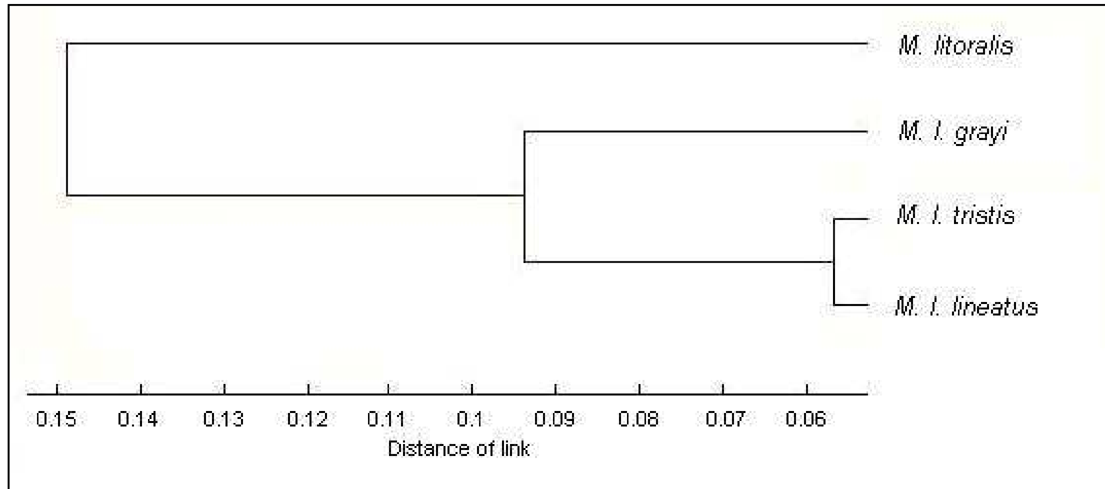

Figure 3.7. Results of the cluster analysis based on the meristic data.

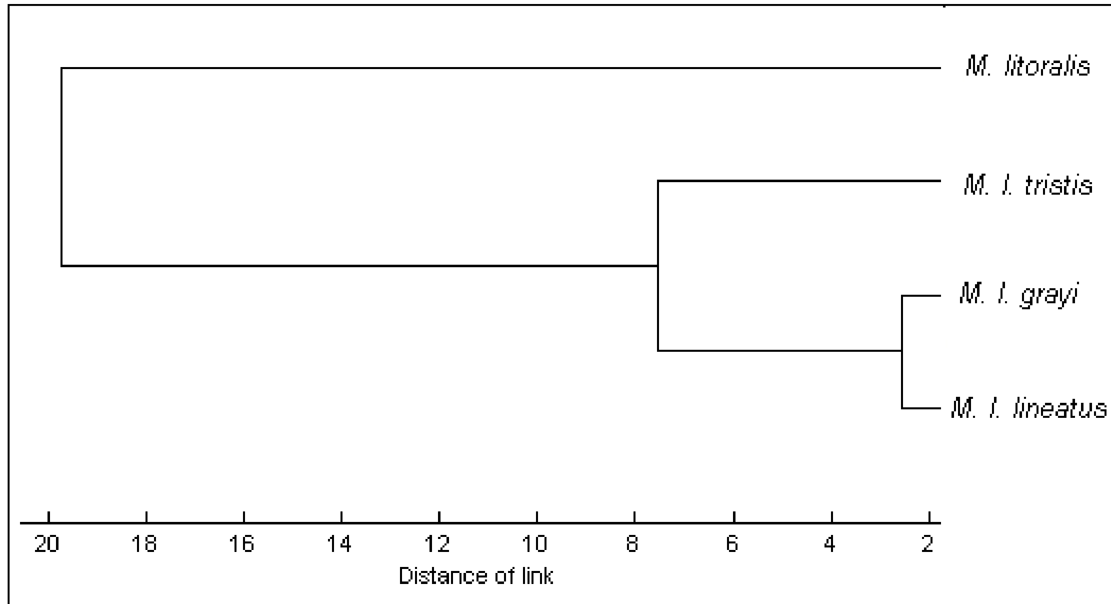

Figure 3.8. Results of the cluster analysis based on the morphometric data for both the head and body measurements.

