

**SYSTEMATICS AND PHYLOGEOGRAPHY OF MORMYRID FISHES
IN SOUTH AFRICA**

by

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A thesis submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

at

DEPARTMENT OF ICHTHYOLOGY AND FISHERIES SCIENCE,
RHODES UNIVERSITY,
GRAHAMSTOWN

September 2014

ABSTRACT

Two genera of mormyrids have been recognised from South Africa. *Marcusenius* is the largest genus of the Mormyridae, comprising 38 species distributed throughout tropical and sub-tropical Africa. In southern and eastern Africa eight species have been recognized, including *M. pongolensis* from South Africa. *Petrocephalus* has about 30 species distributed mainly in tropical Africa, and 15 of these were recently identified in southern and eastern Africa. This thesis integrated several techniques, through the collection of complementary, but separate data sets to investigate the phylogeography of *M. pongolensis* in South Africa, and the systematics, evolutionary history, biogeography of *Marcusenius* and *Petrocephalus* fishes in southern and eastern Africa.

Mitochondrial and nuclear genetic markers consistently demonstrated strong population structuring and identified three divergent lineages within South Africa. The widespread Pongola Lineage occurs partly in the Limpopo River system, and is very common in the Incomati, Pongola (type locality) and Kosi river systems. The Limpopo Lineage endemic only in the Limpopo River system is monophyletic with the Mhlatuze-Nseleni Lineage which is only known from the Mhlatuze and Nseleni rivers in the south. There was strong indication of historical isolation, in the form of allopatric fragmentation between genetically divergent Limpopo and Mhlatuze-Nseleni lineages. High genetic diversity was observed in the Limpopo River system within the Limpopo Lineage and the Pongola Lineage. The occurrence of the Mhlatuze-Nseleni Lineage in the far south cannot be explained by the hypothesis of confluence of rivers, river capture or episodic drainage connections, as the Mhlatuze and Nseleni Rivers would not have had a common confluence or shared low relief terrain with the Limpopo, Incomati, Pongola or Kosi river systems. Morphological studies of these lineages revealed even more differentiations and were described as: *Marcusenius krameri* sp. nov. for the Limpopo

Lineage, and *M. caudisquamatus* sp. nov. from the Mhlatuze and Nseleni river systems. The specimens from the Ruvuma River were also well differentiated from all southern and eastern African species of *Marcusenius*, and were therefore recognised as *M. lucombesi* sp. nov. New diagnosis characters of *M. pongolensis* were re-described and a key to the southern and eastern African species of *Marcusenius* was also provided. The results of all phylogenetic approaches that included all known southern and eastern African *Marcusenius* species are highly congruent and provided very strong support for recognizing *Marcusenius* species from these regions as a monophyletic assemblage that evolved from a single common ancestor. The southern and eastern African *Petrocephalus* is divided into two deeply mitochondrial and nuclear divergent Western and Eastern groups that are separated by the Victoria Falls. The phylogenetic relationships within each group were not supported and future studies incorporating more molecular characters and species will likely help to resolve the relationships. Speciation within *Marcusenius* and *Petrocephalus* ranges in the focus of at least 0.8-5 Million years ago in agreement with geological data, which suggested that vicariant events associated with the formation of the major river systems played a major role in the evolution and distribution of both genera in southern and eastern Africa. The high degree of genetic differentiation and at least four previously undescribed lineages recovered in this study suggested that mormyrid diversity within southern and eastern Africa may be underestimated. Possible threats to the studied mormyrid species and lineages and other freshwater-dependent species were identified during the course of this study. When described, the unidentified lineages would further increase the species diversity of both *Marcusenius* and *Petrocephalus*, and, therefore, the southern and eastern African ichthyofauna.

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DECLARATION

This dissertation is my own unaided work and is being submitted for the degree of Doctor of Philosophy in Fisheries Science at the Department of Ichthyology and Fisheries Science, Rhodes University in Grahamstown. It has not been submitted in whole or in part for any degree or examination at any university, and all material contained herein has been duly acknowledged.

Maake, P. Abram September 2014

DEDICATION

*For my daughter, Bohlale, who celebrated so many birthdays without me while I was away to
complete this work,*

&

*my loving, supportive wife and friend, Audrey, without whose love, encouragement and
support, I would have run short of courage and the will to see this work to completion*

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LIST OF ABBREVIATIONS

1. AMOVA = Analyses of Molecular Variance
2. ANSP = The Academy of Natural Sciences, Philadelphia (USA)
3. BMNH = Natural History Museum [formerly British Museum (Natural History), London]
4. EARS = East African Rift System
5. EODs = Electric Organ Discharges
6. ESC = Evolutionary species concept
7. IPMB = Institute of Pharmacy and Molecular Biotechnology, University of Heidelberg, Germany
8. MRAC = Musée Royal de l'Afrique Centrale, Tervuren
9. mtDNA = Mitochondrial DNA
10. NCPA = Nested clade phylogeographical analysis
11. NMW = Naturhistorisches Museum, Wien [=Vienna]
12. PSC = Phylogenetic species concept
13. PCR = Polymerase Chain Reaction
14. SAIAB = South African Institute for Aquatic Biodiversity
15. USNM = National Museum of Natural History, Smithsonian Institution [formerly United States National Museum, Washington D.C.]
16. ZMB = Museum für Naturkunde [formerly Zoologisches Museum]

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ACKNOWLEDGEMENTS

Firstly, I would like to express my utmost gratitude to Ofer Gon, principal investigator and leader of this project. Among the many things I would like to thank him for believing in me. Without his wisdom, guidance, enthusiasm, encouragement, forethought and support, this research would never have happened. I would equally like to thank Drs. Gavin Gouws and Ernst Swartz, supervisors of this project. Their knowledge of evolutionary biology is astounding, and their abilities to curb my distractions to keep this project on track have been essential to concluding this research.

Special thanks are due to Professor Bernd Kramer (University of Regensburg, Zoology Institute, Germany) for guidance and genuine interest in this study; his discussions and comments on various aspects were very valuable in completing this thesis. I thank Drs Albert Chakona and Wilbert Kadye for their help on various aspects of statistical analyses, and for the fruitful discussions on this work. The National Research Foundation (NRF) of South Africa (NRF, grant no. FA200705100020) and the Rhodes University Travel Grant desk (TSS12/2012) are thanked for their financial support. Collection permits were issued by the Ezemvelo KZN Wildlife (OP 3009/2011) and Mpumalanga Tourism and Parks Agency (MPB. 5277/2). The ethics of the collecting methods were approved by the Ethics Committee of SAIAB (10/2010).

I would like to thank the many field volunteers who – aside from providing me with company, sharing the beautiful outlook and enjoying the attacks from mosquitoes and flies – were able to provide me with mormyrid samples. Without their help, field collecting would have taken far longer than it did. For their help in the field, for driving me to sample sites, providing me with samples from their own projects, and/or facilitating my access to private property, I would like to thank: Ezemvelo KZN Wildlife and Mpumalanga Tourism and Parks Agency, Professor

Bernd Kramer, Drs. Johan Engelbrecht (Deceased), Scotty Kyle, Olaf Weyl and Andre Hoffman, Francois Roux, Mr. Richard Simelane, Mr. Geelbooi Mashilo, Mr. Mpho Ramoejane, Mr. Bernard McKenzie, and all the farmers in KZN, Mpumalanga and Limpopo that allowed us access through their private lands.

For a range of reasons, some target specimens and/or populations were unobtainable from the field, so museum collections were accessed to supplement field collections. Therefore, having access to museum collections was an essential component of this project. For generously allowing access to collections, lending samples, providing information from databases and generating X-ray images, I would like to thank the following: Roger Bills, Bafo Konqobe, Mark Lisher, Nkosinathi Mazungula and Vuyani Hanisi, all from the South African Institute for Aquatic Biodiversity (SAIAB) Collection Department. Sherwyn Mack is thanked for his time in training me in ArcView and DIVA GIS enabling me to produce the maps in this thesis. I also thank the following museums for accepting the type specimens of the newly described species donated to them: Natural History Museum [formerly British Museum (Natural History)], London; Musée Royal de l'Afrique Centrale, Tervuren; National Museum of Natural History, Smithsonian Institution [formerly United States National Museum], Department of Vertebrate Zoology, Washington D.C., and SAIAB.

My parents, Solly and Neo, my brothers, Alfred and Calvin, my wife Audrey and my daughter Bohlale, have been the pillars of my strength, and have provided me with emotional support and encouragement to succeed. They, together with many other close friends and the staff at SAIAB, too many to be mentioned by name, were there for me through all my frustrations. I cherish you all.

RESEARCH OUTPUTS

PEER REVIEWED AND POPULAR ARTICLES

- **Maake, PA**, Gon, O & Swartz, ER. (2014). Descriptions of three new species of *Marcusenius* Gill 1862 (Teleostei: Mormyridae) from South Africa and Mozambique. *Zootaxa* 3780 (3): 455–480.
- **Maake, PA**. 2012. “*Dig down deep and find the spark – unravelling the mystery around elephant fish evolution in southern Africa*” In: NRF/SAIAB 2012 Annual Impact Report. p. 29.

CONFERENCE PRESENTATIONS AND SEMINARS

- **Maake, PA**, Swartz, E, Mwale, M, Kramer, B & Gon, O. 2010. Cryptic species divergence in KwaZulu-Natal fishes. KZN Conservation Symposium, 19-21 October. Pietermaritzburg.
- **Maake, PA**, Swartz, E, Kramer, B & Gon, O. 2011. Phylogeography of *Marcusenius pongolensis* (Mormyridae: Teleostei) in South Africa. Southern African Society for Systematic Biology Conference. 19-21 January. Rhodes University, Grahamstown.
- **Maake, PA**, Swartz, E, Kramer, B & Gon, O. 2012. Molecular phylogeny and biogeography of *Marcusenius* and *Petrocephalus* (Mormyridae) in southern Africa. XIV European Congress of Ichthyology, 3-8 July. Liège, Belgium.

CHAPTER 1: GENERAL INTRODUCTION

1.1. SYSTEMATICS OF THE FAMILY MORMYRIDAE

Fishes of the family Mormyridae are endemic to freshwaters of tropical and subtropical Africa (Hopkins, 1986; Skelton, 2001). They comprise one of the most diverse clades of freshwater fishes from Africa and the single largest group of electric fishes (Alves-Gomes & Hopkins, 1997; Sullivan *et al.*, 2000). The systematics of the family across its distribution has long been known, starting with the older studies by, amongst others, Marcusen (1864), Boulenger (1909, 1916) and Whitehead and Greenwood (1959). Later, Taverne (1969, 1971a, b, 1972) conducted detailed studies of the osteology of all the mormyrid genera. Taxonomic studies at a generic level include, among others, Bigorne's (1987, 1989, 1990) reviews of *Mormyrops* Müller, 1843, *Isichthys* Gill, 1863 and *Brienomyrus* Taverne, 1971a, and *Pollimyrus* Taverne, 1971a from the West African region, respectively, and Boden *et al.*'s (1997) revision of Western-Central and Central African *Marcusenius* Gill, 1862. Morphological variability among specimens of *Petrocephalus* Marcusen, 1854 collected in Gabon (the southern part of the Lower Guinean ichthyofaunal province) was reviewed by Lavoué *et al.* (2004). Recently, Hopkins *et al.* (2007) reviewed all mormyrids of the Lower Guinea and West-Central Africa. In a revision of the genus *Petrocephalus* from southern and eastern Africa, Kramer *et al.* (2012) recognised more diversity, based on morphology, genetics and electric organ discharges.

