The morphological and molecular variation of southern African *Nannocharax* (Characiformes: Distichodontidae), and its taxonomic implications

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Abstract

Nannocharax is the most species rich genus in the family Distichodontidae, being currently represented by 41 species. The genus is widely distributed across much of sub-Saharan Africa, with a range extending from the Zambezi ichthyofaunal province in the south to the Nilo-Sudan ichthyofaunal province in the north. In southern Africa, the genus is currently represented by four species, *Nannocharax dageti* Jerep, Vari, & Vreven, 2014, *N. machadoi* (Poll, 1967), *N. macropterus* Pellegrin, 1926, and *N. multifasciatus* Boulenger, 1923. Each of these species exhibit considerable intraspecific pigmentation pattern variation across their respective distribution ranges, suggesting that the current taxonomy possibly underestimates the taxonomic diversity of *Nannocharax* species in southern Africa. Much pigmentation pattern variation within these southern African species has been observed by both collectors and scientists in the field, prompting an investigation into the extent of this morphological variation as well as what molecular variation may occur as well. The genus displays a high degree of morphological conservatisim, making it difficult to assign external morphological characters as diagnostic. To this end, this study was conducted to determine the extent of diversity of this genus in the region, employing an integrative approach with traditional morphological analysis techniques as well as sequencing the 'barcoding gene', cytochrome oxidase I, testing the hypothesis that there is a greater, hidden diversity of this genus in the region than currently recognised. This study aims to identify these potential lineages and accurately map their distributions.

Phylogenetic analyses were performed using maximum parsimony, maximum likelihood, and Bayesian inference, using the mitochondrial cytochrome oxidase I gene region. Massive genetic divergence was detected between populations of taxa previously considered to be singular, widely distributed species. The three approaches of phylogenetic inference used in this study yielded trees of comparable overall topology, with the exception of the maximum parsimony tree which indicated additional lineages within the southern African *N. multifasciatus* group.

These analyses revealed four deeply divergent (1.3 – 12.3%) lineages within southern African *N. macropterus*, as well as two deeply divergent (0.4-14.6%) populations from the Congo ichthyofaunal region, the lineages here named "*N. macropterus* Congo 1" and "*N. macropterus* Congo 2". Within the southern African region, two deeply divergent (10.3%) lineages of *N.*

macropterus were identified from the Okavango River system, identified as "*N. macropterus* Okavango 2" lineage restricted to the Cuito-Canavale tributary, and "*N. macropterus* Okavango 1" distributed throughout the remainder of the Okavango system. "*N. macropterus* Okavango 2" shares a closer relationship with the unique lineage from the Kwanza ichthyofaunal region, named *N. macropterus "*Kwanza", which itself is deeply divergent from the *N. macropterus "*Okavango 1", *N. macropterus "*Zambezi", *N. macropterus "*Congo 1" and *N. macropterus "*Congo 2" lineages (3.1-14.4%). Principal component analyses (PCA) and discriminant function analyses (DFA) produced overlapping clusters for all identified lineages, with the exception of the *N. macropterus "*Kwanza" lineage, which in all analyses clustered away from the other lineages. Analysis of variance (ANOVA) and Kruskall-Wallis tests indicated significant differences in means between character traits between lineages, however, overlap in measurements and counts occurred in all instances except between the *N. macropterus "*Kwanza" and *N. macropterus* Congo lineages. However the *N. macropterus "*Kwanza" lineages could be distinguished from the other lineages by generally smaller fin lengths (dorsal fin 19.5%SL vs 20.0-22.1%SL in others; pectoral fin 16.5%SL vs 20.6-21.8%SL in others; pelvic fin 18.3%SL vs 21.3-22.4) and pigmentation pattern differences. The *N. macropterus* species group displayed extensive pigmentation pattern variation, to the extent that five pattern grades could be used to classify them. These pattern grades, while not specific to river systems, showed patterns similar to that which was seen in the molecular analyses and could be linked to lineages with only minor overlap between them.

Three lineages of *N. multifasciatus* were identified, with two occurring in the southern African region, each corresponding to a river system, being the *N. multifasciatus "*Zambezi" and *N. multifasciatus "*Okavango" lineages. This species group displayed shallower divergence between lineages than did the *N. macropterus* group, at 2.5% genetic distance. Genetic analysis inferred a closer relationship between the *N. multifasciatus "*Zambezi" and *N. multifasciatus "*Congo" lineages than with the *N. multifasciatus "*Okavango" lineage. Morphological PCA and DFA analyses indicated morphological divergence of the *N. multifasciatus "*Congo" lineage, with generally larger proportional measurements than southern African specimens (body width 12.6%SL vs 9.5-9.7%SL; body depth 26.6%SL vs 21.6-21.9%SL; head width 12.0%SL vs 10.0-10.4%SL). PCA, DFA, and measurements show a near complete overlap between the *N. multifasciatus "*Okavango" and *N. multifasciatus "*Zambezi" lineages. Pigmentation pattern variation occurred within this group, but none that could be assigned to a particular lineage.

The *N. machadoi* species group in southern Africa consists of five lineages: *N. machadoi "*Zambezi 1", *N. machadoi "*Zambezi 2", *N. machadoi "*Kafue 1", *N. machadoi "*Kafue 2", and *N. machadoi "*Okavango". This group displayed shallower genetic divergence between lineages than the other southern African *Nannocharax* species groups (0.4-1.3%). This shallow genetic divergence is paralleled by near complete morphological overlap, with PCA and DFA producing overlapping clusters, and measurements, meristics, and pigmentation pattern metrics consisting of very similar values for the lineages.

These results indicate that what is considered to be "*N. macropterus*" in southern Africa should not be named as such. The *N. macropterus* "Zambezi" and the *N. macropterus "*Okavango 1" lineages, are misidentifications of *Nannocharax dageti*. Other "*N. macropterus*" from the southern African region possesses fewer circumpeduncular scales than the true *N. macropterus* as described by Pellegrin (1926), and require taxonomic re-evaluation, each here being recognised as a unique lineage with species status, here named *N. macropterus "*Okavango 2" and *N. macropterus "*Kwanza". In particular, *N. macropterus* "Kwanza" displays deep genetic divergence as well as morphological dissimilarity with the other southern African "*N. macropterus*" groups.

Nannocharax fasciolaris and *N. monardi* are here placed as junior synonyms of *N. multifasciatus*, owing to vast overlaps in measurements and character counts of these species and *N. multifasciatus*, which is also known to occur within the same geographical distribution, as well as dubious arguments from the original publications in delineating these species from *N. multifasciatus.* Therefore, there is insufficient evidence indicating the presence of multiple species originating from the Okavango system, where it is here indicated that only a single lineage of banded, adipose fin-bearing *Nannocharax* occurs, namely *N. multifasciatus "*Okavango".

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To my friends both near and far, for the support, especially in my life outside of the laboratory and office. Your companionship provided breaths of fresh air in sometimes otherwise difficult patches.

Declaration

This statement is to hereby declare that this is my own work, generated by myself, unaided. It is being submitted for the requirements of a Master of Science for the Department of Ichthyology and Fisheries Sciences, Rhodes University, Grahamstown. It has not been submitted whole or in part for degree or examination to any other university.

Unless otherwise referenced or indicated, figures and images presented in this thesis were produced or taken by the author.

Timothy Smith.

Chapter 1
Introduction

Freshwater ichthyofauna comprises nearly 14000 described species (Lévêque et al. 2008, Nelson et al. 2016), of which about 2800 come from African continental waters (Skelton & Swartz 2011). About 300 freshwater fish species are being discovered yearly (WCMC 1998), of which, in the past decade, about 25 descriptions per year are from Africa (Skelton & Swartz 2011). Projected estimates place the potential number of African freshwater fishes to exceed 3200 species (Lundberg et al. 2000, Paugy 2010), but such diversity may be lost before it has been discovered (Stiassny 2002). Water usage across Africa is predicted to increase dramatically in the near future (Economic Commission for Africa 2003) for agriculture and human consumption uses – and such increases undoubtedly will create conflicts of interest between socioeconomic and conservation needs (Darwall et al. 2011).

As an area that is currently considered to have lower diversity of freshwater fishes, many which are considered to be wide ranging species, southern African freshwaters have been largely overlooked by conservation and taxonomic authorities (Darwall et al. 2011, Alofs et al. 2013). With some excpetions, such as the Cape Fold Ecoregion, areas across most of the subcontinent remain largely unexplored, raising the possibility that undescribed species may exist in these areas. (Alofs et al. 2013).

Compared to the massive diversity known from equatorial Africa, southern African freshwater ichthyofaunal diversity lacks comparatively. Recent publications of molecular studies investigating southern African fish systematics, however, indicate that more widely distributed fish species show high levels of genetic structuring and may in fact may comprise of complexes of cryptic species (e.g. Goodier et al. 2011, van der Bank et al. 2012, Chakona et al. 2015). Many of these newly identified lineages are narrow range endemics, placing them at greater risk of extinction (Harnik et al. 2012). Undiscovered species, or more troubling, difficult to recognise cryptic species, may be overlooked when considering biological diversity, planning conservation measures, or determining the effects of anthropogenic impacts in systems (Bickford et al. 2007. Species which do not have formal taxonomic status are therefore more difficult to conserve – we cannot protect what we do not have knowledge of (Bickford et al. 2007).

The freshwater fish order Characiformes has a Gondwanan distribution as it has representatives in Central and South America and Africa. It is one of the most speciose orders of freshwater fishes with over 2300 currently recognised species (Nelson et al. 2016). The order Characiformes is divided into two suborders, the Citharinoidei and the Characoidei (Nelson et al. 2016; Chakrabarty et al. 2017). The Citharinoidei contains two families, the Citharinidae and Distichodontidae, which are both endemic to the African continent and are thought to be the sister groups to all the other characins (Arroyave & Stiassny 2011). The family Distichodontidae currently consists of 17 genera and about 101 species of highly variable body form and ecology (Froese & Pauly 2015, Nelson 2016).

Nannocharax, known as citharines or African darters, is the most speciose genus within Distichodontidae, with 41 currently recognized species distributed across tropical sub-Saharan Africa and portions of the Nile River (Poll 1973, Jerep & Vari 2014, Eschmeyer et al. 2017). The greatest diversity for the group occurs within the Congo River basin (Poll 1973, Jerep et al. 2014), with species diversity for the genus decreasing away from this region. *Nannocharax* occur within nearly all mainland African ichthyofaunal regions (as delineated by Roberts 1975), with the exceptions of the Maghreb, Abyssinian highlands, East coast, and Southern regions (Fig. 1.1). Some members of the genus were previously assigned to the genus *Hemigrammocharax* Pellegrin 1923, with these species being differentiated in lacking a completely pored lateral line. However, Jerep & Vari (2014) determined that the extent of poring along the lateral line was not a consistent diagnostic character to separate *Nannocharax* and *Hemigrammocharax* into two monophyletic genera. Based on this evidence, these authors placed *Hemigrammocharax* into synonymy with *Nannocharax*. This change has already been implemented in the Catalogue of Fishes (Eschmeyer et al. 2017).

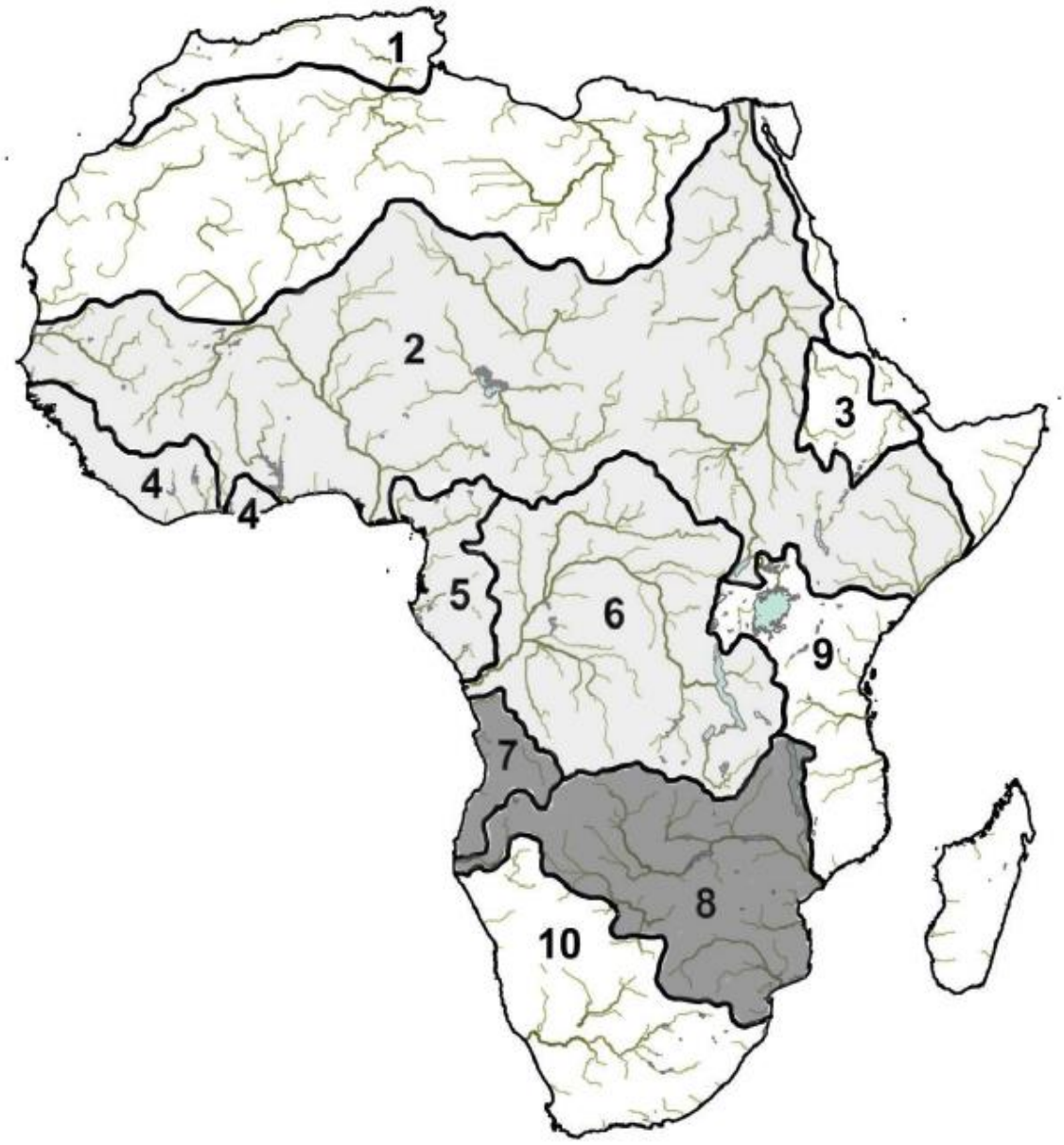

Figure 1.1: Map of the African ichthyofaunal provinces, adapted from Snoeks et al. 2010. The lightly shaded area represents the ichthyofaunal provinces in which *Nannocharax* are known to occur. The present study focused on the *Nannocharax* species in the darker shaded provinces 7 & 8. 1= Maghreb; 2= Nilo-Sudan; 3= Abyssinian Highlands; 4= Upper Guinea; 5= Lower Guinea; 6= Congo; 7= Kwanza; 8= Zambezi; 9= East Coast; 10= Southern

1.1 Characteristics of study group

Members of the Distichodontidae exhibit extremely variable body forms as the family includes both large bodied and very small bodied species. The shared features by species belonging to this family include independently ossified ctenii upon ctenoid scales, bifurcation of the pelvic bone, a lateral ethmoid with posterior extension in contact with the anteromedial edge of the

orbitosphenoid, a mobile premaxilla, loss or reduction and anterior shift of the supraorbital, and attachment of the A_1 adductor mandibulae to the maxilla (Géry 1977, Vari 1979). Species of the genus *Nannocharax* are readily distinguished from other members of Distichodontidae by their distinct morphology, small and overall cylindrical body, flattened ventral surface and a inferior or sub-terminal positioned mouth, that reflects their ecological niche of a primarily benthic micropredatory lifestyle (Géry 1977). As defined by Vari (1979), the clade consisting of *Nannocharax* and *Hemigrammocharax* (now a junior synonym of *Nannocharax*) can be differentiated from other distichodontids by a posteriorly directed dentary process, possession of a single row of teeth on the jaws (premaxillary and dentary), loss of the sensory canal in the dentary, reduction/loss of the premaxillary articular fossa, a lateral ethmoid with a vertically expanded posterior strut, a horizontally expanded hyomandibula, postcleithrum 1 absent, a sphenotic spine structured as a posteroventrally sloping shelf, opercular fenestra opening to its dorsal margin, reduced metapterygoid quadrate-fenestra. The genus also bears a reduced swimbladder with its anterior portion developed into anterior diverticulae (Boulenger 1907, Vari 1979). The teeth in this group are bicuspid, and always arranged in a single row (Géry 1977).

The scale morphology of this group has been described as typical for distichodontids (Vari & Ferraris 2004); the poring of the lateral line scales is variable – even within species – and was previously used to delineate genera (Jerep & Vari 2014). Vari & Ferraris (2004) have described apparent contact organs and breeding tubercles for several species of *Nannocharax*, unique among African characoids. The contact organs described by Vari & Ferraris (2004) were hook-shaped in all observed specimens and present on the pectoral fins and scales immediately surrounding the pectoral insertion point. The extent of the presence of these hooks varied both between specimens and species, but was only present in larger (and assumed male) specimens. The breeding tubercles were present primarily on the head and body, and also on the fins in some specimens, but only among the larger individuals of *N. rubrolabiatus* (Vari and Ferraris 2004). Such features have not been observed in other members of the genus.

The pseudotympanum, an internal structure found in some other characins and Ostariophysians, is known from several members of the genus (Jerep & Vari 2013). In *Nannocharax*, this structure comprises of gaps in muscular tissue between the anterior pleural ribs, with typically three such gaps in specimens studied by Jerep & Vari (2013). To date this structure has been recorded/identified only in South American characins, with the exception of the single African species *Lepidarchus adonis* (Zanata & Vari 2005) and now recently

Nannocharax (Jerep & Vari 2013). Variability in this structure between species and populations has not yet been assessed. The anterior portion of the swimbladder of this genus has also been noted to be hard and globular (Boulenger 1907).

1.2 General biology and ecology of *Nannocharax* species

Little is known about the biology and ecology of *Nannocharax* species (Bell-Cross & Minshull 1988, Marshall 2011). They are generally considered to prefer calm to slow flowing waters and are usually common in shallower waters with vegetation and submerged structures (Géry 1977, Bell-Cross & Minshull 1988, Skelton 2001, Reichard 2008, Marshall 2011). Welcomme & de Merona (1988), Tweddle et al. (2004), and Reichard (2008) found some species of *Nannocharax*, including *N. ansorgii* and *N. macropterus*, to have a preference for sandy substrates, although a *N. macropterus*-like population was collected by Tweddle et al. (2004) in rocky habitats from the Madamyana River within the Zambezi system, noted to be a contrasting habitat preference to other *Nannocharax*. Specimens of *N. macropterus* have been collected in both slow flowing river and pools as well as fast-flowing rivers. The species is most often associated with sandy substrates with vegetation present, but some specimens have also been collected from river reaches with rocky substratum (Tweddle et al. 2004). Specimens of *Nannocharax machadoi* have been found to be frequently associated with vegetated habitats, but have not been recorded from rocky habitats (Hay et al. 1997, Tweddle et al. 2004). *Nannocharax multifasciatus* has been recorded from proximate localities to *N. machadoi*, with the exception of being found in faster flowing, rockier areas where *N. machadoi* does not occur (Hay et al. 1997, Tweddle et al. 2004). *Nannocharax macropterus* has been indicated to have a benthic ecology, while *N. machadoi* and *N. multifasciatus* primarily occupy the water column (Bills et al. 2012). This is reflected in the body forms of these species, with *N. macropterus* having a cylindrical body while both *N. machadoi* and *N. multifasciatus* have laterally flattened bodies. Trophically, they are micropredators, feeding on periphyton and invertebrates from the vegetation among which they live (Skelton 2001).

Both *N. machadoi* and *N. multifasciatus* are known to make migrations upstream and into inundated habitats during the summer floods in the Zambezi (Bell-Cross & Minshull 1988, van der Waal 1996, Bills et al. 2012), although it is unknown if these movements are associated with spawning or feeding. The males of some species are known to become darker during the summer breeding seasons (Skelton 2001). Acquisition of contact organs and development of tubercles have been observed in some mature males at the peak of the breeding season (Vari

and Ferraris 2004). Hauber et al. (2010) observed eggs attached to nearly all the fins of *N. fasciatus* observed in Benin. The relationship between Hauber et al.'s (2010) observations and and the hook-like structures on the fins and body of some *Nannocharax* remains to be explored.

1.3 Currently recognized southern African *Nannocharax*

Skelton (2001) and Jerep et al. (2014) recognise four species of *Nannocharax* occurring in the southern African region (Fig. 1.2), namely *Nannocharax multifasciatus* Boulenger 1923, *N. macropterus* Pellegrin 1926, *N. machadoi* (Poll 1967), and *N. dageti* Jerep, Vari & Vreven (2014). *Nannocharax macropterus* has the widest distribution of the four southern African species, as its range is currently considered to extend from the Okavango and Kwanza River systems in southern Africa, through the Congo system to the Pra River system in west Africa, Ghana (Skelton 2001, Eschmeyer et al. 2016). Specimens from the upper Zambezi and Kafue River were previously identified as *N. macropterus* (e.g. Bell-Cross 1965, Skelton 2001), but these have now been assigned to the recently described species, *N. dageti*, a species whose range extends into the Kasai and Luapula Rivers, tributaries of the Middle and Upper Congo system (Jerep et al. 2014). Both *N. multifasciatus* and *N. machadoi* occur in the upper Zambezi, Kafue, Okavango, Cunene, and Kwanza River systems in southern Africa (Skelton 2001). There are also records of *N. machadoi* from the Zambezi River above Lake Kariba (Marshall 2011), which makes it the only species of this genus found in the Middle Zambezi. Three other species, *N. wittei* (Poll 1933), *N. fasciolaris* (Nichols & Bolton 1927) and *N. monardi* (Pellegrin 1936) were previously reported to occur within the southern African region (Nichols & Bolton 1927, Pellegrin 1936, Anon. 1997, Fig. 1.3), but do not appear to have been collected or identified from these systems since these reports. Confirmation as to the identities of the fishes from these localities is now difficult to confirm, as the types of these specimens have been lost (Eschmeyer et al. 2016).

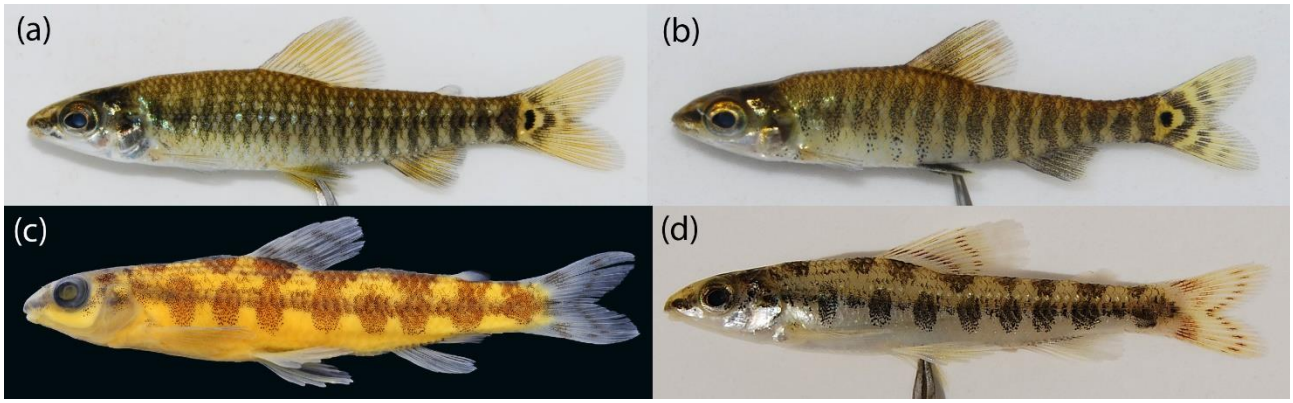

Figure 1.2: The four currently recognised species of *Nannocharax* from southern Africa*:* (a) *N. multifasciatus,* SAIAB95903, Kabampo River, Upper Zambezi River, Zambia; (b) *N. machadoi*, SAIAB186844, Cuito River, Okavango River, Angola; (c) *N. dageti*, CUMV91303, Samfa Rapids, Chambeshi River, Zambia; and (d) *N. macropterus*, SAIAB95807, Musangezhi River, Upper Zambezi River, Zambia. Photo credits (a), (b), (d) Roger Bills. Photo credits (c) Jerep et al. (2014).

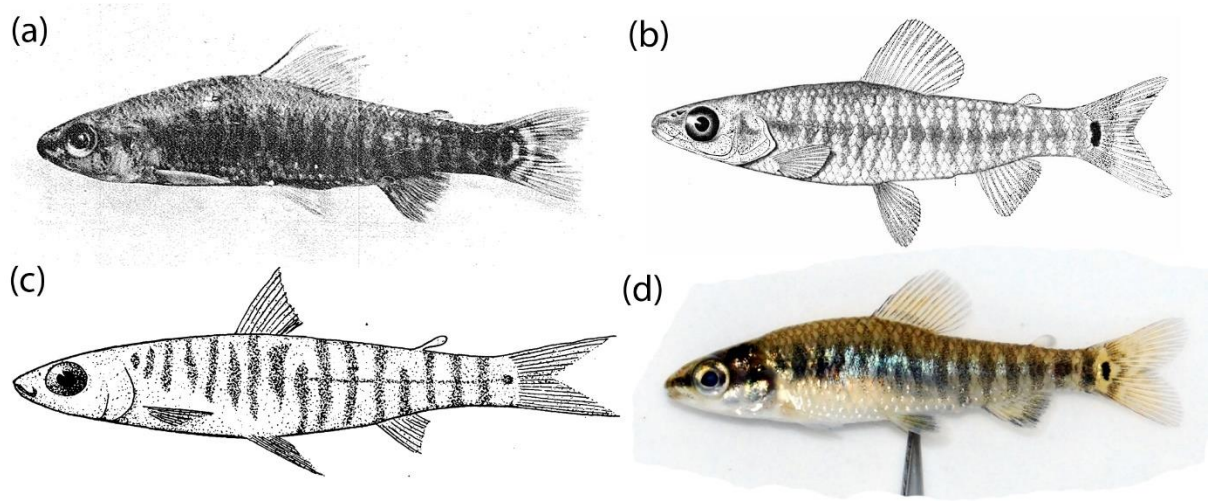

Figure 1.3: additional species which have records of occurring within southern African river systems: (a & b) *N. wittei*, left from original description Poll (1933), right from Poll (1967); (c) *N. fasciolaris* from original description Nichols & Bolton (1927)*;* (d) *N. monardi,* to date, has had no visual representation, and the types have been lost (Eschmeyer et al. 2016). Pictured is a specimen thought to be *N. monardi* that was collected from the Okavango River (Prof. Paul Skelton pers. comm 2016).

1.4 Taxonomic history of southern African *Nannocharax*

In addition to the four species that are currently recognised from southern Africa, there are records of several specimens that have been collected from extensive surveys across the region, but these could not be assigned to any of the currently described species (Table 1.1) that occur within river systems within the selected study area. In the context of the chosen study area of this thesis, the primary species of concern are therefore *N. dageti, N. fasciolaris, N. machadoi, N. macropterus, N. monardi, N. multifasciatus,* and *N. wittei*.

Aside from the original species descriptions, little taxonomic work has been done on *Nannocharax* with the exception of the review done by Boulenger in 1909. The early species descriptions (pre-1930) lack much detail and as such may differ methodologically in that different characters were measured, or can be difficult to compare to more modern works due to the comparative incompleteness (Géry 1977).

Although the type species of the genus was described by Günther in 1867, what would ultimately become *Nannocharax* first appeared in scientific literature about 30 years earlier (Joannis 1835) as *Coregonus niloticus* from the Nile River. It is clear that its relations to other fishes were not understood, and only later did Günther (1867) erect the genus *Nannocharax* with *N. fasciatus* from Gabon as a type species, and corrected the position to *N. niloticus*. The genus was reviewed by Boulenger in 1909, contained within the nine species known at the time.

The first species of *Nannocharax* to be described from southern Africa was *Nannocharax multifasciatus* Boulenger, 1923. This species was described based on specimens that were collected from the Sesheke River, Upper Zambezi. In this description, Boulenger (1923) noted similarity between this species and *N. luapulae* Boulenger, 1915, particularly the close resemblance in the pattern of the vertical bars. However, *N. luapulae*, a species that was described from the Luapula River, can be readily distinguished from *N. multifasciatus* by the spotting on the dorsal surface and blackish spots at the bases of all the fins, while the latter species only has a caudal spot. Fowler (1935) erected a new genus when he described a new species from the Chobe River which was named *Distichodus stigmaturus*. Daget & Gosse (1984) later placed *D. stigmaturus* into synonymy with *N. multifasciatus* (which was identified as *H. multifasciatus* at that time). Bell-Cross & Minshull (1988) note that this species is

frequently confused with *N. machadoi*, and that previous works on either of these two species in the Central African region may refer to both taxa inclusively.

Nannocharax macropterus Pellegrin 1926 was described from the Mongende River, situated on the Kasai River, a left bank affluent of the Middle Congo system (Pellegrin 1926) and its range is considered to extend into southern Africa. Tweddle et al. (2004) and Bills et al. (2012) recognised variation in morphology and habitat of *N. macropterus* in both the Okavango and Upper Zambezi, but to date no further investigation has been conducted.

Nannocharax fasciolaris (Nichols & Bolton 1927) is known only from the type specimen collected from Capelongo, Angola, from the Rio Cubango. This species is morphologically very similar to *N. multifasciatus*, but has more diminutive caudal marking (Nichols & Bolton 1927). van der Waal (1991) and Hay et al. (1997) placed this species in synonymy with *N. multifasciatus* with regards to specimens collected from above the Ruacana Falls of the Cunene River, although this synonymy has not been recognised outside of van der Waal's (1991) and Hey et al.'s (1997) works and both species are still considered valid.

Nannocharax wittei was described from the Kando River, a tributary of the Lualaba River, in the Congo basin (Poll 1933). It was only known from the type locality until a record of this species was reported from Lake Calundo (also known as Lago Cameia), part of the Luena River that flows into the Upper Zambezi. The specimens were collected by the Museu do Dundo and identified by Max Poll (Anon. 1997). However, there are no further reports of this species in subsequent literature on southern African fishes (e.g. Skelton, 2001).

Nannocharax monardi was described based on two specimens that were collected from two river systems, the Kunene and Okavango, and was placed in the genus *Hemigrammocharax* by Pellegrin (1936). Specimens from the Okavango River system were collected from the Kukulakaze River in the Cuando Cubango province of Angola. Géry (1977) and Daget et al. (1984) identified specimens from the Zambezi River as *N. monardi* (*H. monardi* at that time), thus extending the species' distributed into the Zambezi River system. Specimens previously identified as *N. monardi* from the Cunene River were placed in synonymy with *N. machadoi* by Van der Waal (1991), although *N. monardi* bears an adipose fin whereas *N. machadoi* does not. This decision on synonmy has not been recognised by other authors.

Nannocharax machadoi was first described from 8 specimens from the Longa River in Angola by Poll (1967). This was the first and only *Nannocharax* species to be discovered which lacked an adipose fin, but Poll (1967) elected to keep the species within the genus *Nannocharax*. *Nannocharax machadoi* is also the only known species of this genus to occur in the Middle Zambezi as it has been recorded in the Zambezi River below Victoria Falls (Marshall 2011). However, the relationship of the Middle Zambezi specimens with *N. machadoi* above the falls, or with other *Nannocharax* species, has not yet been critically investigated.

Nannocharax dageti was the most recent species to be described from southern Africa (Jerep et al. 2014). The type locality of this species is in the Kando River in the Congo system, but the species' range is considered to extend into the Zambezi River system as Jerep et al. (2014) suggested specimens that were previously identified as *N. macropterus* from the Upper Zambezi and Kafue Rivers should be transferred to *N. dageti*. There are therefore currently seven valid species of Nannocharax in southern Africa (Eschmeyer et al. 2016; Table 1.1), although Skelton (2001) only recognised four species.

Table 1.1: *Nannocharax* species indicated to occur in southern Africa

Species	Type Locality	Known distribution
N. multifasciatus (Boulenger 1923)	Sesheke River, Upper Zambezi system	Chambeshi (Congo) [9] Kunene [8][9] Okavango [9][11] Kafue (Middle Zambezi) [9][11] Upper Zambezi [1][9][11]
N. macropterus (Pellegrin 1926)	Mongende, Tschikapa/Kasai River, Congo system	Kasai (Congo) [2][9][11] Kwanza [11] Okavango [9][11]
N. fasciolaris (Nichols & Bolton 1927)	Capelongo, Okavango system	Cubango [3]
N. wittei (Poll 1933)	Kando, Lualaba River, Congo system	Lualaba (Congo) [4] Upper Zambezi: Lake Calundo/Lake Cameia [7]
N. monardi (Pellegrin 1936)	Kukulakazé, Cubango River, Okavango system and Kuili River, Kunene	Kunene [5] Okavango [5]
N. machadoi (Poll 1967)	Longa, Luena River, Upper Zambezi system	Kunene [8][9][11] Okavango [9][11] Kafue (Middle Zambezi) [9][11] Middle Zambezi [9][11] Upper Zambezi [6][9][11]
N. dageti (Jerep et al. 2014)	Kando, Lualaba River, Congo system	Lualaba, Kasai (Congo) [10] Chambeshi (Congo) [10] Kafue (Middle Zambezi) [10] Upper Zambezi [10]

[1] Boulenger 1923, [2] Pellegrin 1926, [3] Nichols & Bolton 1927, [4] Poll 1933, [5] Pellegrin 1936, [6] Poll 1967, [7] Anon. 1997, specimens identified by M. Poll, [8]Hay et al. 1999, [9] Skelton 2001, [10] Jerep et al. 2014, [11] SAIAB collection data 2015

1.5 Description of study area

The area of study for this thesis is the southern African region as delineated by Skelton (2001), therefore the northernmost limits for consideration in this study will be the Cunene, Okavango, and Zambezi watersheds with the Congo system. In addition, the Kwanza system will be included in the study area, given its affinities with the other southern African systems, in particular the Okavango and Zambezi (Musilová et al. 2013). This region coincides with the northern limits of the Kwanza and Zambezi ichthyofaunal provinces (Snoeks et al. 2010, Fig. 1.1). As such this study will attempt to take into account all available *Nannocharax* (and by extension, *Hemigrammocharax*) specimens stored at the South African Institute for Aquatic Biodiversity (SAIAB) with collection data from the following systems: Cunene, Kwanza, Zambezi, and Okavango, (Fig. 1.4).