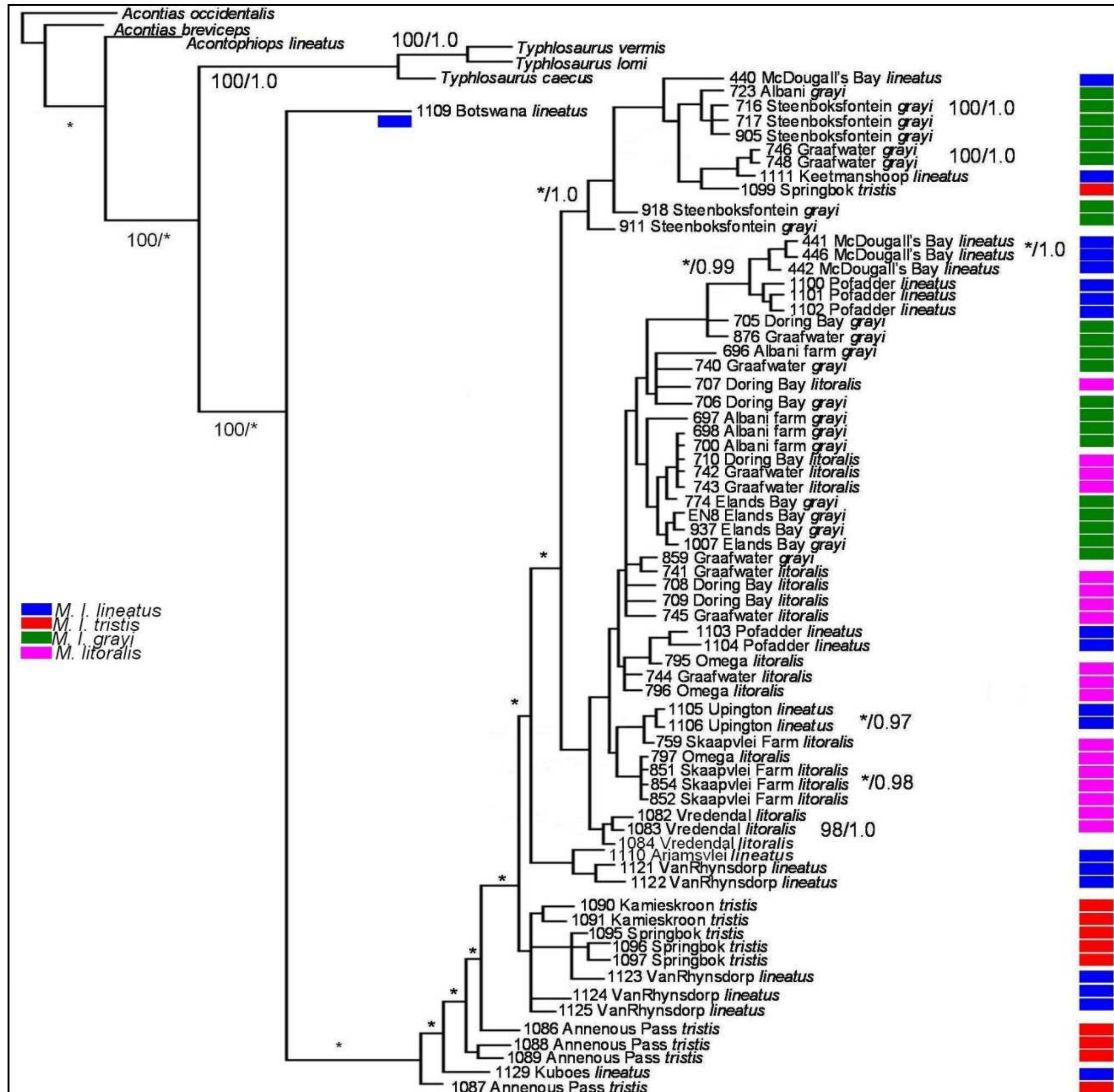

Figure 3.9. Maximum Parsimony phylogram derived from the two mtDNA loci (16S rRNA + cyt *b*) among the *Microacontias*. The first value for each indicated node represents the bootstrap values for the parsimony analyses (%), while the last value for each indicated node represent the posterior probability (*p*P) value derived form the Bayesian inference analyses. Asterices indicate nodes that are