Sullivan *et al.* (2000) reported 19 genera arranged in two sub-families, the Petrocephalinae and the Mormyrinae (Figure 1.1). The taxonomic status of one of the 19 genera, the monotypic genus *Heteromormyrus* Steindachner, 1866, from Angola, is uncertain (Taverne, 1972). The family comprises more than 200 species (Sullivan *et al.*, 2000). With the increased research

interest in mormyrids in recent years, there have been many discoveries of new species (e.g. Kramer & Van der Bank, 2000, 2011; Sullivan & Hopkins, 2005; Kramer *et al.*, 2007, 2012; Kramer, 2013a, b; Lavoué *et al.*, 2008a, b, 2010; Moritz *et al.*, 2009; Kramer & Swartz, 2010; Lavoué, 2011, 2012). In spite of this research, the diversity of many mormyrid groups is still not fully understood (Van der Bank & Kramer, 1996; Boden *et al.*, 1997; Kramer, 2013a) and more species are expected to be described in the future.

The wealth of information now available from morphology and DNA sequence data, and geographical evidence provide strong support for the monophyletic origin of the family Mormyridae within the superfamily Mormyroidea (Taverne, 1971a, b, 1972; Gosse, 1984; Sullivan *et al.*, 2000). Among other characters, the monophyly of this group is supported by the derived presence of the electric organ (Lissmann & Machin, 1958) and a greatly enlarged cerebellum (Taverne, 1971a, b, 1972). The monophyly of each sub-family and of the entire family have been confirmed by more molecular studies (Alves-Gomes & Hopkins, 1997; Lavoué *et al.*, 2000; Sullivan *et al.*, 2000).

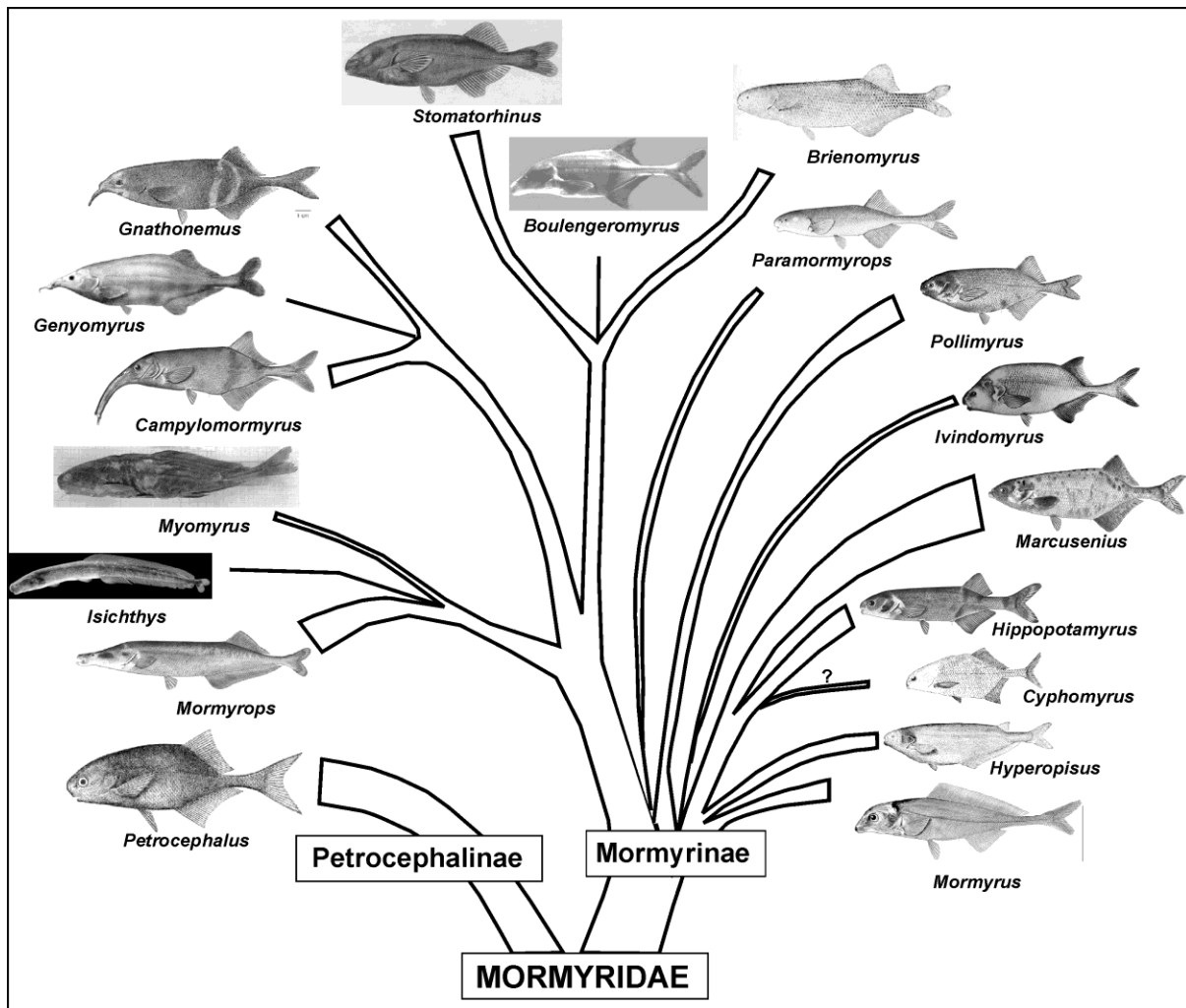


Figure 1.1. A phylogenetic tree modified from Hopkins (1986) and based on Taverne's (1972) anatomical characteristics, showing the two sub-families and 18 genera within the Mormyridae. The recently resurrected *Cyphomorphus* Myers, 1960 (Kramer & Van der Bank, 2011) is shown with a question mark next to *Hippopotamorphus* Pappenheim, 1906, previously its senior synonym. The uncertain monotypic genus *Heteromormyrus* Steindachner, 1866 is excluded from this tree. The width of each branch is proportional to the approximate number of species within a genus. All pictures were taken from <http://mormyrids.lifedesks.org/>.

Mormyrids are known for their characteristic electrogenic and electrosensory systems. All fishes in this family produce weak electric discharges by a highly specialized organ in the caudal peduncle (Lissmann & Machin, 1958; Szabo & Hagiwara, 1967; Kirschbaum, 1975; Hopkins, 1981, 1986, 2009; Kramer & Westby, 1985; Bastian, 1986; Hanika & Kramer, 2000). The role of these systems in the biology of this family has prompted studies that investigated reproductive behaviour (Werneyer & Kramer, 2005; Hopkins, 2009; Crampton *et al.*, 2011)

and the role of communication in speciation (Hopkins, 1986; Arnegard *et al.*, 2005; 2010), as well as studies into the evolutionary patterns of signal diversification (Lavoué *et al.*, 2008a, b; Moritz *et al.*, 2009).

Electric organ discharges (EODs) were shown to be species-specific and have been used, in addition to morphology, as a taxonomic character in many systematic studies (e.g. Van der Bank & Kramer, 1996; Alves-Gomes & Hopkins, 1997; Boden *et al.*, 1997; Moritz *et al.*, 2009, Kramer *et al.*, 2012). EOD recordings revealed a surprising diversity of new taxa in several mormyrid genera in Lower Guinea (Sullivan *et al.*, 2002). Several studies went on to identify and describe cryptic species of *Petrocephalus* using species-specific EODs of species found in sympatry (Lavoué *et al.*, 2004, 2008a, 2010). Feulner *et al.* (2006) showed that reproductive isolation in *Campylomormyrus* Bleeker, 1874 is strongly correlated with divergence in waveform types of the EODs. Kramer (2013a) reported differentiation in morphology and EODs in four para- and sympatric *Marcusenius* species from Côte d'Ivoire, West Africa. EOD recordings are also helpful in interpreting phylogenetic relationships (Sullivan *et al.*, 2000).

1.1.1. South-central African mormyrids

Of the 19 genera reported by Sullivan *et al.* (2000), eight are known to occur in south-central Africa, in the Southern and Eastern African Basins (Figure 1.2) (Skelton, 2001). They are: *Cyphomyrus*, *Hippopotamyrus*, *Marcusenius*, *Mormyrops*, *Mormyrus* Linnaeus, 1758, *Paramormyrops* Taverne, Thys van den Audenaerde and Heymer, 1977, *Petrocephalus* and *Pollimyrus*.

Marcusenius and *Petrocephalus* contain the majority of the mormyrid species in southern and eastern Africa, comprising, respectively, seven and 15 species in the region (Table 1.1) (Kramer & Van der Bank, 2000; Kramer *et al.*, 2007, 2012; Kramer & Wink, 2013). These two genera have overlapping distributions in the Southern and Eastern African Basins (Figure 1.2). There is a need to develop our understanding of these two widespread mormyrid genera further, especially from the Southern and Eastern African Basins, where morphological diversity is highest but little is known about species relationships and their evolutionary history (Kramer *et al.*, 2007, 2012; Skelton & Swartz, 2011).

The Southern African Basin drains major central-southern systems, including the Zambezi, Okavango, Pongola, Incomati and Limpopo river systems that flow east to the Indian Ocean, and the Cunene and Cuanza Rivers, flowing west to the Atlantic Ocean. The Southern African Basin has a moderately rich fauna and the fauna is fairly well documented, but existing information still needs to be synthesised and many new discoveries are anticipated (Skelton, 2001; Skelton & Swartz, 2011). The fish diversity of the Eastern African Basin, which covers the coastal drainages from the Tana River in the north to the Zambezi in the south, is still poorly known (Kramer *et al.*, 2007, 2012), and a new systematic synthesis is needed (Lévêque *et al.*, 2008). The Congo Basin is the largest, dominating Central Africa. Its ichthyofauna is rich and diverse (Lévêque *et al.*, 2008). The three basins mentioned are characterized by a rich and diverse fauna that exhibits high levels of endemism and presents superb systems for studying the effects of geological activity on biological evolution (Skelton & Swartz, 2011).