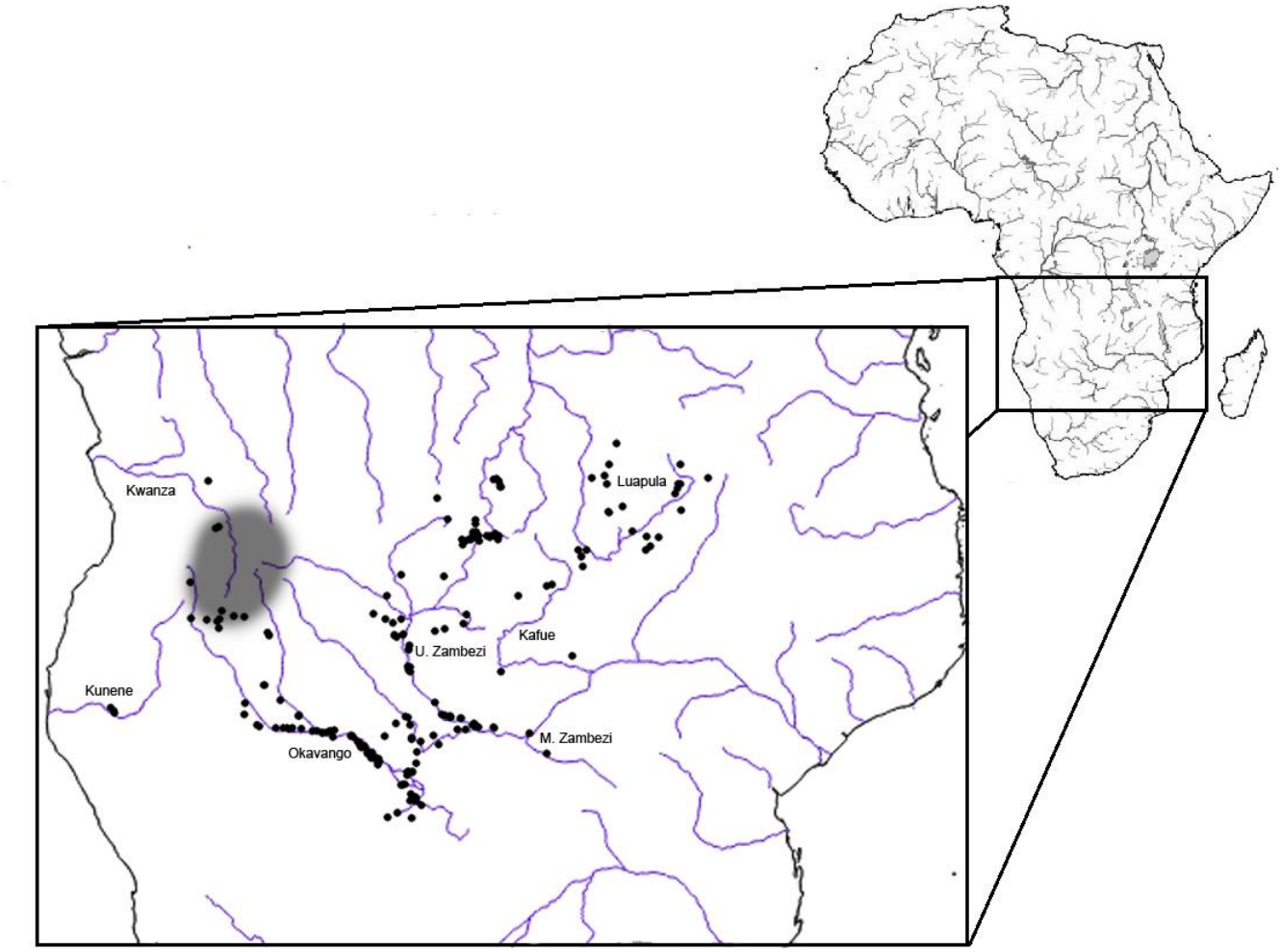

Figure 1.4: The distribution of *Nannocharax* in the southern African region based on distribution records retrieved from the SAIAB collection database (SAIAB 2016). The shaded region represents the Bié Plateau, a region where the headwaters of many southern African rivers originate.

The area delineated by this study contains two ichthyofaunal provinces as defined by Roberts (1975, see Fig. 1.1) – the Cuanza and the Zambezi, with the Congo ichthyofaunal province as an outgroup region. The study area also encapsulates the Cuanza, Kafue, Kalahari, Middle Zambezi – Luangwa, Namib, and Zambezian Headwaters ecoregions as described by Abell et al. (2008), as while these are not the only freshwater ecoregions in southern Africa, they are the only ones to contain *Nannocharax*. Both the ichthyofaunal provinces and the ecoregions are defined by their unique freshwater fish or fauna species, respectively. These groupings are a reflection of not only of species distributions but also the biogeographical patterns that shaped

the distributions and uniqueness of each regions' respective faunas (Roberts 1975, Abell et al. 2008).

The hydrogeography of the southern African region has a rich history, with instances of large changes in landscape and drainage patterns which allowed for periods of contact and exchange between adjacent basins (e.g. Partridge 1997, Goudie 2005, Stankiewicz & de Wit 2006). Samples from the Congo system, which is the centre of *Nannocharax* diversity, were used as outgroups for the present study. Headwaters of all major river systems within the Congo, Kwanza and Zambezi ichthyological provinces drain the Angolan highlands where they arise in close proximity to each other in a relatively flat landscape (Figure 1.4). Intermittent connections between the river systems is likely during periods of heavy flooding. This may facilitate exchange or dispersal of fish species across drainage divides. Evidence of the existence of contact zones between these river systems has been uncovered through use of molecular techniques (e.g. Day et al. 2013, Musilová et al. 2013).
The following is a brief summary of the river systems included in the study, with important hydrogeological events highlighted that may be of importance to any biogeographical considerations of the study species.

The Okavango River

The Okavango River begins in the highlands of Angola (=Cubango and Quito/Cuito rivers, McCarthy & Ellery 1998). It continues as part of the 'panhandle' of the system as it crosses both the Namibia and Botswana borders, forming rapids along the way. Finally, the river drains into the Kalahari Desert, forming an alluvial fan that is the Okavango Delta – an expanse of wetlands and swamps (McCarthy & Bloem 1998). The system experiences seasonal flooding in its lower reaches, forming 'seasonal swamps' (McCarthy & Ellery 1998). The Selinda Spillway, consisting of nonperrenial fluvial features and swampland, is a point of exchange between the Okavango and the Upper Zambezi. Although the fish faunas between the two rivers are extremely similar, with up to 86% of fish species shared (Skelton et al. 1985, Hay et al. 1999), there is growing evidence for differentiation between the two systems (e.g. Kramer et al. 2003, 2014).

The Zambezi River

The Zambezi River is typically divided into three parts delineated by geomorphological barriers: the Upper, Middle, and Lower Zambezi. the Upper and Middle Zambezi are divided by Victoria Falls, and the Middle and Lower Zambezi are separated by the Cahora Bassa rapids (Pinay 1988, Tweddle 2010). Historically, the Upper and Middle Zambezi Rivers were not connected (Balon 1974), but joined approximately 1.8 to 2.5 MYA (Goudie 2005). The Kafue was initially an Upper Zambezi tributary, which itself was fed by the paleo-Chambeshi (Tweddle 2010). The Kafue lost its connection to the Chambeshi, and was subsequently captured by a tributary of the Middle Zambezi (Goudie 2005, Moore et al. 2007)

Historical connections may have included capture of the Congo's Kasai (Veatch 1935) and the Kafue-Chambeshi (Congo) connection (Cotterill & de Wit 2011), both of which are believed to be pathways for faunal distribution expansion in the past (Tweddle 2010). Currently, there is an area of potential contact between the Congo (Kamafwafwa stream) and Upper Zambezi (Kanyita stream) headwaters (Bell-Cross 1965). Although this may only occur for brief periods and exceptionally heavy rainy seasons, it is possible for fish to move across from the Congo side of the watershed to the Zambezi side or vice versa, where *Nannocharax multifasciatus* was found to be among these species (Bell-Cross 1965).

Until recently there was no record of *Nannocharax* occurring below the Victoria Falls, considered to be a major barrier to fish dispersal both up- and downstream. However, sampling has indicated that at least one species (*N. machadoi*) occurs below the Falls just above Lake Kariba (Marshall 2011). It is unknown if this presence at this locality is due to recent or historical events or an artefact of insufficient sampling efforts. *Nannocharax* is unknown from any other portion of the Middle Zambezi or anywhere in the Lower Zambezi, with the exception of the Kafue, which has had a historical connection with the Upper Zambezi and with some 59% of their ichthyofauna shared between them (Skelton 1994, Tweddle 2010).

The Cunene River

Despite being a westward flowing river, the Cunene (or Kunene) lies within the same ichthyofaunal province as the Zambezi and Okavango (Fig. 1.1). The river is unique in being one of a few perennial rivers in an otherwise dry region, presenting with a moderately strong gradient and a moderate flow spaced intermittently with rapids and falls (Kanthack 1921, Hay et al. 1997). The watersheds of the Cunene and Okavango are proximate (Kanthack 1921), with

possible intermittent connection via Colui River (Moore & Larkin 2001). Hay et al. (1999) found that the Cunene shares about 51% of its ichthyofauna with the Okavango, and about 48% with the Upper Zambezi, likely due to its previous connection to the historical group of eastern systems (Bell-Cross 1968). Bell-Cross (1968) suggested that there may have been some degree of faunal exchange between the Kunene and Kwanza Rivers. It is currently isolated from adjacent systems, which explains the existence of several endemic freshwater fish species within the Kunene (van Zyl 1991).

The Kwanza River

The Kwanza (also referred to as Quanza, Cuanza) and nearby affluent rivers form their own ichthyofaunal province due to diversity unique to the region. It is now known that exchanges between this and the Zambezi system can and do occur. A suggested point of contact and therefore interbasin dispersal is between the Okavango tributary Cuchi and the Kwanza tributary Kuquema (Musilová et al. 2013) on the Bié Plateau. Molecular evidence suggests that there has been historical contact between this and the Congo system among cichlid lineages (Schwarzer et al. 2012). This basin has been noted as a region of at least 50% freshwater endemism (Abell et al. 2008), and more new species may yet be discovered in this system with more exploration (Paugy 2010).

1.6 Motivation for this study

Diversity of the genus in the southern African region, currently accepted to be represented by seven species, may be underestimated. Over the past decade seven novel species of *Nannocharax* have been described from a number of regions across the continent (Vari & Ferraris 2004, Dunz & Schliewen 2009, Moritz 2009, Jerep & Vari 2013, Jerep & Vari 2014, Jerep et al. 2014). Furthermore, observations by SAIAB collectors and field workers (see Tweddle et al. 2004) have identified extensive morphological variation in size and pigment patterns within the known species of southern African *Nannocharax*, with some collected specimens that cannot be identified with confidence to species-level. Many of the currently acknowledged species in the region have extensive geographical ranges, being found in multiple river systems both within the southern Africa region as well as in other African river systems. Many African freshwater taxa display broad distributional ranges across several river systems, but it has been strongly suspected that such widely distributed species may be species complexes (Skelton 1988). DNA-based studies utilising mitochondrial gene regions have since uncovered existence of extreme levels of hidden or cryptic diversity in almost all freshwater

fishes studied thus far in Africa (e.g. Goodier et al. 2011, van der Bank et al. 2012, Chakona et al. 2015). Patterns from these studies suggest that almost all widely distributed African freshwater fishes may contain species that are yet to be scientifically documented. The genetic differentiation seen in these studies often reveal typical species-level divergence between the newly identified lineages. It is important to employ a multifaceted approach towards taxonomic problems, and in particular to employ both morphological and molecular data when approaching these problems, as while molecular approaches have proven efficient in identifying hidden diversity, morphological data provide identifiable characters that allow for determinable distinction between given species or populations (Schlick-Steiner et al. 2007). Populations isolated from each other by distance or barriers, or inhabiting differing environments, might be expected to differ from one another morphologically and/or genetically (Wright 1943, Schluter 2009). Such differences may result in reproductive barriers being formed between these populations, and incipient speciation may occur. Finally, the interrelationships between species is a critical and necessary step in the process of developing accurate biogeographical theories (Greenwood 1983).

1.7 Study objectives

The objectives of this study were to: (1) explore the molecular diversity of southern African *Nannocharax* using a portion of the mitochondrial COI gene region, in order to identify potentially unique lineages within the known species; (2) use morphometric, meristic and pigmentation pattern to determine the degree of morphological divergence between the identified lineages within each species complex and make decisions on their taxonomic status; (3) to determine the distribution ranges of the lineages or candidate species identified in this study, and (4) to briefly comment on the biogeographic patterns of the species.

1.8 Thesis outline

The present study used an integrative approach to determine the extent of genetic and morphological divergence within the currently recognised southern African *Nannocharax* species. The study used voucher specimens and DNA tissue samples available at the South African Institute for Aquatic Biodiversity (SAIAB) and the Royal Museum for Central Africa (MRAC), and the genetic sequences already uploaded to the online database, BOLD. The samples were collected from multiple localities encompassing the known geographic ranges of the study species. The study thus facilitated a more accurate mapping of the distribution ranges of new lineages and/or species.

The second chapter of this thesis covered the molecular and morphological work of the study, encompassing the methods and results of the study. From this information, unique lineages were identified. Both internal and external morphological methods were employed to identify new useful characters in differentiating the lineages and potentially identifying new species to science. The third and final chapter summarized the findings of the study, what conclusions were drawn from the results, and highlights the opportunities for further work on this group in the future.

Chapter 2

Integrative taxonomy reveals cryptic diversity in the genus *Nannocharax* in southern Africa

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2.1 Introduction

Previous research focussing on *Nannocharax* (and its synonym *Hemigrammocharax*) taxonomy has been limited to morphological studies. Studies involving the broader scope of Distichodontidae, however, have seen a suite of techniques to determine the interrelationships of the group, and have included both morphological and genetic techniques using mitochondrial and nuclear DNA. Within such studies (e.g. Calcagnotto et al. 2005, Arroyave et al. 2013), representatives of *Nannocharax* and *Hemigrammocharax* have been included, but such inclusions have only so far yielded results inferring relationships between distichodontids, and not necessarily the species-level relationships of the clades. The genus *Nannocharax* has received sparse attention in taxonomic literature, with the last comprehensive review on this group having been done more than a century ago by Boulenger (1909). This lack of taxonomic attention may impede discovery and accurate documentation of species diversity (Fontaine et al. 2012). Despite having a wide distribution, much of the range of *Nannocharax* lies in remote areas, with the centre of *Nannocharax* diversity being within the enigmatic Congo rainforest region. Such areas have only infrequently visited by institutions, especially in recent times (Alofs et al. 2013), and even if specimens have been acquired they may be held in storage long before they receive taxonomic attention (Fontaine et al. 2012).

This group has not been the focus of many studies perhaps because of its limited use to man. Although Moelants (2010) and van der Waal et al. (2011) have indicated that fishes from this group may be taken by subsistence fisheries, there is no evidence that these fishes significantly feature in fisheries across their distribution. Some species are known to be kept by aquarium hobbyists (Skelton 2001, Axelrod et al. 2007, Lalèyè & Moelants 2010) although these endeavours do not appear to have contributed further to the research on the genus.

Currently there is poor understanding of the species boundaries, incomplete knowledge of the true diversity of *Nannocharax*, including among its southern African distributed species, and a lack of systematic knowledge of the southern African group as a whole. These gaps in

knowledge have important implications for other fields of biological research including biogeography, conservation, and ecology (Bickford et al. 2007). Other African freshwater fish taxa share similar problems, and recent research has indicated that almost all freshwater fishes that were previously considered to be widely distributed species comprise multiple lineages with distributions limited to only one or more isolated river systems, as seen in other characoids (Goodier et al. 2011), cyprinids (Bloomer & Impson 2000; Swartz et al., 2009), mormyrids (Kramer & Swartz 2010; Maake et al. 2014), and catfishes (Day et al. 2013). Accurate documentation of this currently underestimated diversity is therefore critical in the face of increasing environmental degradation surrounding increased resource usage across the African continent (Darwall et al. 2011).

In many small freshwater fish taxa, some complexes may comprise species that have highly conservative morphologies, rendering them superficially morphologically indistinguishable from each other. Such similar morphologies may arise from, for example, non-visual mating systems, allowing species divergence while maintaining similar morphological characteristics (e.g. Lamml & Kramer 2007). This raises the issue of true species diversity among certain species groups. The integration of morphological and genetic data has been found to be an effective approach for identifying and delineating species, particularly in groups that exhibit extreme morphological conservatism (e.g. Dayrat 2005, Wishart et al. 2006, Ball et al. 2016,, Chakona & Skelton 2017). Molecular data is important in understanding the relationships between populations and species, but cannot alone replace the need for traditional taxonomy determining diagnostic characters and testing species hypotheses (Wiens & Penkrot 2002, Sites & Marshall 2003, Wheeler et al. 2004, Nwani et al. 2011). Morphological characters of species remain important tools to taxonomy (Wiens 2004), and visible external characters are among the most useful identification tools for scientists and others out in the field. Cryptic species groups that are identified through molecular techniques may remain in crypsis following discovery if identifiable morphological characters are not explored and applied to each lineage (Schlick-Steiner et al. 2007). A greater understanding of morphology in respect to the phylogeny of a group can in turn help researchers understand the ecomorphology within a given group (Franssen et al. 2015).

Previous field work had identified that there was significant morphological variation among the currently recognised southern African *Nannocharax* (Tweddle et al. 2004, Bills et al. 2012, Bills pers. comm. Skelton pers. comm.). The aim of this study was to combine molecular,

morphometric, and pigmentation pattern data, in order to test the hypothesis that the widely distributed and highly morphologically variable southern African *Nannocharax* species may in fact be comprised of multiple lineages containing higher taxonomic diversity than is currently recognised. The study will determine the extent of hidden diversity within each of the four species that are currently recognised in southern Africa, determine the taxonomic status of any new lineages that may be uncovered/identified and update the distribution ranges of the species or lineages. The study will also briefly comment on the evolutionary history and biogeographic patterns of the group in southern Africa.

2.2 Materials and methods

2.2.1.1 Molecular techniques and data collection

The study used genetic samples that were collected from surveys conducted between May 1998 and November 2013 by various researchers at the South African Institute for Aquatic Biodiversity (SAIAB). These tissue samples were deposited in the biomaterials bank at SAIAB. Additionally, available sequences of *Nannocharax* or sequences recorded as *Hemigrammocharax* that are stored on the online database the Barcode of Life Database (BOLD) were also used. A total of 10 samples of the *N. machadoi* species group were used in the present study. These samples were collected from five localities across the three river systems, which represents the known geographic range for this species in southern Africa, with the exception of the Kunene River, for which no genetic material was available. A total of 15 samples of the *N. multifasciatus* species group were used in the present study. These samples were collected from seven localities across the three river systems, which represents the known geographic range for this species in southern Africa. A total of 19 samples of the *N. macropterus* species group were used in the present study. These samples were collected from ten localities across the three river systems, which represents the known geographic range for this species in southern Africa. An additional two samples of *N. multifasciatus*, one specimen of *N. machadoi*, four specimens of *N. macropterus*, and three specimens of *N. fasciatus*, were used as outgroup material for this study. The locality information and list of genetic samples used in the present study are presented in see Appendix 2.

DNA was extracted from muscle tissue or fin clips stored in ethanol, using the salting out method (Sunnucks & Hales 1996). Extracted samples were placed under a NanoDrop 2000 (Thermo Scientific) to ensure a sufficient amount of DNA material had been extracted, and verified using agarose gel electrophoresis. A fragment of the mitochondrial cytochrome

oxidase I gene region was amplified using the primers VF2-T1 (TGCCACCTGACGTCTAAGAA) and VR1-T1 (ATTACCGCCTTTGAGTGAGC) (Ivanova et al. 2007) using polymerase chain reaction (PCR).

The 22µl PCR solutions were prepared using 12.8 µl water, 2.5 µl KAPAtaq 10x Buffer A, 1 µl MgCl2, 2.5 µl 8mM dNTPS, 0.2 µl KAPA Taq, 2 µl of the template DNA, and 0.5 µl of each the aforementioned primers. The PCR profile were as follows: 1 cycle of initial denaturation at 95°C for 1 minute; 5 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 40 seconds, and extension at 72°C for 1 minute; and 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 40 seconds, and extension at 72°C for 1 minute; and 1 cycle for final extension at 72°C for 10 minutes. The quality and concentration of the resultant PCR was determined using a NanoDrop 2000 (Thermo Scientific). Successful PCR products were purified using the ExoSAP protocol (ThermoFisher Scientific) as per Werle et al. (1994). The samples then had their concentrations determined, and were then appropriately diluted prior to cycle sequencing. These products were then purified following an ethanol/EDTA precipitation protocol. Sequencing was conducted at SAIAB using a 3500 Genetic Analyzer (Applied Biosystems). A total of 42 sequences were generated: *N. machadoi* (n = 9), *N. multifaciatus* (n = 14), and *N. macropterus* (n = 19). An additional 11 sequences were downloaded from BOLD (see Appendix 2).

2.2.1.2 Sequence and phylogenetic analyses

Sequences for all species groups were analyses together for all following phylogenetic analyses. Sequences were aligned using MEGA7 (Kumar et al. 2016) using the Clustal alignment method. DNA sequences were subsequently trimmed and edited using MEGA7 (Kumar et al. 2016) and GBlocks (Castresana 2000) where the sequences were made to be equal lengths, to 560 base pairs (BP).

Unique haplotypes were identified using DnaSP (Librado & Rozas 2009). Models of substitution for the maximum likelihood analysis were determined using jModelTest (Posada 2008), and the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985) was selected as the best choice based on its Akaike's Information Criterion (AIC) weighting (Akaike 1981, Posada & Buckley 2004). Percent pairwise distances between groups calculations, maximum likelihood, and maximum parsimony analysis were conducted using MEGA7 (Kumar et al. 2016), utilising heuristic tree searching methods of nearest neighbour interchange, applying a moderate branch

swapping filter. Both the maximum likelihood and maximum parsimony analyses were supported using bootstrap replicates (Felsenstein 1985) for 1000 replicates in each case.

Bayesian inference analysis was conducted using MrBayes (Huelsenbeck & Ronquist 2001), using four Monte Carlo Markov chains (MCMC) to detect the tree topology with the greatest likelihood. The tree was inferred from 1,000,000 generations, and sampled every 100th generation; 10% of the resultant trees were discarded as burn-in, and the remaining trees were used likelihood represented by posterior probabilities.

In trees resulting from the maximum parsimony and maximum likelihood analyses, branches with bootstrap values below 50% were considered not supported; branches with 50% to 69% as a weakly supported; branches with 70% to 89% as a moderately supported; and branches with 90% and greater as a well-supported. In the Bayesian inference analysis, branches with posterior probabilities below 0.90 were considered weakly supported, while those with values equal to or greater than 0.95 were considered to be well supported (Felsenstein 1985).

Tree nodes in were collapsed, representing the lineages that occurred within each river system. The resultant branches (clades) were labelled according to the the species morphotype and the river basin from which they originated.

2.2.2.1 Morphological analyses

Specimens stored and accessioned at the SAIAB under the names *Nannocharax* and *Hemigrammocharax* and collected from river systems from within the selected study area were used (see Appendix 3). These specimens were collected between November 1977 and November 2013 by SAIAB collectors and their various collaborators.

Specimens were selected to ensure that every *Nannocharax* species occurring in the designated study area and currently stored in SAIAB were analysed. Some specimens of the aforementioned species from outside the study area were examined as comparative material. Finally, specimens used in the genetic analyses were included to compare the patterns observed between both datasets.

For the ease of grouping and comparison in this study, specimens were grouped according to the species that they were most visually similar to, in ‘species groups’ (morphotypes), as defined here. Specimens that bore bars on the lateral surface of the body and bore an adipose

fin were included in the *N. multifasciatus* species group. Those bearing lateral body bars but lacked an adipose fin were placed in the *N. machadoi* species group. Specimens that bore midlateral blotches, dorsal saddling, and/or a prominent midlateral stripe were placed in the *N. macropterus* species group. The final group included *N. dageti* specimens – this was due to the superficial similarity to *N. macropterus,* allowing a comparative analysis of these similar species.

Within these aforementioned groups, the specimens were further classified according the river basin from which they were collected. Other *Nannocharax* specimens used as outgroup taxa, *Nannocharax gracilis* and *Nannocharax fasciatus,* were not assigned these group identifiers and hydrogeographic localities. For the purposes of this study, *N. machadoi* specimens from the Kunene were grouped with Okavango specimens, and Kafue specimens were grouped with the Zambezi specimens. This was done for statistical purposes, as in some species the small sample sizes for those particular regions had no effective degrees of freedom, and had to be grouped with others. However, given the biogeographical uniqueness of these regions, the specimens from these catchments are taken into consideration, and are included separately in analyses where possible.

2.2.2.2 Morphological techniques

Measurements and meristic counts were taken following Fink & Weitzman (1974), under the conditions and adjustments as per Tables 2.1 and 2.2, and as illustrated in Figure 2.1. Measurements were taken point-to-point using a pair of digital callipers, and recorded to the nearest 0.01mm. Measurements and counts were taken on the left side of the fish's body, unless the body had been damaged to the extend of obscuring measurement points or did not allow for accurate counting of the meristic characters. Notes on the general appearance of the specimen, pigmentation, body pattern, and presence/absence of modified scales were also recorded.

Measured specimens were subsequently photographed on the left side, except for only six specimens that were photographed on the right side for the reasons stated above. The photographs were taken using a Canon EOS 450d 12 MP camera, with the specimens laid perpendicular to the camera lens alongside a graduated ruler for scale. Images were uploaded to a computer, and grouped by species and then by locality. Species sharing localities within the same river basins and similar pigmentation patterns were again grouped together. These

photographs were employed for accurate comparison of different population pigmentation patterns.

Specimens were not measured if the specimen was particularly distorted during preservation and storage, therefore not allowing for accurate measurements; similarly meristic data may not have been collected if the body part had been too damaged to accurately count the character. Pigmentation patterns were only photographed and recorded if the specimen presented a clear pigmentation pattern without fading, and could be assigned accurately to a pattern grade. Individual specimen data may have been eliminated if errors were later detected.

Table 2.1: Descripts of the morphometric measurements of *Nannocharax* used in the present study, following Fink & Wietzman (1974).

Measurement	**Description/notes**
Standard length	Anteriormost point of the snout to the middle point of end of the caudal peduncle
Head length	From the anteriormost point of the snout to the bony edge of the operculum
Head width	Measured in line with the centremost point of the eyes
Head depth	The distance from the dorsal surface of the head to the ventral surface of the head, measured in line with the centremost point of the eyes
Snout length	From the anteriormost point of the snout to the nearest bony edge of the orbit
Upper jaw length	From the anteriormost point of the snout to the furthest visible distal point of the upper jaw (maxillary)
Horizontal eye diameter	The longest horizontal distance between the anterior and posterior bony orbits
Interorbital width	The least distance between the dorsalmost points of the left and right orbits
Predorsal length	From the anteriormost point of the snout to the insertion point of the dorsal fin
Prepectoral length	From the anteriormost point of the snout to the insertion point of the pectoral fin
Prepelvic length	From the anteriormost point of the snout to the insertion point of the pelvic fin
Preanal length	From the anteriormost point of the snout to the insertion point of anal fin
Body width	The widest point of the body past the head, usually measured from in line with the dorsal fin insertion
Body depth	The horizontal distance measured from the deepest part of the body, usually from directly in front of the dorsal fin origin
Caudal peduncle length	From the posteriormost insertion point of the anal fin to the midmost point of the end of the caudal peduncle
Caudal peduncle depth	The horizontal least distance between the dorsalmost and ventralmost points of the caudal peduncle
Dorsal fin length	From the anteriormost point of insertion of the dorsal fin to the tip of the longest ray
Pectoral fin length	From the anteriormost point of insertion of the pectoral fin to the tip of the longest ray
Pelvic fin length	From the anteriormost point of insertion of the pelvic fin to the tip of the longest ray
Anal fin length	From the anteriormost point of insertion of the anal fin to the tip of the longest ray

Table 2.2: counts used as meristic values in this study, as per Fink & Weitzman (1974), with descriptions and notes about the counts used

Meristic count	**Description/notes**
Longitudinal scale count	Scales counted horizontally along a single row that includes the pored lateral line series, from the first scale proceeding the end of the operculum
Lateral line scale count	Count of pored scales that occur in a horizontal row, from the first scale proceeding the end of the operculum to the base of the caudal fin
Caudal scale count	Counted vertically as a ring of scales around the caudal region
Predorsal scale count	Counted from the first scale on the dorsal surface, posteriorly until the dorsal fin origin
Scale count between dorsal fin origin and lateral line	Counted diagonally from the dorsal fin origin to the lateral line, not including the middorsal scales or lateral series scales
Scale count between anal fin origin and lateral line	Counted diagonally from the anal fin origin to the lateral line, not including midventral scales or the lateral series scales
Unbranched dorsal fin rays	All dorsal fin rays without branching
Branched dorsal fin rays	All dorsal fin rays with branching, with the any posterior rays sharing a split base being counted as separate rays
Unbranched pectoral fin rays	All pectoral fin rays without branching
Branched pectoral fin rays	All pectoral fin rays with branching
Unbranched pelvic fin rays	All pelvic fin rays without branching
Branched pelvic fin rays	All pelvic fin rays with branching
Unbranched anal fin rays	All anal fin rays without branching
Branched anal fin rays	All anal fin rays without branching, with the any posterior rays sharing a split base being counted as separate rays
Caudal fin rays	Counts inclusive of the two procurrent rays (dorsal and ventral), and the branched rays in between

.

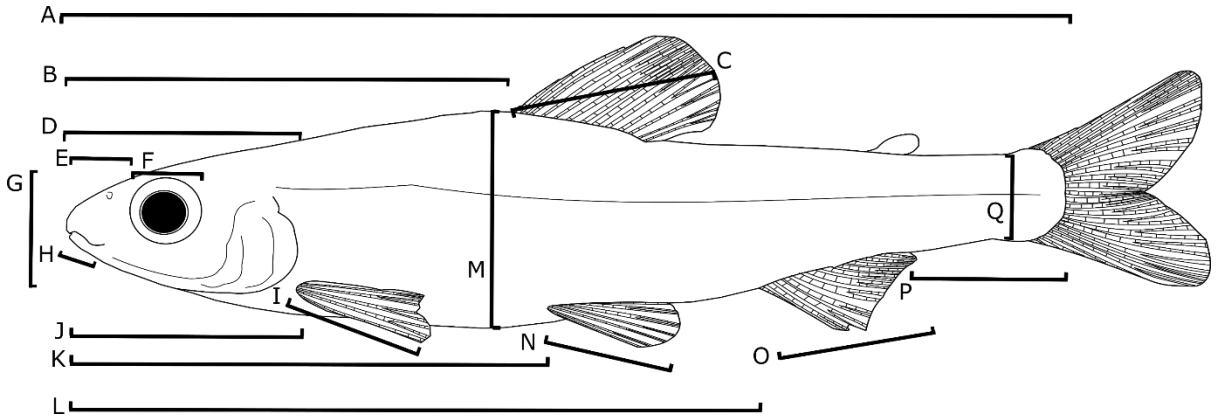

Figure 2.1: Linear measurements of *Nannocharax* that were used for this study. A, standard length; B, predorsal length; C, dorsal fin length; D, head length; E, snout length; F, horizontal eye diameter; G, head depth; H, upper jaw length; I, pectoral fin length; J, prepectoral length; K, prepelvic length; L, preanal length; M, body depth; N, pelvic fin length; O, anal fin length; P, caudal peduncle length; Q, caudal peduncle depth. See Table 2.1 for an explanation of these measurements.