unsupported with bootstrap values < 75% and posterior probabilities < 0.95 *p*P.

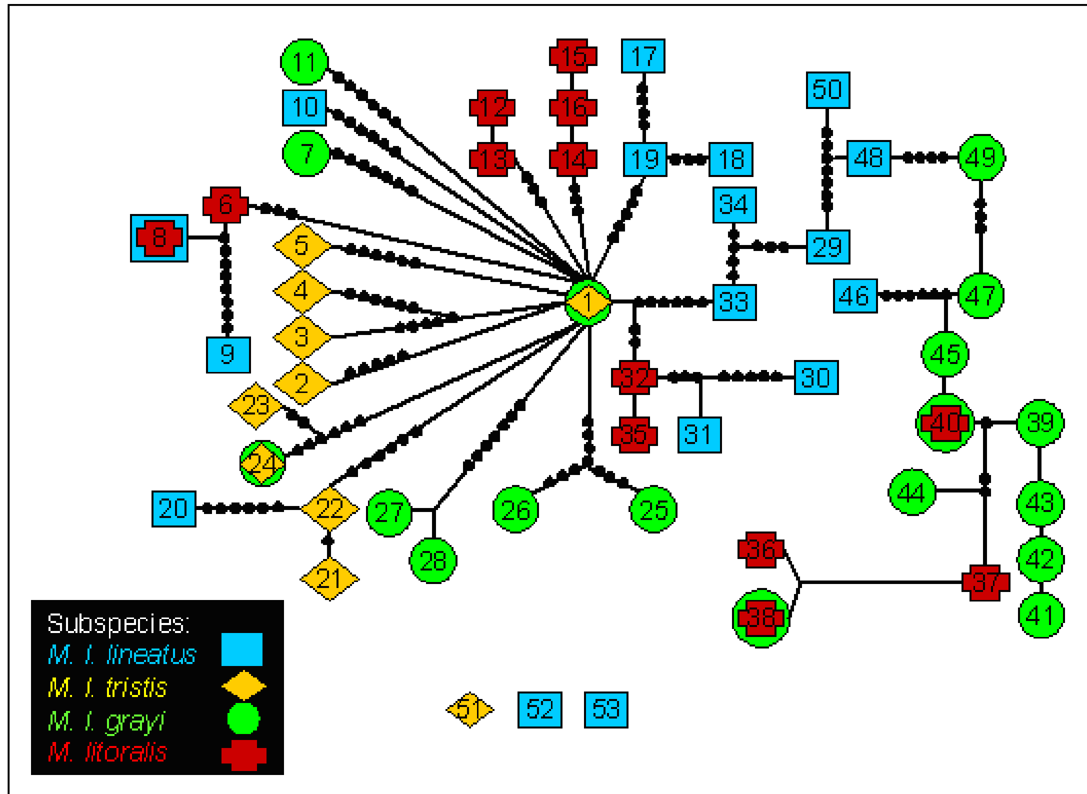

Figure 3.10. Minimum spanning network of the 53 haplotypes derived from the combined analyses of the two mtDNA loci for the *M. lineatus* species complex.

Microacontias, *Acontias*, Subspecies boundaries, Fossorial skinks, Mitochondrial DNA Sequencing, mtDNA, Molecular Systematics, Morphological Systematics, DFA, PCA.