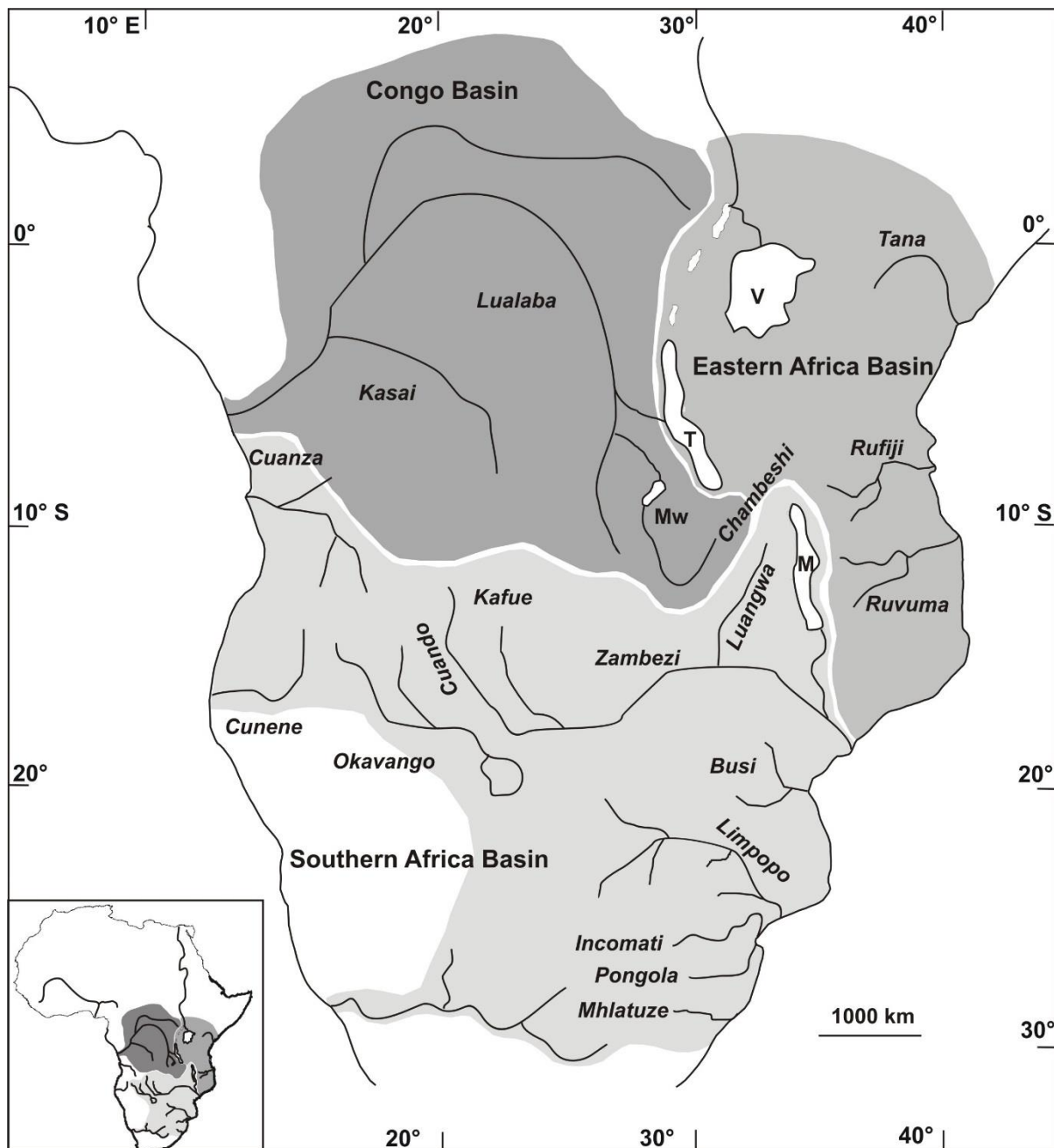


Table. 1.1. Type material of *Marcusenius* and *Petrocephalus* species known from the Southern African Basin (SAB) and Eastern African Basin (EAB). Those from the Congo Basin (CB) were previously synonymised with *P. catostoma* are also indicated. Abbreviations used to represent institutions and collections cited follow Sabaj Pérez (2013).

Species name and author(s)	Type locality and river system	Type species	Basin
<i>Marcusenius</i>			
<i>M. altisambesi</i> Kramer, Skelton, Van der Bank & Wink, 2007	Caprivi strip, Lisikili River, Upper Zambezi	SAIAB 79135 (specimen L39isi)	SAB
<i>M. angolensis</i> (Boulenger, 1905)	Quanza River, Angola	BMNH 1905.5.29.64	SAB
<i>M. devosi</i> Kramer, Skelton, Van der Bank & Wink, 2007	Tana River, Kenya	SAIAB 79138 (TA13NA)	EAB
<i>M. livingstonii</i> (Boulenger, 1989)	Ruvuma River, Tanzania-Mozambique border	BMNH 1864.7.9.30	SAB
<i>M. macrolepidotus</i> Peters, 1852	Lower Zambezi, Tete, Mozambique	ZMB 3678	SAB
<i>M. multisquamatus</i> Kramer & Wink, 2013	Cunene, Namibia	SAIAB 78781 (KUNE24)	SAB
<i>M. pongolensis</i> (Fowler, 1934)	Pongola River, South Africa	ANSP 54950	SAB
<i>Petrocephalus</i>			
<i>P. catostoma</i> (Günther, 1866)	Ruvuma River, Tanzania/Mozambique	BMNH 1863.10.12.4	EAB
<i>P. congicus</i> (David & Poll, 1937)	Mukishi River, DRC	MRAC 30807-30808	CB
<i>P. degeni</i> Boulenger, 1906	Katonga River, Lake Victoria, Uganda	BMNH 1906.5.30.84	EAB
<i>P. haullevillii</i> Boulenger, 1912	Lower Congo.	BMNH 1912.4.1.181–183	CB
<i>P. longianalis</i> Kramer, Bills, Skelton & Wink, 2012	Luapula System, Luongo River DRC	SAIAB 76758	CB
<i>P. longicapitis</i> Kramer, Bills, Skelton & Wink, 2012	Katima Mulilo, Upper Zambezi River, Namibia	SAIAB 85916	SAB
<i>P. magnitrunci</i> Kramer, Bills, Skelton & Wink, 2012	Okavango Delta, Botswana,	SAIAB 67069	SAB
<i>P. magnoculis</i> Kramer, Bills, Skelton & Wink, 2012	Cunene River, Namibia	SAIAB 78788	SAB
<i>P. okavangensis</i> Kramer, Bills, Skelton & Wink, 2012	Okavango, Thoage River, Botswana	SAIAB 030046	SAB
<i>P. petersi</i> Kramer, Bills, Skelton & Wink, 2012	Zambezi River, Mozambique	SAIAB 60486	SAB
<i>P. squalostoma</i> (Boulenger, 1915)	Lukinda River, Lake Moero, DRC	BMNH 1920.5.26.1	CB
<i>P. steindachneri</i> Fowler, 1958	Ulanga River system, Tanzania	NMW 55.118:1–3	EAB
<i>P. stuhlmanni</i> Boulenger, 1909	Kingani River, Tanzania.	BMNH 1907.12.3.1	EAB
<i>P. tanensis</i> Whitehead & Greenwood, 1959	Garsen, Tana River, Kenya.	BMNH 1963.11.29.1	EAB
<i>P. wesselsi</i> Kramer & van der Bank, 2000	Sabie River, South Africa,	ZSM 28556	SAB

Many recent studies of the fishes of these basins which have utilised either a molecular and/or a morphological approach have uncovered differentiation within traditionally-recognised species, indicating that hidden or cryptic diversity may be a common phenomenon (Kramer & Van der Bank, 2000; Koblmüller *et al.*, 2006; Katongo *et al.*, 2007; Kramer *et al.*, 2007, 2012; Dorn *et al.*, 2011; Goodier *et al.*, 2011; Lavoué, 2012; Maake *et al.*, 2013; Pinton *et al.*, 2013). Some examples of work studying mormyrids include Tweddle and Willoughby's (1982) morphological comparison of the large-scaled *Marcusenius* species of Malawi, Van der Bank and Kramer's (1996) study of phylogenetic relationships between eight southern and eastern African species of mormyrid genera, the revision of the genus *Petrocephalus* in southern and eastern Africa by Kramer and Van der Bank (2000) and Kramer *et al.* (2012), Kramer *et al.*'s (2003) work on parapatric speciation in *Pollimyrus castelnaui* (Boulenger, 1911), a revision of the *Hippopotamyrus ansorgii* (Boulenger, 1905) species complex by Kramer *et al.* (2004), the multi-disciplinary studies of the genetics, morphometrics and EODs of allopatric populations of *M. macrolepidotus* (Peters, 1852) by Kramer *et al.* (2007) and Kramer and Wink (2013), and the reinstatement of the genus *Cyphomyrus* by Kramer and Van der Bank (2011).

1.1.2. *Marcusenius* Gill, 1862

The genus *Marcusenius* comprises 38 valid species (Kramer *et al.*, 2007; Kramer, 2013a; Kramer & Wink, 2013), distributed widely from the Nilo-Sudan (Boden *et al.*, 1997) to South Africa (Kramer *et al.*, 2007). Taverne's (1971b) diagnosis of the genus *Marcusenius* includes the following characters, as translated by Boden *et al.* (1997: p. 1646): "an elongate body; a less well-developed snout, usually shorter than the postorbital segment; a rounded sub-mental swelling; a lower jaw slightly longer than the upper jaw; nostrils midway between the tip of the snout and the anterior margin of the eye; depth of caudal peduncle two to five times into its length; 19 to 36 dorsal-fin rays; 25 to 43 anal-fin rays; dorsal fin about the same length as the

anal fin; 10-12 pectoral-fin rays; distance between pectoral and pelvic fins shorter than the distance between pelvic and anal fins; 36 to 46 caudal-fin rays; 38 to 98 lateral line scales; 8-26/12-28 transversal scales on the body; 7-21/7-21 transversal scales between dorsal and anal fins; 8 to 18 caudal-peduncle scales; 3-8/3-10 conical or bicuspid teeth on the jaws; absence of a basisphenoid; lateral ethmoid present, median ethmoid reduced; caudal skeleton with a parhypural and four hypurals; and five circumorbitals". Overall, the genus *Marcusenius* has simple and clear diagnostic characters that can easily separate it from other genera occurring across its wider distribution from the Nilo-Sudan to South Africa.

In the Southern and Eastern African Basins, the genus ranges from the equatorial Tana River in Kenya (Seegers, 1996) to the Mhlathuze River in South Africa's KwaZulu-Natal Province (Skelton, 2001; Kramer *et al.*, 2007). It is also recorded from the Cunene, Okavango and Upper Zambezi systems (Figure 1.2). In a comparison of populations from different watersheds across southern and eastern Africa, Kramer *et al.* (2007) divided the formerly widespread *M. macrolepidotus* into five species. *Marcusenius macrolepidotus* was re-described and is now only known to occur in the Lower Zambezi. Kramer *et al.* (2007) resurrected *M. angolensis* (Boulenger, 1905) from the Cuanza River and *M. pongolensis* (Fowler, 1934) from South Africa, while describing *M. altisambesi* from the Upper Zambezi and Okavango and *M. devosi* from the equatorial Tana River as new species. A sixth species in this region is *M. livingstonii* (Boulenger 1898), which occurs in parts of the Malawi-Shire and the Ruvuma system. Later, Kramer & Wink (2013) described *M. multisquamatus* from the Cunene River. More *Marcusenius* diversity is still reported from South African river systems while the *Marcusenius* population from a tributary of the Ruvuma River system, Lucombe River, is somehow different from the topotypical *M. macrolepidotus* (Kramer *et al.*, 2007).