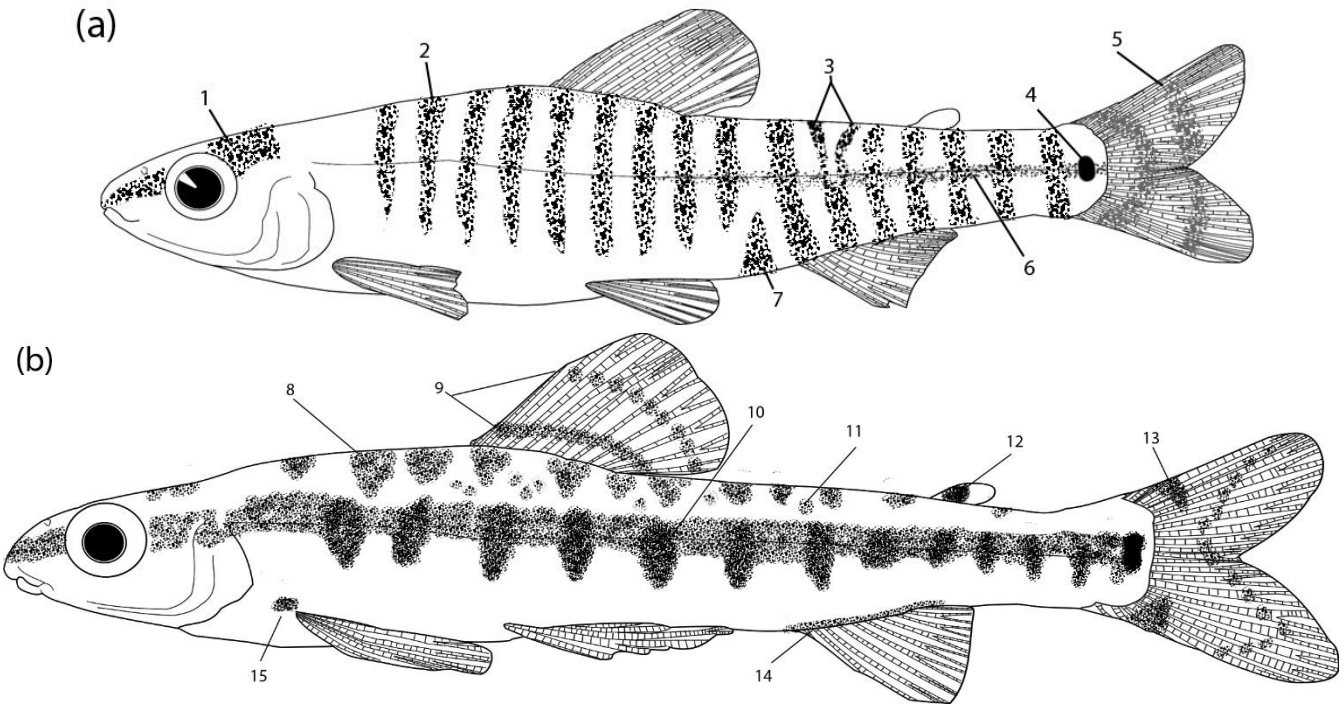

Figure 2.2: Images representing the body areas and generalized pigmentation patterns and other pigmented areas on the study species for (a) the *N. multifasciatus*, *N. machadoi*, *N. monardi*, and *N. wittei* species groups and (b) the *N. macropterus* and *N. dageti* species groups. 1: head pigmentation; 2: body bar; 3: split Y bar (may be inverted); 4: caudal spot; 5: caudal fin bars; 6: midlateral stripe 7: deviant body bar (originates ventrally); 8: saddle; 9: dorsal lines; 10: body blotch; 11: dorsal speckling; 12: adipose spot; 13: caudal fin spot (may present as first caudal bar if the dorsal and ventral spots join); 14: ventral pigmentation; 15: pectoral spot.

The use of colours in fish taxonomy is somewhat limited, particularly given that colour is a feature that is soon lost once specimens have been preserved (Steedman 1976a). However, melanin colour patterns remain in preserved specimens even decades after their death, as the darker pigments that form the shapes in the patterns are not lost due to its greater resistance to bleaching effects of preservation and fixation (Steedman 1976b). Pigmentation patterns can vary greatly even within a single species, due to factors such as sex, season, ontogeny, diet, and environmental effects (Endler 1980, McMilian et al. 1999, Sugimoto 2002). Colour and colour pattern are extremely useful tools when dealing with cryptic or recently evolved groups which may otherwise show little morphological divergence. For example, colour pattern has been used to successfully separate species of cichlids (Couldridge & Alexander 2002) and frogs (Wollenberg et al. 2007). Colours and colour patterns have proven to be useful identifiers among *Nannocharax* species (e.g. Dunz & Schliewen 2009, Moritz 2009). Owing to the range of pigmentation patterns seen among southern African *Nannocharax*, such variability warrants investigation into the function of these pigmentation patterns taxonomically.

Pigmentation patterns were assessed using counts. All counts were performed on the left-hand side of the specimen, unless otherwise impossible due to damage to the left-hand side of the specimen. For barred species groups, *N. machadoi* group and *N. multifasciatus* group, a bar is defined as a single, thin, vertical pigmentation pattern on the lateral surfaces on the body of the specimen. A bar was counted whether it originated from the dorsal or ventral surface. A bar that splits at any point (as per a 'split Y bar', see Fig. 2.2a 3) is counted as a single bar. For the *N. macropterus* group, both body blotches and saddles were counted, separately (Fig. 2.2 numbers 8 and 10). A blotch is a circular to oval shaped area of pigmentation, found midlaterally along the flanks of the specimen, clearly visually distinguishable from other such midlateral pigmentation (such as the midlateral stripe), but not inclusive of the caudal spot (Fig 2.2a 4). A saddle is defined as a circular to oval shaped area of pigmentation that lies across the dorsal surface of the specimen, often visible from both the left and right-hand side of the specimen (Fig. 2.2b 8). Smaller areas of pigmentation near the dorsal surface, termed dorsal speckling (Fig. 2.2b 11), that do not cross the dorsal ridge, were not included in this count.
Notes on the general arrangements and distribution of pigmentation were recorded for all assessed specimens. Terminology used for these descriptions are as indicated in Figure 2.2. For the *N. macropterus* group, specimens with similar pigment patterns were placed into pattern grades, as defined in the results section (Table 2.21).

2.2.2.3 Statistical analyses

Morphological measurement data were converted in one of three manners prior to statistical analysis – first as a percentage of standard length (for body measurements) or as a percentage of head length (for the remaining head measurements); second, with the data adjusted for the effects of allometric growth on the bodily measurements as per Wells (1978). The first method is commonly employed by taxonomists in order to view that data as proportions of the standard lengths of the specimen. For the purpose of this study, this type of data conversion is particularly useful in that it provides more meaningful information, being comparable to the previous works conducted on the genus in which proportional measurements were used, thereby standardising the data in concordance with previous works. Adjustment for allometric growth is a useful tool reducing the effects of proportional growth and size differences from the raw data, especially since different populations may differ in size, either naturally or as an artefact of sampling (Reimchen et al. 1985). Adjustment for allometric growth was done using the formula (as per Wells 1978):

$$\log Y'_{ci} = [\log Y_{ci} - (u_c(\log x_i - \log \bar{x})$$

Where Y' is the adjusted value, for c character and i individual. Y is the character value in question. u represents the pooled regression coefficient for logY on the log of the unadjusted standard length. x_i is the standard length of the individual, where $\bar{x}$ is the mean standard length, or head length for head measurements, of all individuals used in the analysis.

Statistical analyses were performed in Statistica 64 v13 (StatSoft 2015), JMP 13 Software (SAS 2016), and NCSS 12 (NCSS 2017). Analyses were performed separately for each species group, each of which is defined above. Morphological and meristic data sets were analysed using principal component analyses (PCA), for which the data met the assumptions. In order to analyse which components contributed most to variation, only eigenvectors with a value greater than 1 were extracted, under the Kaiser criterion (Kaiser 1960). The first two principal components were extracted as they contribute the most to variation. Eigenvalues within each of these components were considered important contributors to variation if their values were greater than 3.0 or lesser than -3.0, following (McGarigal, 2000)..

Both the morphological and meristic data sets were analysed using forward discriminant function analyses (DFA). DFA attempts to identify which variables aids in discrimination

between given groups, and if these variables can be used in the prediction of assignment to these groups. Using the stepwise function, the features (either measurements or counts) most important in discriminating the populations from one another could be identified. Those characters with the smallest Partial Wilk's Lambda (λ) scores were the greatest contributors in discriminating populations.

Following the DFA the data were placed in a classification matrix, and the probability of classification success of specimen to each system was recorded. The canonical scores were calculated, and the first two canonical scores were plotted against each other to visualise the importance of each root. In cases in which only two groups were to be analysed, only one root was produced from the analysis, and therefore no graphical plot could be constructed. For both (or singular) roots, the three standardised coefficients with the greatest or least values were considered to have the greatest loadings on discrimination between the groups.

The proportional data, meristic data, and pigmentation pattern data were checked for normality using the Shapiro-Wilk test. Datasets with normal distributions compared using one-way analysis of variance (ANOVA), or, in the cases where only two systems were comparable, a t-test was used. Datasets with non-normal distributions were compared using a Kruskal-Wallis test. If the null hypothesis was rejected and a significant difference was found between groups ($p<0.05$), a post hoc Tukey's HSD test for normally distributed data or a Man-Whitney U test for non-normal datasets were conducted to determine between which groups (systems) the differences could be found.

A hierarchal cluster analysis (HCA) was conducted on the proportional morphometric, meristic, and both data sets together. Using morphometric and meristic characters only aids in identifying where shortfalls in classification may occur in the combined analysis, and also allows one to view the relationships between the effects certain characters have on species similarity. Input values were based on two bases: the species 'group' to which the sample belonged, and the river system to which the sample belonged. The averages of these groups were the values employed in the cluster analyses. The clusters were measured through their Euclidean distances. Multivariate cluster analyses were conducted using PAST (Hammer et al. 2001).

The results produced from the cluster analyses here are not necessarily a reflection of the phylogeny of the group, or present a theory of the interrelationships of the species and populations, although it has uses in assisting as a first step in distinguishing new species (Ye & Robbins 2004). Instead this is a summary of the measure of the similarity of these groups to each other based on the characters used in each analysis, presenting natural groupings. This is a good measure of variability as the more similar (less varied) groups will present closer on the resultant topology.

2.3 Results

2.3.1 Molecular analyses

Of the 560 base pairs (BP) that were used from the mitochondrial DNA in the molecular analyses, 355 (63.4%) were conserved, while 205 (36.6%) BP sites were variable; 178 BP sites (31.9%) were parsimony informative. When excluding the outgroups, however, the *Nannocharax* group had 388 (69.3%) conserved BP sites, 172 (30.7%) variable sites, and 164 (29.3%) BP sites that were parsimony informative. Eight of these sites among the *Nannocharax* were autapomorphic (1.4%), being unique to individual specimens.

32 unique haplotypes were identified among the study species; 10 belonging to the *N. multifasciatus* species group, 7 belonging to the *N. machadoi* species group, and 15 belonging to the *N. macropterus* species group. From these and the resultant tree topologies (Figs. 2.3-2.5), clades could be identified: 3 lineages in the *N. multifasciatus* species group, at least 6 lineages in the *N. machadoi* species group, and 8 lineages in the *N. macropterus* group.

2.3.1.1 Maximum Parsimony (MP) analysis

The analysis revealed eight lineages within the *N. macropterus* group (Fig. 2.3). These lineages could not be identified by river basin alone: the *N. macropterus "*Okavango 2" lineage was only distributed in the Cuito-Canavale drainage of the Okavango system, whereas the *N. macropterus* "Okavango 1" lineage was distributed throughout the rest of the Okavango. The clade containing the groups identified as *Nannocharax* cf. *gracilis, Nannocharax* cf. *macropterus,* and *Nannocharax macropterus "*Congo 2", represent a group that, while not having a well-supported bootstrap value, exhibited some of the largest sequence divergence from the other *N. macropterus* groups (Table 2.3), greater than 12.0% in all instances. This Congo group also forms a separate clade form the Congo/Zambezi clade, but share similar

distributions within the Congo system. The *N. macropterus* "Kwanza" lineage is confined entirely to the Kwanza river basin.

Unlike the other molecular analyses, the ML analysis indicates at least three lineages of *N. multifasciatus* group in southern Africa, with an additional two in the Congo. This tree shows moderate support for the divergence of two groups from the Okavango system. An interesting aspect of this tree is that *N. multifasciatus* specimens from the Okavango, while overall forming their own clade, show a moderately supported split, with specimens from the Cuito River forming their own group in *Nannocharax multifasciatus* Okavango 2.
Nannocharax machadoi cannot be considered to have gained resolution from the results of this analysis. While receiving varying support (from unsupported through to well supported), the Zambezi and Kafue groups do not form their own monophyletic groups, despite having specimens sharing geographically close localities.

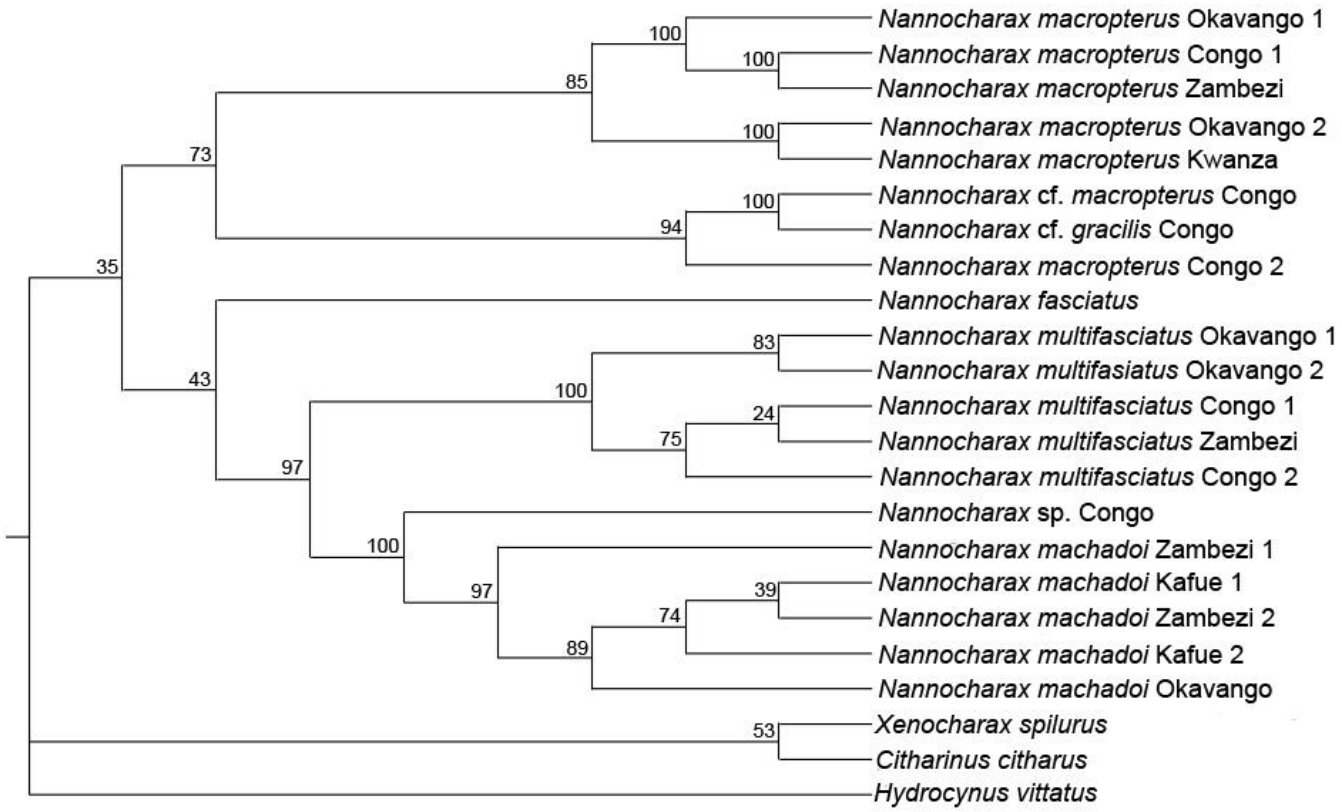

Figure 2.3: maximum parsimony tree of the phylogenetic relationships of southern African *Nannocharax* and outgroup species, inferred from mtDNA cytochrome oxidase I with 1000 bootstrap pseudoreplicates. Numbers on branches represent bootstrap support values.

2.3.1.2 Maximum Likelihood (ML) analysis

The tree resulting from the ML analysis shares a similar topology to that of the MP tree, with a few exceptions (Fig. 2.4). Within the *N. multifasciatus* group, both the Okavango and Congo system groups form their own groups, indicating a single unique lineage for each river system, as is also seen the the Bayesian inference analysis. The previously mentioned issue of *N. machadoi* from the Zambezi, Kafue, and Okavango not forming a well-resolved group persists in this tree but presents stronger bootstrap support values. In common with the MP analysis, the *N. machadoi* "Zambezi 1" group is shown as a strongly supported sister group to the other *N. machadoi* southern African groups. While not presenting as a polytomy (as in the BI analysis analysis), the relationship among southern African *N. machadoi* remains somewhat unclear. The relationships inferred from the pattern seen in the ML and MP analyses may be a result of the complex geological history of southern African river systems, although similar patterns are not shared by other southern African *Nannocharax* species. In the past the Upper Zambezi

(then with the Kafue as a tributary) and the Okavango both drained inland into the Kalahari basin (Moore 1999), forming a singular system that would have allowed for this pattern to occur, despite rather low genetic divergences.

The *N. macropterus* species group present the same number and geographical distribution of lineages as in the MP analysis, with stronger support for the split of the separation of the *N. macropterus* "Congo 2" group from the other Congo and southern African groups.

It is interesting to note that in the resulting topology, *Nannocharax fasciatus* falls within the group containing *N. multifasciatus* and *N. machadoi* – two species which were previously placed in the genus *Hemigrammocharax* whereas *N. fasciatus* was not (though receiving no bootstrap support). This result occurred in both the MP and ML analysis, however on both occasions having little to no bootstrap support (35.0 and 54.0%, respectively).

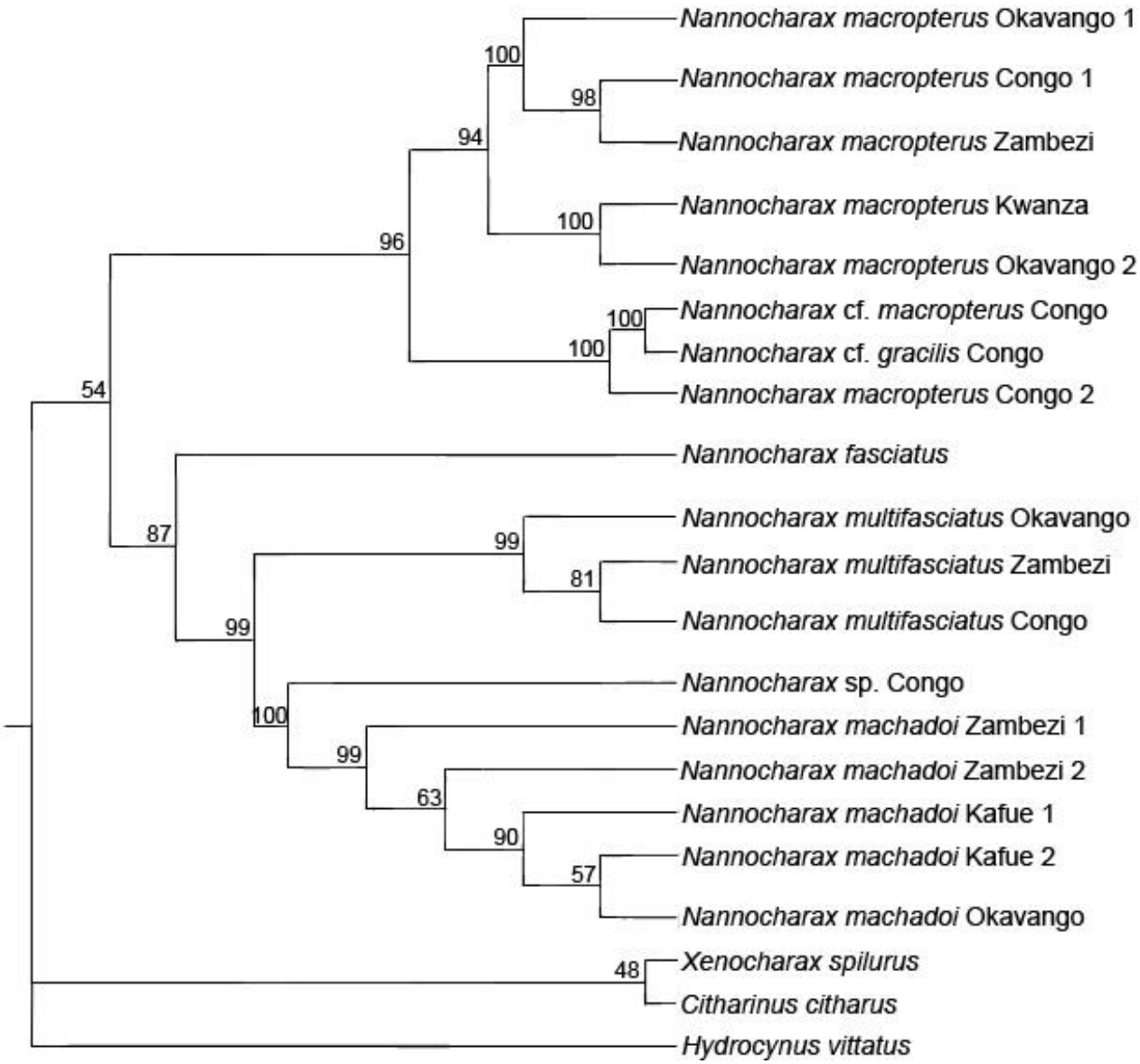

Figure 2.4: maximum likelihood tree of the phylogenetic relationships of southern African *Nannocharax* and outgroup species inferred from mtDNA cytochrome oxidase I, with 1000 bootstrap pseudoreplicates, using the HKY+G+I substitution model (Hasegawa et al. 1985). Numbers on branches represent bootstrap support values.

2.3.1.3 Bayesian Inference (BI) analysis

The tree resulting from the BI analysis, while bearing a few similarities to the MP and ML trees, has some notable differences (Fig. 2.5). The *N. fasciatus* group allies with neither the *N. macropterus* group nor the *N. multifasciatus/N. machadoi* (previously both

Hemigrammocharax) group. The former *Nannocharax/Hemigrammocharax* split mentioned in the previous trees, while manifesting much as before, is not well supported by the BI analysis. The *N. macropterus* clade presents itself in the same fashion as in the previous analyses, once again showing support for the *N. macropterus "*Congo 2" clade as well as a unique *N. macropterus "*Okavango 2" lineage that more closely allies with *N. macropterus* "Kwanza" lineage than other southern African *N. macropterus.* The *N. multifasciatus "*Congo" and *N. multifasciatus "*Zambezi" groups, while appearing as a clade, appears as a polytomy. The previously mentioned arrangement of the Zambezi-Kafue-Okavango *N. machadoi* clade likewise presents itself as a polytomy, again weakly supported.

An unusual aspect of this tree is the placement of the distantly related *C. citharus* and *X. spilurus* not only within *Nannocharax*, but also that it is nested within the *N. macropterus* group. This is despite massive (>19.0%) genetic difference from all ingroup taxa.

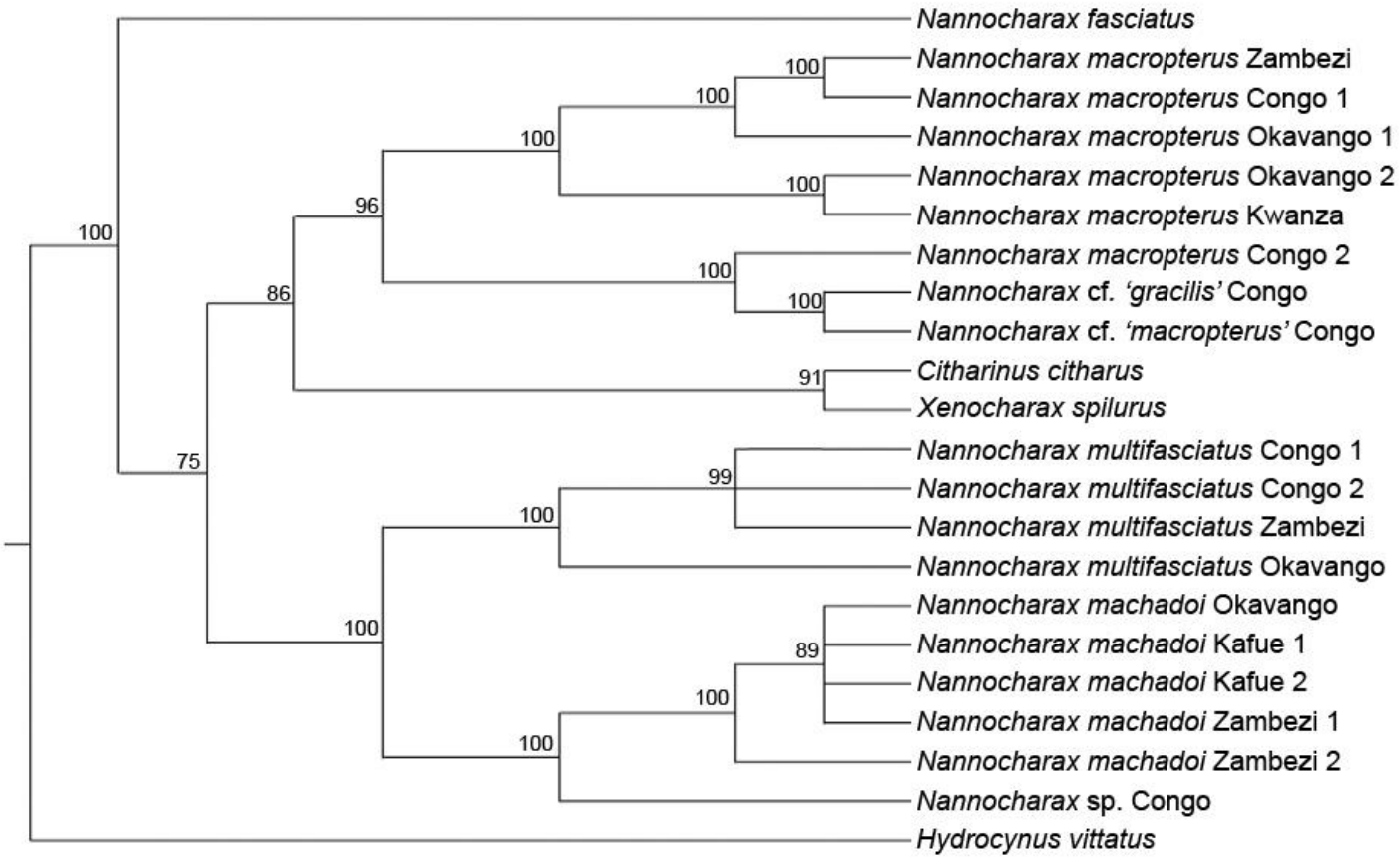

Figure 2.5: topology of the phylogeny of southern African *Nannocharax* and outgroup species, from a Bayesian inference analysis of 1,000,000 generations, inferred from mtDNA cytochrome oxidase I sequences. Numbers on branches are Bayesian posterior probabilities.

Species/Population	Species number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Citharinus citharus	1																					
Hydrocynus vittatus	2	25.4																				
Xenocharax spilurus	3	21.5	29.4																			
Nannocharax fasciatus	4	23.2	27.3	25.2																		
Nannocharax cf. *gracilis*	5	20.2	28.3	20.9	18.2																	
Nannocharax cf. *macropterus*	6	20.2	27.9	20.9	18.2	0.4																
Nannocharax macropterus Congo 1	7	22.9	29.1	23.1	20.3	14.4	14.1															
Nannocharax macropterus Congo 2	8	19.9	27.8	20.3	18.7	5.9	5.7	14.6														
Nannocharax macropterus Zambezi	9	22.8	28.9	22.4	19.3	13.2	13.0	1.3	13.4													
Nannocharax macropterus Okavango 1	10	23.4	29.8	21.4	20.0	12.8	12.6	3.2	12.6	2.2												
Nannocharax macropterus Okavango 2	11	19.6	28.0	21.3	18.4	13.8	13.7	12.3	13.3	11.2	10.3											
Nannocharax macropterus Kwanza	12	19.0	27.3	21.4	18.9	14.4	14.3	12.1	12.9	11.0	10.8	3.1										
Nannocharax multifasciatus Congo 1	13	21.1	26.0	22.5	15.1	17.7	17.4	20.7	17.5	20.2	20.5	18.1	17.9									
Nannocharax multifasciatus Congo 2	14	21.8	25.8	22.7	15.4	17.5	17.2	20.9	17.3	20.5	20.7	18.4	18.1	0.6								
Nannocharax multifasciatus Zambezi	15	23.2	26.2	23.7	16.2	19.0	18.7	21.6	18.8	21.1	21.4	18.8	18.5	1.7	1.5							
Nannocharax multifasciatus Okavango	16	22.0	25.7	21.8	15.8	17.4	17.1	18.9	18.1	18.8	19.0	18.2	17.9	1.9	1.7	2.5						
Nannocharax sp. Congo	17	25.0	27.2	24.1	17.9	19.3	19.0	19.9	19.3	19.5	19.7	20.2	20.8	9.7	9.5	10.2	9.3					
Nannocharax machadoi Zambezi 1	18	22.0	26.6	22.4	17.4	18.6	18.3	21.0	17.7	20.0	19.8	19.5	21.0	9.3	9.5	10.2	9.9	4.3				
Nannocharax machadoi Zambezi 2	19	21.4	26.5	23.4	17.4	19.3	19.1	21.2	18.0	20.8	20.0	20.0	21.5	9.3	9.5	10.2	9.9	4.5	1.3			
Nannocharax machadoi Kafue 1	20	21.4	27.1	23.1	17.7	19.6	19.4	21.5	18.3	20.5	20.3	20.3	21.8	9.7	9.9	10.6	10.3	4.1	0.9	0.7		
Nannocharax machadoi Kafue 2	21	21.9	27.0	23.4	17.9	19.6	19.3	21.7	18.2	20.7	20.5	20.5	21.5	9.7	9.9	10.6	10.3	4.1	0.9	0.7	0.4	
Nannocharax machadoi Okavango	22	21.5	27.2	23.6	18.1	20.0	19.7	21.9	18.6	20.9	20.6	20.6	22.0	9.8	10.0	10.7	10.4	4.2	1.0	0.8	0.5	0.5

Table 2.3: percent pairwise genetic distances of the groups used in this study, with 19 groups and 3 outgroups.

2.3.1.4 Estimated pairwise genetic distances

The highest and lowest estimated sequence divergences between the established species complex groups give a reference point as to what approximate point of sequence divergence could be expected to occur between the different species of *Nannocharax*. Identifying inter- and intraspecies variation allows one to establish a benchmark for species-level delineation (Hubert et al. 2008). Southern African *Nannocharax* groups' sequences differed from a geographically distanced relative, *N. fasciatus*, between 15.1% and 20.3%; *N. multifasciatus* species groups and *N. machadoi* species groups differed from each other an estimated 9.3-10.7%; *N. multifasciatus* species groups differed from *N. macropterus* species groups an estimated 17.1-21.6%; and *N. machadoi* species groups differed from *N. macropterus* species groups 17.7-22.0%. Distances between currently established species groups therefore fell between a mean low and high of 14.8 and 18.7%, average 16.9%, and minimum and maximum values of 9.3 and 22.0%. These values fell within what is known for divergences between species not only among other vertebrate groups [0.0-20.0% Johns & Avise (1998); 9.6% Hebert et al. (2003b)], but also between fish species [5.0% Johns & Avise (1998), 9.9% Ward et al. 2005] in mitochondrial gene regions.

Within the *N. multifasciatus* group, the lowest and highest divergences between river basin populations were 0.5% and 2.5%, respectively (Table 2.3). The mean within group difference was 1.6%. The *N. machadoi* group presented the lowest and highest divergences within the group to be 0.4% and 4.5% respectively. The mean within group difference was 1.9%. However, it should be noted that these values are when '*Nannocharax* sp. Congo' were included among this group, as it appeared to be most closely allied with *N. machadoi*. Its identity cannot be confirmed as no physical specimen was accessible. Excluding this sample, the lowest and highest divergences were 0.5% and 1.0% respectively, and a mean within group difference of 0.8%. The *N. macropterus* species group presented the low and high percent divergences to be 0.4% and 14.6% respectively. The mean within group difference was 10.5%. The higher sequence divergences within this group, typically 13.0% or greater, were paired with individuals from the 'Congo 2' group - as well as individuals best identified as *N.* cf. *gracilis* and *N.* cf. *macropterus*, as they were identified on BOLD (although the institution housing the material only identified the samples down to *Nannocharax* sp., without a specific name). Should this "Congo 2" group represent a separate species group and not be included among the *N. macropterus* group proper, then the mean within group difference was 7.8%, with the highest and lowest differences at 1.3% and 12.3%, respectively.

2.3.2 Morphological analyses

2.3.2.1 *Nannocharax multifasciatus* group

2.3.2.1.1 Principal component analyses

Morphometrics

The first two principal components were extracted from the allometry-adjusted data according to the Kaiser criterion (1960), with PC 1 contributing 80.6% toward variation, and PC 2 contributing 5.3% (Table 2.4, Fig. 2.6a) For the proportion-adjusted data; the first principal component contributed to 28.3% of variation and the second contributed 18.3% of variation (Table 2.5, Fig. 2.6b).

The allometry-adjusted data's first principal component (Table 2.5, Fig. 2.6b) had all characters with nearly equal loading, and the second principal component characters having low loadings, therefore indicating no particular characters that were of significance according to the McGarigal et al. (2000) methods. The second principal component highlighted head length as an important character contributing to variation. The first principal component of proportion-adjusted data presents prepelvic length, predorsal length, preanal length, and caudal peduncle depth characters with the highest loadings. The second principal component presents head length, eye horizontal diameter, snout length, upper jaw length, and interorbital width with the greatest loadings.

The clustering of Zambezi specimens as seen on the top of the graph in Fig. 2.6 (a) is of particularly small specimens. As mentioned by Reimchen et al. (1995), the formula by Wells (1978) does not completely remove the effects of size on the measurements, but only reduces it. Given a strong enough contrast seen here, the difference in size was significant enough to allow for clustering of a group. The same cluster cannot be seen in Fig. 2.6 (b). In both data adjustments there is a strong overlap of Zambezi and Okavango clusters, and only in the proportional adjusted data (Fig. 2.6 (b)) can the Congo cluster be made out from the group, although slightly overlapping with the southern African agglomerate.