Table 2.1. Number of specimens used for the morphometric and meristic analyses for each *Microacontias* taxon.

Taxa	Morphometric analysis	Meristic analysis
M. l. lineatus	11*	27*
M. l. tristis	35	94
M. l. grayi	54	97
M. litoralis	54	82

*The small sample size of *M. l. lineatus* specimens used in the analyses was due to the difficulty of finding specimens in the field as well as the unsuitability of museum specimens that were brittle due to long storage.

Table 2.2: List of *Microacontias* taxa sampled per locality. *N* represents the sample size per locality.

Taxon	Locality	*N*	GPS Co-ordinates	
M. l. lineatus	Pofadder road to Kakamas	5	29°05' 463" S	19°30' 5 27" E
	McDougall's Bay	4	29°11' 160" S	16°52' 480" E
	Upington	2	28°26' 024" S	21°18' 323" E
	Leseding Research station, Botswana*	1	18°25' 832" S	21°53' 765" E
	Ariamsvlei, Namibia	1	28°05' 600" S	19°36' 380" E
	Keetmanshoop, Namibia	1	26°36' 324" S	18°07' 951" E
	Van Rhynsdorp	5	31°39' 160" S	18°34' 369" E
	Kuboes	1	28°27' 289" S	16°59' 751" E
M. l. tristis	Anenous Pass	4	29°13' 093" S	17°36' 943" E
	Springbok road to Steinkopf	4	29°31' 017" S	17°51' 524" E
	Kammieskroon	2	30°12' 041" S	17°56' 679" E
M. l. grayi	Doring Bay*	2	31°49' 090" S	18°14' 280" E
	Albani Farm, near Lamberts Bay*	5	32°09' 501' S	18°18' 902" E
	Steenboksfontein Farm, near Lamberts Bay*	5	32°09' 966" S	18°19' 902" E
	Graafwater	5	32°09' 372" S	18°36' 467" E
	Elands Bay*	4	32°18' 557" S	18°20' 760" E
M. litoralis	Doring Bay*	4	31°49' 090" S	18°14' 280" E
	Graafwater*	5	32°09' 372" S	18°36' 467" E
	Omega Farm, near Lutzville*	3	31°30' 934' S	18°20' 760" E
	Vredendal road to Van Rhynsdorp*	3	31°39' 157" S	18°34' 371" E
	Skaapvlei Farm, near Lutzville*	4	31°29' 212" S	18°04' 789" E

* Indicates new locality records.

Table 3.1. Variation in head and body scale counts in the *Microacontias* taxa. MSR = Mid-body Scale Row count, SD = Subcaudals, SO = Supraoculars, SC = Supraciliaries, Sb = Suboculars, UL = Upper labials, and VC = ventral scale counts. N refers to sample size.

Taxa	N	MSR	SD	SO (%)		SC (%)		Sb (%)		UL (%)		VC
				1	2	2	3	2	3	4	5	
M. l. lineatus	27	14	36 – 45	0	100	78	22	15	85	0	100	161 – 181
M. l. tristis	94	14	34 – 40	100	0	18	82	100	0	100	0	164 – 181
M. l. grayi	97	14(15)*	34 – 39	69	31	0	100	100	0	36	64	161 – 172
M. litoralis	82	14	34 – 40	100	0	23	77	100	0	100	0	147 – 160

*A single specimen was found with an MSR count of 15.

Table 3.2. Variation in absolute head and body measurements expressed as ratios of SVL in the *Microacontias* taxa. SVL = Snout-Vent length, TL = Tail length, HW = Head width, HL = Head length, HH = Head height, ML = Mental scale length, and RL = Rostral scale length.