1.1.3. *Petrocephalus* Marcusen, 1854

Petrocephalus is the only genus of the sub-family Petrocephalinae and currently comprises 30 species distributed throughout the more tropical regions of Africa through to southern Africa (Lavoué, 2012; Kramer *et al.*, 2012). The systematics of *Petrocephalus* is known to be problematic (Bigorne & Paugy, 1990; Kramer & Van der Bank, 2000; Lavoué *et al.*, 2004). The distinction between species is often based upon slight differences in combinations of morphological characters. Taverne (1969), as translated to English by Kramer *et al.* (2012), indicated that *Petrocephalus* can easily be distinguished from the Mormyrinae genera by several unique morphological characteristics (p. 2228-2231, Kramer *et al.*, 2012): “Fairly short body; eye medium to large; snout much shorter than the postorbital portion of the skull and rounded; mouth under-slung and located below the eye; nostrils slightly closer to the eye than to the end of the snout; caudal peduncle 2.5 to 3 times as long as high; 19 to 33 rays on the dorsal fin; two initial unbranched spines on the dorsal fin instead of one like the other Mormyridae; 25–39 rays on the anal fin; 9 or rarely 10 rays on the pectoral fin; 6 rays on the ventral fin; 39–44 rays on the caudal fin; pelvic fins a bit closer to the pectorals than to the anal fin; scales more or less round and 90–105% as wide as they are long; 35–50 scales in lateral series; 6–15 or 11–15 scales in a transverse line on the body; 7–14 or 8–16 scales in a transverse line between the dorsal and anal fins; 8–16 scales around the caudal peduncle; jaws have 7–24/15–36 bicuspid teeth...”. Recent studies on EODs have also indicated that EOD waveforms of *Petrocephalus* are always of short duration in comparison to those of the sub-family Mormyrinae (Lavoué *et al.*, 2008; Moritz *et al.*, 2009; Kramer *et al.*, 2012).

In their review of *Petrocephalus* species from the Congo, Southern and Eastern African Basins, Whitehead and Greenwood (1959) regarded *P. degeni* Boulenger, 1906 and *P. stuhlmanni* Boulenger, 1909 from eastern Africa as synonyms of *P. catostoma* (Günther, 1866); they also

considered South African populations as members of this species. Moreover, acknowledging that the species from the distant and unconnected Congo Basin seemed clearly different from those in the Southern and Eastern African Basins, they further recognised *P. haullevillii* Boulenger, 1912, *P. congicus* (David and Poll, 1937) and *P. squalostoma* (Boulenger, 1915), all from the Congo Basin, as synonyms of *P. catostoma* originally described from the Eastern African Basin. Seegers (1996) added *P. steindachneri* Fowler, 1958 from eastern Africa to the synonymy list. Following this, *P. catostoma*, originally described from the Ruvuma River in Tanzania, ranged from the Katonga River, Lake Victoria, in the north to the Pongola River in South Africa in the south, and from the Congo and Cunene Rivers in the west to the rivers draining into the Indian Ocean in the east (Whitehead & Greenwood, 1959; Gosse, 1984; Seegers, 1996; Kramer *et al.*, 2012) (Figure 1.2).

Systematic investigations of the *P. catostoma* complex using morphology, EODs and molecular characters found that the South African populations of *Petrocephalus* represent a new species, *P. wesselsi* Kramer and Van der Bank, 2000, ranging from the Limpopo River system southwards, with the Pongola River (KwaZulu-Natal) as its southernmost limit. Kramer *et al.* (2012) were in agreement with previous studies (Skelton, 1993; Kramer & Van der Bank, 2000) and revealed that *Petrocephalus* species diversity in southern and eastern Africa was greatly underestimated and that the hypothesis of a widespread *P. catostoma* could not be supported. They re-instated all the previously-synonymised species from the three basins and described *P. okavangensis* and *P. magnitrunci* from the Okavango Delta, *P. magnoculis* from the Cunene River, *P. petersi* from the Lower Zambezi River, *P. longicapitis* from the Upper Zambezi and *P. longianalis* from the Lufubu River in northern Zambia. Areas of higher *Petrocephalus* species diversity than previously anticipated have since been discovered in other regions of Africa, suggesting a high prevalence of cryptic species (Lavoué *et al.*, 2004, 2008a, b, 2010;

Lavoué, 2011). For example, five species were found and described from the northwest Congo Basin (Odzala National Park), where eleven sympatric species are now known (Lavoué *et al.*, 2008a). More *Petrocephalus* diversity is still reported within *P. wesselsi* in South African river systems where each system is believed to be inhabited by a distinct *Petrocephalus* species (Kramer & Van der Bank, 2000; Kramer *et al.*, 2012). Kramer and Swartz (2010) indicated that more diversity in southern and eastern African systems is still expected, especially from river systems such as the Cunene and Cuanza, where diversity is still poorly known, and a new systematic synthesis is needed.

1.2. MOLECULAR SYSTEMATICS

The rapid development of molecular systematics over the last three to four decades has opened up a powerful new approach to understanding the patterns and processes of speciation fundamental to explaining the diversity of life (Barracough & Nee, 2001). Molecular data have proven very useful for recovering information on heredity and for quantitatively measuring levels of genetic diversity, historical biogeography, for dating divergences among species and lineages, and for resolving the taxonomic status of species (Koblmüller *et al.*, 2006; Katongo *et al.*, 2007; Teske *et al.*, 2009; Dorn *et al.*, 2011; Goodier *et al.*, 2011; Chakona *et al.*, 2013a, b; Pinton *et al.*, 2013).

Hillis (1987) indicated that DNA sequences of mitochondrial genes are suitable for systematic studies because of their efficacy in resolving molecular phylogenetic relationships within and among species. Meyer (1993) also indicated that mitochondrial DNA is particularly attractive because of its high mutation rate. Analysis of DNA molecules provides a large number of observable characters for use in molecular analysis (Hou *et al.*, 2007). As such, molecular

systematic studies can analyse up to several hundred or thousand characters compared to morphological analysis, which rarely includes more than a few hundred characters with an average of three characters that can clearly define a taxon (Hillis & Wiens, 2000). Mitochondrial DNA limitations, such as hybridization, maternal inheritance, the absence of informative mutational events over certain time scales and the influence of locus-specific patterns related to the randomness of the coalescence process and/or lineage sorting (Hillis, 1987; Gadagkar *et al.*, 2005), can distort mtDNA lineage ancestry and phylogenies (Maddison, 1997). Consequently, a growing number of phylogeographic, phylogenetic and biogeographic molecular studies have used multiple unlinked genes and have integrated and compared the results of analyses based on mtDNA with those based on nuclear genes (Guo & Chen, 2010; Maake *et al.*, 2013).

The non-coding and theoretically neutral regions of the nuclear DNA introns are a potentially informative class of DNA markers (Chow & Hazama, 1998; Cooke & Beheregaray, 2007; Cooke *et al.*, 2009). However, the use of introns has presented mixed results. Some studies revealed significant levels of intron variability and, thus, patterns of genetic structure that parallel and complement results based on other markers (Palumbi & Baker, 1994; Lavoué *et al.*, 2003; Maake *et al.*, 2013). For example, a molecular study of South African gobiid species using a complete mitochondrial cytochrome *b* fragment and a fragment of the nuclear S7 intron 1 showed that the recognized taxonomy underestimated the diversity of the genus *Glossogobius* Gill, 1862 in South Africa (Maake *et al.*, 2013). However, other studies have detected low levels of intron variability, which precluded the effective use of these markers for population level analyses (Gay *et al.*, 2004; Guo & Chen, 2010). A molecular approach was used in the current study mainly because of its potential to provide information about the fundamental

genetic basis of evolutionary change that is generally inaccessible through traditional taxonomic techniques (Hillis, 1987; Hillis & Wiens, 2000).

1.2.1. Phylogeography

Phylogeography considers the evolutionary relationships among populations in conjunction with geographic distribution to explain biogeographic patterns (Avice, 2009). In this way, phylogeographic investigations using mtDNA test hypotheses about whether the genetic structure of the taxa in question is related to their geographical distributions and whether they conform to an expected pattern (Meyer, 1993; Stepien & Kocher, 1997). Extensive genetic gaps can develop, for example, when a long-term barrier to gene flow, such as an area of unsuitable habitat, causes populations to diverge in isolation (Hewitt, 2000; Qi *et al.*, 2007).

The ability of phylogeographic studies to estimate the timing of diversification provides a tool for describing the timeline for gene flow among populations and, hence, provides an insight into the processes that were most influential in shaping present biodiversity patterns (Avice, 2000, 2009). Mitochondrial DNA (mtDNA) sequence data have been used successfully in phylogeographic studies and have facilitated the discovery of cryptic diversity within several traditionally-recognized species (e.g. Swartz *et al.*, 2007, 2009; Teske *et al.*, 2009, 2011; Kramer & Swartz, 2010; Maake *et al.*, 2013; Mwale *et al.*, 2013). Some studies revealed information regarding population history and evolutionary processes (Waters & Cambray, 1997; Bermingham & Martin, 1998; Daniels *et al.*, 2006; Chakona *et al.*, 2013a, b). Using extensive surveys, a phylogeographic study can be a powerful tool for providing a good foundation for taxonomic and biogeographic investigations (Beheregaray, 2008). In this way, phylogeographic structure is explored by partitioning variation in a hierarchical manner in order to reveal patterns of gene flow at a finer scale (Stepien & Kocher, 1997). Phylogeographic

investigations, thus, identify and distinguish between historical and contemporary patterns and the processes that influence genetic connectivity among populations and their distribution (Templeton, 1998; BurrIDGE *et al.*, 2008).

There has been an increase in the number of intraspecific phylogeographic studies of endemic freshwater fishes in South Africa. Most studies have focused on species from the Cape Floristic Region of South Africa (Bloomer & Impson, 2000; Swartz *et al.*, 2007, 2009; Chakona *et al.*, 2013a, b). These studies have revealed both highly-restricted and widely-distributed genetic lineages of freshwater-dependent fish species in this region, suggesting that some species have been isolated for long periods of time, while others have been able to maintain gene flow despite the apparent isolation of contemporary river systems in this part of the country. Extrinsic geological characters, such as river capture and the confluence of river systems, as well as intermittent freshwater connections have been inferred as the possible processes shaping the observed distribution of biodiversity. In sub-tropical South African river systems (such as the Limpopo, Incomati, Pongola, Kosi and Mhlatuze Rivers) (Figure 1.2), several phylogeographic studies have reported distinct phylogeographic breaks and morphological differentiation of freshwater fishes among these systems (Oosthuizen *et al.*, 2009; Matlala *et al.*, 2010; Van der Bank *et al.*, 2012; Bartáková *et al.*, 2013). Nevertheless, the finding of minimal numbers of shared haplotypes in some studies suggested a close linkage, possibly explained by historical drainage connections (Oosthuizen *et al.*, 2009; Matlala *et al.*, 2010; Skelton, 2001). However, few biogeographic or phylogeographic explanations have been proposed for the observed genetic divergences and morphological differences in these river systems.

Little phylogeographic information is available for the South African mormyrid species of the genera *Marcusenius* and *Petrocephalus*. One exception was a study conducted by Kramer *et al.* (2007), who found intraspecific variation in genetics, morphology and EODs between populations of *M. pongolensis*. Populations from all major river systems were differentiated to some degree and those from the Limpopo River system markedly so. Kramer *et al.* (2012) also reported that the differentiation of *P. wesselsi* populations seems to follow a north–south geographic cline, with genetic and morphological separation of the two populations farthest apart (e.g. the Incomati vs Kosi river systems). The lack of adequate sampling thus prevented detailed insight into the phylogeographic structure of *M. pongolensis* or that of *P. wesselsi*, which subsequently led to the present study investigating the variation and phylogeography of the species. The wide distribution of *M. pongolensis* from the Limpopo River system in the north to the Mhlatuze River in the south makes it a particularly suitable group for studying phylogeographic patterns and evolutionary processes in riverine species across this geographical area. Information on the phylogeography and systematics of South African *Marcusenius* should help to understand the events that have resulted in speciation and the current distribution patterns within this genus.