Table 2.4: eigenvector factor loadings for the principal components of the allometry-adjusted morphometric characters of southern African *N. multifasciatus* No values were indicated to be important contributors to variation

	Factor 1	Factor 2
Head length	-0.23	-0.03
Predorsal length	-0.23	0.10
Prepectoral length	-0.23	0.02
Prepelvic length	-0.24	0.10
Preanal length	-0.24	0.06
Head width	-0.22	-0.04
Body width	-0.23	-0.05
Head depth	-0.23	-0.05
Body depth	-0.21	-0.04
Dorsal fin length	-0.23	0.04
Pectoral fin length	-0.23	-0.04
Pelvic fin length	-0.24	-0.05
Anal fin length	-0.23	0.02
Caudal peduncle length	-0.22	0.11
Caudal peduncle depth	-0.24	-0.02
Eye horizontal diameter	-0.23	-0.05
Snout length	-0.21	-015
Upper jaw length	-0.23	-0.02
Interorbital width	-0.23	0.00

Table 2.5: eigenvector factor loadings for principal components of the proportion-adjusted morphometric characters of southern African *N. multifasciatus.* No values were indicated to be important contributors to variation

	Factor 1	Factor 2
Head length	-0.02	0.46
Predorsal length	-0.32	0.08
Prepectoral length	-0.21	0.04
Prepelvic length	-0.34	0.02
Preanal length	-0.32	0.02
Head width	-0.19	0.06
Body width	-0.16	0.06
Head depth	-0.28	0.05
Body depth	-0.25	0.07
Dorsal fin length	-0.26	0.01
Pectoral fin length	-0.24	0.15
Pelvic fin length	-0.25	0.12
Anal fin length	-0.24	0.10
Caudal peduncle length	-0.05	0.00
Caudal peduncle depth	-0.31	0.11
Eye horizontal diameter	-0.10	-0.45
Snout length	-0.13	-0.44
Upper jaw length	-0.19	-0.42
Interorbital width	-0.14	-0.35

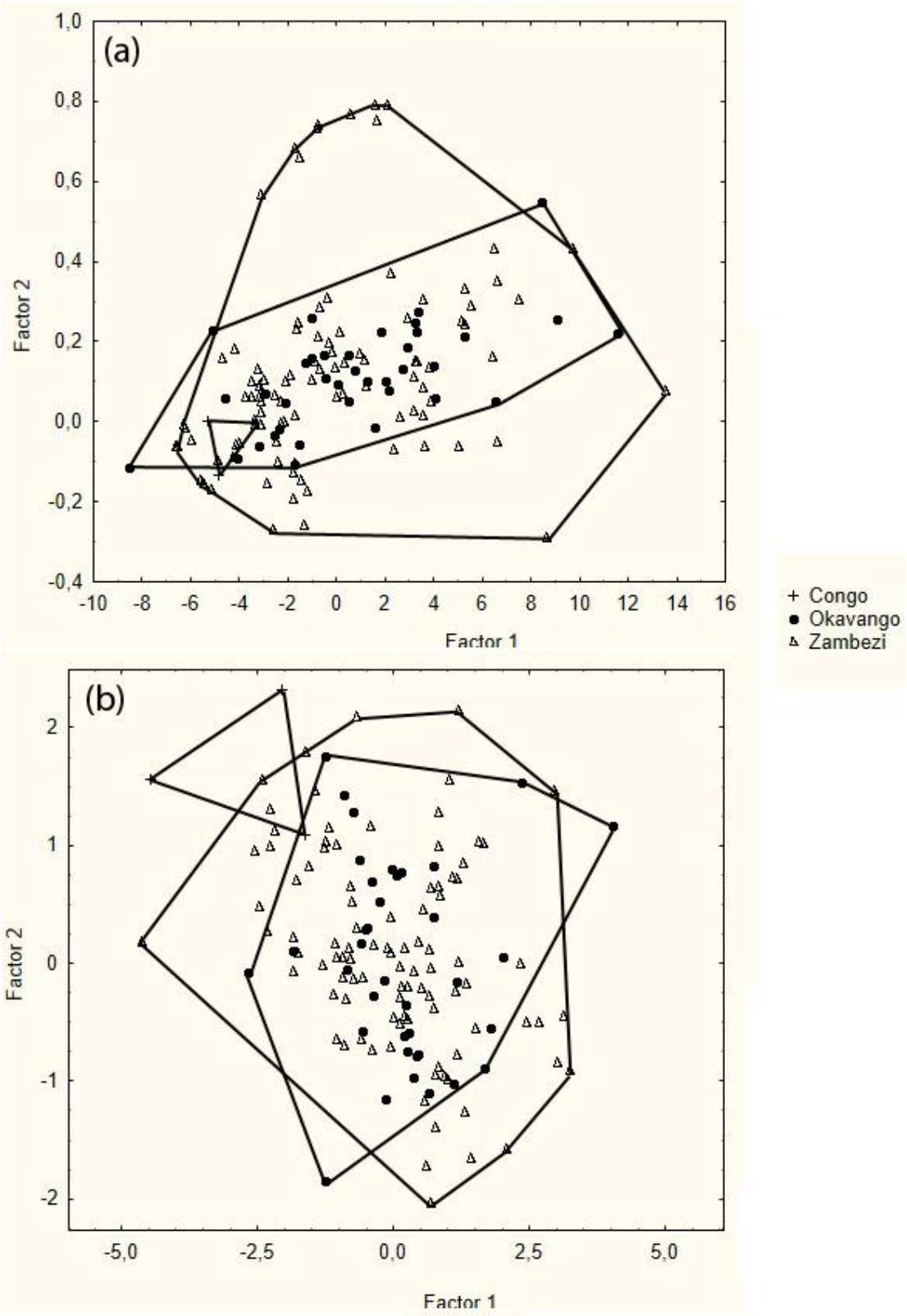

Figure 2.6: scatterplot of cases for PC1 and PC2 from the PCA of the morphometric measurements of *N. multifasciatus* using (a) measurements corrected for allometry and (b) percentage of standard length measurements; Zambezi n=94, Okavango n=43, Congo n=3.

Meristics

The first and second principal components comprised 21.9% and 13.1% of observed variability respectively, 35.0% overall. The characters with the highest loadings on the first principal component were the caudal scale count, scales between the dorsal fin origin and the lateral line, and the scales between the pelvic fin origin and the lateral line. The second principal component had greater loadings on the branched pectoral ray count, unbranched pectoral ray count, horizontal scale count, predorsal scale count, and the number of scales between the dorsal and adipose fins.

The meristic characters are, graphically, not more informative than the morphometric measurements, with overlaps between all three groups used in the analysis. The vast overlap may be in part due to the similar contributions to variation by the first two principal components.

Table 2.6: eigenvector factor loadings for principal components of meristic characters of southern African *N. multifasciatus.* Bold values indicate important contributors to variation

	Factor 1	Factor 2
Number of body bars	0.15	0.15
Horizontal scale count	0.24	**0.40**
Caudal scale count	**0.43**	-0.07
Predorsal scale count	0.23	**0.38**
Dorsal/lateral line scale count	**0.39**	-0.10
Pelvic/lateral scale count	**0.36**	-0.08
Scales between dorsal/adipose	0.25	**0.30**
Unbranched dorsal rays	-0.15	-0.11
Branched dorsal rays	0.25	-0.17
Unbranched pectoral rays	0.29	**-0.43**
Branched pectoral rays	-0.19	**0.52**
Branched pelvic rays	0.25	0.25
Unbranched anal rays	-0.07	-0.06
Branched anal rays	0.25	0.00

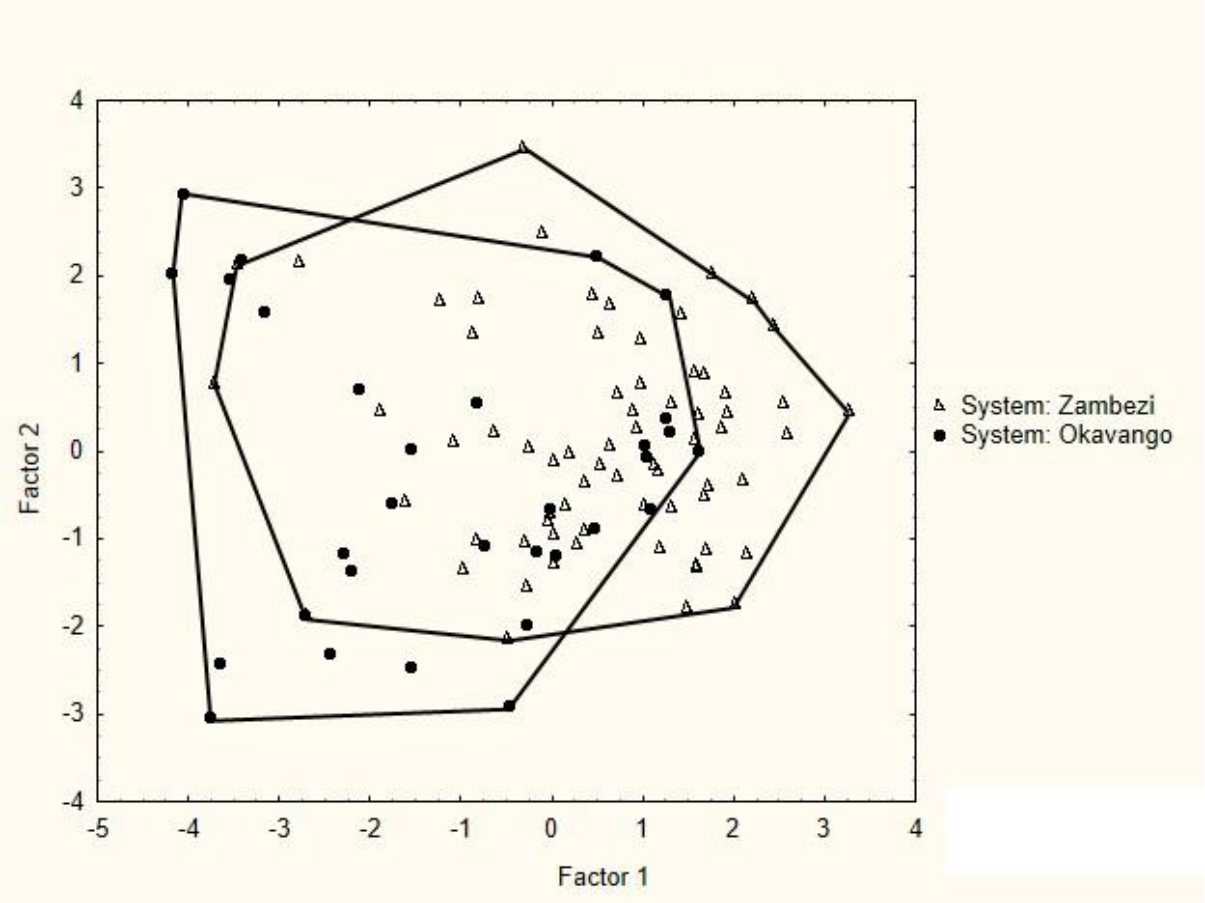

Figure 2.7: scatterplot of cases of PC1 and PC2 from the PCA of the meristic characters of *N. multifasciatus;* Zambezi n=78, Okavango n=38.

2.3.2.1.2 Discriminant Function Analyses

Morphometrics

The discriminant function classification matrix of morphometric data was moderately successful in classifying specimens to the correct river system in both the allometry- and percent-adjusted datasets, with 79.3% and 81.4% success respectively. Major shortfalls were seen in both datasets in identifying specimens from the Okavango system, with 52.5% of allometry-adjusted and 50.0% of proportion-adjusted data Okavango specimens instead being assigned to the Zambezi system. Specimens from the Congo system were assigned with 100.0% accuracy in both classification matrices.

The lowest partial Wilk's λ scores for the allometry-adjusted data were caudal peduncle length, head width, and interorbital width; the lowest scores for the proportion-adjusted data were head width, interorbital width, and caudal peduncle length.

Two roots were produced in the discriminant function analysis for both datasets. In the allometry-adjusted dataset, the first and second roots accounted for 60.5% and 39.5% of

discriminatory power respectively. The proportion-adjusted data placed more emphasis on the first root, with 69.7%, while the second root had only 30.3% contribution. The allometry-adjusted dataset root 1 had the greatest loadings on interorbital width, head width, and snout length, while root 2 placed emphasis on preanal length, eye horizontal diameter, and caudal peduncle length. The proportion-adjusted dataset root 1 identified interorbital width, head length, and head width as important characters, while root 2 identified eye horizontal diameter, caudal peduncle length, and upper jaw length as important.

Meristics

The discriminant function classification using the meristic data for this group yielded moderate success in correctly assigning 75.2% of the specimens to the correct population. The majority of the inaccuracy arose from specimens from the Okavango system being incorrectly identified as Zambezian (51.3%); conversely, few (13.2%) Zambezi specimens were assigned to the Okavango system.

Meristic characters having the lowest partial Wilk's λ scores were the dorsal unbranched rays, predorsal scale count, and the dorsal branched rays. These same characters had the greatest loadings resulting from the canonical analysis.

Similar results are seen for both the allometry and proportion adjusted data, although appearing mirrored (Fig. 2.8 a and b). The large overlap between Zambezi and Okavango groups persists as in other species groups (Figs. 2.10 & 2.15). These populations can mostly be clustered apart by characters on the second canonical root. Although in both cases having lower discriminatory power than the first roots, both of these second canonical roots included eye horizontal diameter and caudal peduncle length as important characters in discrimination. In both datasets the Congo group could be discriminated from the Okavango groups by characters within the second root, while being discriminated from the Zambezi and Okavango specimens by characters on the first root, both indicating interorbital width and head width as important characters.

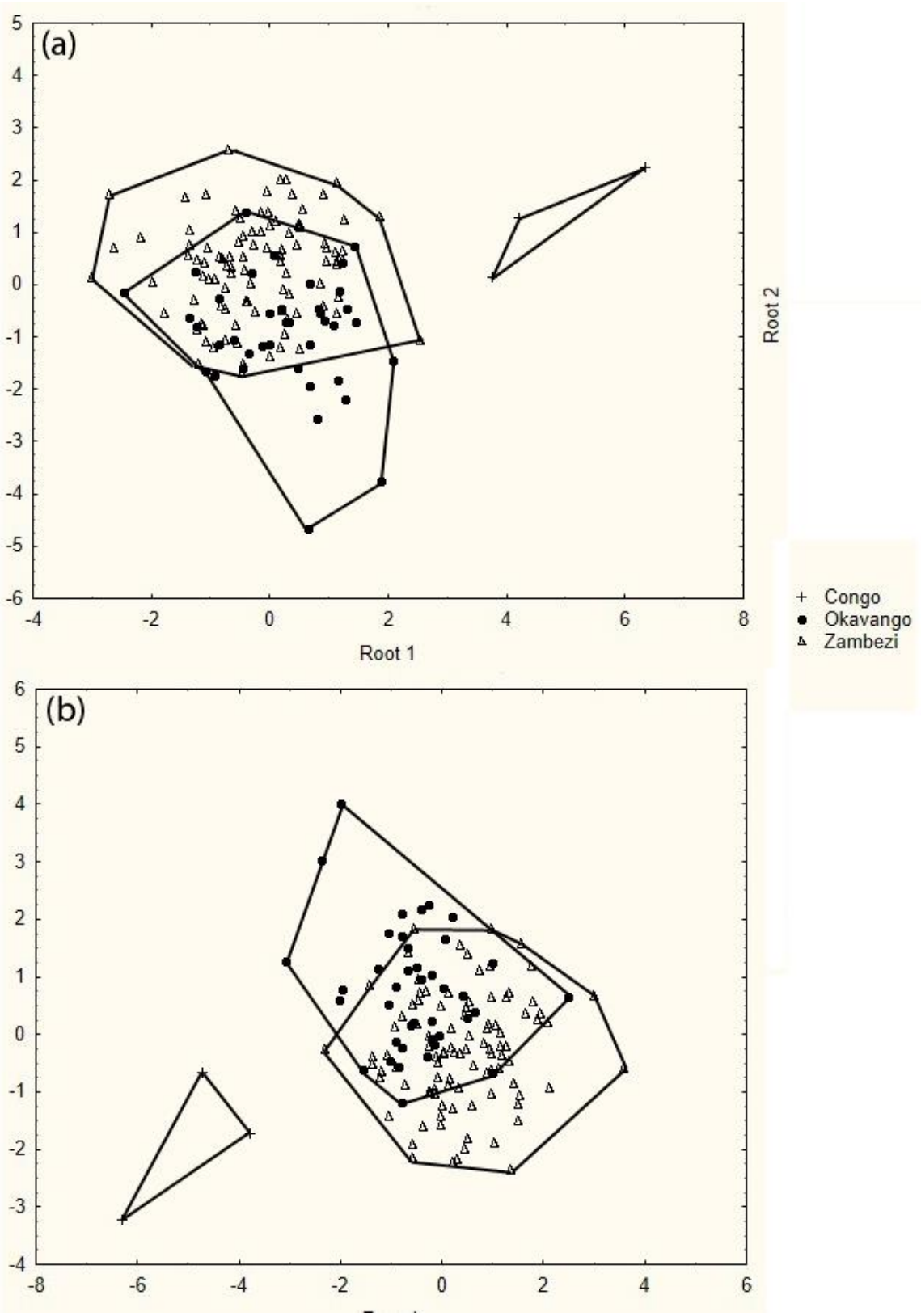

Figure 2.8: scatterplot of canonical scores of root 1 and root 2 from the discriminant function analysis of the morphometric measurements of *N. multifasciatus* using (a) measurements corrected for allometry and (b) percentage of standard length measurements; Zambezi n=94, Okavango n=43, Congo n=3.

2.3.2.1.3 Traditional Morphometrics and meristics

Morphometrics

Measurements with significant differences between them, as indicated by ANOVA and Kruskal-Wallis tests, are those between the Congo and the two southern African systems (Zambezi and Okavango, Table 2.7), in particular body width, body depth, and head width which were greater than those of the southern African specimens: body width mean 9.5-9.7%SL in southern African specimens vs 12.6%SL in Congo specimens; body depth mean 21.6-21.9%SL in southern African specimens vs. 26.6%SL in Congo specimens; head width mean 10.0-10.4%SL in southern African specimens vs 12.0%SL in Congo specimens. While these measurements overlap with those of the southern African populations to some extent, the Congo mean proportions tend to be larger than those of their southern African counterparts. Such differences may be explained by the small sample size of Congo specimens examined (n=3). Zambezian and Okavango specimens showed significant differences between some measurements (viz. head width, caudal peduncle length, snout length; Table 2.7), although large overlaps in values rendered these traits non-diagnostic.

Table 2.7: morphometric measurements of the *N. multifasciatus* group, expressed as a percentage of standard length. The last four (head) measurements are expressed as a percentage of head length. Values shown are the range, with the mean value displayed in brackets. The p column indicates the p-values for the measurement, where * indicates a significant difference ($p<0.05$), and the letters represent the following system pairings between which a significant difference occurred: CO=Congo-Okavango; CZ=Congo-Zambezi; ZO=Zambezi-Okavango; Zambezi n=94, Okavango n=43, Congo n=3.

	Zambezi	Okavango	Congo	p
Standard length	17.0-40.8 (30.5)	18.7-41.8 (29.2)	31.6-36.4 (34.3)	-
Head length	22.5-29.0 (25.3)	22.4-28.5 (25.4)	25.3-27.4 (26.5)	-
Predorsal length	41.7-50.5 (46.2)	38.5-49.7 (46.3)	45.8-52.3 (48.1)	-
Prepectoral length	24.1-33.7 (28.1)	24.6-31.9 (27.9)	26.0-28.4(27.2)	-
Prepelvic length	45.1-55.5 (50.9)	42.0-54.2 (50.4)	49.7-53.3 (51.9)	-
Preanal length	68.5-79.3 (72.8)	61.2-77.4 (72.9)	75.3-79.3 (77.5)	-
Head width	8.6-11.6 (10.0)	8.5-12.2 (10.4)	11,.7-12.6 (12.0)	* CO CZ ZO
Body width	6.8-13.0 (9.5)	6.5-11.5 (9.7)	11.7-13.1 (12.6)	* CO CZ
Head depth	10.4-14.2 (12.4)	8.6-13.8 (12.2)	13.1-14.0 (13.6)	-
Body depth	17.7-25.6 (21.9)	16.7-26.2 (21.6)	26.1-27.1 (26.6)	* CO CZ
Dorsal fin length	17.9-27.0 (22.2)	17.8-24.3 (21.6)	17.8-21.9 (20.5)	-
Pectoral fin length	11.0-18.5 (14.9)	9.0-16.1 (14.0)	14.4-15.5 (14.8)	-
Pelvic fin length	12.2-19.5 (15.0)	11.8-17.6 (15.2)	15.2-17.5 (16.2)	-
Anal fin length	11.7-18.2 (14.6)	11.2-17.7 (14.5)	15.5-15.8 (15.7)	-
Caudal peduncle length	8.6-15.9 (13.1)	9.4-16.2 (13.6)	10.8-12.8 (11.8)	* CO ZO
Caudal peduncle depth	7.5-10.5 (9.2)	7.6-10.5 (8.9)	9.1-10.6 (10.0)	-
Eye horizontal diameter	28.5-40.9 (34.4)	31.3-40.8 (35.6)	27.0-30.0 (28.9)	-
Snout length	20.3-30.6 (25.0)	16.8-28.0 (24.1)	20.0-22.2 (21.1)	*
Upper jaw length	17.1-24.4 (21.0)	16.5-25.4 (21.1)	19.5-22.2 (20.8)	-
Interorbital width	16.7-27.5 (23.0)	16.6-28.8 (23.3)	25.4-32.3 (27.7)	-

Meristics

The Zambezi and Okavango specimens showed significant differences mostly in scale counts, in that Zambezi specimens tended to have greater counts than the Okavango specimens (Table 2.8); mean caudal scale and predorsalscale counts there were 13 in Zambezian fishes vs 12 in Okavangan specimens. There is considerable overlap between the two populations, and these traits cannot be considered diagnostic. The number of scales between the dorsal and adipose fin could be considered a diagnostic character to differentiate specimens from the Congo from those from the southern African systems, having significantly fewer than their southern African counterparts, with a mean of 7 scales in Congo specimens vs. 10 in southern African specimens. These characters can therefore be used to identify the populations apart from one another on a species-level.

Table 2.8: meristic counts for the *N. multifasciatus* group. Values shown are the range, with the mean value displayed in brackets. The p column indicates the p-values for the count, where * indicates a significant difference ($p<0.05$), and the letters represent the following system pairings between which a significant difference occurred: CO=Congo-Okavango; CZ=Congo-Zambezi; ZO=Zambezi-Okavango; Zambezi n=78, Okavango n=38, Congo n=3.

	Zambezi	Okavango	Congo	p
Horizontal scale count	36-41 (38)	36-41 (38)	32-40 (37)	-
Lateral line scale count	12-35 (23)	19-39 (28)	17-40 (25)	-
Caudal scale count	9-15 (13)	10-14 (12)	10 (10)	* ZO
Predorsal scale count	9-15 (13)	10-15 (12)	12-15 (13)	* ZO
Dorsal fin to lateral line scale count	4-6.5 (5)	3-6.5 (5)	3-5 (4)	* CO CZ
Pelvic fin to lateral line scale count	2.5-5.5 (5)	3.5-5.5 (4)	3-5 (4)	* CZ
Scales between dorsal/adipose	9-12 (10)	8-12 (10)	6-7 (7)	* CO CZ
Dorsal unbranched rays	3-4 (3)	3-4 (3)	3 (3)	* ZO
Dorsal branched rays	10-13 (12)	10-13 (11)	11-12 (11)	-
Pectoral unbranched rays	1-3 (2)	1-3 (2)	1 (1)	* CZ
Pectoral branched rays	6-10 (8)	6-10 (8)	9-10 (10)	* CO CZ ZO
Pelvic unbranched rays	1 (1)	1-2 (1)	1 (1)	-
Pelvic branched rays	7-8 (8)	6-8 (7)	7 (7)	* CO CZ
Anal unbranched rays	2-3 (3)	2-4 (3)	3 (3)	-
Anal branched rays	7-10 (9)	7-10 (9)	7-8 (8)	* CO CZ

2.3.2.1.4 Pigmentation pattern variation

Figure 2.9: unique pigmentation pattern variation for *N. multifasciatus*, enclosed within the systems within which forms of variation could be found. Not to scale. (a) Zambezi River system specimens: (i) SAIAB 72785, Njoko River, Zambia; (ii) SAIAB 98175, Chisolo River, Zambia; (iii) SAIAB 122409, Linyanti River, Namibia; (iv) SAIAB 73568, Madamyana River, Zambia; (v) SAIAB 73449, Mwombezhi River, Zambia; (b) Okavango River system specimens: (i) SAIAB 187051, Thamalakane River, Botswana; (ii) SAIAB 186713, Cacuchi River, Botswana; (iii) SAIAB 68448, Thaoge River, Botswana.

No particular patterns or counts could be attributed to any one genetic lineage. *Nannocharax multifasciatus* from the Congo system appear to display a significantly lower body bar count than their southern African counterparts, although slight overlap does occur (Table 2.9). The sample size of the Congo *N. multifasciatus* was very small (n=3), so these counts may not have captured the full variation of this population.

Some pigmentation pattern variation exists within southern African *N. multifasciatus* (Fig. 2.9)*;* however, not necessarily to the extent or bearing much similarity to what Fowler (1935) had described for the species (there referred to as *Distichodus stigmaturus).* The range of body bars is greater than described by Boulenger (1923) when this species was first described, although that was described from a single specimen, and would not have captured the full extent of the variation of this species.

Aberrant patterning was more common among Zambezi specimens than it was among Okavango specimens. In Okavango specimens, the 'split Y bar' condition was seen more regularly, while broken barring was more common among Zambezi fishes.

Table 2.9: range of pigmentation pattern character counts for *Nannocharax multifasciatus,* with the numbers in brackets indicating the mean value; Zambezi n=74, Okavango n=36, Congo n=3.

	Zambezi	Okavango	Congo
Number of body bars	13-23 (17)	13-21 (17)	13-14 (13)
p	*, C	*, C	*, ZO

2.3.2.2 *Nannocharax machadoi* group

2.3.2.2.1 Principal component analyses

Morphometrics

The first two principal components provided the greatest contribution to variation at 13.8% and 11.6% for principal components 1 and 2 respectively. The characters with the highest loadings on the first principal component were, in descending order, anal fin length, body depth, and pelvic fin length. The second principal component's highest loadings were on head length, predorsal length, prepelvic length, interorbital length, and preanal length.

The proportion-adjusted dataset had much greater emphasis on the first principal component, contributing to 67.9% of the variance, as opposed to 4.0% contributed by principal component 2. Due to the higher contribution by the first principal component, however, no single eigenvector of this component could be said to have a higher loading as compared to the other eigenvectors. On the second principal component, prepectoral length, pectoral fin length, and preanal length, had the greatest loadings.

A similar pattern of large overlap is seen here as in the *N. multifasciatus* group morphometric graphical results. As per those results, but perhaps better illustrated in Fig. 2.10a is the closer nearest neighbour points between specimens. Therefore, despite showing a similar pattern to

the proportion adjusted data, greater clustering and less spread is seen in the allometry adjusted data.

Table 2.10: eigenvector factor loadings for the principal components of the allometry-adjusted morphometric characters of southern African *N. machadoi.* Bold values indicate important contributors to variation

	Factor 1	Factor 2
Head prop	-0.16	**0.48**
Predorsal length	-0.15	**0.43**
Prepectoral length	-0.01	0.05
Prepelvic length	-0.27	**0.39**
Preanal length	-0.17	**0.30**
Head width	-0.24	0.11
Body width	**-0.30**	-0.16
Head depth	-0.19	0.17
Body depth	**-0.33**	-0.07
Dorsal fin length	-0.21	-0.03
Pectoral fin length	-0.20	0.04
Pelvic fin length	**-0.32**	-0.15
Anal fin length	**-0.37**	-0.09
Caudal peduncle length	-0.18	-0.15
Caudal peduncle depth	-0.28	-0.23
Eye horizontal diameter	0.12	-0.01
Snout length	-0.18	-0.06
Upper jaw length	-0.22	-0.23
Interorbital width	-0.18	**-0.30**

Table 2.11: eigenvector factor loadings for the principal components of the proportion-adjusted morphometric characters of southern African *N. machadoi*. Bold values indicate important contributors to variation

	Factor 1	Factor 2
Head length	-0.25	-0.04
Predorsal length	-0.24	-0.10
Prepectoral length	-0.17	**-0.59**
Prepelvic length	-0.25	-0.15
Preanal length	-0.19	**-0.41**
Head width	-0.24	-0.01
Body width	-0.24	-0.12
Head depth	-0.22	0.03
Body depth	-0.24	-0.01
Dorsal fin length	-0.23	0.05
Pectoral fin length	-0.19	**0.54**
Pelvic fin length	-0.24	0.21
Anal fin length	-0.24	0.23
Caudal peduncle length	-0.21	0.07
Caudal peduncle depth	-0.24	0.15
Eye horizontal diameter	-0.23	-0.05
Snout length	-0.22	-0.04
Upper jaw length	-0.24	0.03
Interorbital width	-0.23	0.10

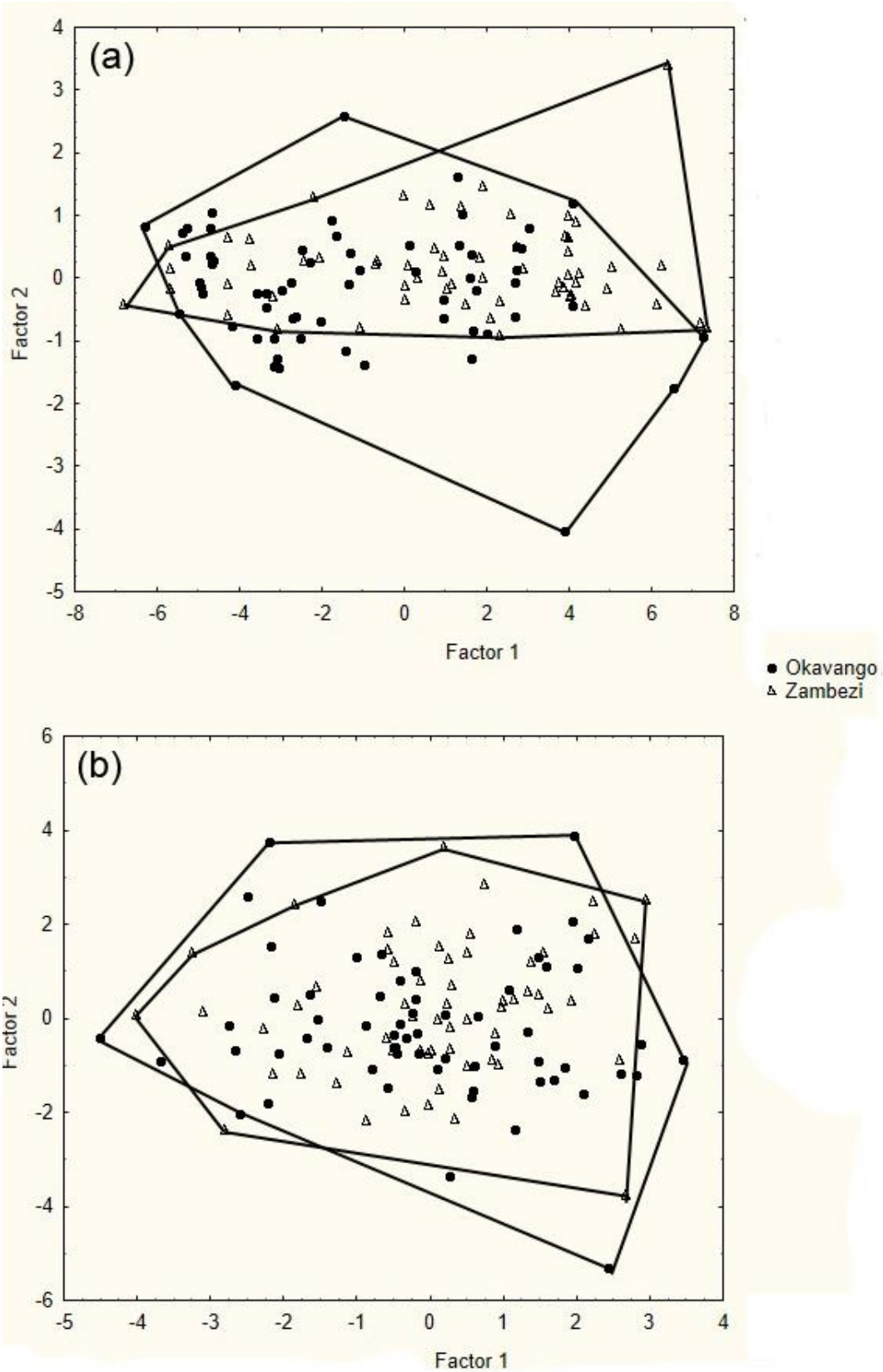

Figure 2.10: scatterplot of PC1 and PC2 from the PCA of the morphometric measurements of *N. machadoi* using (a) measurements corrected for allometry and (b) percentage of standard length measurements*;* Zambezi n=59, Okavango n=67.

Meristics

The first two principal components contributed the most to variation, contributing 21.4% and 16.1% respectively, 37.5% of variation combined. The characters with the highest loadings on principal component 1 were scales between the dorsal fin origin and lateral line, scales between the pelvic fin origin and the lateral line, unbranched pectoral rays, branched pectoral rays, and unbranched anal rays. The characters with the highest loadings on the second principal component included the caudal scale count and branched dorsal rays, in addition to sharing were scales between the dorsal fin origin and lateral line, scales between the pelvic fin origin and the lateral line, and branched pectoral rays with principal component 1.