Taxa		TL/SVL	HW/SVL	HL/SVL	HH/SVL	ML/SVL	RL/SVL
M. l. lineatus	Min	0.231	0.029	0.021	0.021	0.020	0.013
	Max	0.257	0.053	0.049	0.049	0.046	0.017
	Average	0.243	0.034	0.034	0.032	0.014	0.020
M. l. tristis	Min	0.065	0.026	0.022	0.022	0.017	0.011
	Max	0.281	0.039	0.045	0.045	0.045	0.019
	Average	0.213	0.031	0.035	0.027	0.014	0.020
M. l. grayi	Min	0.081	0.016	0.021	0.022	0.017	0.010
	Max	0.255	0.039	0.074	0.074	0.050	0.037
	Average	0.211	0.032	0.039	0.033	0.017	0.022
M. litoralis	Min	0.071	0.020	0.016	0.016	0.020	0.008
	Max	0.264	0.036	0.046	0.046	0.044	0.019
	Average	0.226	0.030	0.0289	0.034	0.014	0.021

Table 3.3. Results of a one-way multivariate analysis with the meristic data of all the taxa, $p < 0.001$ is indicative of significant differentiation.

	Test	Value	F	Effect	Error	*P*
Intercept	Wilks lambda	0.000	1912515	7	290.000	0.000
Species	Wilks lambda	0.045	77	21	833.274	0.000

Table 3.4. Results of a one-way multivariate analysis with the morphometric data of all the taxa, $p < 0.001$ is indicative of significant differentiation.

	Test	Value	F	Effect	Error	*P*
Intercept	Wilks lambda	0.000	66762.16	7	144.000	0.00
Species	Wilks lambda	0.075	28.87	21	414.040	0.00

Table 3.5. Results of the PCA using the meristic data. See Table 3.1 for abbreviations.

Factor	Eigen value	% Total variance	Cumulative Eigen value	Cumulative %	Eigenvectors of Factor weights						
					MSR	SD	SO	SC	Sb	UL	VC
1	2.2276	31.82	2.2276	31.82	-0.003	-0.076	-0.374	0.217	-0.383	-0.324	-0.072
2	1.115	15.93	3.3426	47.75	-0.476	0.462	-0.119	-0.478	0.098	-0.334	-0.305
3	1.0108	14.44	4.3534	62.19	0.516	-0.216	-0.002	-0.241	0.021	0.038	-0.785
4	0.9679	13.83	5.3213	76.02	0.639	0.741	-0.166	-0.006	-0.017	-0.019	0.217
5	0.8636	12.34	6.1849	88.36	-0.275	0.435	0.181	0.680	-0.103	0.378	-0.494
6	0.4544	6.49	6.6393	94.85	0.191	-0.016	0.605	0.550	0.623	-1.049	-0.074
7	0.3607	5.15	7	100	-0.072	-0.133	1.119	0.353	1.149	0.217	-0.075

Table 3.6. Results of the PCA using the morphometric data. See Table 3.1 for abbreviations.

Factor	Eigen value	% Total variance	Cumulative Eigen value	Cumulative %	Eigenvectors of Factor weights						
					SVL	TL/ SVL	HW/ SVL	HL/ SVL	HH/ SVL	ML/ SVL	RL/ SVL
1	2.593	37.050	2.593	37.050	-0.488	-0.007	-0.473	0.161	-0.338	-0.484	-0.406
2	1.600	22.858	4.195	59.908	-0.189	0.132	-0.289	-0.699	0.597	-0.094	-0.102
3	0.995	14.220	5.189	74.128	-0.085	-0.986	-0.042	-0.115	0.049	0.017	0.061
4	0.711	10.15	5.900	84.282	0.033	-0.049	0.236	-0.091	-0.045	0.452	-0.852
5	0.465	6.65	6.365	90.929	-0.709	0.073	-0.099	0.185	0.068	0.619	0.246
6	0.404	5.768	6.769	96.700	-0.447	0.033	0.748	-0.319	-0.196	-0.307	0.069
7	0.231	3.304	7.000	100.000	0.125	0.048	-0.258	-0.573	-0.694	0.274	0.174

Table 3.7. Results of the DFA based on the meristic data. See Table 3.1 for abbreviations. DFA summary: approximate Wilks' lambda: 0.04543; $F_{(21,833)} = 76.778$; $p < 0.001$.