1.2.2. Molecular phylogeny

The role of phylogenetics in molecular systematics is to define ancestor and descendant relationships, while providing a hierarchical foundation for species taxonomy (Hou *et al.*, 2007). Together with information on the biogeography and ecology of species, molecular phylogenetics can provide information on the causes of speciation within groups (Hillis *et al.*, 1996; Barraclough & Nee, 2001). DNA markers, such as sequences of mtDNA cytochrome *b*, have been widely employed and considered useful in reconstructing phylogenies of closely-

and distantly-related species of various fish taxa across the Southern and Eastern African Basins, e.g. mormyrids (Kramer *et al.*, 2007, 2012; Kramer & Swartz, 2010), cichlid fishes (Katongo *et al.*, 2007) and tigerfishes (Goodier *et al.*, 2011).

Despite strong evidence for the monophyly of the mormyrid family, the existing morphological and molecular data sets supported conflicting phylogenies at the generic level (Lavoué *et al.*, 2000; Sullivan *et al.*, 2000). The phylogeny of Taverne (1972) (Figure 1.1) proposed that *Stomatorhinus*, *Brienomyrus* and *Boulengeromyrus* form a monophyletic group based on osteological similarity, but did not indicate a position for *Pollimyrus* within the subfamily Mormyrinae. More recently, Sullivan *et al.* (2000) indicated a close relationship between *Stomatorhinus* and *Pollimyrus* based on molecular data and similarities in their electrosensory organs. Taverne (1971a) had also synonymized *Cyphomyrus* with *Hippopotamyrus* based on osteological similarity and their ill-defined morphology. Van der Bank and Kramer (1996) and Sullivan *et al.* (2000) suggested that the genus *Hippopotamyrus* is polyphyletic based primarily on molecular data. *Cyphomyrus* was later reinstated as a valid genus (Kramer & Van Der Bank, 2011), probably as a sister genus to *Hippopotamyrus* (see Figure 1.1). At a generic level, relationships are also not always clear and very little information is available in the mormyrid literature to describe the phylogenetic relationships and biogeographic hypotheses that might have caused the high level of species diversification currently observed in the Southern and Eastern African Basins. This is especially true for the genera *Marcusenius* and *Petrocephalus*, which had complex taxonomic histories as evident from the fluctuations in their species numbers over the years.

Although *Marcusenius* is regarded as the most species-rich and widespread genus of the Mormyrinae (Kramer, 2013a), a molecular phylogeny that includes all known species is not

available and the phylogenetic position of the southern and eastern African species within the genus is unknown. According to Lavoué *et al.* (2000, 2003) and Lavoué and Sullivan (2004), the genus *Marcusenius* is polyphyletic, but they all stated that this hypothesis has to be supported through the analysis of a greater number of taxa. So far, only three molecular phylogenetic studies have been conducted on *Petrocephalus*, focused elsewhere in Africa (Lavoué *et al.*, 2010; Lavoué, 2011, 2012). These have included representatives of *P. catostoma* from only the Rufiji River system in the Eastern Africa Basin (Figure 1.2). Southern African species (such as *P. wesselsi*) were not included, while the phylogenetic position of the eastern African *P. catostoma* was not consistent. Kramer *et al.* (2012) recently suggested that southern African *Petrocephalus* is divided into two major clades, separated by the Victoria Falls in the Middle Zambezi River system. However, this remains to be tested as these authors based their suggestion on the analysis of only four of the 15 *Petrocephalus* species they detailed as occurring in the Southern and Eastern African Basins.

Given the paucity of material and the sampling gaps across the Southern and Eastern African Basins, the phylogenetic relationships and hypotheses proposed by Kramer *et al.* (2007) for *Marcusenius* and Kramer *et al.* (2012) for *Petrocephalus* should be regarded as preliminary and these await testing with a well-represented dataset. This study aims to conduct intensive sampling in South Africa and, combined with the previous data from Kramer *et al.* (2007, 2012), will contribute to an improved estimate of phylogenetic relationships within these genera. To fully understand the biogeography and evolutionary relationships within these two widespread genera in southern and eastern Africa, this study included most known topotypical populations of all recognised southern and eastern African species of the genus *Marcusenius* and nearly all *Petrocephalus* species, and previously unknown populations and/or lineages across the distribution. The inclusion of previously unknown lineages will increase the

accuracy and confidence of the results in comparison to the analyses of the currently described species, as lineages are the appropriate units for understanding the evolution of any widespread taxon (Waters & Wallis, 2000; Swartz *et al.*, 2009).

1.2.3. Biogeography

Biogeography is the study of the distribution of organisms across geographical space (Avise, 2000). Historical biogeography is phylogenetically-informed biogeography that aims to explain the past and present geographic distributions of organisms, as deduced by biological and geological data (Lundberg, 1993). Therefore, historical biogeography recognises that, if populations of a species became fragmented and isolated by vicariance, evolution would proceed independently in each population. Such separation requires that the exchange of genes among populations be restricted, often by some form of physical barrier, such as a river, a mountain range, waterfalls or an expanse of unsuitable habitat (Avise, 2000). To the extent that the geological history and associated vicariant events contributed to the species diversity, the signature of this history will be evident in the resulting biogeographic pattern and the timing of species divergence (Skelton, 1994; Cotterill, 2000, 2003a; Koblmüller *et al.*, 2006; Brown *et al.*, 2010).

Recent geological studies revealed dynamic histories for many African river systems (e.g., Moore & Larkin, 2001; Cotterill, 2003a; Stankiewicz & de Wit, 2006). These histories include a number of river reversal and river capture events driven by tectonics and glaciation that involved the transfer of streams between adjacent river catchments (Cotterill, 2003a; Cotterill & de Wit, 2011). Such river evolution effects the vicariant isolation of freshwater fish populations that were previously connected and can lead to cladogenesis and the emergence of new species (e.g., Katongo *et al.*, 2007; Kramer *et al.*, 2007; Goodier *et al.*, 2011; Pinton *et al.*,

2013). Furthermore, the extent of genetic divergence among populations and species is strongly linked with the period of physical isolation of the systems they inhabit, as determined using geological and molecular techniques.

Understanding the historical biogeography and origins of southern and eastern African *Marcusenius* and *Petrocephalus* requires knowledge of the geological history of the region. The evolution of modern drainages has been controlled by the complex interplay of a variety of geological processes (Skelton, 1994; Cotterill, 2000, 2003a). The hypothesis of drainage rearrangement patterns in eastern Africa proposes that the central Congo Basin was submerged during much of the Cretaceous and, after being uplifted, it drained eastwards through a Paleo-Congo River towards the Indian Ocean, ending at the present Rufiji Delta, Tanzania (Stankiewicz & de Wit, 2006). This pattern was subsequently disrupted by the uplift associated with the East African Rift System (EARS). This disruption is evident in the geographical patterns and divergence times of *Mastacembelus* eels (Brown *et al.*, 2010) and *Synodontis* catfishes (Pinton *et al.*, 2013), which parallel the formation and the activities of the EARS and the formation of Lake Malawi and Lake Tanganyika Plateau, geologically dated to have started between 4 and 12 million years (Ma) ago. Concurrently, the resultant landlocked Central Congo River system was captured by short rivers draining into the Atlantic Ocean, establishing the westward draining Congo River (Figure 1.2) in the Miocene (Stankiewicz & de Wit, 2006).

Some evidence also indicates that the south-central Africa drainage systems, including the Okavango, Cuando and Zambezi-Luangwa Rivers, drained into the Indian Ocean. These formed the headwaters of the proto-Limpopo River during the Miocene, before uplift along the Okavango-Zimbabwe Axis severed the links between the Limpopo and the Okavango, Cuando and Zambezi-Luangwa Rivers (Moore & Larkin, 2001; Cotterill, 2003a; Kramer *et al.*, 2007).

Moore & Larkin (2001) recently proposed a drainage evolution model for the Southern African Basin. They suggest that in the Early Pleistocene, the Lower Zambezi was captured, forming a link with the Upper Zambezi. Around the same time the Chambeshi River, formerly part of the Zambezi, was captured by the Luapula tributary of the Congo. During the Middle Pleistocene, the Cuando and Kafue Rivers were captured by the main Zambezi system and produced the pattern observed today (Figure 1.2). There would, therefore, have been opportunities for the historical isolation of and/or for the transfer of fish species between currently-separated basins and between rivers within basins. The occurrence and timing of these events are corroborated by molecular studies of various taxa, such as antelopes (Cotterill, 2000, 2003a, b), mole rats (Van Daele *et al.*, 2007; Faulkes *et al.*, 2011) and freshwater fishes (e.g. Koblmüller *et al.*, 2006; Katongo *et al.*, 2007; Brown *et al.*, 2010; Goodier *et al.*, 2011; Pinton *et al.*, 2013), which have revealed concordant patterns of historical biogeography and regional genetic structuring. For example, the recent geomorphological evolution of the Luapula and Zambezi drainages has been an important influence on the region's biodiversity, as indicated by the biogeography of *Damaliscus* and *Kobus* antelopes (Cotterill, 2000, 2003b), tiger fishes (Goodier *et al.*, 2011) and *Synodontis* catfishes (Pinton *et al.*, 2013).

The biogeographic history is still incomplete and the precise timing of key events awaits finer resolution (Cotterill, 2003a). While much remains to be learnt, especially about the precise timing and sequence of events, a synthesis of current knowledge about drainage evolution is possible. Given the geological history and the associated vicariance that contributed to species diversity in the affected area, biogeographic investigations are required to understand the impact of these events on the diversity and distribution of *Marcusenius* and *Petrocephalus*, both widespread genera in the Southern and Eastern African Basins. This study could shed

some light on the important factors and events in the evolution of *Marcusenius* and *Petrocephalus* in the Southern and Eastern African Basins.

1.3. MORPHOLOGICAL SYSTEMATICS

The morphology of fishes has historically been the primary source of information for taxonomic and evolutionary studies (Hillis, 1987). Species have characteristic shapes, sizes, pigmentation patterns, position of fins and other external features that aid in recognition, identification and classification (Strauss & Bond, 1990). However, the presence of cryptic taxa has confounded many traditional studies, since these may appear morphologically similar yet be phylogenetically distinct (Wiens & Penkrot, 2002; Wiens *et al.*, 2003; Bickford *et al.*, 2006). Advances in obtaining and analysing DNA sequences now facilitate the delineation of cryptic species and aid in the identification of distinct lineages within species for further research, frequently leading to descriptions of new taxa (Skelton & Swartz, 2011). Often subtle morphological differences, as well as behavioural differences, are identified after cryptic diversity has been recognised through molecular studies (Stepien & Kocher, 1997).