The left-to-right divergence of clusters of the respective river basin groups is evident in Fig. 2.11 despite a large overlap being present. This is more so as an effect of the first principal component, although only slightly more than the second principal component, and include differences primarily in unbranched anal and pectoral ray counts, as well as the transverse scale counts.

Table 2.12: Factor loadings for the principal components of the meristic characters of southern African *N. machadoi*. Bold values indicate important contributors to variation

	Factor 1	Factor 2
Horizontal scale count	-0.24	0.17
Caudal scale count	0.15	**0.38**
Predorsal scale count	-0.23	0.29
Dorsal/lateral line scale count	**0.39**	**0.33**
Pelvic/lateral line scale count	**0.35**	**0.34**
Unbranched dorsal rays	-0.07	-0.16
Branched dorsal rays	-0.13	**0.50**
Unbranched pectoral rays	**0.47**	-0.07
Branched pectoral rays	**-0.38**	**0.31**
Branched pelvic rays	-0.10	0.25
Unbranched anal rays	**-0.43**	-0.07
Branched anal rays	0.01	0.29

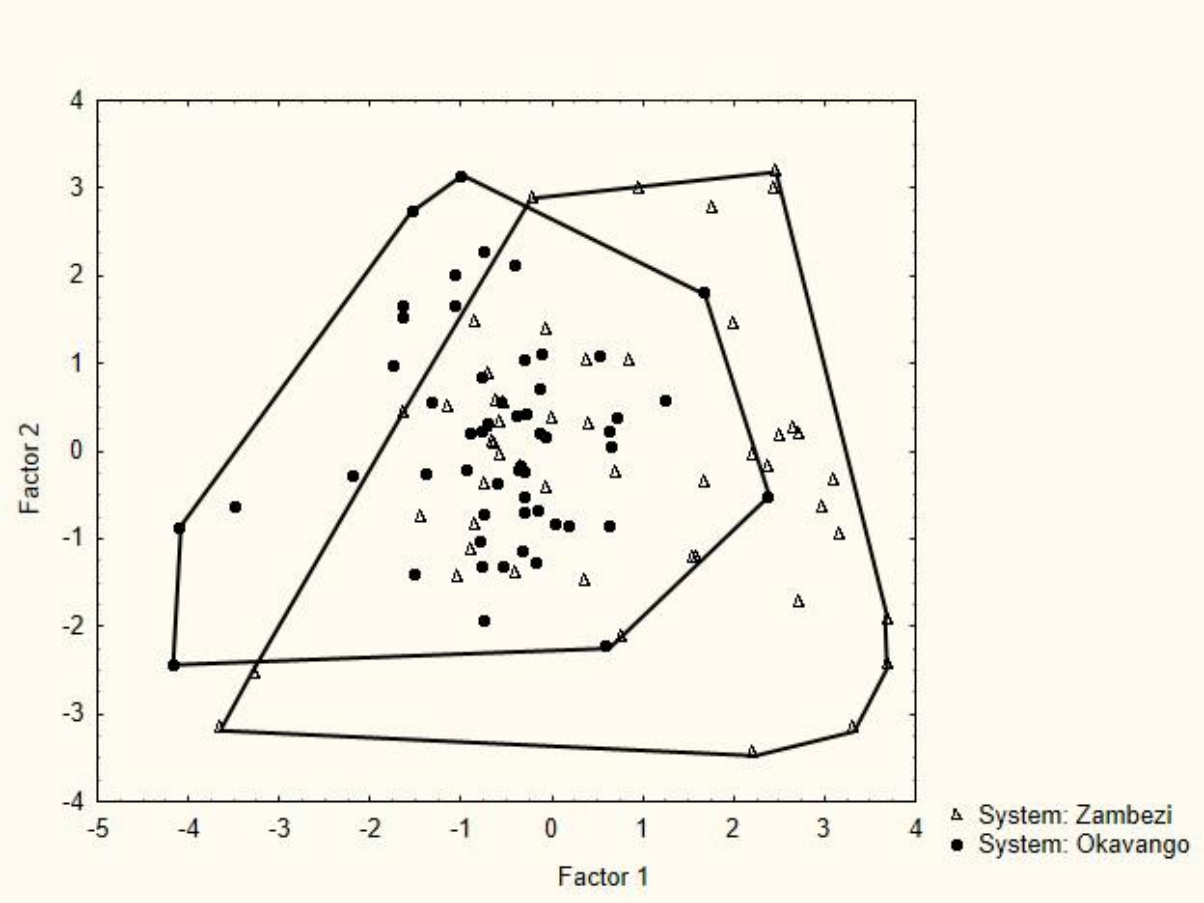

Figure 2.11: scatterplot of cases of PC1 vs PC2 from the PCA of the meristic characters of *N. machadoi*; Zambei n=51, Okavango n=62.

2.3.2.2.2 Discriminant function analyses

Morphometrics and meristics

In both the morphometric and the meristic discriminant function analyses, a single discriminant function was produced. No scatterplot visualising the results could therefore be produced. The classification matrix could correctly identify the specimens according to river system with 79.8% and 77.4% accuracy for the proportional morphometric data and the allometry-adjusted morphometric data respectively. Using meristic data, the classification matrix only managed to correctly identify specimens to the correct river system with 73.0% accuracy.

For the proportion-adjusted morphometric data, the eye horizontal diameter, caudal peduncle length, and head length had the lowest partial Wilk's λ values, indicating more pronounced contribution to variation in this analysis. Conversely, the allometry-adjusted data indicated that snout length and pectoral fin length were more important variables in discrimination, but included caudal peduncle length as well. These same results were seen in the canonical analysis value loadings for both the proportion-adjusted and the allometry-adjusted datasets.

The meristic variables with the lowest partial Wilk's λ were the unbranched pectoral rays and the number of bars on the body. The canonical analysis values with the greatest loadings (by the standardised coefficients) were again the unbranched pectoral rays, the number of bars on the body, and including the horizontal scale count, in descending order.

2.3.2.2.3 Traditional morphometrics and meristics

Morphometrics

Although significant differences occur between several of the measurements of *N. machadoi,* none could be considered diagnostic in differentiating the populations (Table 2.13). Eye horizontal diameter was the measurement with the greatest differences between populations, with Zambezi specimens having a greater eye diameter than the Okavangan specimens (mean 37.0%HL in Zambezi vs 35.2%HL in Okavango).

Table 2.13: morphometric measurements of the *N. machadoi* group, expressed as a percentage of standard length. The last four (head) measurements are expressed as a percentage of head length. Values shown are the range, with the mean value shown in brackets. The p column indicates the p-values for the measurement, where * indicates a significant difference ($p<0.05$); Zambezi n=59, Okavango n=67.

	Zambezi	Okavango	p
Standard length	16.6-29.1 (21.8)	17.3-29.1 (24.1)	-
Head length	22.4-28.9 (26.1)	22.6-29.4 (25.6)	*
Predorsal length	41.6-52.6 (48.0)	40.8-51.8 (47.2)	*
Prepectoral length	23.6-34.0 (29.4)	24.9-33.6 (28.6)	-
Prepelvic length	46.1-56.3 (49.8)	41.7-54.7 (49.7)	-
Preanal length	68.5-77.3 (72.4)	67.6-80.4 (73.2)	-
Head width	7.6-12.0 (10.2)	8.7-11.8 (10.3)	-
Body width	6.1-11.0 (8.4)	6.4-11.8 (8.9)	*
Head depth	9.5-15.9 (12.7)	10.5-15.0 (12.6)	-
Body depth	19.0-24.7 (21.7)	18.0-26.0 (21.7)	-
Dorsal fin length	17.4-25.4 (21.4)	17.1-25.3 (21.3)	-
Pectoral fin length	10.7-16.5 (13.0)	8.6-15.5 (12.4)	*
Pelvic fin length	11.6-17.0 (14.1)	11.5-19.1 (14.3)	-
Anal fin length	11.4-16.4 (14.3)	11.0-16.4 (14.2)	-
Caudal peduncle length	11.6-16.4 (14.2)	10.5-17.1 (13.6)	*
Caudal peduncle depth	7.3-10.2 (8.8)	7.0-10.2 (8.7)	-
Eye horizontal diameter	31.2-41.5 (37.0)	28.9-42.7 (35.2)	*
Snout length	16.4-27.9 (23.0)	19.2-28.5 (23.7)	-
Upper jaw length	14.1-20.8 (18.1)	12.4-22.0 (18.4)	-
Interorbital width	17.4-29.2 (23.5)	17.4-29.5 (23.3)	-

Meristics

No meristic character could be considered to be diagnostic in differentiating the *N. machadoi* populations (Table 2.14), despite the statistical tests detecting significant differences in some of these characters. Considerable overlap occurs between the two populations, as was the case with the morphological measurements.

Table 2.14: meristic counts for the *N. machadoi* group. Values shown are the range, with the mean value displayed in brackets. The p column indicates the p-values for the counts, where * indicates a significant difference ($p<0.05$); Zambei n=51, Okavango n=62.

	Zambezi	Okavango	p
Horizontal scale count	31-38 (34)	31-43 (35)	-
Lateral line scale count	3-10 (8)	3-10 (8)	-
Caudal scale count	9-12 (10)	9-14 (10)	-
Predorsal scale count	10-13 (12)	10-15 (12)	-
Dorsal fin to lateral line scale count	2.5-5.5 (4)	2.5-5.5 (4)	*
Pelvic fin to lateral line scale count	2.5-4.5 (4)	2-5 (4)	-
Scales between dorsal/adipose	-	-	-
Dorsal unbranched rays	2-4 (3)	2-4 (3)	-
Dorsal branched rays	8-11 (10)	8-12 (10)	-
Pectoral unbranched rays	1-2 (1)	1 (1)	*
Pectoral branched rays	4-9 (7)	5-10 (7)	*
Pelvic unbranched rays	1 (1)	1 (1)	-
Pelvic branched rays	5-8 (7)	5-8 (7)	-
Anal unbranched rays	1-3 (3)	2-3 (3)	*
Anal branched rays	7-8 (8)	7-9 (8)	-

2.3.2.2.4 Pigment pattern variation

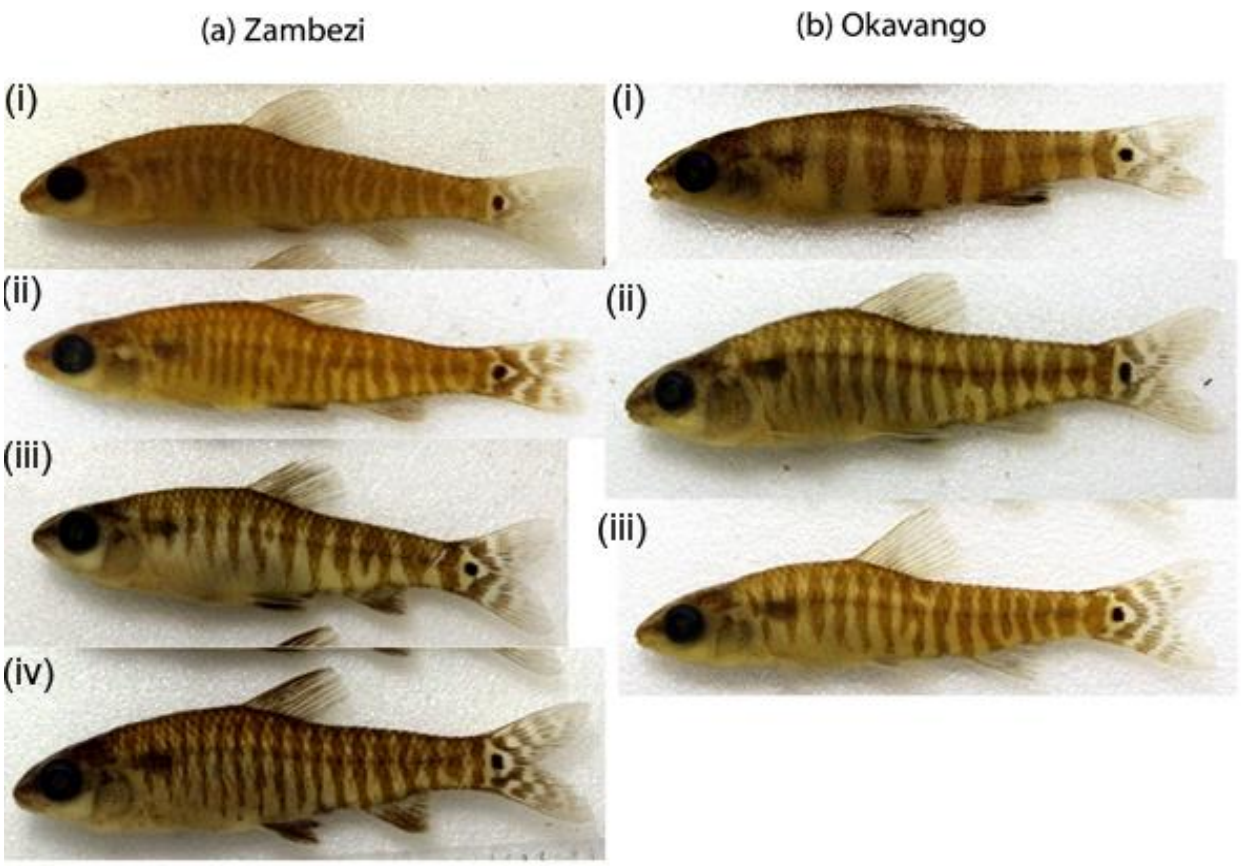

Figure 2.12: unique pigmentation pattern variation for *N. machadoi,* enclosed within the systems within which forms of variation could be found. Not to scale. (a) Zambezi River system specimens: (i) SAIAB 72317, Luena River, Zambia; (ii) SAIAB 73258, Lalafuta River, Zambia; (iii) SAIAB 83833, Kafue River, Zambia; (iv) SAIAB 83833, Kafue River, Zambia; (b) Okavango River system specimens: (i) SAIAB 29498, Thamalakane River, Botswana; (ii) SAIAB 71507, Thaoge River, Botswana; (iii) SAIAB 124919, Cuito River, Namibia.

Statistically, there is a significant difference between the number of bars between *N. machadoi* from the Zambezi and the Okavango, where the Okavango specimens bar count ranges into the lower numbers. The considerable overlap between these counts, however, renders this traint non-diagnostic for river system population identification. Deviations from the regular pattern were common and not particular to any system, although as per the first Okavango specimen (Fig. 2.12), some Okavango fishes bore fewer (<10) and broader bars than any other populations, and were found primarily within the lower Okavango main river ('panhandle' and 'pan' areas, see Chapter 1.5).

In the species original description, Poll (1967) likened *N. machadoi* to *N. wittei,* although noting 12 to 15 bars on *N. machadoi* [vs. the 15-17 in his original description for *N. wittei* (Poll 1933)]. Here we see a much broader range of bar numbers within this species, which may have been limited by the small sample size Poll diagnosed the species from (at 8 specimens).

Table 2.15: range of pigmentation pattern character counts for *Nannocharax machadoi,* with the numbers in brackets indicating the mean value; Zambezi n=48, Okavango n=58.

	Zambezi	Okavango
Number of body bars	12-20 (15)	9-21 (14)
p	*	*

2.3.2.3 *Nannocharax macropterus* species group

2.3.2.3.1 Principal component analyses

Morphometrics

The allometry-adjusted morphometric data, only the first principal component could be extracted as per the Kaiser criterion (1960), being the only principal component having an eigenvalue greater than 1, and contributing to 79.4% of the total variation in the analysis. However, the eigenvalues within this principal component had very similar values, with none meeting the requirements as per McGarigal et al. (2000), therefore all having similar loading. The second principal component, however, showed greater effects from the pectoral and pelvic fin lengths. The proportion-adjusted data presented differently, the first two components contributing 17.0% and 13.3% to variation. The first component presented higher loadings on head length, prepectoral length, and pectoral fin length, while the second had high loadings on pectoral fin length, pelvic fin length, anal fin length, dorsal fin length, and body depth.

In the allometry adjusted data Fig. 2.13a, the Zambezi group differs from the Okavango group partially by the first component and partially by the second component, although still overlaps somewhat. The Congo group lies within this zone of overlap between these two groups. The Kwanza group shows no overlap with any of the other systems, and appears to be differentiated from the other groups largely by the second principal component, despite this principal component having a low eigenvalue by Kaiser's (1960) standards. This second principal component had important loadings on fin size (pectoral and pelvic fin length), which are important characters as seen in the traditional morphology section (Table 2.19).

In Fig. 2.13 (b), using proportional data, shows a much greater overlap in the Zambezi and Okavango groups than in the previous dataset, although the Congo group is still situated within

this overlap. The *N. macropterus "*Kwanza" lineage is once again clustered away from other southern African groups, although the nearest neighbour distances are much greater. Characters useful in distinguishing this group lie within the first principal component, indicating prepectoral length, body width, and dorsal fin length as important in differentiating this group. This is reflected in the traditional morphometric data (Table 2.19), in which prepectoral length significantly differs from all other *N. macropterus* species group populations, although the values overlap somewhat, and the dorsal fin length differs significantly from the other southern African species group populations, in being shorter. None of these characteristics are diagnostic, however.

Table 2.16: Factor loadings for the principal components of the allometry-adjusted characters of southern African *N. macropterus.* Bold values indicate important contributors to variation

	Factor 1	Factor 2
Head length	-0.21	0.29
Predorsal length	-0.24	0.24
Prepectoral length	-0.24	0.15
Prepelvic length	-0.24	0.15
Preanal length	-0.23	0.27
Head width	-0.24	0.02
Body width	-0.24	-0.02
Head depth	-0.24	0.01
Body depth	-0.24	0.03
Dorsal fin length	-0.24	-0.24
Pectoral fin length	-0.23	**-0.37**
Pelvic fin length	-0.21	**-0.62**
Anal fin length	-0.23	-0.27
Caudal peduncle length	-0.22	0.06
Caudal peduncle depth	-0.24	-0.06
Eye horizontal diameter	-0.21	0.19
Snout length	-0.22	0.09
Upper jaw length	-0.23	0.17
Interorbital width	-0.22	-0.08

Table 2.17: eigenvector factor loadings for the principal components of the proportion-adjusted characters of southern African *N. macropterus.* Bold values indicate important contributors to variation

	Factor 1	Factor 2
Head length	**0.46**	0.21
Predorsal length	-0.01	-0.09
Prepectoral length	**0.42**	0.07
Prepelvic length	0.21	0.16
Preanal length	0.10	0.19
Head width	0.18	0.06
Body width	**-0.30**	0.15
Head depth	0.23	0.24
Body depth	0.02	**0.32**
Dorsal fin length	-0.12	**0.36**
Pectoral fin length	**-0.32**	**0.40**
Pelvic fin length	-0.13	**0.36**
Anal fin length	-0.02	**0.34**
Caudal peduncle length	-0.09	0.09
Caudal peduncle depth	-0.20	0.20
Eye horizontal diameter	0.23	-0.16
Snout length	-0.22	-0.24
Upper jaw length	-0.19	-0.11
Interorbital width	-0.25	-0.13

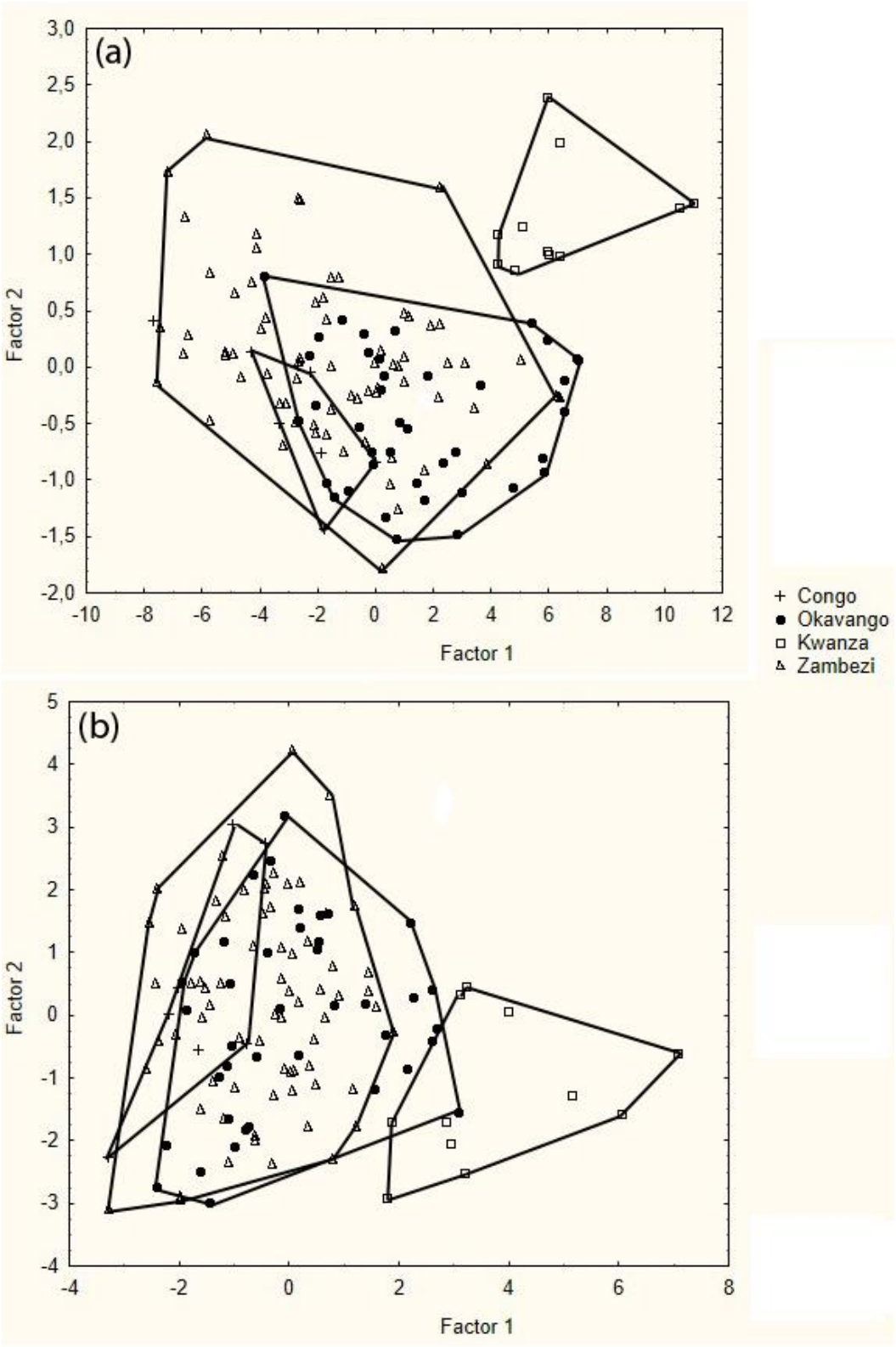

Figure 2.13: scatterplot of cases of PC1 vs PC2 from PCA of the morphometric measurements of *N. macropterus* using (a) measurements corrected for allometry and (b) percentage of standard length measurements; Zambezi n=71, Okavango n=42, Congo n=8, Kwanza n=11.

Meristics

The first two principal components contributed the most to variation, 22.4% and 12.4% respectively, 34.8% combined. The characters in principal component 1 with the highest loadings were the scales between the dorsal fin origin and lateral line, scales between the pelvic fin origin and lateral line, scales between the dorsal and adipose fins, and the branched anal ray count. Principal component 2 had highest loadings on the number of bodily blotch patterns, unbranched dorsal rays, and unbranched anal fin rays.

Graphical representation of these results (Fig 2.14) again presents an overlap between Zambezi and Okavango groups, although (with the exception of two errant Okavango specimens), the Zambezi population can in part be grouped about on the first principal component. The Congo group is clearly clustered away from the southern African groups, being strongly influenced by the characters on the single principal component – primarily scale counts.

Table 2.18: eigenvector factor loadings for the principal components of the meristic characters of southern African *N. macropterus* species group. Bold values indicate important contributors to variation

	Factor 1	Factor 2
Blotch pattern count	-0.22	**0.32**
Saddle pattern count	0.07	-0.07
Horizontal scale count	0.29	0.02
Caudal scale count	0.20	-0.18
Predorsal scale count	0.24	-0.06
Dorsal/lateral line scale count	**0.32**	-0.22
Pelvic/lateral line scale count	**0.37**	-0.26
Scales between dorsal fin/adipose fin	**0.39**	0.18
Unbranched dorsal rays	-0.05	**-0.49**
Branched dorsal rays	0.27	0.27
Unbranched pectoral rays	-0.17	-0.13
Branched pectoral rays	0.28	-0.03
Branched pelvic rays	0.09	-0.12
Unbranched anal rays	-0.08	**-0.59**
Branched anal rays	**0.42**	0.16

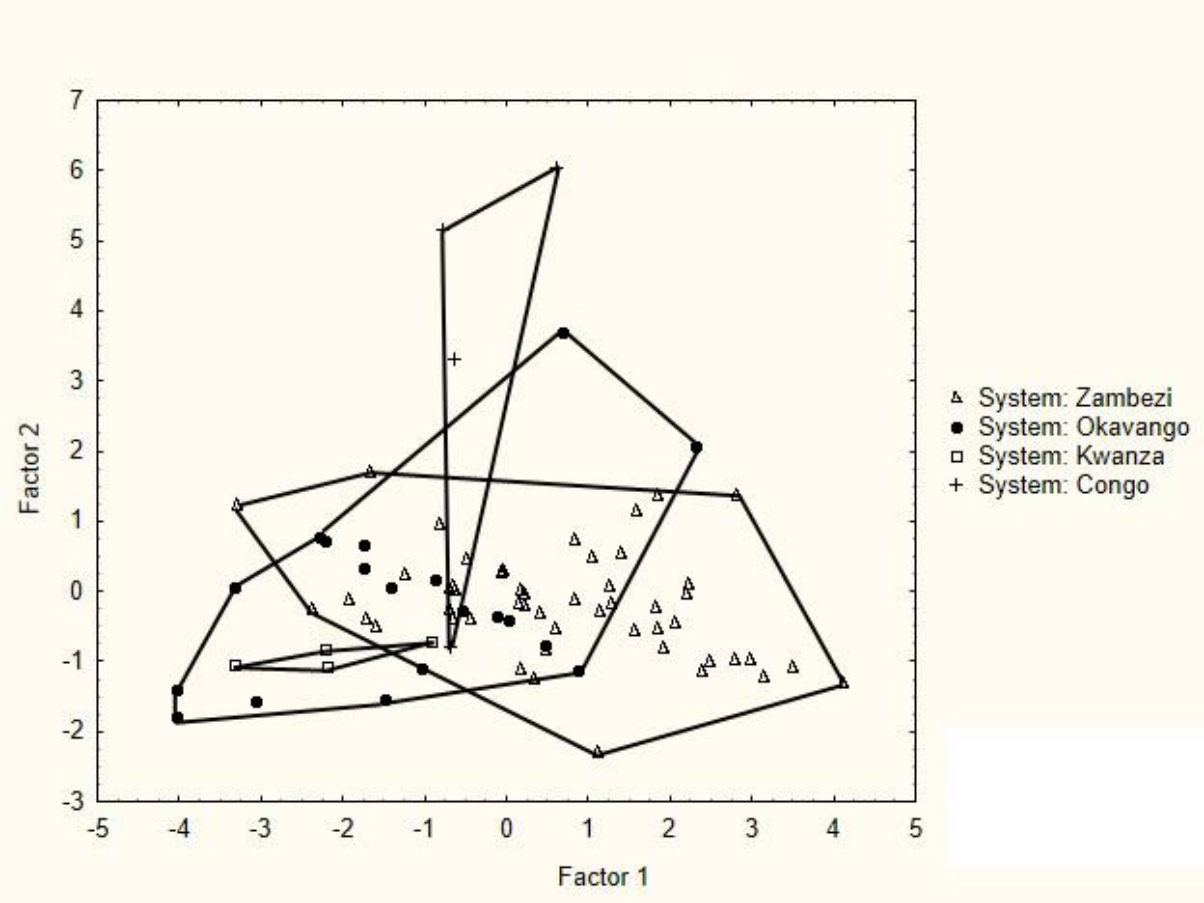

Figure 2.14: scatterplot of cases of PC1 vs PC2 from the PCA of the meristic characters of *N. macropterus*; Zambezi n=58, Okavango n=25, Congo n=8, Kwanza n=6.

2.3.2.3.2 Discriminant function analysis

Morphometrics

In the proportion-adjusted data, root 1 contributed 61.1% of variation, with root 2 only contributing 26.1%. The allometry adjusted data showed a similar ratio of contribution to variation, at 6.8% and 23.7% for roots 1 and 2 respectively.

The classification matrix utilizing morphometric data in both the proportion-adjusted and the allometry adjusted yielded similar results, identifying the specimens to the correct river systems with 82.9% and 85.4% respectively. In the analysis with the allometry adjusted data, head width, caudal peduncle depth, and anal fin length presented to lowest partial Wilk's λ values. Head width and anal fin length were similarly highlighted as important in the proportional adjusted morphometric data; however, eye horizontal diameter was another character with one of the lowest partial Wilk's λ values.

The canonical analysis for the allometry-adjusted data indicated that the highest loadings were (in descending order) on the caudal peduncle depth, interorbital width, and predorsal length on

the first root, and on head width, anal fin length, and head depth on the second root. Proportion-adjusted data indicated highest loadings on eye horizontal diameter, interorbital width, and dorsal fin length on the first root, and head width, anal fin length, and eye horizontal diameter on the second root.

Similar graphical patterns as seen in the PCA analyses can be seen in the *N. macropterus* species group DFA, with Fig. 2.15 (a) and (b) depicting similar results. The overlap between the Zambezi and Okavango groups, while still present, is not as pronounced as in the previous analyses. The Congo group is again clustered within the overlap between the Zambezi-Okavango cluster. The Kwanza group again stands out, although it presents mild overlap with the large Zambezi-Okavango cluster. The Kwanza group can be discriminated from the other groups on the first canonical root, with head width and anal fin length indicated as important characters for discrimination in both datasets.

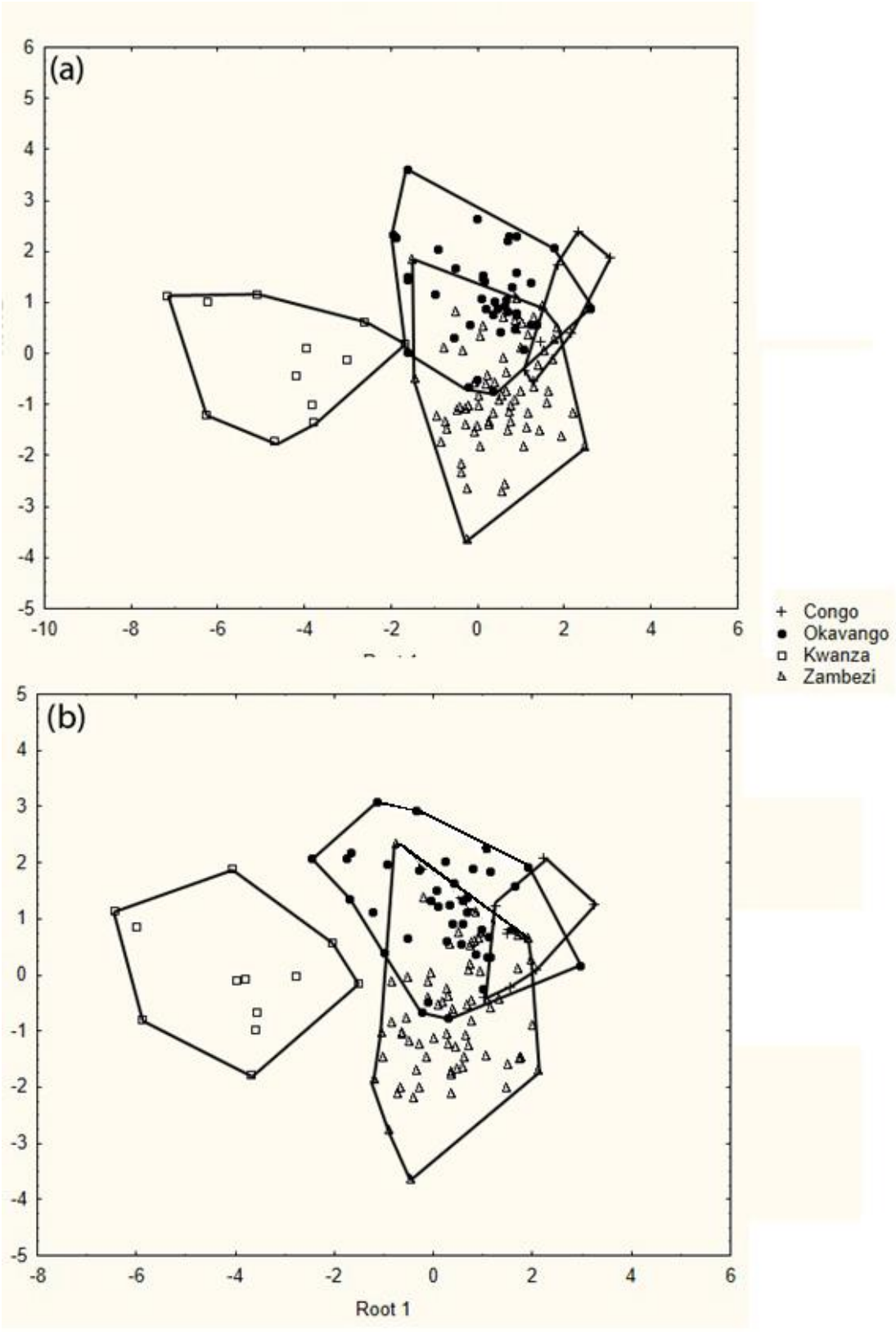

Figure 2.15: scatterplot of canonical scores of root 1 and root 2 from the discriminant function analysis of the morphometric measurements of *N. macropterus* using (a) measurements corrected for allometry and (b) percentage of standard length measurements, Zambezi n=71, Okavango n=42, Congo n=8, Kwanza n=11.