Scales	Wilks' lambda	Partial lambda	F-remove 3,290	*p*	Tolerance	R^2
Sb	0.152	0.299	226.683	0.000	0.993	0.007
UL	0.085	0.536	83.671	0.000	0.994	0.006
SO	0.056	0.816	21.817	0.000	0.992	0.008
SC	0.052	0.869	14.600	0.000	0.988	0.012
VC	0.050	0.905	10.096	0.000	0.995	0.005
SD	0.046	0.995	0.486	0.692	0.996	0.011
MSR	0.045	0.999	0.059	0.981	0.989	0.0113

Table 3.8. Results of the DFA based on the morphometric. See Table 3.1 for abbreviations, all measurements are given as ratios of SVL. DFA summary: approximate Wilks' lambda: 0.07504; $F_{(21,414)} = 28.870$; $p < 0.001$.

Measurements	Wilks' lambda	Partial lambda	F-remove 3,290	*p*	Tolerance	R^2
HW	0.250	0.300	111.934	0.000	0.845276	0.155
HH	0.113	0.663	24.385	0.000	0.307288	0.693
HL	0.096	0.779	13.617	0.000	0.381382	0.619
ML	0.092	0.816	10.803	0.000	0.855538	0.144
TL	0.077	0.970	1.465	0.227	0.994852	0.005
RL	0.076	0.990	0.455	0.715	0.869352	0.131

Table 3.9. The DFA classification matrix based on the meristic data.

	Taxa	Percent correctly classified	1	2	3	4
1	*M. l. lineatus*	85%	23	4	0	0
2	*M. l. tristis*	75%	0	73	24	0
3	*M. l. grayi*	94%	0	0	89	5
4	*M. litoralis*	82%	0	0	14	68

Table 3.10: The DFA classification based on the morphometric data.

	Taxa	Percent correctly classified	1	2	3	4
1	*M. l. lineatus*	45%	5	2	4	0
2	*M. l. tristis*	80%	3	28	4	0
3	*M. l. grayi*	91%	0	5	49	0
4	*M. litoralis*	100%	0	0	0	54

Table 3.11. Euclidean distances of the meristic and morphometric data. The top right values represent the distances for the meristic data and the bottom left for the morphometric data.

		Euclidean distances			
		1	2	3	4
1	*M. l. lineatus*	0	7.5295	2.5673	19.7228
2	*M. l. grayi*	0.0938	0	6.3895	12.5374
3	*M. l. tristis*	0.0567	0.0747	0	18.8351
4	*M. litoralis*	0.1488	0.0944	0.1310	0

Table 3.12: List of the haplotype (combined 16S and cyt *b*) frequencies for each sampled locality for the *Microacontias lineatus* species complex.

Haplotype		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Localities	Subspecies																				
Anenous	*M. l. tristis*	1	1	1	1																
VanRhynsdorp	*M. l. lineatus*	1																	1	1	1
Keetmanshoop	*M. l. lineatus*	1																			
Springbok	*M. l. tristis*					1															
Skaapvlei farm	*M. litoralis*						1						1	2							
Steenboksfontein	*M. l. grayi*							1				1									
Upington	*M. l. lineatus*								1	1											
Doring Bay	*M. l. grayi*																				
	M. litoralis								1												
McDougall's Bay	*M. l. lineatus*										1										
Omega farm	*M. litoralis*												1								
Kamieskroon	*M. l. tristis*														1	1	1				
Ariamsvlei	*M. l. lineatus*																	1			

Table 3.12 Continued.