In an attempt to stimulate a debate on the cryptic species confusion, Knowlton & Weigt (1997) asked how much variability should a single species encompass? The taxonomic response to this question depends upon the nature of the species concept employed. Numerous species concepts are described in the literature (Wiley & Mayden, 2000; Mayden, 2002; de Queiroz, 2007) and two of these predominate systematic studies: the Phylogenetic Species Concept (PSC: Mishler & Theriot, 2000) and the Evolutionary Species Concept (ESC: Wiley & Mayden, 2000). The ESC defines a species as a group of organisms with its own independent evolutionary trajectory separate from others in space and time. The PSC defines a species as

the least inclusive unit within a phylogenetic classification as evidenced by monophyly. While Mishler & Theriot (2000) argued that the PSC provides robust operational criteria for delimiting species (i.e. monophyly), the ESC also provides diagnosability of what constitutes a species (i.e. qualitative, fixed differences) (de Queiroz, 2007). Therefore, the use of both molecular data for the PSC and morphological data for the ESC will provide results independent of each other to evaluate the presence of cryptic species and species boundaries within both *Marcusenius* and *Petrocephalus* genera in the Southern and Eastern African Basins.

Morphological measurements have been widely used to discriminate species of various mormyrid genera in southern Africa, e.g. *Petrocephalus* (Kramer & Van der Bank, 2000; Kramer *et al.*, 2012), *Hippopotamyrus* (Kramer *et al.*, 2004; Kramer & Swartz, 2010), *Pollimyrus* (Taverne, 1971b; Kramer *et al.*, 2003), *Marcusenius* (Kramer *et al.*, 2007; Kramer & Wink, 2013) and *Cyphomyrus* (Kramer & Van Der Bank, 2011). Some of these studies (e.g. Kramer *et al.*, 2007, 2012) have shown that the lack of comprehensive characters or taxonomic keys made the identification of some species difficult or problematic in the past, with results from morphology, EODs and genetics sometimes giving mixed results. For example, the degree of similarity among EODs of *Petrocephalus* species, *P. tanensis*, *P. longicapitis*, *P. okavangensis* and *P. wesselsi*, has been noted and the identification of species was dependent strongly on qualitative morphological characters (Kramer *et al.*, 2012).

Despite the recent attention directed at *Marcusenius* in the Southern and Eastern African Basins (Tweddle & Willoughby, 1982; Kramer *et al.*, 2007), the taxonomy is still obscure and collected material in museums remains unidentified, due to absence of species identification keys. Designation of species of southern and eastern African *Marcusenius* has, therefore, been a confusion of synonyms and uncertainty. For example, some morphologically-distinct

Marcusenius species, such as *M. moeruensis* Boulenger, 1915 from Lake Mweru, Luapula drainage, and *M. graeverti* Steindachner, 1914 from the Ruaha River, Tanzania remain synonyms of *M. macrolepidotus*, which is now only known from the Lower Zambezi River system and its tributaries (Kramer *et al.*, 2007). The latter study shows that *Marcusenius* in the Eastern African Basin has still more differentiation at a morphological level than presently recognised and might contain even more un-described taxa.

This is also true for *M. pongolensis*, the southernmost occurring species of the genus, and the only species from South Africa. It was recently discovered that geographically-separated populations of *M. pongolensis* exhibited unusual morphology and EODs (Kramer *et al.*, 2007). This implied that species-level differentiation may exist within this species, but little was known of the extent of this differentiation. Samples from the Limpopo system could also not be identified with certainty using the taxonomic characters detailed for *M. macrolepidotus* and *M. pongolensis*, and comparisons of morphology and EODs indicated interspecific differentiation between the Limpopo system and other systems in South Africa (Kramer *et al.*, 2007). Nevertheless, the name *M. pongolensis* has been applied to all the South African populations. In the present study, morphological characters will be analysed as one of the chosen tools to study *Marcusenius* from South Africa (as well as Swaziland) and from northern Mozambique, particularly the Ruvuma system. This study will also aim to source original or updated descriptions of all known southern and eastern African *Marcusenius* species to provide the first identification key for this region.

1.4. AIMS AND OUTLINE OF THE THESIS

The primary aims of this study are to re-evaluate the taxonomic status, to investigate the phylogeography, phylogenetic relationships and biogeography of *Marcusenius* and *Petrocephalus* fishes throughout their range in southern and eastern Africa, by applying both molecular and morphological approaches.

The second chapter of this research thesis will present the study of the phylogeography of South African *Marcusenius* to better understand the historical events that may have led to the emerging species composition and the geographical distribution of genetic variation within the species. This will be accomplished with materials collected from extensive field surveys and the sequencing of mitochondrial DNA [cytochrome *b* and cytochrome c oxidase subunit I (COI)] genes and the ribosomal nuclear S7 intron 1 marker (S7 intron 1). The results of this study could have strong implications for the conservation and management of endemic freshwater fish species in these sub-tropical river systems. These key questions will be investigated:

- What is the genetic diversity within and among South African sampling sites of *M. pongolensis*?
- What are the events that might have led to the separation and current distribution of South African populations of *Marcusenius*?

The aim of the third chapter will explore the diversity among *Marcusenius* populations in South Africa using morphometrics and meristic data, in comparison to a previously identified lineage from the Ruvuma River system, northern Mozambique. To obtain maximum geographical

coverage in sampling, both museum and field-collected samples will be included. Samples will be grouped according to the geographical distribution of divergent mitochondrial DNA lineages identified in Chapter 2 and by Kramer *et al.* (2007). This study will also develop a comprehensive view of morphological variation across the distribution of *M. pongolensis* in South Africa. The following key questions are addressed in this chapter:

- What is the level of morphological variation among *Marcusenius* specimens from different South African systems and from the topotypical *M. pongolensis*?
- To what extent are the Limpopo populations of *Marcusenius* morphologically different from those of the Incomati, Pongola, Kosi and Mhlatuze river systems.
- To what extent are the Ruvuma populations of *Marcusenius* morphologically different from the southern African species?

The main objective of Chapter 4 is to formulate a hypothesis concerning the evolution and phylogenetic relationships of the Southern and Eastern African Basin species of the genera *Marcusenius* and *Petrocephalus*, using mitochondrial cytochrome *b* and nuclear S7 intron 1 sequences. This study will evaluate how *Marcusenius* and *Petrocephalus* species perform as biotic indicators of landscape evolution by considering their phylogenies to resolve details of river system evolution. This chapter evaluates how rearrangements of Africa's drainages - as reviewed by Stankiewicz & de Wit (2006) and Moore & Larkin (2001) - interfaced throughout the Miocene with the evolution of these mormyrid genera. Given geographic coverage from the tropical Lower Congo to the eastern Lower Zambezi, and from the equatorial Tana River in Kenya to the Mhlatuze River in South Africa in the south (the southernmost distribution of any known mormyrid), this study will analyse the largest dataset of the genera *Marcusenius* and *Petrocephalus* in southern and eastern Africa. The following key questions are asked:

- Are the South African species genetically different from the other southern and eastern species of each genus?
- What are the levels of genetic divergence among all the species and historically-isolated lineages within each genus?
- What are the divergence time estimates for the speciation and evolution within the genera and among the South African species?

The final chapter (Chapter 5) is a synthesis of the results and a consideration of the implications from all the analyses of the previous chapters. This chapter also attempts to discuss the expectation that the proposed study will enhance the taxonomy, the understanding of distribution, and diversification of both *Marcusenius* and *Petrocephalus* in the Southern and Eastern African Basins. Furthermore, this will help shift the conservation focus from individual species, which is the current approach, to communities and will help determine the processes that influence the observed diversity of mormyrids in southern and eastern Africa.

CHAPTER 2: THE DISCOVERY OF TWO DISTINCT LINEAGES OF *MARCUSENIUS*
PONGOLENSIS (TELEOSTEI: MORMYRIDAE) IN SOUTH AFRICA WITH
SIMILAR EVOLUTIONARY HISTORIES

ABSTRACT

The present study analyzed the phylogeographic history of populations of *Marcusenius pongolensis* Fowler, 1934 *sensu lato* that spans five major sub-tropical South African river systems. A total of 194 individuals were sequenced for the mtDNA *cyt b* (857 nucleotides), COI (639 nucleotides) and nuclear S7 intron (612 nucleotides). Three divergent major lineages (1.5-2.9% mtDNA divergences), that separated during the Pleistocene, were identified through maximum likelihood, Bayesian and network analyses of mitochondrial DNA datasets. The widespread Pongola Lineage occurs partly in the Limpopo River system, and is very common in the Incomati, Pongola (type locality) and Kosi River systems. The Limpopo Lineage which occurs only in the Limpopo River system in the north is monophyletic with the Mhlatuze-Nseleni Lineage which is only known from the Mhlatuze and Nseleni rivers in the south. The Pongola and Limpopo lineages occur in sympatry in the Olifants River of the Limpopo River system. In contrast to the mitochondrial data, the S7 intron did not show the divergence between the lineages, probably due to the slow mutation rate of the marker. The mitochondrial evolutionary history of the Pongola Lineage revealed that at least two distinct isolated clades can be identified, the Limpopo and an Incomati-Pongola-Kosi clade. There was strong indication of historical isolation, in the form of allopatric fragmentation between genetically divergent Limpopo and Mhlatuze-Nseleni lineages. High genetic diversity was observed in the Limpopo River system within the Limpopo Lineage and the Pongola Lineage. During the Late Pleistocene, the populations were isolated in different systems, followed by population expansion and secondary contact during more humid periods during the Holocene. The occurrence of the Mhlatuze-Nseleni Lineage in the far south cannot be explained by the hypothesis of confluence of rivers, river capture or episodic drainage connections, as the Mhlatuze and Nseleni Rivers would not have had a common confluence or shared low relief terrain with Limpopo, Incomati, Pongola or the Kosi river systems. The results of this study require that the taxonomic status of all *Marcusenius* lineages be investigated.

2.1. INTRODUCTION

Phylogeographic studies have contributed to a better understanding of intraspecific genetic diversity, its distribution and the evolutionary forces (extrinsic and intrinsic) that influenced the diversification of freshwater fishes in South Africa (Bloomer & Impson, 2000; Swartz *et al.*, 2004, 2007, 2009, 2014; Chakona & Swartz, 2012; Chakona *et al.*, 2013a, b). These studies inferred that extrinsic geological and climatic events (e.g. river captures and sea level fluctuations) and the taxon's intrinsic characteristics (e.g. physiological adaptations, dispersal abilities or reproductive biology) played a role in the distribution and diversity of freshwater fishes. For example, river capture and the confluence of river systems when the South African coastline was about 130 m below the present level were inferred as the main routes of dispersal of some lineages of the cyprinid genus *Pseudobarbus* (Swartz *et al.*, 2007, 2009; Chakona *et al.*, 2013b). Alternative mechanisms may also play a role, such as intermittent freshwater connections across low relief terrain during wetter periods, which may have allowed an air-breathing galaxiid species to disperse across multiple systems (Chakona *et al.*, 2013a, b). These studies indicated that the evolution of freshwater fishes in the temperate south-western region of South Africa is tightly linked to the geological and climatic history of the drainage basins they inhabit, resulting in a high degree of phylogeographic structure and genetic differentiation between lineages.