Meristics

The discriminant function analysis for the *N. macropterus* meristic data produced 3 discriminant functions (roots), although the first two comprised the most variation (39.7% and 37.3% respectively). The discriminant function classification matrix utilising meristic data could assign specimens to the correct river system with moderate accuracy (84.3%), with the shortfall of this classification primarily lying with specimens from the Okavango system, with 33.3% being incorrectly classified, mostly being incorrectly placed among specimens from the Zambezi system. Conversely, 9.7% of Zambezian fishes were incorrectly classified as being from the Okavango system. Specimens from the Kwanza system were identified with 100.0% accuracy.

In the discriminant function analysis for the meristic data, the characters with the lowest Wilk's λ values were, in order of importance for discrimination, unbranched anal rays, branched dorsal rays, and predorsal scale count. The canonical analysis found the highest factor loading on the first discriminant function to be unbranched anal rays, branched anal rays, and branched pectoral rays. The second root, contributing a similar degree of variation, highlighted the horizontal scale count, unbranched anal rays, and the predorsal scale count to be important contributors. The Zambezi and Okavango groups had large overlaps of their clusters. Some separate clustering occurred in this analysis, mostly with the Kwanza and Congo groups, although the Congo group overlapped somewhat with the Zambezi/Okavango group.

A much stronger overlap between the Zambezi and Okavango groups is present in the meristic data than when compared to the morphometric data. As per the PCA meristic results, the Congo and Kwanza groups form clusters away from the other groups, although the Congo cluster is spread out wide (possibly due to consisting of specimens from two deeply divergent lineages, see molecular analyses and the discussion section). Both of these groups can be discriminated from the other southern African *N. macropterus* groups on both the first and second canonical roots, with the important characters in each highlighted above.

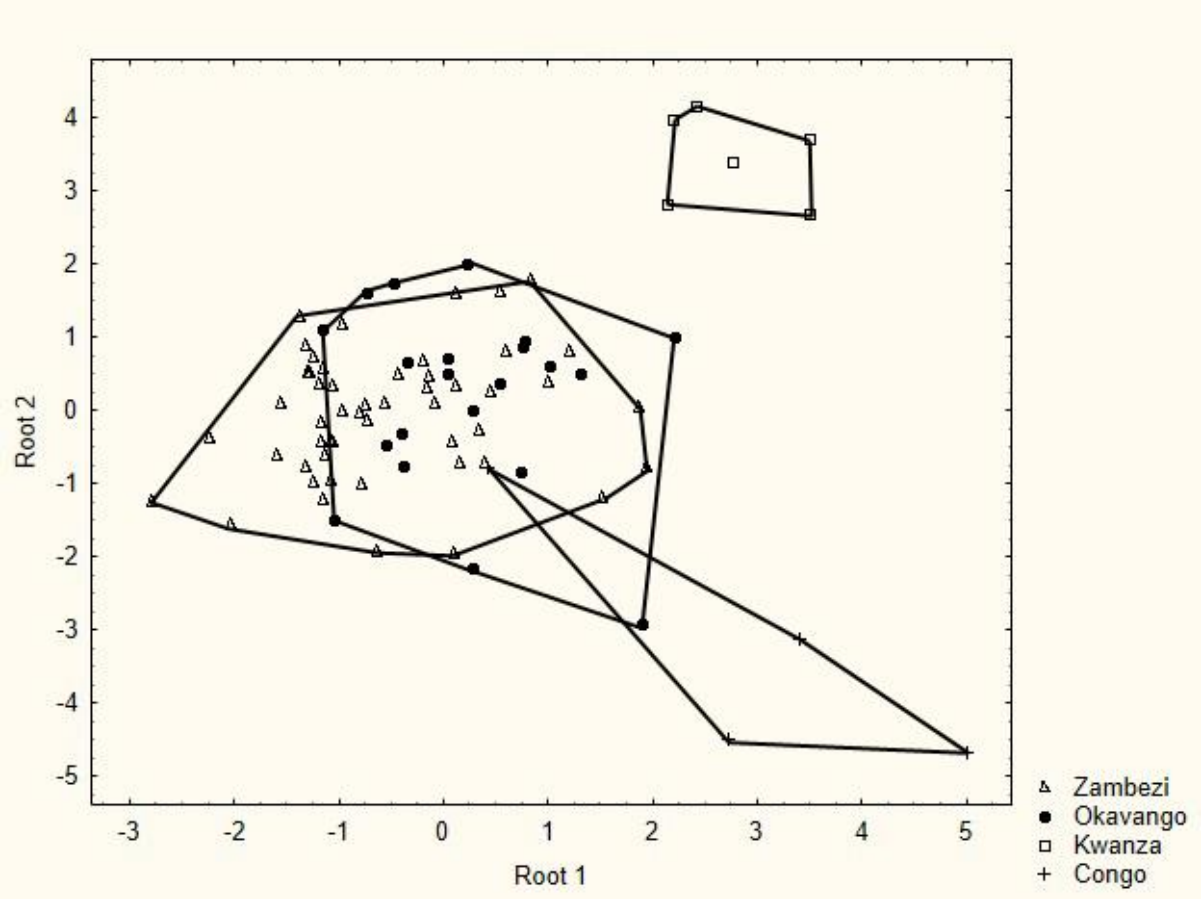

Figure 2.16: root 1 vs root 2 scatterplot of the canonical scores of the discriminant function analysis of the meristic characters of *N. macropterus* species group; Zambezi n=58, Okavango n=25, Congo n=8, Kwanza n=6.

2.3.2.3.3 Traditional morphometrics and meristics

Morphometrics

Kwanza system specimens indicated several measurements that aid in differentiating them from fishes from other systems. Of importance is the head length, which appears larger than those from other systems, and in turn would appear to affect the predorsal, prepectoral, and prepelvic lengths (head length mean 26.8%SL vs 23.5-24.1%SL in others). Fin length measurements of Kwanza specimens are lesser than those from other systems, with the exception of the anal fin length; dorsal fin length mean 19.5%SL vs 21.9-22.1%SL, pectoral fin length mean 16.5%SL vs 20.6-21.8%SL, pelvic fin length mean 18.3%SL vs 21.3-22.4%SL, as compared to other southern African river system populations. Okavango specimens differed from Zambezi fishes in having a shorter preanal length (67.0-77.9% O vs 66.0-76.8% Z), larger anal fin (15.4-20.2% O vs 11.8-18.9 Z), and larger eyes (26.5-36.8 Z vs 29.1-39.7% O).

Table 2.19: morphometric measurements of the *N. macropterus* species group, expressed as a percentage of standard length. The last four (head) measurements are expressed as a percentage of head length. Values shown are the range, with the mean value shown in brackets. The p column indicates the p-values for the measurement, where * indicates a significant difference ($p<0.05$), and the letters represent the following system pairings between which a significant difference occurred: KC=Kwanza-Congo; KZ=Kwanza-Zambezi; KO=Kwanza-Okavango; CO=Congo-Okavango; CZ=Congo-Zambezi; ZO=Zambezi-Okavango; Zambezi n=71, Okavango n=42, Congo n=8, Kwanza n=11.

	Congo	Zambezi	Okavango	Kwanza	p
Standard length	24.8-56.5 (35.5)	20.6-46.5 (32.5)	19.5-37.5 (27.3)	16.8-25.5 (21.3)	-
Head length	21.8-26.6 (23.5)	18.5-27.2 (24.0)	20.9-29.0 (24.1)	24.3-29.0 (26.8)	* KC KZ KO CO CZ
Predorsal length	38.4-42.7 (40.8)	37.1-44.7 (41.1)	37.4-45.2 (41.5)	43.2-47.9 (45.8)	* KC KZ KO CO CZ
Prepectoral length	22.7-27.6 (25.4)	20.7-30.8 (25.9)	23.1-30.6 (26.7)	27.6-30.3 (28.9)	* KC KZ KO CO
Prepelvic length	40.5-49.1 (45.8)	42.5-50.1 (46.1)	42.0-49.6 (46.0)	45.2-52.1 (48.4)	* KC
Preanal length	67.9-78.0 (73.1)	66.0-76.8 (72.4)	67.0-77.9 (71.2)	68.7-78.3 (73.4)	* KC KO ZO CZ
Head width	8.1-11.7 (10.4)	8.5-11.8 (9.9)	9.1-12.1 (10.4)	8.7-12.4 (10.5)	* ZO
Body width	8.3-11.8 (10.1)	7.7-13.0 (10.5)	8.4-11.8 (10.1)	7.9-11.0 (9.2)	* KZ
Head depth	7.9-12.2 (10.8)	9.4-13.6 (11.7)	9.7-13.2 (11.7)	11.3-14.2 (12.6)	* KC KZ KO
Body depth	14.4-21.5 (17.4)	15.4-23.3 (19.1)	16.2-20.4 (18.4)	16.8-22.0 (19.6)	-
Dorsal fin length	13.3-24.4 (20.9)	18.0-25.3 (21.9)	18.2-25.2 (22.1)	15.3-22.4 (19.5)	* KZ KO
Pectoral fin length	14.6-26.4 (20.6)	16.7-25.8 (21.8)	14.4-27.6 (21.3)	12.4-22.0 (16.5)	* KC KZ KO
Pelvic fin length	20.4-25.9 (22.0)	15.4-28.5 (21.3)	17.8-25.6 (22.4)	13.4-24.8 (18.3)	* KZ KO
Anal fin length	11.1-20.1 (16.2)	11.8-18.9 (16.1)	15.4-20.2 (17.3)	12.2-18.5 (16.1)	* ZO
Caudal peduncle length	13.6-17.3 (15.1)	10.7-16.3 (13.3)	9.1-17.1 (12.8)	9.6-16.5 (13.2)	-
Caudal peduncle depth	7.7-9.8 (8.6)	7.6-10.8 (8.8)	7.6-9.7 (8.7)	6.6-8.9 (7.7)	* KZ KO
Eye horizontal diameter	24.6-34.3 (30.1)	26.5-36.8 (31.0)	29.1-39.7 (34.3)	30.7-39.8 (35.5)	* KC KZ ZO CO
Snout length	17.8-29.3 (24.6)	16.5-28.1 (24.1)	17.6-29.4 (23.8)	12.3-29.1 (23.0)	-
Upper jaw length	12.4-23.2 (19.0)	15.4-21.1 (18.3)	15.3-21.6 (18.9)	12.8-20.0 (17.6)	-
Interorbital width	22.5-24.7 (27.1)	19.3-29.6 (24.4)	17.3-30.4 (22.9)	22.3-30.8 (26.5)	*KC KZ KO

Meristics

Many meristic characters showed significant differences between Kwanza specimens and those from other river systems (Table 2.20). Kwanza specimens indicated a greater predorsal scale count (mean 12 vs 10-11 in other systems), lesser dorsal/adipose scale count (mean 10 vs 11-12 in other systems), and fewer branched pectoral rays than those from other systems (mean 8 vs 9-10 in other systems). Okavango and Zambezi specimens though overlapping in counts did display some differences, but none could be considered diagnostic. Pelvic fin to LL scale count and anal branched ray counts indicated significant differences in the ANOVA, however, the post-hoc test did not indicate where these differences occurred.

Table 2.20: meristic counts for the *N. macropterus* species group. Values shown are the range, with the mean value displayed in brackets. The p column indicates the p-values for the counts, where * indicates a significant difference ($p<0.05$), and the letters represent the following system pairings between which a significant difference occurred: KC=Kwanza-Congo; KZ=Kwanza-Zambezi; KO=Kwanza-Okavango; CO=Congo-Okavango; CZ=Congo-Zambezi; ZO=Zambezi-Okavango; Zambezi n=58, Okavango n=25, Congo n=8, Kwanza n=6.

	Zambezi	Okavango	Congo	Kwanza	p
Horizontal scale count	37-42 (40)	36-41 (39)	39-43 (41)	36-38 (37)	* KC KZ ZO CO
Lateral line scale count	25-41 (38)	30-41 (35)	37-42 (39)	34-37 (35)	* ZO CO
Caudal scale count	8-12 (10)	8-12 (10)	10-11 (10)	10 (10)	-
Predorsal scale count	8-13 (11)	9-12 (10)	10-14 (11)	11-12 (12)	* KO ZO CO
Dorsal fin to lateral line scale count	3-5.5 (5)	3-5.5 (4)	3-4.5 (4)	4.5-5 (5)	-
Pelvic fin to lateral line scale count	2-5.5 (4)	3-4.5 (4)	3-4 (3)	4-4.5 (4)	*
Scales between dorsal/adipose	8-14 (12)	10-14 (11)	11-13 (12)	9-10 (10)	* KC KZ
Dorsal unbranched rays	2-4 (3)	2-4 (3)	2-3 (3)	3 (3)	* CZ
Dorsal branched rays	10-12 (11)	9-11 (10)	10-12 (11)	9-11 (10)	* KC KZ ZO CO
Pectoral unbranched rays	1 (1)	1-2 (1)	1 (1)	1 (1)	-
Pectoral branched rays	8-12 (10)	7-11 (9)	9-10 (9)	8 (8)	* KC KZ KO
Pelvic unbranched rays	1 (1)	1 (1)	1 (1)	1 (1)	-
Pelvic branched rays	6-8 (7)	6-7 (7)	6-8 (7)	7 (7)	* CZ
Anal unbranched rays	2-3 (3)	2-3 (3)	1-3 (2)	3 (3)	*KC CZ CO
Anal branched rays	6-10 (8)	6-9 (8)	7-9 (8)	7-8 (7)	-

2.3.2.3.4 Pigmentation pattern variation

The *N. macropterus* species group displayed a large amount of pigment pattern variation between the river basin populations, to the extent that it was possible to grade recurring patterns into pattern grades (Table 2.21). Some pattern grades were localised to particular river basins, such as pattern grades 1 and 4, while the other pattern grades were more widespread. While the pattern grades could be assigned to the lineages identified in the genetic analyses of this study, the patterns did not appear to strictly adhere to phylogenetic patterns (Fig. 2.17).

Table 2.21: pattern grades of southern African *Nannocharax macropterus* species group. See Fig. 2.2 for descriptions of pigmentation placement

Image	Pattern grade	Pattern grade description	System(s)	River(s)
	1	• Large, elongate, oval blotches • Saddles present, interspersed with speckling • Pigmentation may be present on adipose but sparse • Up to two lines present on dorsal fin • Large spots on dorsal- and ventral-most proximal area of caudal fin, rarely joining • Ventral pigmentation extends into anal fin, sometimes scarcely • Head pigmented • Pectoral spot may or may not be present • Caudal spot may be large, often resembling body blotches • One to two caudal bars, with the proximal bar not connecting and forming a dorsal- and ventralmost spot	Congo	Kabumba Chambeshi Kakulu Kisanfu Kilebe Luele

	2	• Blotches small, more or less circular • Saddles either absent or small and restricted to posterior region • Area of dark pigmentation under dorsal fin • Up to two lines present on dorsal fin • First (proximal) bar of caudal fin may connect, forming sideways 'M' • Head pigmentation • Pectoral spot may or may not be present • No pigment on adipose fin • Ventral pigmentation may extend onto anal fin, but not to the extent of pattern grade 1 • Caudal spot may be large, resembling body blotches	Congo Zambezi	Kisansrala Kisanfu Kando Zambezi East Lumwana Kabampo

	3	• Saddles small, beginning under dorsal fin or slightly anterior of • Body blotches may or may not be discernible from midlateral stripe; if so, do not extend above midlateral stripe • No clear dorsal stripes • One to two caudal bars, proximal connected in middle and forming semicircle • Ventral pigmentation does not extend onto anal fin • Pectoral spot may or may not be present • Pigmentation on adipose, only forming a spot in Okavango specimens • Caudal spot small, often darker and distinguishable from body blotches	Kwanza Okavango Zambezi	Kuquema Luando Kwanza Lower Okavango Canavale Cuito Kwando

	4	• Body blotches more oval than circular, 'elongate', and plentiful (>12), often distinct from midlateral stripe and may extend above it • Saddles often small • Adipose spot present • 2 caudal bars, often interspersed with speckled pigmentation • Dorsal fin stripes present • Pectoral spot present • Ventral pigmentation not extending into anal fin region	Okavango	Cuito

	5	• Saddles present, large, and may begin anterior to the dorsal fin • Blotches circular or oval, often extending above midlateral stripe • Pectoral spot most often present • Adipose spot may or may not be present • 2 to 3 dorsal stripes present • 2 caudal bars, with the proximal bar often not connecting, forming a dorsal and ventral spot. • Caudal spot large and often similar to body blotches • Ventral pigmentation scarcely extending into anal fin region, if at all • Dorsal speckling presents more often in Zambezi specimens	Okavango Zambezi	Lower Okavango Cuito Quebe Longa East Lungu Mutundu Lumwana Kabompo Njoko Mwombezhi Madamyana Maheba Kasanjiku Musangezhi Lwalaba Chisolo Muzhimbezhi Lungwebungu

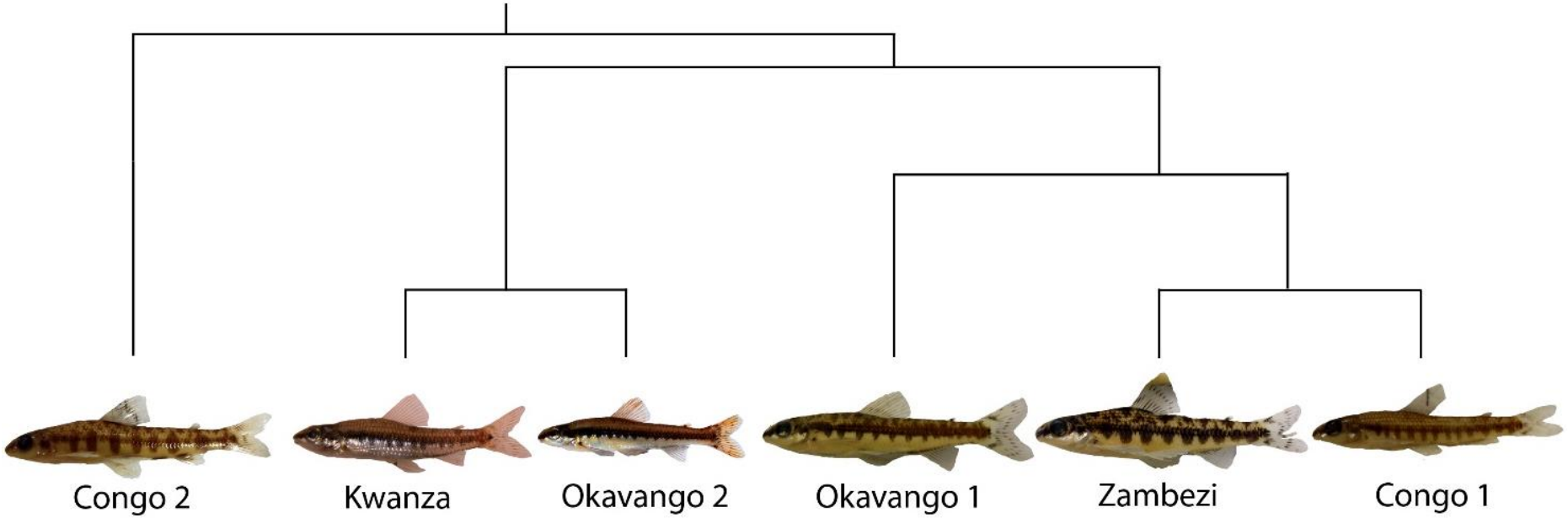

Figure 2.17: pattern grades displayed alongside the topology of the maximum likelihood tree from the genetic results for *N. macropterus*. *N. macropterus* 'Congo 2' SAIAB 76506, Lwela River, Luapula System, Zambia; *N. macropterus* 'Kwanza' SAIAB 85813, Luando River, Kwanza System, Angola; *N. macropterus* 'Okavango 2' SAIAB 186886, Canavale River, Okavango System, Angola; *N. macropterus* 'Okavango 1' SAIAB 186915, Longa River, Okavango System, Angola; *N. macropterus* 'Zambezi' SAIAB 185661, Kasanjiku River, Zambezi System, Zambia.

The only statistically significant differences in pigmentation patterns are the differences in number of blotches between the Okavango and Zambezi populations, in which case Zambezian specimens generally have fewer bodily blotches than the Okavango specimens (Table 2.22). The pattern described in some northern Zambezi populations ("with brown horizontal lines on the dorsal surface marking the edges of scale rows", Tweddle et al. 2004) was noted not only in some specimens from this area, but also among several other Zambezi and Congo specimens. These patterns when viewed from a moderate distance make the dorsal surface appear darker, and when viewed up close (especially under a microscope), the horizontal line from one scale edge joins the next, forming a horizontal zig-zag pattern. The patterns were viewed in some specimens in a given population, but not necessarily others – it cannot be determined at this point whether this pattern is unique to a particular (possibly cryptic) species, sex, age group, or what effects preservation have on maintaining this pattern over long storage periods. These patterns were not observed in any Okavango or Kwanza specimens, and were only identified from fishes from the *N. macropterus* Zambezi and *N. macropterus* Congo lineages.

The pattern grades (Table 2.21) presented some form of pattern across the southern African landscape (Fig. 2.17). Pattern grade 1 was only found within the Kwilu, Lualabla, and Chambeshi, all Congo waters. Pattern grade 2 was distributed within the Congo (only the Lualaba) and Zambezi systems, although not within the Kafue. Pattern grade 3 appeared to be a character maintained only by western populations, found only within the Kwanza and Okavango systems. Pattern 4 was only present in a single specimen, but was unique enough to be considered its own pattern grade, currently known only from the Okavango system. The final pattern grade, 5, appeared to be the most widespread, and may be the most basal form. It was found to occur among all southern African *N. macropterus* populations with the exception of the Kwanza specimens.

The original description for *N. macropterus* (Pellegrin 1926) indicates that the specimen in question has at least 12 blotches. Jerep et al. (2014) described *N. dageti* as having 6 to 10 saddles, or otherwise with "diffuse" saddles (as seen in many *N. macropterus* specimens), and 7 to 12 midlateral blotches. These values alone overlap with all the other systems, and other pigmentation pattern characters (as well as morphological and meristic characters) are needed to differentiate *N. dageti* from *N. macropterus* from other systems.

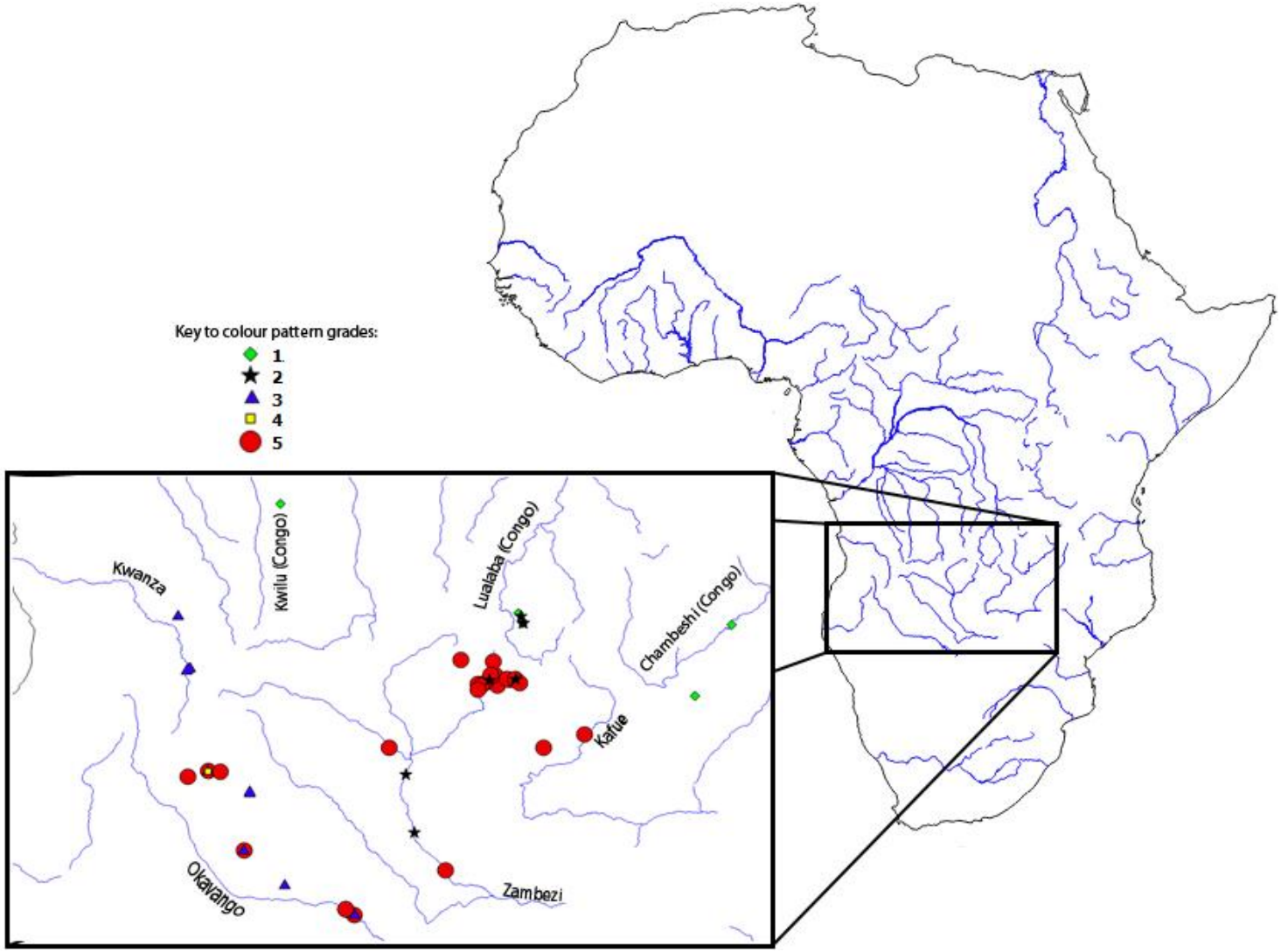

Figure 2.18: map showing the distribution of the identified pattern grades (see Table 2.22) of *N. macropterus* in southern African river systems, as well as some of the southern Congo tributaries.

Table 2.22: range of pigmentation pattern character counts for *Nannocharax macropterus*, with the numbers in brackets indicating the mean value; Zambezi n=56, Okavango n=26, Congo n=8, Kwanza n=6.

	Zambezi	Okavango	Congo	Kwanza
Number of blotches	5-12 (9)	7-15 (10)	6-14 (10)	8-10 (9)
p	*, O	*, Z, K	-	*, O
Number of saddles	4-10 (7)	2-12 (7)	4-8 (7)	3-9 (6)
p	-	-	-	-

2.3.3 Hierarchal Cluster Analyses (HCA)

In general, the HCA dendrogram topologies similarly reflect those presented by the molecular analyses. With few exceptions, the *N. macropterus* group, *N. machadoi* group, and *N. multifasciatus* group all appear as their own clusters more similar to each other than other groups. HCA utilizing only morphometric measurements yields the species groups as applied above. The exception lies with the *N. macropterus "*Kwanza" lineage, which clusters with *N. machadoi/N. multifasciatus* rather than the other *N. macropterus* groups. In all three species group clusters, the Zambezian and Okavango populations present as being more similar to one another than those in the Kafue. The Kunene *N. machadoi* is placed as being dissimilar to all other *N. machadoi* populations as well as all *N. multifasciatus*. The Congo specimens bearing superficial similarity to *N. multifasciatus* and *N. machadoi* appear most dissimilar to all other groups. These outgroupings of Congo specimens reflects patterns seen in the molecular results for *N. machadoi* and *N. multifasciatus* groups.

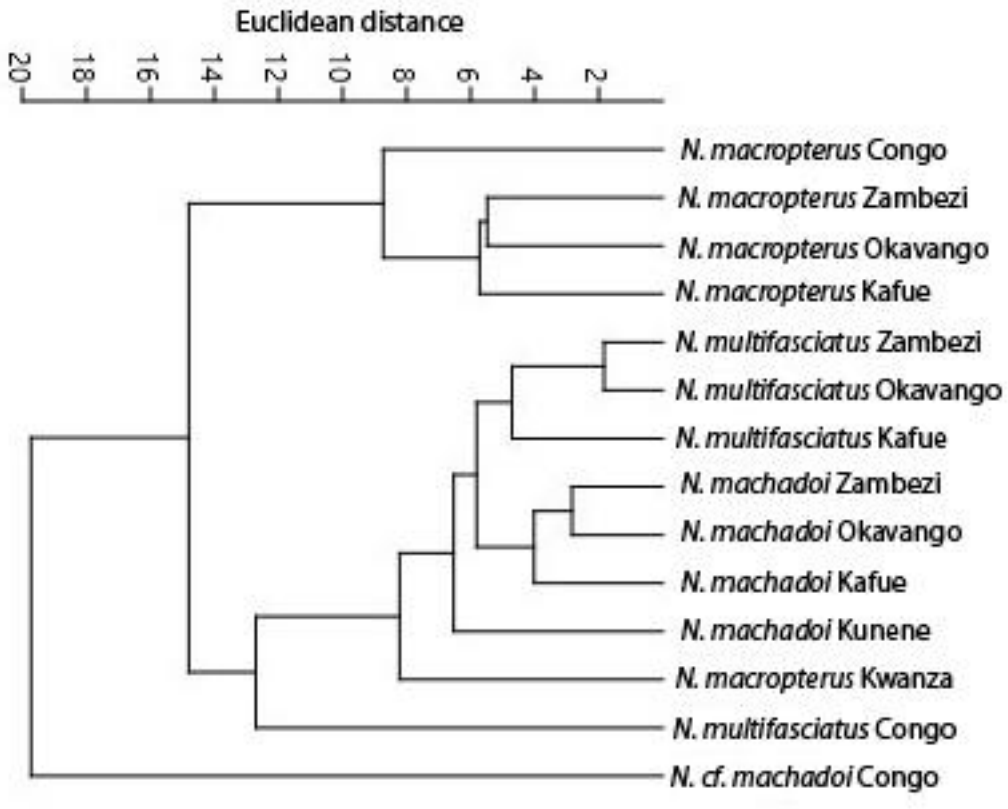

Figure 2.19: hierarchal cluster analysis UPGMA dendrogram using the proportional morphometric characters of southern African *Nannocharax* using UPGMA, grouped by species group and river system.

The HCA utilizing only the meristic characters presents the *N. macropterus* and the *N. multifasciatus* groups as most similar to each other, as the removal of morphometric characters (and therefore body shape) that may better reflect ecology no longer group the ecologically similar species. The *N. macropterus* groupings closely reflect those presented in the molecular results, with the Okavango specimens being most similar to those from the Kwanza, and the Zambezian specimens being more similar to those from the Congo.

Again, the Kunene *N. machadoi* shows less similarity to the other *N. machadoi* populations. The Congo specimen most superficially resembling *N. machadoi* shows similarity in its meristic characters to the southern African groups, although once body measurements are used (both Figs. 2.19 and 2.21) it is dissimilar to all other groups.

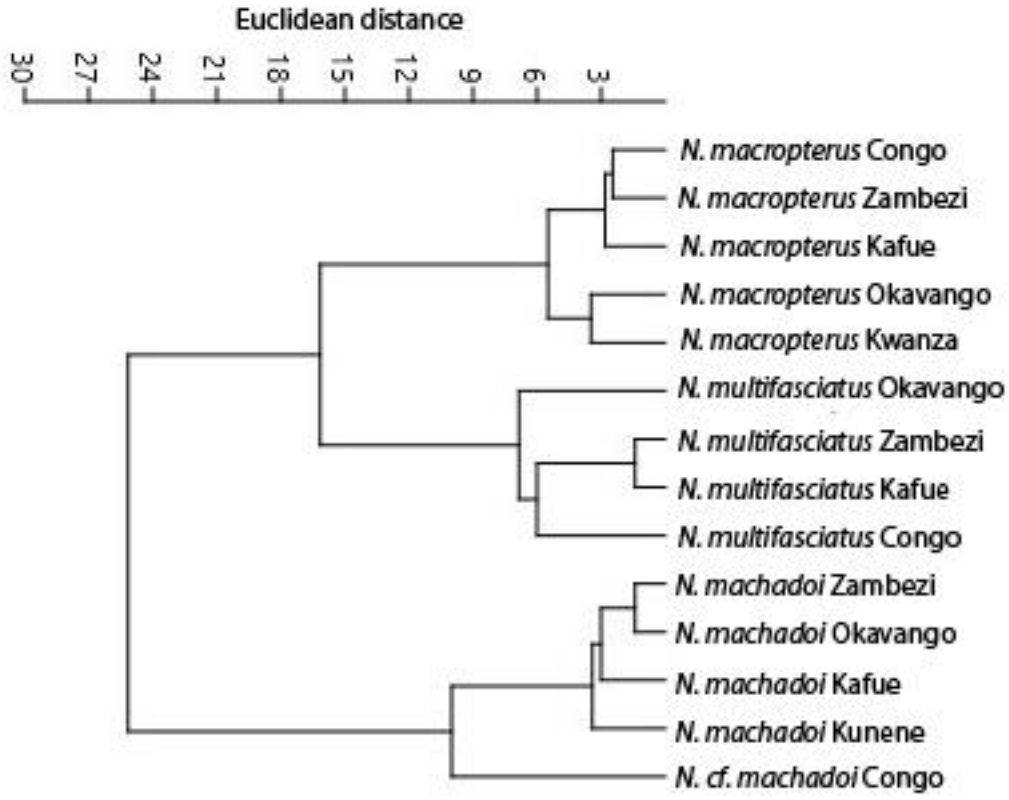

Figure 2.20: hierarchal cluster analysis UPGMA dendrogram using the meristic characters of southern African *Nannocharax*, grouped by species group and river system.