Haplotype		21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
Localities	Subspecies																			
Springbok	*M. l. tristis*	1	1																	
Kamieskroon	*M. l. tristis*			1	1															
VanRhynsdorp	*M. l. lineatus*				1															
Steenboksfontein	*M. l. grayi*					3														
Albani farm	*M. l. grayi*						1													
Graafwater	*M. l. grayi*							1	1										1	
	M. litoralis															1	1		1	
McDougall's Bay	*M. l. lineatus*									1				1	1					
Pofadder	*M. l. lineatus*										1	1								
Omega farm	*M. litoralis*												1			1				
Doring Bay	*M. l. grayi*																	2		
	M. litoralis																			
Elands Bay	*M. l. grayi*																			1

Table 3.12 Continued.

Haplotype		40	41	42	43	44	45	46	47	48	49	50	51	52	53
Localities	Subspecies														
Albani farm	*M. l. grayi*	2				1	1								
Graafwater	*M. l. grayi*								1		1				
	M. litoralis	2													
Doring Bay	*M. l. grayi*							1			1				
	M. litoralis	1													
Elands Bay	*M. l. grayi*		1	1	1										
Pofadder	*M. l. lineatus*							1		1		1			
Springbok	*M. l. tristis*												1		
Kuboes	*M. l. lineatus*													1	
Leseding, Botswana	*M. l. lineatus*														1

Table 3.13: The nested contingency results based on 10000 permutations in GeoDIS. Significant support values for the used clades are $p < 0.05$. Inferences were made by using Templeton's key (2004).

Clade	χ^2	Probability	Inference chain	Inferred chain
3-6	20.77	0.002	1-2-3-4	Restricted gene-flow with isolation by distance
4-9	64.87	0.002	1-2-3-5-6-7	Restricted gene-flow with dispersal, but long distance dispersal
5-3	14.60	0.016	1-2-3-5-6-7-8	Sampling design inadequate to describe isolation by distance vs. long distance dispersal
Total	112.66	0.023	1-2-3-5-6-7-8	Sampling design inadequate to describe isolation by distance vs. long distance dispersal

Table 3.14: Diversity measures for the *M. lineatus* subspecies sampled. *N* represents the sample size, *Nh* the number of haplotypes, *Np* the number of polymorphic sites, *h* the haplotype diversity and π_n the nucleotide diversity.

Locality	Subspecies	*N*	*Nh*	*Np*	*h*	π_n
Elands Bay	*M. l. grayi*	4	4	4	1.0000 +/- 0.1768	0.002008 +/- 0.001659
Steenboksfontein	*M. l. grayi*	5	3	36	0.7000 +/- 0.2184	0.016960 +/- 0.010646
Albani farm	*M. l. grayi*	5	4	28	0.9000 +/- 0.1610	0.010751 +/- 0.006882
Graafwater	*M. l. grayi / M. litoralis*	10	8	42	0.9556 +/- 0.0594	0.013655 +/- 0.007562
Doring Bay	*M. l. grayi / M. litoralis*	6	5	22	0.9333 +/- 0.1217	0.007600 +/- 0.004747
Omega farm	*M. litoralis*	3	3	11	1.0000 +/- 0.2711	0.006796 +/- 0.005465
Skaapvlei farm	*M. litoralis*	4	3	9	0.8333 +/- 0.2224	0.004171 +/- 0.003091
Vredendal	*M. litoralis*	3	3	2	1.0000 +/- 0.2722	0.001236 +/- 0.001270
Kamieskroon	*M. l. tristis*	2	2	6	1.0000 +/- 0.5000	0.005556 +/- 0.006001
Springbok	*M. l. tristis*	4	4	35	1.0000 +/- 0.1768	0.016204 +/- 0.010962
Anenous Pass	*M. l. tristis*	4	4	11	1.0000 +/- 0.4768	0.010721 +/- 0.007783
McDougall’s Bay	*M. l. lineatus*	4	4	26	1.0000 +/- 0.1768	0.012203 +/- 0.008349
VanRhynsdorp	*M. l. lineatus*	5	3	13	1.0000 +/- 0.1265	0.011696 +/- 0.007846
Pofadder	*M. l. lineatus*	5	5	20	1.0000 +/- 0.1265	0.009453 +/- 0.006095
Upington	*M. l. lineatus*	2	2	9	1.0000 +/- 0.5000	0.008341 +/- 0.008792
Ariamsvlei	*M. l. lineatus*	1	1	N/A	N/A	N/A
Keetmanshoop	*M. l. lineatus*	1	1	N/A	N/A	N/A
Leseding, Botswana	*M. l. lineatus*	1	1	N/A	N/A	N/A
Kuboes	*M. l. lineatus*	1	1	N/A	N/A	N/A