Similarly, several studies have reported distinct phylogeographic breaks and morphological differentiation of freshwater fishes among sub-tropical river systems of South Africa (Engelbrecht & Mulder, 1999; Oosthuizen *et al.*, 2009; Matlala *et al.*, 2010; Dorn *et al.*, 2011; Van der Bank *et al.*, 2012; Bartáková *et al.*, 2013). Oosthuizen *et al.*'s (2009) genetic and morphological assessment of allopatric populations of *Labeobarbus polylepis* showed that

consistent differences exist between the Incomati and Pongola river systems (Figure 2.1). In a systematic and phylogeographic study of the widespread sucker-mouth catfishes of the genus *Chiloglanis*, Matlala *et al.* (2010) found a significant degree of morphological and genetic variation among populations between the Limpopo and Incomati river systems. Similar studies of a short-lived killifish, *Nothobranchius furzeri*, also revealed deeply-divergent mitochondrial lineages between the Limpopo and Incomati river systems, originating as a consequence of a long-period of isolation since the Late Pleistocene (Dorn *et al.*, 2011; Bartáková *et al.*, 2013). These studies suggest that primary freshwater fishes may not be able to disperse between the Limpopo, Incomati and Pongola river systems and that diversity across and within these systems may be much higher than previously thought. However, the sharing of some alleles among systems also indicated that some dispersal may have occurred, possibly explained by historical drainage connections (Oosthuizen *et al.*, 2009; Matlala *et al.*, 2010). These systems are thought to have come into contact across large, flat floodplains in southern Mozambique during wet periods during the Pleistocene (Jubb, 1971; Skelton, 2001), possibly allowing dispersal between river systems. Given the above results, it is unclear whether other freshwater fish species were isolated or were able to disperse between these river systems.

The South African bulldog mormyrid, *Marcusenius pongolensis* Fowler, 1934, was described from a single specimen from the upper Pongola River system (Figure 2.1: Pongola 1). The distribution of *M. pongolensis* is regarded as that ranging widely from the tropical Limpopo, Incomati, Pongola and Kosi river systems, as well as the southernmost population from Mhlatuze River in KwaZulu-Natal (Figure 2.1). In a recent taxonomic study of the southern African species of the genus *Marcusenius*, it was established that *M. pongolensis* was not monophyletic (Kramer *et al.*, 2007). This study, although based on relatively limited geographic sampling and a small sample size, indicated that *M. pongolensis* was heterogeneous

for mitochondrial cytochrome *b*, morphology and electric organ discharge signals. For example, Kramer *et al.* (2007) showed that the Limpopo specimens of *M. pongolensis* were morphologically differentiated, most significantly in body depth, from the Incomati, Pongola and Kosi specimens of the same species. Furthermore, the genetic analyses, which did not include the Limpopo samples, identified two genetically-divergent lineages, one distributed in the southernmost locality, the Mhlatuze River or the Mhlatuze lineage, and the other in the Incomati and Pongola river systems or the Pongola lineage. This suggested that intraspecific differentiation exists in South African *Marcusenius*, opposed to the single widespread *M. pongolensis* currently accepted. Despite these results, Kramer *et al.* (2007) indicated that, with the possible exception of the Limpopo samples, morphology supports allopatric variation within a single species, similar to other freshwater species in the same systems (e.g. Oosthuizen *et al.*, 2009; Matlala *et al.*, 2010; Dorn *et al.*, 2011).

Southern Africa has been characterised by the formation of physical barriers to gene flow in the past (Moore, 1999) and strikingly complex climatic conditions defined by extremely wet and dry periods (Partridge *et al.*, 1999; Ramsay & Cooper, 2002; Finch & Hill, 2008). These conditions are thought to have produced the recurrent connection and separation of adjacent river systems (Partridge & Maud, 1987). As detailed by Moore (1999) and authors therein, the geomorphological processes responsible for landscape evolution in South Africa have been interpreted to reflect lines of flexuring associated with the formation of the Escarpment and Ciskei-Swaziland Axis that are inferred to be of Plio-Pleistocene age and have separated the coastal (e.g. Kosi and Mhlatuze) and inland (e.g. Limpopo) river systems (Partridge & Maud, 1987; Moore, 1999; Moore *et al.*, 2009). The relatively drier, local conditions proposed to have persisted across the interior plateau of South Africa during the Pleistocene (Partridge *et al.*, 1997, 1999; Finch & Hill, 2008) probably further reduced the possibility of gene flow and thus

produced strong population genetic structure, such as that observed within *Chiloglanis* species (Matlala *et al.*, 2010). However, some of these systems are thought to have come into contact during wetter periods of the Late Pleistocene to Early Holocene (Jubb, 1971; Skelton, 2001). With the knowledge of the evolutionary mechanisms underpinning the diversification and distribution of freshwater taxa in these systems still developing, *M. pongolensis* provides an interesting species with which to study drainage evolution in sub-tropical South Africa.

The present study extends the previous research of Kramer *et al.* (2007) with more geographic sampling that spans five major sub-tropical South African river systems (Figure 2.1) and more genetic markers for analysis to study the phylogeography of *M. pongolensis*. The aims of the present study were to examine the genetic differentiation in the mtDNA and nuclear DNA sequences of *M. pongolensis* throughout its known distribution in South Africa in an attempt to infer which contemporary and historical processes have influenced populations currently isolated in isolated river systems. This phylogeographic study will allow comparison to other similarly distributed freshwater fish species to gain a better understanding of drainage evolution of the eastward flowing rivers in sub-tropical South Africa. The taxonomic status of the Limpopo population remains uncertain and systematic research is needed to confirm any conclusions (Kramer *et al.*, 2007); samples from this area are therefore included in the present study. The aims of this study will be accomplished with the analysis of mitochondrial DNA [combined fragments of cytochrome oxidase subunit I (COI), as used in DNA barcoding (Hebert *et al.*, 2003), and cytochrome *b* (cyt *b*)] and nuclear DNA [S7 ribosomal protein gene intron 1 (S7 intron 1)] markers which provide information on both paternal and maternal gene flow and can be used to identify reproductively-isolated lineages in sympatry (Chow & Hazama, 1998; Zhang & Hewitt, 2003; Cooke *et al.*, 2009; Guo & Chen, 2010; Maake *et al.*, 2013).

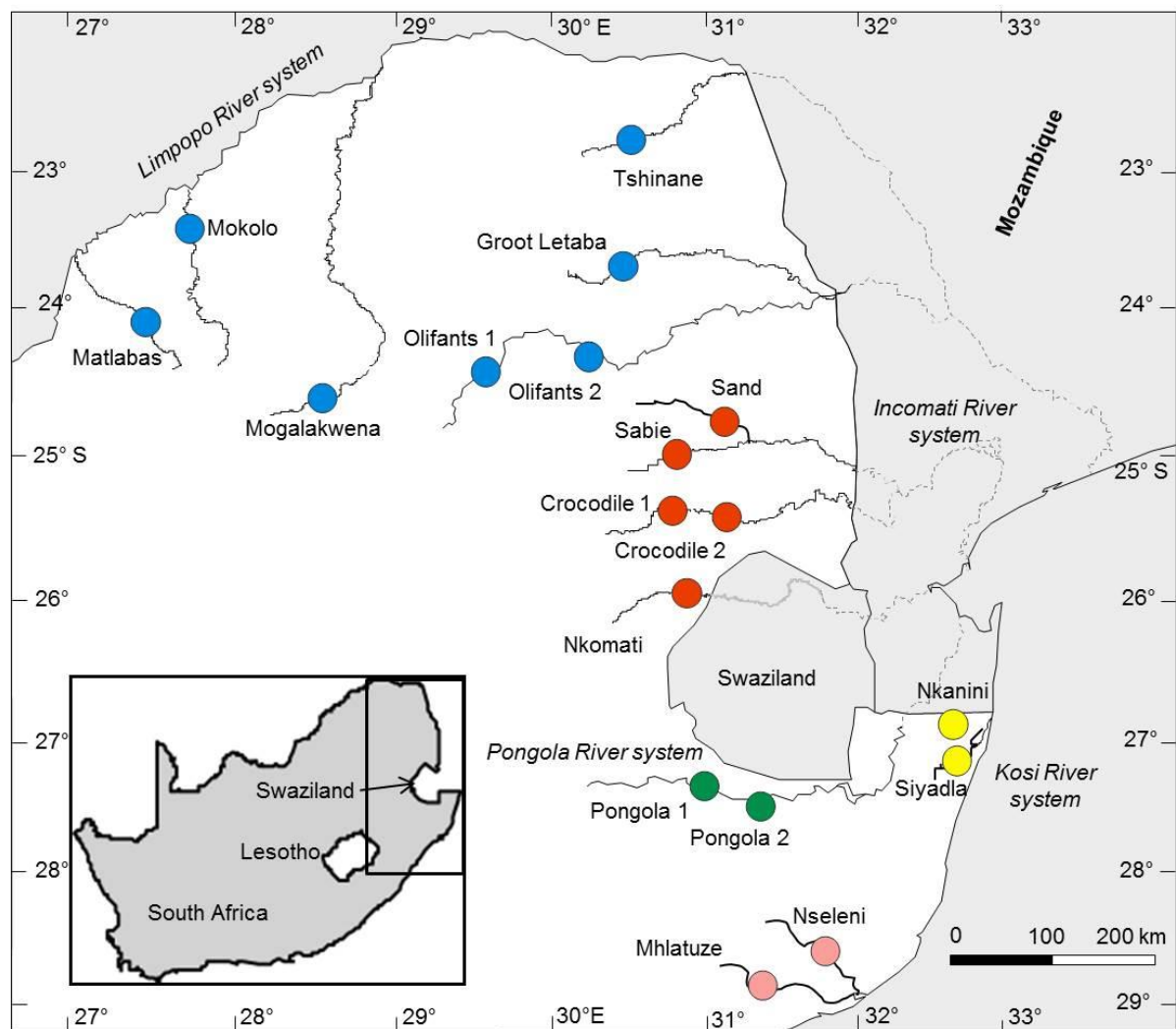


Figure 2.1. The collection localities of *Marcusenius pongolensis* across South Africa. Major river systems are shown and indicated in italics. Colour codes indicate the different river systems. Latitudes and longitudes of the localities are given in Table 2.1.

2.2. MATERIALS AND METHODS

2.2.1. Sample collection and DNA extractions

Specimens of *M. pongolensis* were collected from 18 localities across the species' distribution range during three surveys (September-November 2009, October-November 2010 and October-November 2011), using electrofishing and gill nets. These included rivers or tributaries of the Limpopo, Incomati, Pongola, Kosi, Nseleni and Mhlatuze river systems (Table 2.1; Figure 2.1). Muscle tissue samples were dissected and placed in 99% EtOH in the field and frozen at -20 °C upon returning to the laboratory. In addition, the specimens from which muscle tissue was dissected were fixed in formalin and deposited in the National Fish Collection of the South African Institute for Aquatic Biodiversity (SAIAB), Grahamstown, as voucher specimens.

Total DNA extractions were performed following the protocol of the Promega Wizard DNA purification kit (Madison, New York). Amplification of a fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene was achieved by Polymerase Chain Reaction (PCR) with the primers VF2-T1 5' TGT AAA ACG ACG GCC AGT CAA CCA ACC ACA AAG ACA TTG GCA C 3' and VR1-T1 5' CAG GAA ACA GCT ATG ACT AGA CTT CTG GGT GGC CAA AGA ATC A 3' (Ivanova *et al.*, 2007) and for the partial mitochondrial cytochrome *b* (cyt *b*) fragment with the primers GluF 5' AAC CAC CGT TGT ATT CAA CTA CAA 3' (Machordom & Doadrio, 2001) and H15917 5' TAG TTG GCC AAT GAT GAT GAA TGG GTG TTC TAC TGG TT 3' (Kramer *et al.* 2003).