The HCA incorporating both datasets includes some interesting clusters. In *N. macropterus*, the Kwanza lineage is shown to be most dissimilar to all other such *N. macropterus* groups, while all other the southern African groups being more similar to each other than to the Congo group. This latter pattern is seen in the *N. multifasciatus* and is not seen among the *N. machadoi* groups, should the Congo '*N.* cf. *machadoi'* be considered among 'true' *N. machadoi*. The *N. machadoi* Kunene population is again seen to be to the other southern African *N. machadoi* than they are to each other.

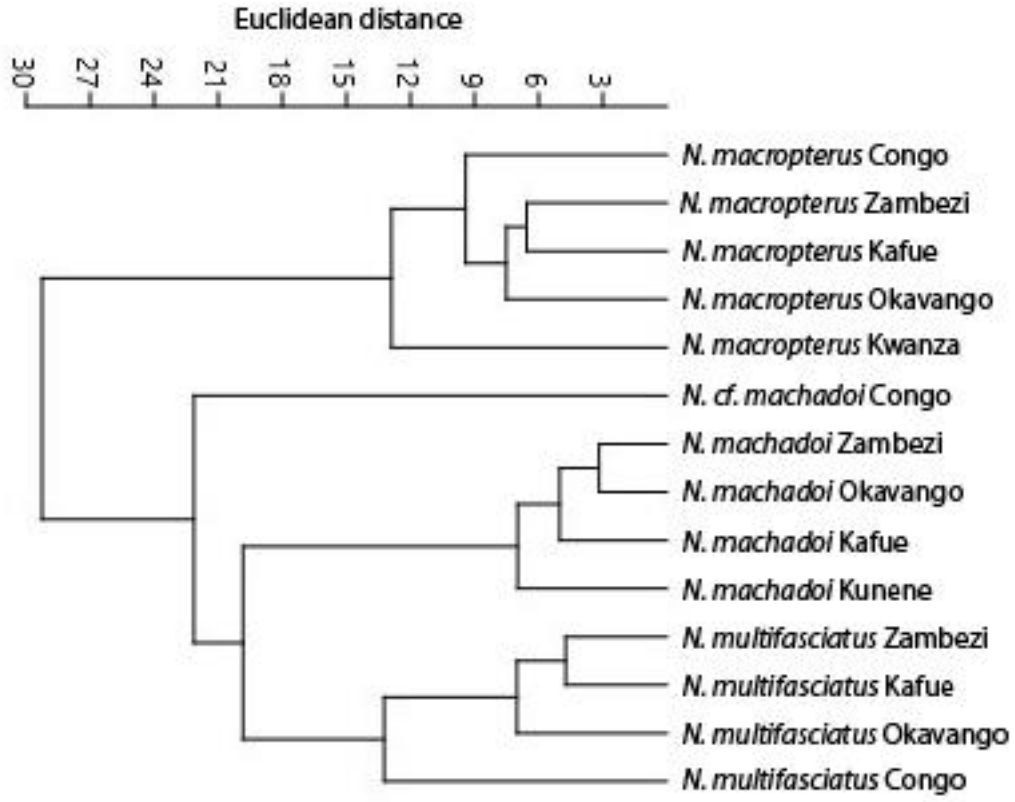

Figure 2.21: hierarchal cluster analysis UPGMA dendrogram using both the proportional morphometric and meristic characters of southern African *Nannocharax*, grouped by species group and river system.

2.4 Discussion

Southern African *Nannocharax* species display a large range of morphological variation both within and between populations. Given the extensive range of what is currently considered four species, such diversity may be expected. Such diversity had been previously noted (Tweddle et al. 2004, Bills et al. 2012, Bills pers. comm. Skelton pers. comm.) however no such analysis had been conducted to date. Furthermore, this is the first study to conduct an investigation into the genetic variation of COI mtDNA in any *Nannocharax* species. Although it was understood that the species occurring across multiple river systems would have little (if any) recent contact with neighbouring systems, interesting and repeated patterns can be seen in the three species groups.

2.4.1 Commentary on the analysis of COI mtDNA

Attempting to detect species-level differences among widely distributed taxa can be particularly revealing to the real diversity of organisms across the given area. Besides the importance of this task in conservation, this can also aid taxonomists in discovering species new to science and providing resolution to interspecies relationships. In the context of this study, detecting species-level differences may be a useful tool in determining the presence or absence of species that have historically been recorded in the region, but may have long been misidentified as similar species. Among the methods of doing so is using the genetic distance between species to delimit what scientists may consider separate taxa, and this requires strict definitions as there is no true "hard limit" that defines universal species differentiation. Establishing limits in the context of the study group can better reflect the true relationships between species.

The genetic distances seen among the established *Nannocharax* species groups is similar to what is seen among other groups vertebrate and fish groups (Johns & Avise 1998, Hebert et al. 2003 Ward et al. 2005; see Chapter 2.3.1.4), although these are broad in terms of taxa specificity. A low 2.0% genetic distance cut-off point has been proposed for species delineation (Johns & Avise 1998), although this is based on taxa largely unrelated to this study group, and is rather low for freshwater taxa expected to have been isolated for some time. Among freshwater taxa, varying degrees of congeneric interspecific distances occur, ranging from 9.9-20.6% (Ward 2005), 7.5-8.3% (Hubert et al. 2008), 0.0-25.0% (Ward 2009), and 1.6-23.7% (Dahruddin et al. 2016). Among characin groups, the ranges are also rather large: 0.4-24.8% (Pereia et al. 2013), 2.0-30.9% (Rossini et al. 2016), and 2.2-4.0% (Resende et al. 2016) as examples. The morphologically and ecologically similar Neotropical counterpart to *Nannocharax*, *Characidium* (Reinhardt, 1867), presented between 0.9-18.7% (mean 13.9%) genetic distances (Pereia et al. 2013), and perhaps may reflect the best possible data to compare these results to.

For the species as currently established, distances between *Nannocharax* species are estimated to be between 9.3% and 22.0% (mean 16.9%, Table 2.3), in most cases agreeing with the ranges of values, if not far greater, than what has been established for freshwater fishes and characins above. Among the species groups, some populations of *N. macropterus* present intraspecific distances within this range, indicating that there may be species-level divergence within the *N. macropterus* group, rendering *N. macropterus* a species complex (Table 2.3). The other species groups conversely show comparatively low intraspecific distances, and are discussed below.

The reason that similar, smaller genetic distances in populations of *N. machadoi* and *N. multifasciatus* as compared to the more strongly divergent lineages as seen within the *N. macropterus* species group could be because of the differing ecologies of these species. The former two species are pelagic, and are known to make migratory movements during rainy seasons and floods, and have been noted to occupy inter-system habitats within permeable or semi-permeable system watersheds (see Chapter 1; Bell-Cross 1965). Similar observations have not been reported for *N. macropterus*. The contrast in ecologies between these groups may mean that there is greater gene flow between populations and river systems within *N. machadoi* and *N. multifasciatus*, thereby facilitating gene flow between populations and river systems in these groups.

2.4.2 *Nannocharax dageti* in the context of this study

As there were no specimens housed at SAIAB that were labelled as *N. dageti*, it is important to recognise which groups from among the morphological and molecular analyses may represent this recently described species, and how it may be accurately differentiated from the superficially similar *N. macropterus*, especially in the context of this study. The paper describing *N. dageti* states that the species occurs in the Congo and Zambezi systems, with previous indications that *N. macropterus* noted to have occurred within these systems being misidentifications of *N. dageti* (Jerep et al. 2015). Within the molecular analyses, it is inferred that at least two lineages from the *N. macropterus* species group occur in the Congo system (Figs. 2.3, 2.4, 2.5), with one of these lineages, *N. macropterus* Congo 1 group, sharing a more recent common ancestry with the Zambezian '*N. macropterus'* ('*N. macropterus* Zambezi' group). The *N. macropterus* Congo 1 lineages is a monophyletic group consisting of three lineages (*N. macropterus* Congo 2, *N.* cf. *macropterus,* and ''*N. gracilis*''), which are separated by 5.7 – 5.9% divergence. The specimens have been identified by their host institutions variously as *N. macropterus, N. gracilis*, *N.* cf. *macropterus*, *N.* cf. *gracilis*, and *Nannocharax* sp., but could not be verified morphologically as these sequences were acquired from the BOLD datatbase. This lineage, however, only has one specimen representative that was measured (the others being housed in other institutions). Its measurements did not fall within the range of presented values expected for *N. dageti* as per Jerep et al. (2014).

Fishes from the Kabompo, Kasanjiku, Lumwana, and Mwombezhi rivers (all northern tributaries of the Zambezi) and, curiously, the Njoko River (a south-eastern tributary connecting directly to the Zambezi main channel), from the *N. macropterus* Zambezi group, share very similar proportional measurements to the '*N. macropterus* Congo 1' group. Tweddle

et al. (2004) indicated that the so-called '*N. macropterus'* from these same rivers (with the exception of the Njoko, which was not included in that study) did appear to be larger and different to other Zambezian *N. macropterus*, and I propose here that those specimens may have represented *N. dageti.*

Jerep et al. (2014) further distinguished *N. dageti* from *N. macropterus* in that *N. dageti* possessed fewer circumpeduncular scales than *N. macropterus* (12 in *N. dageti* vs 15-16 in *N. macropterus*), and bore an incomplete lateral line (vs. complete in *N. macropterus).* None of the observed specimens in the *N. macropterus* group from any of the systems had a peduncle scale row count greater than 12 (mean value was 10.0 scale rows for all systems). Skelton (2001) and Marshall (2011) also noted this to be true for Zambezian specimens of '*N. macropterus'* in having 12 scales around the caudal peduncle. This difference between southern African "*N. macropterus"* and the original description for *N. macropterus* in this characteristic indicates that these southern African specimens do not represent *N. macropterus* sensu stricto, but represent a currently unrecognised complex of 4 distinct lineages or candidate species, with one of those lineages being *N. dageti.*

The completeness of the lateral line was variable not only between localities, but between individuals of the same populations as well. The Okavango and Kwanza specimens more frequently displayed a full lateral line or falling one or two scales short, whereas Zambezian and Congo specimens more frequently displayed an incomplete lateral line. Completeness/incompleteness was not consistent within systems or populations.

Given the information presented here, the '*N. macropterus* Congo 1' and '*N. macropterus* Zambezi' lineages, represent what is now known as *N. dageti.* Those most closely allied with '*N. macropterus* Congo 2' – *N.* cf. *gracilis* and *N.* cf. *macropterus* Congo (Figs. 2.3-2.5) – should not be included in the group identified as *N. dageti* as they cannot be verified morphologically (the sequences were downloaded from the BOLD database), and show moderate divergence from '*N. macropterus* Congo 1' up to 5.9% genetic distance. These may represent an already established species, as it was identified as closely resembling *Nannocharax gracilis* by Stiassny (NDG, AMNH), or otherwise may represent a new species altogether. Further investigation is warranted.

The results of this study, based on the molecular data as well as diagnostic meristic characters, indicate that the southern African specimens currently recognised as '*N. macropterus'* from the Zambezi system (including the Kafue) are misidentifications of *N. dageti* (i.e. the lineages here identified as *N. macropterus* "Zambezi"). The other southern African populations of '*N. macropterus'* – the lineages *N. macropterus "*Kwanza", *N. macropterus* "Okavango 1", and *N.*

macropterus "Okavango 2" – do not match the descriptions for either *N. dageti* or *N. macropterus* and the implications for this are discussed below.

2.4.3 Relationship of the Congo specimens to southern African specimens

The Congo groups being isolated from the southern African groups among all three species groups reflects ideas by Roberts (1975) that the Zambezi and Congo form separate ichthyofaunal regions. Despite molecular evidence (Table 2.3, Figs. 2.3-2.5) that the species shared by these systems are diverging from the southern African lineages, considerable overlaps in the morphology and pigmentation patterns exist.

The morphological overlap and shared pattern grades between the two *N. macropterus* Congo genetic groups is interesting. In particular, pattern grade 1 is only found among Congo specimens, regardless of lineage. Despite the large divergence (14.6%, Table 2.3), the groups share a similar phenotype. Congo specimens cluster in the PCA and DFA plots for morphometrics and meristics. It may be possible that the similar environments experienced by these groups, or perhaps the phylogenetic limitation that favoured particular environmental niches for this group, have allowed for this phenotype to emerge in both populations. Each of these genetic populations occur within separate ecoregions – those most closely allied with the Zambezi group from the Upper Lualaba ecoregion, while the group indicated above to possibly represent *N. dageti* was sampled from the Kasai ecoregion, a region from where this species was described.

Several morphological samples resembling *N. macropterus* have been collected by SAIAB from the Bangweulu-Mweru region (Congo system), but due to the limited sampling of Congo specimens in the scope of this study, they were not analysed. Given the patterns seen in this study with *N. macropterus*-like fishes from the different Congo ecoregions, it may be worthwhile investigating their relationships with other southern African *Nannocharax* species.

The *N. machadoi* group relationships between the Congo and southern African systems remain unclear, mostly surrounding the confusion about the identities of what may be considered "*N. machadoi*" occurring in the Congo. The specimens used in the molecular analysis, accessioned as *Hemigrammocharax* sp., were most closely allied with the *N. machadoi* group in all three molecular analyses (Figs. 2.3, 2.4, 2.5). The physical voucher specimen was unavailable for analysis. Conversely, the only "Congo *N. machadoi"* specimen available for the morphological analysis (again assigned only as ''*Hemigrammocharax* sp.'') had no genetic voucher specimen available for analysis. As a singleton specimen, it could not be subjected to any statistical

analyses, although in the HCA (Figs 2.19-2.21) its placement leaves clues to its relations. Using morphology alone, it allies with none of the southern African species; morphology and meristics ally it more closely with *N. multifasciatus* and *N. machadoi;* and meristics alone cluster the specimen alongside *N. machadoi*. The genetic and morphological groups should not be considered one-and-the-same, as while they are both from the Congo, they originate from different sampling localities (Upper Lualaba vs. Bangwuelu for genetic and morphological respectively). Despite this mismatch, it is apparent from the results that *N. machadoi*-like fishes occur within the Congo system, and whether they are an already-established species or undescribed taxa is worthwhile investigating.

2.4.4 *Nannocharax* of the Kwanza system

One group that could be repeatedly discriminated from others was the Kwanza lineage of *N. macropterus*. The specimens in question at first glance appear to be most similar to *N. macropterus* sensu lato, owing to the presence of a dark midlateral stripe and rounded blotches on the body, as in other *N. macropterus* populations. The hierarchal cluster analysis (Fig. 2.19), when considering morphological measurements alone, however, places the group among *N. machadoi* and *N. multifasciatus*. However, when meristics are taken into account, as well as in the molecular analyses, this population is placed among the other population of *N. macropterus*. The principal component analysis and the canonical scores for both the proportional data and the allometry adjusted data both formed clusters of the Kwanza lineage away from the other populations of *N. macropterus*. The proportional data also showed significant differences in this group from the other systems in many of the measurements (Table 2.20); only predorsal length and prepectoral length could be considered diagnostic characters, however, in distinguishing the Kwanza lineage from the Congo lineage (43.2-47.9%SL vs 38.4-42.7%SL and 27.6-30.3%SL vs 22.7-27.6%SL respectively). While the meristic characters overlapped with other populations in the principal component and discriminant function analyses, statistically significant differences between the Kwanza and other systems were detected in several characters using Kruskall-Wallis tests. The Kwanza lineage possessed fewer scales in the lateral series than other specimens (36 to 38, mean 37 in Kwanza vs. 36-43, mean 40 in others), a discontinuously pored lateral line, fewer scales between the dorsal and adipose fins (9-10, mean 10 in Kwanza vs. 8 to 14, mean 12 in others), and fewer branched pectoral rays (8 in Kwanza vs. 9 in others).

This group bore smaller fins proportionate to body size than all the other southern African *N. macropterus* groups. The pattern grade among the Kwanza specimens was shared primarily

with specimens from the '*N. macropterus* Okavango 2' lineage, although this pattern grade could be found in both the '*N. macropterus* Okavango 1' as well.

This group shows deep genetic divergence from the other *N. macropterus* groups. The phylogenetic analyses in this study indicate a close relationship between the *N. macropterus "*Kwanza" and the *N. macropterus* Okavango 2 group. There is evidence that the Okavango and Kwanza systems have historically had contact, and this pattern of relationships has been seen in other groups, such as cichlids, occurring in these systems (e.g.: Schwarzer et al. 2012, Musilová et al. 2013). All other southern African and Congo groups show a great genetic distance from this group, in excess of 10.0%. This is rather high not only among fishes (see section 2.4.1) but also in the context of this study, higher than the intraspecific genetic distance of both the *N. machadoi* and the *N. multifasciatus* groups (1.9% and 1.6% respectively, 1.6% average for both groups). Given the evidence of deep genetic divergence of this population from the other '*N. macropterus*' lineages, as well as unique morphological and pigmentation pattern characters, this population represents a species that is distinct from the other southern African "*N. macropterus".* The Kwanza system already shows remarkable endemicity for its fish fauna as high as 50% (Abell et al. 2008), and it has been acknowledged that the region's ichthyofauna is poorly known (Lévêque 2008, Snoeks et al. 2010).

2.4.5 Other species of *Nannocharax* reported to have occurred in southern African river systems

Nannocharax monardi was originally described as occurring in the Okavango River (Pellegrin 1936). A recent survey of the fishes of the Okavango by Prof. Skelton and others (pers. comm. 2016) came across a specimen believed to be *N. monardi* (Fig. 1.2 (d)). This specimen strongly resembles *N. multifasciatus*, aside from the seemingly shorter body and a lesser longitudinal scale count (approx. 37-38, vs. the reported 40-44 and for *N. multifasciatus*). The actual specimen could not be accessed, and the number of pored lateral line scales could not be determined from the photo, but the original description places this number between 8 and 11. In this study, the range of scales for fish within the *N. multifasciatus* group had a range of longitudinal scale counts ranged from 36 to 41 in the southern African populations, and seven specimens bore a rather short lateral line, as in *N. monardi*, but did not bear other characters that suggested that these specimens may be this species, such as a shorter head length (8.3% SL vs 9.5% SL in *N. multifasciatus*), or greater body depth (8.3% SL vs 7.5% SL in *N. multifasciatus*) from the original descriptions. The shorter appearance of the body was noted in some Okavango specimens, although these appeared to be younger fish, this effect was

removed once the data were adjusted for allometry. Pellegrin (1936) indicated that *N. monardi* differed from *Distichodus stigmaturus* (now a synonym of *N. multifasciatus)* in having an incomplete lateral line, contrasting this with the fact that Fowler (1935) had not mentioned an incomplete lateral line. What was mentioned was "Scales 39-42 in lateral line to caudal base and 1 or 2 more on latter" (Fowler 1935), and this lateral line would rather have referred to the longitudinal series, as *N. multifasciatus* does not bear a full lateral line, although it may appear as almost complete in some specimens. Pellegrin's *N. monardi* also bore several meristic characters that typically mismatched those of *N. multifasciatus*, although in the course of this study (as well as others, e.g. Skelton 2001) these characters overlap strongly between both species. Given this information, it is unlikely that *N. monardi* exists as a valid taxon, but rather what was previously identified as *N. monardi* were misidentifications of *N. multifasciatus.*

Nannocharax fasciolaris is only known from its type locality within the Kunene river. The distinctions between this species and *N. multifasciatus* given by Nichols & Bolton (1927) are questionable at best. The high scale counts given for the single specimen are dubious given the state of the specimen at the time of description:"… somewhat damaged small specimen", "Scales (lacking)", "As its higher scale-count is somewhat uncertain, due to the poor condition of our only specimen…", (Nichols & Bolton 1927). The only other feature given to distinguish this species from *N. multifasciatus* is that of the caudal spot, which was said to be smaller than in *N. multifasciatus*. Given the small size and therefore probably younger age of the specimen (34mm SL), the small caudal spot size is unsurprising, and many such instances of smaller, circular caudal spots were seen among the smaller *N. multifasciatus* of this study. This evidence infers that *N. fasciolaris* is not a valid species, and should be considered a junior synonym of *N. multifasciatus.*

Nannocharax wittei (Poll 1933), although described from the Congo system (Kando, Lualaba system), has been recorded as occurring within the Zambezi and therefore warrants investigation (Anon. 1997). This single specimen of this species was found in Lake Cameia (or Lac/Lagundo Culando), located in Angola within the Zambezi basin. Given that the specimen in question was identified by the same person who described the species, the accuracy of the identification of this specimen bears some weight. *Nannocharax wittei* has a rather extensive distribution across the southern Congo and its tributaries, and given the proximity of the Zambezian locality to the Kasai River (Anon. 1997), it is not unreasonable to assume the possibility of this species occurring within this region.

The original description for *N. wittei* indicates that this species has 11 to 14 lateral line scales (Poll 1933), however Géry (1977) indicated that *N. wittei* bears "rarely more than 18 lateral line scales", implying that the number of lateral line scales could be as high although it is not known which specimens he examined for this publication. No specimens from the Lake Cameia region were observed during this study, and no specimens from any of the southern African populations could be identified as *N. wittei* using diagnostic characteristics from the original description. Sampling from the Lake Cameia region would be ideal for confirming the presence of this species in the southern African region, but at currently the presence of *N. wittei* in southern African river systems is doubtful.

2.4.6 Ichthyofaunal affinities between Okavango and Zambezi systems

In all species groups, but most clearly in the *N. macropterus* and *N. multifasciatus* groups, there is a divergence between the Zambezi and Okavango populations. Although it has been long held that the Zambezi and Okavango systems share similar ichthyofaunas, recent evidence indicates that isolation of the systems may be leading toward divergence of the populations by system, and in some instances speciation (Kramer et al. 2003, Kramer et al. 2014). That being said, the Zambezi and Okavango still have points of connectivity that allow passage between the two systems via the Selinda Spillway, although this corridor is seasonal and intermittent (Shaw 1984).

The morphological analyses indicate that the Zambezi and Okavango populations, while clustering with their own groups and show some signs of divergence, there is a strong overlap in the morphologies of the Zambezi and Okavango *Nannocharax* of all species groups. In the instances where genetic divergence is evident but morphological evidence is lacking, several possibilities may exist. The split between the groups may have only recently occurred, and such a brief geological period may not have allowed phenotypic characters to establish on a given population even if such characters are beginning to appear, as evidenced by some degree of divergence of population morphology. Another hypothesis is that despite reproductive isolation, the environmental pressures exerted upon *Nannocharax* in both river systems are similar enough to allow for the similar phenotypes to endure in both systems.

Nannocharax machadoi populations from the Okavango and Zambezi systems do not appear to differ morphologically, although statistical analysis of these characters (Figs 2.10, 2.11; Tables 2.13, 2.14) indicate there is some degree of differentiation, although they do not contribute strongly as discriminatory characters. The molecular results are unclear on the

relationships of this species between systems, except for perhaps the genetic distances between populations – with a mean of 0.8%, the lowest intraspecific distances among the southern African *Nannocharax* groups. Whether these patterns are a result of a recent dispersal across their range, the gene dispersal and migratory ability of the species, slowly evolving traits, or any combination of these is beyond the scope of the study and would only be speculation.

The genetic distance between *N. multifasciatus* from the Okavango and Zambezi is the largest shared between any two groups in this species. The lower genetic distance between the Congo and Zambezi could be expected with the examples of contemporary interbasin contact (Bell-Cross 1965) and the relatively recent connection between the systems (Goudie 2005). The Okavango system specimens showing only 1.7 to 1.9% genetic distance (Table 2.3) from the Congo was more surprising. This is especially since *N. multifasciatus* is known to occur within the Selinda Spillway region, the point of interbasin contact between the Zambezi and Okavango systems (Merron 1989), and these groups would be expected to share a closer relationship than was seen in these results (Fig. 2.3).

2.4.7 Divergences within the Okavango system

Nannocharax macropterus from the Okavango system displayed as two lineages with fairly large divergence (10.3%, Table 2.3) in the molecular analyses (Figs 2.3, 2.4, 2.5). This split was noted previously by Bills et al. (2012) between the eastern and western Okavango groups, inhabiting sandy and rocky habitats, respectively. Morphologically these groups are extremely similar, with HCA (not included) not clustering individuals with their own kind from the same parts of the system. Some pattern grades are shared between the groups however, and this may be evidence of convergent morphologies of related species adapting to similar environmental pressures (Endler 1982). Other morphological differences may occur, given the difference in habitat as stated by Bills et al. (2012), but such characters were not captured in this study.

The molecular analysis indicated that the specimens from the Cuito-Canavale tributaries were most closely allied with the Kwanza specimens (the relationships are explained in the Kwanza paragraph above). *N. macropterus* “Okavango 2” lineage from the Cuito-Canavale may have entered southern Africa through a Kwanza-Congo pathway (Musilová et al. 2013). The *N. macropterus* “Okavango 1” group, found in the Okavango main river through to the ‘panhandle’, may have entered southern Africa through a different route, likely via the Zambezi-Congo (Goudie 2005) historical connections. This theory bases on the fact that the

"Okavango 1" group is most closely allied with the Zambezi and Congo system *N. macropterus* populations in the gene trees (Figs. 2.3, 2.4, 2.5).

Bills et al. (2012) suspected that the patterns observed in *N. macropterus* in the Okavango might be seen in *N. multifasciatus* as well, although noted that their differing ecologies may influence such relationships differently. In all but the MP analysis, *N. multifasciatus* presented itself as a single lineage within the Okavango. The instance where two lineages were detected was a similar scenario to that seen in *N. macropterus,* in that one lineage was found to occur within the Okavango mainstream while the others were sampled only from the Cuito. However, the divergence between these groups was not as deep as seen in the Okavango *N. macropterus,* with a mean of 0.8% genetic difference between the two Okavango *N. multifasciatus* groups (min. 0.4%, max 1.3%). While it is apparent that the processes that structured the Okavango *N. macropterus* groups into the different lineages did not have a similar influence on the two Okavango *N. multifasciatus* groups, it is apparent that the fishes distributed within the Cuito differ from the Okavango mainstream populations, and separation of these populations are maintained by some mechanisms, whether biological or geological.

2.4.8 *Nannocharax* of the Kunene system

Only a few Kunene specimens were available for *N. machadoi*, and despite *N. multifasciatus* is also known to occur in this system, no such specimens were available. No genetic vouchers were available for either species groups. As a result, no general conclusions can be drawn about the relationship of this system to the other southern African systems can be made. In the case of *N. machadoi,* the HCA indicated that both the morphological and meristic characters were sufficiently different from those of the other southern African groups. The low sample sizes excluded the group from statistical analyses, but the comparing the values of the characters side by side with the other groups does not produce particularly contrasting results, with the exception of generally lower bodily bar counts – 10 to 13 (mean 11) in the Kunene specimens, vs. 9 to 21 and 12 to 20 in the Okavango and Zambezi specimens, respectively. The results indicate that this group most likely shares closer relations to Okavango fishes than to other systems; this would make sense given the historical and possible current points of contact between these systems (see Chapter 1.6).

2.4.9 Diagnostic characters for southern African *Nannocharax* species and populations

In both the morphometric measurements and the meristic counts in all 3 species groups, there are overlaps in many of the characters, leaving few diagnostic characters that alone can identify a particular population. As such one would rely on a combination of characters in order to successfully identify to which population any southern African *Nannocharax* would belong to. Even among the many statistically significant differences discovered during the analyses, there are many examples where the overlap is still too extreme for the character(s) to be useful identifying characteristics.

*N. macropterus "*Kwanza*"* specimens showed remarkable differences from their southern African relatives. Greater head length (K 26.8% SL vs. 24.0-24.1%SL), likely increasing predorsal length, prepectoral length, prepelvic length of Kwanza specimens; greater head depth (K 12.6%SL vs. 11.7% SL), smaller dorsal fin (K 19.5% SL vs. 21.9-22.1% SL), smaller pectoral fins (K 12.4% SL vs. 14.4-27.6% SL), smaller pelvic fins (K 16.5% SL vs. 21.3-21.8% SL), a shallower caudal peduncle (K 7.7% SL vs. 8.7-8.8% SL), fewer longitudinal scales (K 37 vs. 39-41), more predorsal scales (K 11-12 vs. 8-12), fewer scales between the dorsal and adipose (K 10 vs. 11-12), and fewer pectoral branched rays (K 8 vs. 9) were the primary characters that differentiated the Kwanza from the other southern African systems. Such distinctive morphology alongside the molecular evidence indicates that this group may represent a separate species from *N. macropterus* (see Chapter 3.1).

The two Okavango *N. macropterus* lineages could not be discriminated from each other with the methods employed in this study. This group could be differentiated from the Zambezi *N. macropterus* through the slightly wider head (O 9.1-12.1% SL vs. Z 8.5-11.8% SL), longer anal fin (O 15.4-20.2% SL vs. Z 11.8-18.9% SL), larger eyes (O 29.1-39.7% SL vs. Z 26.5-36.8% SL), generally fewer branched dorsal fin rays (O 9-11 vs. Z 10-12) and fewer branched pectoral rays (O 7-11 vs. Z 8-12); these were not diagnostic tools but rather indicate differentiating populations.

Okavango *N. multifasciatus* differed from Zambezi *N. multifasciatus* with shorter preanal length (O 61.2-77.4& SL vs. Z 68.5-79.3% SL) and shorter pectoral fin length (O 9.0-16.1% SL vs. Z 11.0-18.5% SL), and had fewer lateral line scales (6-39 vs. 12-35). The results presented here may indicate that the lateral line variability may be much greater than anticipated, with this species (or at least this population) bearing a variably discontinuous lateral line as seen in other *Nannocharax* species (Jerep & Vari 2013). The lateral line of *N.*

multifasciatus has been described as both incomplete (e.g. Skelton 2001) and (occasionally) complete (e.g. Géry 1977) in literature.

Nannocharax machadoi displays a rather homogenous morphology across its range, with no characteristics being particularly useful in differentiating populations. Despite evidence of divergent lineages, this species group displayed the lowest overall sequence divergences of all *Nannocharax* used in this study, with a mean within group difference of 0.8%, much lower than the expected interspecies genetic distance, and lower than the other observed interpopulation distances for other *Nannocharax* species.

2.4.10 Usefulness of pigmentation pattern as a species identifier

The variation in pigmentation patterns in both the *N. multifasciatus* and *N. machadoi* groups was largely uninformative in distinguishing fishes from different systems from one another. *Nannocharax multifasciatus* from the Congo could be differentiated from the southern African river basin populations by the generally lower bar count, though as mentioned above, may be as a result of this study not capturing the full variation of the population due to not having a sufficiently large sample size of this population. The number of bars in *N. multifasciatus* have been described variously as 15 (Boulenger 1923), 13 to 20 (Fowler 1935), 13 or more (Géry 1977), and 16 to 25 (Skelton 2001) in literature. The results of the study concur with these descriptions, with the bars never being fewer than 13, and none of the bars exceeding 23 in number (Table 2.9). Other 'barred' species recognised from the region include *N. monardi* with "about 15 bars" (Pellegrin 1936), *N. fasciolaris* with 17 bars (Nichols & Bolton 1927), 15 to 17 in *N. wittei* (Poll 1933), and 12 to 15 (Poll 1967) or 16 (Skelton 2001) in *N. machadoi*, although in this study specimens were noted to have as low as 9 and as many as 21 bars on the body (Table 2.15). All species bear an ocellus at the base of the caudal, and midlateral pigmentation extending from the caudal area was present for all species except *N. monardi*. In this, pigmentation patterns are not useful in diagnostically identifying species, with the exceptions that *N. monardi* was not mentioned to bear a midlateral stripe, and that *N. machadoi* may have few bars on the body, but may have as many or more than the other southern African species.

The number of deviant patterns (atypical from the 'regular' vertical bodily barring) in both *N. multifasciatus* and *N. machadoi* were slightly more common among Zambezi specimens than in Okavango fishes, but occurred in moderate numbers in all groups, and patterns different from the more common forms could be found in specimens among more 'regularly' patterned

individuals. That is to say, no single given population expressed unique patterning, nor was any unique patterning unique to one population.

The pigmentation patterns within the *N. macropterus* group are varied and widespread. The patterns, which can be loosely grouped by similarity into five grades, are not useful in delimiting different river systems populations or genetic lineages from each other. From what evidence can be gathered from this study, the evolutionary relationships of the populations cannot be inferred from shared or differing pigmentation patterns, as markedly divergent genetic lineages may share similar enough patterns such that they fall within the same pattern grades (Fig 2.17). Nonetheless, sharing of patterns by different lineages may be indicative of similar environmental pressures on these divergent groups, resulting in a convergence in adaptations, in this case, pigmentation patterns.

2.4.11 Summation

The currently recognised species of *Nannocharax* show an immense amount of variation within all three species groups established by this study. This could be expected in such species spanning across 4 or 5 river systems, many of which are isolated from one another. The molecular results perhaps give the best insight to the extent of variation and the relationships among southern African *Nannocharax*, especially in identifying unique lineages that would have more than likely been missed if this study had relied on morphology.

Molecular results infer that a group, currently considered "*N. macropterus"*, represents a unique lineage within the Kwanza, and represents a novel species not only deeply genetically divergent from other southern African populations, but displays distinctive morphological characteristics as well. Two deeply divergent lineages within the Okavango that likely have different origins in that system were also detected in the morphological analyses. Two *N. macropterus*-like lineages occur in the Congo basin, with only one more closely allying itself with the southern African, Zambezian group.

Nannocharax multifasciatus indicated a close relationship between the Congo and Zambezi, and the Congo and Okavango, but most interestingly a greater genetic distance between the adjacent Okavango and Zambezi systems, for reasons yet unclear. Two shallow divergent lineages occur within the Okavango, interestingly sharing a similar geographical distribution of lineages as seen in the two *N. macropterus* Okavango lineages. *N. machadoi* shows little divergence between all lineages, and the relationships between the groups across its distribution remain unclear.