Table 3.15: Pair-wise sequence divergences. The highlighted values indicate the significant pair-wise difference between haplotypes. Values are relative as they indicate haplotype distances, regardless of sample quantity and distribution congruency ($p < 0.05$).

	1	2	3	4	5	6	7	8	9	10	11
1	0.00000										
2	-0.71930	0.00000									
3	0.22185	-0.74242	0.00000								
4	**0.32009**	-0.52174	**0.19097**	0.00000							
5	0.65057	-0.05882	**0.55145**	**0.50562**	0.00000						
6	**0.60208**	-0.12500	0.50226	0.42624	**0.50345**	0.00000					
7	**0.58982**	-0.29474	**0.52250**	**0.49601**	**0.41802**	0.33628	0.00000				
8	**0.61826**	-0.06250	**0.56807**	**0.54334**	**0.56160**	**0.40159**	**0.15525**	0.00000			
9	**0.49402**	-1.28750	**0.47683**	**0.41478**	**0.57498**	0.45950	**0.51801**	**0.56019**	0.00000		
10	**0.51377**	-0.81609	**0.50000**	**0.48023**	0.55274	0.43316	**0.35718**	**0.27729**	**0.48014**	0.00000	
11	**0.56782**	-0.69231	**0.46323**	0.40757	0.18848	0.45244	0.07565	0.30951	**0.50259**	**0.36118**	0.00000
12	0.54630	1.00000	0.02542	0.14634	0.79310	0.61702	0.64088	0.60769	0.44207	0.48867	0.66154
13	0.70644	0.71429	**0.59111**	**0.53658**	**0.70805**	0.75806	**0.58369**	**0.64115**	0.55646	**0.61826**	0.62500
14	**0.42664**	-1.37634	**0.39015**	**0.37760**	0.29936	0.26754	-0.02198	0.19878	**0.37885**	**0.26318**	0.03309
15	**0.55277**	-0.52632	**0.48712**	**0.44369**	**0.46026**	0.38485	0.06988	**0.29949**	**0.30787**	**0.31950**	**0.20357**
16	0.89727	1.00000	0.86597	0.82968	0.95373	0.90863	0.91399	0.89613	0.81056	0.86356	0.92414
17	**0.74564**	0.69048	**0.64539**	**0.58377**	**0.72222**	**0.71527**	**0.20530**	**0.43190**	**0.52443**	**0.48675**	**0.48110**
18	0.46315	0.14286	0.21569	0.06118	0.71917	0.63415	**0.59599**	0.63300	**0.50923**	0.57551	0.60426
19	0.76156	1.00000	0.69895	0.59302	0.90955	0.82857	0.84078	0.81915	0.68448	0.76276	0.85235

Table 3.15 Continued:

	12	13	14	15	16	17	18	19
11								
12	0.00000							
13	0.93651	0.00000						
14	0.41765	0.38693	0.00000					
15	0.54688	**0.55826**	-0.02956	0.00000				
16	1.00000	0.98611	0.84889	0.87815	0.00000			
17	0.90877	**0.87606**	0.07048	0.07323	0.97676	0.00000		
18	0.64706	0.84585	**0.41201**	0.54180	0.93970	0.83779	0.00000	
19	1.00000	0.97279	0.72358	0.78439	1.00000	0.95813	0.85714	0.00000