The morphological results best inferred the interrelationships of the groups through hierarchal cluster analyses, which mostly resembled the patterns seen in the gene trees. The morphology of the Zambezi and Okavango specimens strongly overlap in all three species groups, and in many the Congo outgroups cluster well within the southern African clusters. The *N.* macropterus "Kwanza" lineage showed clustering outside of the other groups in the HCA, PCA, and DFA, except for the analysis using meristic characters in the PCA. Traditional morphometrics were useful in identifying the extent of the overlaps between the different southern African systems, and further exemplified the extent of the morphological variation of the southern African groups.

Pigmentation pattern was not identified as a particularly useful tool in the *N. multifasciatus* and *N. machadoi* groups as a character by itself. In the *N. macropterus* group, pigmentation pattern could be graded, but no pigmentation pattern could be considered unique to any individual system, population, or lineage. However, there were some patterns that could be divided in a more or less east to west or north to south basis, but again did not reflect lineage, but rather may be a reflection of another influence, such as differing environmental pressures.

Findings of with particularly interesting implications are that of the group currently labelled as "*N. macropterus",* but should not be named as such, since none of the southern African specimens bear 15-16 circumpeduncular scales as per the true *N. macropterus* (Pellegrin 1926). Specimens occurring in the Zambezi system (inclusive of the Kafue), as well as the lineage *N. macropterus "*Okavango 1" may best be identified as *N. dageti*, while the identities of the *N. macropterus "*Kwanza" and *N. macropterus "*Okavango 2" specimens has not yet been established.

Chapter 3
General Discussion

The primary aim of this study was to explore the morphological and genetic diversity of the four currently known species of southern African *Nannocharax.* To this end, the data presented in the previous chapter presents a new evidence of this region's species richness and their respective variation. Furthermore, this investigation provides new information of what the taxonomical implications of this observed variation may be.

The results in the previous chapter agree with the notion that widespread southern African freshwater fish species can be expected to display a wide range of morphological and genetic diversity (e.g. Goodier et al. 2011, van der Bank et al. 2012), but that this may not always be the case, with the *N. machadoi* group being an example of this, in having shallow genetic divergence across its range (< 1.0% difference between populations, Table 2.3) and little morphological variability between lineages (Tables 2.13 and 2.14).

Patterns of hydrogeographical change may be useful in explaining the genetic patterns seen among the species groups, with points of interbasin connectivity – both contemporary and historical – being key points in explaining current distributions and intraspecies relationships. This was not true for all species groups, however, as current understanding of the historical processes of southern African hydrogeography do not aid in explaining the patterns of relationships of the Kafue-Zambezi-Okavango *N. machadoi,* nor do they aid in explaining the unexpected divergence between Zambezi and Okavango *N. multifasciatus.*

In conclusion to this study, this chapter will explore:

(i) the taxonomic implications of this research; (ii) a brief look at the distributions of these species and populations as they are now understood, with a discussion on biogeography; (iii) and finally problems encountered with this research and the results.

3.1 Taxonomic implications of this study

The biological species concept (Mayr 2000) defines a species as populations of organisms that actually, or have the potential to, interbreed in nature. This concept rests on the foundations that the populations in question reproductively isolated from other similar populations through isolation mechanisms. Following the genetic distance thresholds discussed in Chapter 2.4.1, and assuming a reasonable distance for speciation (or incipient speciation) to have occurred to

be approximately 9.0% or greater (as per Chapter 2.3.1.4), several populations in this study are of interest. Genetic distances between populations should not be considered alone in the case of speciation of population from its congeners without consideration of the morphological characters, but such levels of genetic divergence indicate populations of interest where significant differences from related populations may have taken place.

Within these parameters, the *N. macropterus* Kwanza lineage and their closest relations from the Okavango Cuito-Canavale (designated as *N. macropterus* Okavango 2 in this study) and the *N. macropterus* Congo 2 group show sufficient levels of genetic difference to fall into the category where these groups could likely be considered different species. The *N. macropterus* Congo 1 group, along with *N. macropterus* Zambezi and *N. macropterus* Okavango 1, represents *N. dageti* (as per Chapter 2.4.2). The *N. macropterus* Okavango 2 group is morphologically very similar to the *N. macropterus* Okavango 1 group. The *N. macropterus* "Kwanza" group is indicated in nearly all the morphological analyses to have a phenotype distinct from all other southern African *N. macropterus.* The methods used in this study were perhaps insufficient to determine the differences between the two Okavango lineages, as such differences between these lineages may lie in the ecology, reproduction, or other factors not considered in the scope of this study.

In the morphological analyses of this study, the groups could be seen to cluster according to river basin lineage, in many cases with a degree of overlap. Statistical analyses identified characters useful in discriminating populations from one another with highly significant differences. In this regard, it can be said that the populations, perhaps with the exceptions of those among *N. machadoi*, have at least begun to differ morphologically, such that a combination of characters can aid in identification from which system a particular specimen came from. As briefed on above and in Chapter 2.4.4, the *N. macropterus "*Kwanza" group presented the greatest number (and degree) of differences between itself and its congeners, to the extent of species-level differences.

The results of this study indicate that the lineages of *N. macropterus* Okavango 1, *N. macropterus* Zambezi, and *N. macropterus* Congo 1, are misidentifications of *N. dageti*. The fishes from this system are not "true" *N. macropterus,* instead bearing characteristics more closely resembling those of *N. dageti*: 8-12 circumpeduncular scales (vs. 15-16 in *N. macropterus),* as many as 41-42 horizontal scales (vs 48 in *N. macropterus*), and a (usually) incomplete lateral line (vs complete in *N. macropterus*). This would extend the range of what

is currently known for *N. dageti* to include the Okavango system, with the exception of the Cuito-Canavale which extends up to the Bié Plateau (see Fig. 1.4), which contains a different lineage of *Nannocharax*, outlined below.

The group identified in this study as *N. macropterus "*Kwanza" displays both unique morphological characteristics that allow for differentiation from other southern African *Nannocharax,* as well as deep genetic divergence from all other southern African *Nannocharax* with the exception of the *N. macropterus "*Okavango 2" lineage. This group can be differentiated from "true" *N. macropterus* by the following features: horizontal scale count (35-38 vs 48 in *N. macropterus),* branched pectoral rays (8 vs 10), dorsal fin length (15.3 to 22.4% SL vs 26.6%SL); pectoral fin length (18.1-22.0% SL vs 26.7% SL), and pelvic fin length (13.4-24.8% SL vs 26.6% SL); the Kwanza group can be distinguished from *N. dageti* by its horizontal scale count (35-28 vs 40-43), circumpeduncular scale count (10 vs 12), branched pectoral rays (8 vs 10), and head depth (11.3-14.2% SL vs 15.8-17.6% SL). Given this evidence, this population may represent a novel species currently unknown to science. The Kwanza specimens are the only *Nannocharax* specimens to fall within the Cuanza ichthyofaunal province, and the characteristics seen within this group do not appear in any other populations from other geographical regions, with the exception of pigmentation pattern similarity with individuals from the *N. macropterus* "Okavango 2" lineage. Following the definitions of the freshwater ecoregions by Abell et al. (2008), however, the specimens observed in this study span two freshwater ecoregions, namely the Zambezian Headwaters and the Cuanza ecoregions. The Kwanza headwaters have been grouped with the Zambezian headwaters due to historical records (Musilová et al. 2013). The specimens from the Zambezian Headwaters ecoregion are those from the Kwanza mainstream, and Kwiva and Kuquema tributaries, while those from the Cuanza ecoregion are from the Luando tributary further downstream. This is reflected somewhat in the findings of this study, as the group from the upper Kwanza (Zambezian Headwaters ecoregion) presents a 1.9% genetic distance from the lower Kwanza (Cuanza ecoregion), likely maintained by the series of rapids and waterfalls within the Kuquema that act as a barrier to dispersal. Although the waterfalls and rapids within the Kwanza have proven as effective barriers in other groups (Musilová et al. 2013), the *N. macropterus* "Kwanza" do not appear to share similar patterns or degrees of divergence.

The origins of Kwanza ichthyofauna have been speculated by multiple authors, spanning the evidence gathered from multiple groups. Darwall et al. (2009) suggested that Kwanza freshwater fauna shows distinctness from both the Congo and Zambezi faunas. Catfishes of the genus *Synodontis* from this system appear to originate from the Congo (Day et al. 2013), while

some cichlid groups appear to arise from the Okavango system (Musilová et al. 2013) through theorized periods of contact between the headwaters of these systems, which lie in proximity to each other in the Bié Plateau. The genetic results of this study (Table 2.3, Figs 2.3-2.5) imply the Kwanza population shares closer relationship with the *N. macropterus* Okavango 2 lineage than it does with any other, hinting at an Okavangan origin of the Kwanza population.

The *N. macropterus* "Okavango 2" lineage should not bear the *N. macropterus* name, since the morphological and genetic evidence provided by this study indicate that it does not bear the characteristics of the "true" *N. macropterus.* Morphologically, this lineage can be differentiated from the "true" *N. macropterus* by its lesser horizontal scale count (36-40 in *N. macropterus* Okavango 2 vs 48 in *N. macropterus*), circumpeduncular scale count (10 vs 15-16), dorsal fin length (21.9% SL vs 26.7 %SL), and pectoral fin length (21.1% SL vs 26.7% SL). The Okavango 2 lineage can be differentiated from *N. dageti* primarily by its pored lateral line scale count (30-38 vs 39 to 40), circumpeduncular scale count (10 vs 12), and head depth (11.8% SL vs 15.8-17.6% SL). Morphologically and genetically, this group shares a close relationship with the *N. macropterus* Kwanza group, with 3.1% genetic divergence. However, it can be differentiated from the Kwanza group by the branched pectoral rays (9-11 in the Okavango 2 group vs 8 in the Kwanza group), and generally the presence of more midlateral bodily blotches on the body (more than 10 in Okavango 2 group vs less than 10 in Kwanza group).

It would appear that this lineage is restricted to the Cuito-Canavale River, a right-bank effluent of the Okavango River, within the Okavango system. Brooks (2012) noted that several species from the Cuito, particularly cyprinids, differed morphologically from other Okavangan populations of the same species; no mention was made of any *Nannocharax* or *Hemigrammocharax*, however. There was no indication as to what sort of barrier is present that would isolate the Cuito ichthyofauna from the rest of the Okavango.

The additional three species with scant literature and collection records from the southern African region (*N. fasciolaris, N. monardi,* and *N. wittei*, Fig. 1.1) were not seen amongst the samples used in this study. Two of these species, *N. fasciolaris* and *N. wittei*, did not have the collection sites where these species had been recorded from covered by the sampling area by this study, namely Lake Culando (Upper Zambezi) for *N. wittei;* the only species with samples from the Kunene system was *N. machadoi*, with no samples among these resembling the description of *N. fasciolaris*.

Both *N. monardi* and *N. fasciolaris* were described from the Cubango, from the Okavango system. The original descriptions for these species, however, include meristic counts and

measurements that overlap those of both the *N. multifasciatus* holotype (Boulenger 1923) from the Zambezi, as well as the Okavangan specimens observed during the course of this study. To add further to the issue, the measurements and counts from the Zambezian specimens, too, overlap many of the characteristics of all the aforementioned groups. This similarity in morphology between the Zambezi and Okavango *N. multifasciatus* populations is unexpected due to the two lineages observed in the molecular results, with one lineage being found in the Okavango system, and the other being found in both the Zambezi and Congo systems. These groups show moderate genetic divergence, between 1.7% and 2.9% (Table 2.3), although not as large divergence as seen between other groups in this study. This comparatively low divergence and stasis in morphological form may have been maintained by interbasin connectivity through the Selinda Spillway within which *N. multifasciatus* is known to reside (Merron 1989).

It is somewhat doubtful that *N. fasciolaris* represents a unique species. While no material for the species was examined, the description of characteristics for *N. fasciolaris* and the overlapping distribution with the morphologically similar *N. multifasciatus* raises some questions. The original description of *N. fasciolaris* provides little information on the meristic characters, except 48 longitudinal scales, 13 dorsal fin ray elements, and 10 anal fin ray elements (Nichols & Bolton 1927). The authors cast doubt on the true number of longitudinal scales due to the extent of damage to the specimen, as discussed in Chapter 2.4.5. The meristic and morphometric characters described in that paper otherwise fall within the range of values seen for southern African *N. multifasciatus* (but not the *N. multifasciatus* Congo lineage specimens), with the exception of the higher scale count and shorter dorsal and anal fins, although all three of these characters may have been compromised by the damage done to this single specimen. The authors elected not to use the scale count as a diagnostic character in differentiating this species from *N. multifasciatus*, but rather by the smaller caudal spot (Nichols & Bolton 1927). Many of the *N. multifasciatus* specimens observed in this study bore a smaller caudal spot, even individuals collected among others with larger caudal spots, including among *N. multifasciatus* Zambezi specimens. Given the variability of this character in *N. multifasciatus*, the vast morphological overlap of the original descriptions of *N. monardi* and *N. fasciolaris* with *N. multifasciatus*, as well as no evidence of deep genetic structuring between the Okavango and Zambezi populations, I concur with the ideas of van der Waal et al. (1991) and Hay et al. (1997) that what is currently understood to be *N. fasciolaris* instead represents *N. multifasciatus*. *Nannocharax fasciolaris* should therefore be placed in synonymy with *N. multifasciatus*, and is therefore considered a junior synonym of *N. multifasciatus.*

The status of *N. monardi* remains uncertain. The characteristics that differentiate this taxon from *N. multifasciatus*, as discussed in Chapter 2.4.5, were not identified among any specimens observed from the Okavango region. The characteristics that differentiate *N. monardi* (Pellegrin 1936) from *N. multifasciatus* fell into the range of measurements and counts as seen in both Okavangan and Zambezian specimens of *N. multifasciatus* used in this study. Alongside no morphological characteristics that would identify this species, the moderate genetic divergence between Zambezian and Okavangan *N. multifasciatus* (as discussed above for *N. fasciolaris*) does not seem sufficient to infer speciation between river systems; that is, it is not enough evidence to say that *N. multifasciatus* Okavango represent a unique lineage, namely, *N. monardi.* Given the evidence provided by this study, I tentatively place *N. monardi* in synonymy with *N. multifasciatus*, with *N. monardi* being considered a junior synonym of *N. multifasciatus.*

Due to none of the specimens observed in this study bearing the diagnostic characteristics that would identify *N. wittei,* I consider the collection record of *N. wittei* in the southern African region, specifically in the Zambezi system, to be erroneous based off a misidentification of the specimen. Further sampling within the Lake Cameia region may yet reveal this species' presence in the region, but until then this species should not be considered a part of the southern African ichthyofauna.

A singular specimen (SAIAB 77237) from the Kalungwishi system, an affluent of Lake Mweru, within the greater Congo system, in north-eastern Zambia, was particularly interesting. With the exceptions of a slightly deeper body and head, morphologically it most closely resembles *N. machadoi,* although unlike this species it bears an adipose fin. Hierarchical cluster analyses indicated this this specimen may be morphologically similar to *N. machadoi*, with the exception of the presence of an adipose fin. Genetic samples were not available for this specimen or population. Given that *N. machadoi* does not occur in the Congo basin, investigation into the *N. machadoi*-like fishes in this region is warranted, as this may represent a currently unknown taxon.

3.2 Comments on geographical distribution and biogeography

The results of this study indicate that that Okavango and Zambezian fish faunas have diverged from one another. This is demonstrated best in the molecular data, in that all three species groups had distinct lineages for each system, although this notion is less clearly supported in the case of *N. machadoi* (Figs. 2.3, 2.4, 2.5). Morphological results, while overlapping between the two groups, still allowed for some degree of distinction between the systems, particularly if characters are combined (see Chapter 2.4.9). Reasons for this apparent morphological stasis may include shared environmental constraints between populations, nonvisual sexual selection, or the process of morphological disparity may be slow or recent (Lavoué et al. 2010, Bocxlaer & Hunt 2013) During high water events when the Selinda Spillway connects the Okavango and Zambezi systems, both *N. multifasciatus* and *N. machadoi* have been known to occupy this region (Merron 1989). This would imply that these species, at least potentially, have contact between these two systems. The shallower genetic divergence between these systems for *N. machadoi* as opposed to those for *N. multifasciatus* may be explained by habitat preference of *N. machadoi*. Merron (1989) found *N. machadoi* to prefer quieter, swampy waters, characteristics that he said defined the Selinda Spillway. In this, *N. machadoi* would be more likely to be found right across this environment.

It is unknown if *N. macropterus* could make use of this interbasin connection, as no records exist of this species occupying this region. Furthermore, *N. macropterus* has a very different body shape to that of *N. multifasciatus* and *N. machadoi*, which indicates a differing ecology that may perhaps prevent *N. macropterus* from using the spillway as a means of dispersal (Burridge et al. 2008, Frannsen et al. 2015); Bills et al. (2012) believed that this species being less migratory than the other two species may have shaped the distribution patterns seen.

The present-day Kafue is connected to the Zambezi River at the Middle Zambezi (see Fig. 1.4), although historically this system was connected to both the Upper Zambezi as well as the Upper Congo system (Goudie 2005). The molecular and morphological results of this study indicate that the Kafue specimens are to be more closely allied with the Upper Zambezi fauna than with the Congo, with Kafue specimens from all three species groups clustering within, or sharing a more recent common ancestor, with Zambezian specimens than any Congo specimens. None of the species groups included in this study appear to have successfully invaded the Middle, and further on the Lower, Zambezi River. This is with the exceptions of *N. machadoi,* which has been sampled below the falls both upstream and downstream of Lake Kariba, and all three species groups having been collected within the Kafue River and its tributaries. The occurrence

of these species within the Kafue is not surprising given the historical connection of this river with the Upper Zambezi and Congo (Goudie 2005, Cotterill & de Wit 2011), where these species groups are known to occur widely within these systems. However, no samples of any of the species were found to occur below the Kafue Gorge, considered an ecological barrier between the Kafue and Middle Zambezi (Bell-Cross 1965), indicating the origins for the species in the Kafue to be via the historical connection Upper Zambezi, or the Congo, further backed by the evidence of the molecular studies of this project. *Nannocharax machadoi* has been sampled in localities near the gorge, but apparently not within the rapids or gorge itself, perhaps implying that this sort of environment acts a barrier to dispersal with the species group. Whether the *N. machadoi* from the Middle Zambezi below the Victoria Falls are linked to the southern Kafue distribution of *N. machadoi* is uncertain, as no specimens were attainable for this study.

The relationship between the Kwanza groups and the other southern African populations is built upon the results of this study as well as biogeographical theories presented using evidence from other fish taxa (e.g. cichlids in Musilová et al. 2013). The *N. macropterus* "Kwanza" lineage appears to be most closely related to the *N. macropterus* "Okavango 2" lineages, which in turn is deeply divergent from the *N. macropterus* "Okavango 1" lineage. This latter lineage appears to be more closely allied with the *N. macropterus* from the Zambezi and the Congo (2.2% to 3.2% difference, Table 2.3), and alongside morphological evidence, indicate that these three groups represent *N. dageti*. This may be due to the differing origins of the two Okavango *'N. macropterus'* groups – one originating from the Congo-Zambezi through the historical contact between these three systems (Goudie 2005), and the other possibly dispersing into the Okavango system via the Kwanza between the headwaters of these systems (Musilová et al. 2013). Further analyses would be required to confirm or disprove these pathways.

It is interesting to note that *N. machadoi* is the only species to occur below two large waterfalls in systems where it co-occurs with other species of *Nannocharax,* i.e. below Ruacana Falls on the Kunene, and below Victoria Falls on the Zambezi. According to Hay et al. (1997) the Ruacana Falls do not present a formidable barrier to downstream dispersal, but instead differences between above- and below falls fauna to be a result of differing habitats. Given that both falls are of considerable and comparable height (Ruacana Falls 120m, Victoria Falls 108m), I feel that they contribute similar challenges to downstream dispersal. In both these instances, the gradient of the river is less below the falls than above them (Hay et al. 1997,

Flugel et al. 2014), perhaps providing a contrasting environment that is better filled by *N. machadoi* than by the other southern African species. No specimens of either of these below-falls populations could be obtained. Should the falls present a sufficient boundary to upstream gene flow, as it has in other species such as *Cyphomyrus* spp. in the Zambezi (Kramer & van der Bank 2011) and endemics in the Kunene (Hay et al. 1999), it would be well worthwhile to investigate the differences between these populations and those above the falls, alongside the other systems investigated in this study.

3.3 Problems and conflicts between results

A result that was apparent itself in all three molecular analyses was that of *N. machadoi* forming an ill-resolved relationship among the specimens from the Kafue, Zambezi and Okavango systems (Figs. 2.3, 2.4, 2.5). The results presented in these figures provide little resolution, possible due to strong gene flow between the populations. Given the shallow morphological and genetic divergence (<1.0%) between all the *N. machadoi* population groups, the southern African *N. machadoi* species group as defined by this study, should be considered as a single species across its range.

The hierarchal cluster analysis, though not strictly reconstructing a phylogeny of the taxa subjected to the analysis, does aid in providing clues to the relationships between these taxa in terms of the similarity between their morphologies. The results of the HCA in many respects generated topologies that mimicked the topologies of the molecular results, although exceptions did occur. Within the *N. macropterus* species group, the Kwanza lineage was either placed among other species groups based on morphology, which was most different from the molecular groupings. Using meristic characters, however, this group is placed among the other *N. macropterus* groups, and matches well with the molecular results in clustering with Okavango specimens rather than other southern African groups. Using both morphometric and meristic datasets, however, it conflicts with the molecular results in placing Kwanza specimens sister to all other *N. macropterus.* The Zambezi and Congo appear to have a similar relationships to the molecular results in the HCA.

Despite the molecular results consistently placing the Congo *N. multifasciatus* alongside the Zambezi group, the HCA analyses often placed the Congo group as sister to all other *N. multifasciatus*. Similar placements were seen with the 'Congo *N.* cf. *machadoi'.* The conflicts between the morphological and molecular topologies are like the results of homologies between closely related species, obscuring the results somewhat. Overall, the topology of the HCA utilizing both the morphometric and meristic characters perhaps best reflected the

molecular results, and indicates that the use of a larger suite of characters is important if one wishes to infer relationships of closely related groups through the use of morphology. HCA analyses, in this instance, provide insight to morphologically diverging populations, but cannot be used as a proxy for true phylogenetic analyses or to infer taxonomic status of populations.

When data were adjusted using the formula used to account for allometry (as per Wells 1978), the graph results produced from the principal component and discriminant function analyses presented better resolution and clustering compared to the proportionally adjusted data. The changes in body shape as *Nannocharax* species develop are evidently significant enough to produce such a result. Although in many instances the two adjusted results were very similar, those resulting from the PCA rarely matched characters between data adjustment procedures. It is therefore important to take the effects of allometry into account prior to subjecting the data to analysis in order to better understand population- or species-level differences between groups.

The DFA results (using canonical scores) appear to be able to better distinguish groups better than the PCA results (using eigenvectors), as evidenced by the better clustering of populations as well as the highlighting of useful characteristics in discriminating the populations. This may be explained by the fact the in discriminant analysis, the groups are predefined before the analysis, allowing the analysis to recognise to which groups data points belong, decreasing the within-group variance (Fabrigar et al. 1999). The discriminant function analysis in this instance was a better tool to use for identifying clusters between populations and species groups, and can be an important step to take when investigating potentially speciated groups.

The use of pigmentation pattern as a tool is somewhat problematic with this genus for a number of reasons. Some authors (e.g. Tweddle et al. 2004) note interesting pigmentation patterns in particular populations, but such observations have not been placed into the broader context of intraspecies variation. In the context of this study, pigmentation pattern variation could be graded an applied to populations in the "*N. macropterus*" species group, now identified as multiple species. While pigmentation pattern could only rarely be applied to single populations and hence was not useful as a diagnostic in species identification. The distribution of each pattern grade could be assigned approximately by genetic lineage (Fig. 2.17), allowing one to visualise that pattern grade may be shared by closely related lineages, as in "*N. macropterus*" Kwanza and "*N. macropterus"* Okavango 2, but in other instances may instead be a reflection of similar environments or evolutionary pressures in more distantly related lineages, as in the

two *"N. macropterus"* Congo 1 and "*N. macropterus*" Congo 2 lineages. Secondly, closely related species and populations within this genus show remarkably similar pigmentation patterns, with distinction between species sometimes coming down other less superficial characters such as meristic counts or proportion of measurements to body length. This is evident in the less genetically divergent groups as in *N. multifasciatus* and *N. machadoi* species groups, in which pigmentation pattern did not differ significantly between lineages or between geographical localities. Finally, some level of pigment deterioration may have occurred in preserved specimens, especially older specimens. This limits the use of specimens in such aspects of pigmentation pattern studies, which can be problematic in instances where only a few specimens are available from a dated collection trip, as is the case for many isolated African collection sites.

The pigmentation traits explored within this study could not accurately assign any particular pigmentation patterns by sex, and therefore sexual dimorphism, if present in any of the southern African species, has not been accounted for in this study.

This was the first study to use molecular techniques to investigate the intra- and interspecific differences between members of the genus *Nannocharax.* This molecular aspect was especially useful in identifying multiple lineages that may have otherwise gone unnoticed using traditional morphological techniques alone. The applications of this methodology to other widespread *Nannocharax* species, as well as distichodontids and African groups as a whole, may reveal similar patterns and help uncover the extent of African biodiversity across the continental waters. Despite the applicability of the barcoding gene, the addition of more gene regions may present a clearer relationship of between the studied taxa, and in particular aid in providing resolution among poorly resolved relationships (such as the Kafue-Zambezi-Okavango *N. machadoi* group in this study).

3.4 Final conclusions and further research

Limited aspects of this research

An aspect of this research that would have provided not only greater understanding of the relationships of the groups to one another, but also aided in understanding the presence or absence of certain species within southern African systems, is the inclusion of more comparative material, including type material. In terms of comparative material, this would include more specimens from poorly sampled areas within the designated study area, as well as more specimens of the study species from outside the study area. In particular, more

specimens from all species groups (where possible) from the Congo region would have allowed for better comparison between these and the southern African populations, especially in the cases where few specimens from particular ecoregions were the only representatives of the Congo outgroup. Not all regions of the Congo share similar histories with southern African systems (Goudie 2005), and these additional relationships may have provided additional clarity as to how the current southern African taxa came to their current distributions, and clarified their interrelationships.

Type materials were not available for analysis within this study for any of the species indicated to occur within southern African systems. The inclusion of type specimens would help especially in identifying the 'additional species' - *N. fasciolaris, N. monardi,* and *N. wittei* - and allow for clarity in their identities and whether or not these species do exist within southern African systems.

Future research

Further investigation into the type material of the species included in this study, as well as those species which have been inferred to reside within the study area, would aid in the definitive identification as to what species are occurring within the southern African region. Additional techniques that may aid in the identification and discrimination of lineages, such as the inclusion of an additional gene region, referring to internal structures (e.g.: skeletal morphology, vertebral counts, pseudotympanum variability, etc.) as characteristics of taxonomic importance, and finer-scale sampling, would all perhaps provide greater resolution to the interrelationships of this group than what could be seen from the results of this project. In particular, the addition of a nuclear gene region, such as the ribosomal S7 region, would reduce the phylogenetic bias generated by the use of a single mitochondrial gene, and may perhaps reflect a more accurate phylogeny.

The distinctness of certain faunas, such as those from the Kunene system, as well the presence of *N. machadoi*-like fishes occurring in the southern tributaries of the Congo system, warrants investigation. Designation of the *N. macropterus "*Kwanza" and *N. macropterus "*Okavango 2" lineages as species status is the next step of this research.

Conclusion

The use of both morphological and molecular data in this study have proven useful in identifying the variability and relationships of the populations of the southern African *Nannocharax* species. In the previously established "*N. macropterus*" species group, it was

determined that none of the river system populations or genetic lineages represent the true *N. macropterus*. Instead, the Congo, Zambezi, and "Okavango 1" lineages were determined to represent another southern African species: *N. dageti*. The "Kwanza" and "Okavango 2" lineage from this species group have been determined in this study to represent species new to science, with each of these closely related lineages being both morphologically and genetically distinct from other such southern African populations. The *N. multifasciatus* species group was found to consist of a single species across the southern African distribution, only displaying little morphological variation, but with moderate genetic divergence between the river basins. *Nannocharax fasciolaris* and *N. monardi* were placed in synonymy with *N. multifasciatus* due to strong morphological overlaps no molecular evidence for as many lineages in the systems from which they were described. The *N. machadoi* species group was determined to represent a morphologically and genetically homologous group across its southern African range, with very little difference between river system populations.

In conclusion, it can be said that the southern African species of *Nannocharax* have a greater degree of variability than initially imagined at the conception of this project, with the morphological and molecular variability being as important, if not more so, than the recognised variation in the pigmentation patterns originally noted for *Nannocharax*. The results of this study indicate that there is a greater degree of diversity within the southern African freshwater regions than is currently acknowledged, with the future descriptions of the highlighted novel species mentioned above hopefully not only drawing more attention to the diversity of this group, but to other widespread African species as well, and what hidden diversity may lie within.

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Appendix 1

River systems and rivers in which the specimens in this study were collected from

Species group	River system(s)	River(s)
Nannocharax machadoi	Congo*	Kalungwishi*
	Kunene	Kunene Olushandja Dam
	Okavango	Boro Botletle Cuito Nxamaseri Okavango Thamalakane Thaoge Zibadiaja
	Zambezi	Chobe Kafue Kataba Kavombo Kwando Lalafuta Luambimba Luampa Luanginga Luena Lumwana Lungwebungu Mashi Mininga Mundestream Njoko
Nannocharax macropterus/N. dageti	Congo	Chambeshi Kakulu Kando Kilebe Kisanfu Kisansrala Luele Lulimala
	Kwanza	Kuquema Kwanza Kwiva Luando
	Okavango	Canavale Cuito Longa Okavango Quebe

	Zambezi	Chisolo Kabompo Kasanjiku Kwando Lumwana Lungu Lungwebungu Lwalaba Madamyana Maheba Makondu Musangezhi Mutundu Muzhimbezhi Mwombezhi Njoko Zambezi
N. multifasciatus	Congo	Bona
	Okavango	Boro Cacuchi Chissombo Cuito Kabumbe Lake Ngami Longa Maunachira Okavango Quebe Thamalakane Thaoge Zibadiaja
	Zambezi	Chisolo Chobe Lumwana Kabampo Kafue Kataba Kwando Luanginga Lububa Luena Lungu Lungwebungu Madamyana Maheba Manyinga Mininga Musangezhi Mwombeshi Mwekera

		Mwombezhi Njoko Zambezi

* = a single specimen, most resembling *N. machadoi* in measurements, meristics, and pigmentation pattern; however, bears an adipose fin

Appendix 2

List of genetic specimens used the analyses of this study

Institution	Species identifier	Collection number(s)
South African Institute for Aquatic Biodiversity (SAIAB/RUSI)	*Nannocharax fasciatus*	188414, 188494
	Nannocharax macropterus	68765, 182187, 185661, 186659, 186825, 186886, 187020, 187060
	Nannocharax cf. *macropterus*	82878
	Nannocharax sp.	118789, 186845
	Hemigrammocharax multifasciatus	186630, 186667, 186713, 186844, 187051, 187066, 193510
	Hemigrammocharax machadoi	68626, 83819, 83868, 83833, 187048
	Hemigrammocharax cf. *multifasciatus*	99050
	Hemigrammocharax sp.	82880, 85009, 85023, 85031, 85183, 101121
American Museum of Natural History (AMNH) *From BOLD*	*Nannocharax macropterus*	254901
	Nannocharax gracilis	254902
	Hemigrammocharax multifasciatus	91291
	Xenocharax spilurus	242511
	Citharinus citharus	226441
	Hydrocynus vittatus	245529

Appendix 3

List of specimens used for morphological analyses in this study

All material is accessioned at the South African Institute for Aquatic Biodiversity (SAIAB)

Species identifier	Collection number
Nannocharax macropterus	18817, 40070, 40093, 41020, 42904, 68765, 71048, 72145, 72586, 73551, 81179, 97212, 97961, 119147, 119152, 124791, 126745, 140172, 182187, 182854, 185661, 186659, 186825, 186886, 186909, 187020, 188213, 189973, 193552, 193680
Nannocharax cf. *macropterus*	81185, 82861, 82878
Nannocharax gracilis	40121
Nannocharax fasciatus	188414, 188494
Nannocharax sp.	81517, 81538, 82908, 118789, 186845, 186915
Hemigrammocharax multifasciatus	18914, 19105, 22212, 24206, 26675, 29962, 40044, 40075, 41177, 41180, 41202, 66685, 66833, 68448, 68452, 68712, 71010, 71114, 71730, 71927, 72318, 72585, 73158, 73296, 73316, 73398, 73421, 73449, 80572, 97956, 98175, 101184, 119150, 122010, 122409, 122432, 124817, 124830, 126744, 140156, 186630, 18667, 186713, 186844, 186925, 187051, 189967, 190624, 190925, 193510, 193634, 193687
Hemigrammocharax cf. *multifasciatus*	81539, 99050
Hemigrammocharax machadoi	18627, 18876, 19109, 28883, 29498, 29953, 39000, 41021, 41218, 66456, 66889, 68447, 68689, 71507, 71855, 71926, 72317, 72600, 72946, 73157, 73225, 73258, 83833, 122025, 124907, 124919, 125045, 125131, 187048, 189999, 193590, 193625, 193669, 193706
Hemigrammocharax cf. *machadoi*	71893
Hemigrammocharax sp.	73377, 73568, 77237, 85009, 85023, 85031, 85183, 101121, 186851, 190586

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