The first intron of the S7 gene was amplified using the primers S7RPEX1F 5' TGG CCT CTT CCT TGG CCG TC 3' and S7RPEX2R 5' GCC TTC AGG TCA GAG TTC AT 3' (Chow & Hazama, 1998). Each 25 µl reaction contained: ~1-3 µl template DNA (depending on the DNA concentration), 1 x reaction buffer, 25 mM MgCl₂, 0.125 mM of dNTPs (Promega), 0.2 units of Super-therm® Taq DNA polymerase, 25 pmol of each of the forward and reverse primers and topped to 25 µL with DNAase free water.

Conditions for the amplification of COI were 3 minutes at 94 °C, then 38 cycles of 30 seconds at 94 °C, 30 seconds at 54 °C and 50 seconds at 72 °C, finishing with 7 minutes at 72 °C. For *cyt b*, the PCR conditions were 4 minutes at 94 °C and 31 cycles of 45 seconds at 94 °C, 1 minute at 52 °C and 2 minutes at 72 °C, finishing with 5 minutes at 72 °C. The S7 intron reaction had initial denaturation at 95 °C for 4 minutes, followed by 35 cycles of amplification (1 minute denaturation at 95 °C, 1 minute annealing at 64 °C and 90 seconds extension at 72 °C), with a final extension step at 72 °C for 7 minutes.

PCR products were purified with the QIAquick PCR purification kit (QIAGEN, Valencia, California) before sequencing, using the BigDye Terminator 3.1 cycle-sequencing ready reaction kit (Applied Biosystems, Foster City, California). The end products of cycle-sequencing were precipitated using an ethanol precipitation procedure (Sambrook & Russell, 2001). Sequences were screened on an ABI 3730xl DNA Genetic Analyzer (Applied Biosystems) at Macrogen Inc. (Seoul, South Korea), and on an ABI 3100 at Rhodes University (Grahamstown, South Africa).

Table 2.1. Sample information of *Marcusenius* localities collected in South Africa.

Locality name	Locality code	River system	Latitude	Longitude	SAIAB accessioned no.	Sample size	
						mtDNA	S7 intron 1
Matlabas	MT	Limpopo	24° 13' 35.9"S	27° 28' 43.4"E	188289	12	13
Mokolo	MK	Limpopo	23° 22' 01.7"S	27° 41' 09.6"E	188294	16	15
Mogalakwena	MG	Limpopo	24° 42' 04.2"S	28° 24' 40.8"E	188295	9	9
Tshinane	TN	Limpopo	22° 53' 56.3"S	30° 31' 28.7"E	188290	12	12
Groot Letaba	GL	Limpopo	23° 50' 22.5"S	30° 13' 01.4"E	188302	17	17
Olifants 1	OL1	Limpopo	24° 31' 41.6"S	29° 32' 47.2"E	188291	13	12
Olifants 2	OL2	Limpopo	25° 26' 15.5"S	29° 28' 32.6"E		4	4
Sand	SD	Incomati	24° 42' 53"S	31° 12' 14"E	188304	9	9
Sabie	SB	Incomati	25° 01' 48.9"S	31° 01' 31.2"E	188280,	11	11
					188279,		
					188293		
Crocodile 1	CC1	Incomati	25° 32' 10.0"S	31° 18' 41.4"E	188297	21	25
Crocodile 2	CC2	Incomati	25° 32' 36.7"S	31° 19' 57.5"E	188300	4	4
Nkomati	SZNK	Incomati	25° 46' 04"S	31° 28' 28"E	65118, 69893, 69907	4	0
Pongola 1	PG1	Pongola	27° 18' 33.7"S	30° 53' 51.7"E	188296	19	21
Pongola 2	PG2	Pongola	27° 20' 28.4"S	31° 44' 45.1"E	188277	2	2
Nkanini	NK	Kosi	26° 59' 36.0"S	32° 44' 34.3"E	88629, 88637	13	13
Siyadla	SY	Kosi	27° 04' 53.0"S	32° 47' 11.0 "	88615, 88692, 96007,	11	11
Nseleni	NS		28° 42' 57.8"S	31° 59' 34.4"E	96543,	7	7
					96603,		
					96730, 96771		
Mhlatuze	MZ		28° 50' 44.2"S	31° 51' 59.6"E	88684	12	11

2.2.2. Sequence diversity analyses

Prior to analysis, samples from the two stations in the Olifants River were combined and analysed as one population. In a similar way, the two sampling stations from Crocodile River were combined, and those from the Pongola River system were also combined before analysis. Original sequence chromatograms were manually checked and edited for ambiguities using SeqMan version 11 (Lasergene 11.2.1, DNA Star, Inc., Madison, WI). Alignments of each gene region were performed using ClustalX 2.1 (Larkin *et al.*, 2007). Phylogeographic analyses were based on a combined mitochondrial dataset (cyt *b* and COI) and the nuclear dataset (only the S7 intron 1), separately. A combined mitochondrial and nuclear dataset was only used for the phylogenetic analysis. Prior to the phylogeographic analyses of the S7 intron data, all

sequences were phased with PHASE 2.1 (Stephens *et al.*, 2001), implemented in DnaSP (Librado & Rozas, 2009), to infer the gametic phases of alleles of the S7 intron sequences. DnaSP was used to identify shared and unique alleles, to determine the number of polymorphic sites and the number of alleles (N_A) in the entire sample, within river systems and also for each locality per dataset. The same program was used to calculate allelic (A_D) and nucleotide (π_D) diversity. To test for evidence of recent population expansion, Fu's F_s (Fu, 1997) statistics were calculated for the entire sample and for major river systems (Limpopo, Incomati, Pongola and Kosi) using the mtDNA dataset in DnaSP. The significance of the Fu's F_s parameters was assessed using 20 000 random permutations. Significant negative values of Fu's F_s indicate an excess of low-frequency haplotypes, a signature characteristic of either selection or a recent population expansion (Fu, 1997). The expansion of particular populations was estimated for the mtDNA dataset with the formula $T = \tau/2u$ (Rogers & Harpending, 1992), where T = time since expansion, τ is expansion time in coalescent units, and $u = (\mu, \text{mutation rate}) \times (\text{generation time}) \times (\text{number of bases sequenced})$. Due to the lack of a calibrated mutation rate for the genus *Marcusenius*, a general conservative cyt *b* mutation rate of 1.4 – 2.5% per million years, used in other mormyrid studies (Sullivan *et al.*, 2002), was used here. The age of female maturity of *M. pongolensis*, estimated at 1.25 years (Booth & Khumalo, 2010), was used. Demographic and historical inferences were not determined for the S7 intron data because the phylogeographic sorting of nDNA polymorphisms is slower than for mtDNA (Hoelzer, 1997). MtDNA is particularly attractive because of its high mutation rate and sensitivity to recent demographic changes (Meyer, 1993).

2.2.3. Population genetic structure and evolutionary history

Prior to analysis, models of sequence evolution that fitted the mtDNA and the S7 intron 1 set best were estimated in Modeltest 3.7, using Akaike's Information Criterion (AIC) (Posada &

Crandall, 1998). Phylogenetic analyses proceeded with only unique alleles of the combined mtDNA cyt *b* and COI data set. *Marcusenius altisambesi* and *M. angolensis* were chosen as the outgroup taxa, based on the phylogenetic analyses in Chapters 3 and 4. Phylogenetic relationships were reconstructed using maximum likelihood (ML) in PAUP* (Swofford, 2002) and Bayesian inference (BI) in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). For ML, the parameters of the chosen model were implemented in a heuristic tree search, which employed TBR-branch-swapping and 100 random stepwise taxon-addition replicates. Nodal support was not determined for the ML phylogeny.

Two independent BI analyses were performed. The Markov chain Monte Carlo process was set so that four chains (three heated and one cold) ran simultaneously for ten million generations. Parameter values and trees were sampled every 2000 generations. Stationarity was determined by examining graphical plots of sampled likelihood scores from each of the analyses. The first 20% of sampled generations in each run were discarded as 'burn in'. The 50% majority-rule consensus trees were calculated from the remaining 4000 trees from the posterior probability distribution of each analysis. Posterior probability values greater than or equal to 95% were considered to represent significant support for the relationships reflected in the trees (Ronquist & Huelsenbeck, 2003). Model-corrected sequence divergences among alleles were determined using parameters of the chosen model in PAUP.

Estimates of the time of divergence of particular phylogroups were made using the mtDNA dataset and the Bayesian MCMC approach implemented in BEAST 1.7.1 (Drummond *et al.*, 2012). The analysis was conducted with a relaxed, lognormal clock. Sequences were analysed with default priors. The MCMC chains were run for 60 million generations with trees sampled every 1000th generation. The first 10% of the iterations were discarded as burn-in. TRACER

1.5 (Rambaut & Drummond, 2007) was used to confirm the stationarity and to determine whether effective sample sizes were adequate for all parameters. The divergence at 1.6 million years ago of *M. pongolensis* from its sister species, as estimated in Chapter 3, was used to calibrate the root age of the tree.

Nested clade phylogeographical analysis (NCPA) was used to make inferences about the evolutionary history of *M. pongolensis* across its distributional range. Even though the method has been criticized (e.g. Panchal & Beaumont, 2007; Petit, 2008), its use in phylogeography is still warranted, especially when used in conjunction with other methods (Templeton, 2008, 2009; Faulks *et al.*, 2010). TCS 1.21, which implements a 95% minimum-spanning parsimony network, was used to accommodate non-bifurcating relationships among alleles (Clement *et al.*, 2000). Loops or ambiguities in the networks were resolved using a combination of criteria, following Posada & Crandall (2001) and Pfenninger & Posada (2002). The frequency criterion states that alleles are more likely to be connected to older ones (those with higher frequency in the population) than to younger ones (or singletons); a topological criterion explains that singletons are more likely to be connected to interior alleles than to those on the tips, and are also more likely to be connected to present rather than to hypothetical haplotypes/alleles; and, lastly, a geographical criterion explains that alleles are more likely to be connected to alleles from the same geographic region or population than to those from geographically-distant populations. Alleles in the respective networks were manually nested into a hierarchical nested clade design following the rules of Templeton (1998) and Cunningham (2002). Distances between populations were measured along river courses and along the current coastline using ArcView GIS 3.3 (ESRI, Redlands, CA). Using Geodis 2.5 (Posada *et al.*, 2000), these distances were used to calculate the different nested clade distances and to test whether the null hypothesis of no association between alleles or clades and their geographical distribution (i.e.

panmixia) could be rejected. The clad distances (which determines the geographical range of a given clad), nested clad distances (a measure of how a clad is geographically distributed relative to its closest evolutionary sister clades), average interior versus tip clad distances and average interior versus tip nested clad distances were calculated in Geodis 2.5 (Posada *et al.*, 2000) . The most recent inference key by Posada and Templeton (2011) was used to infer the processes responsible for the observed distributions of alleles for each clad.

To test for genetic structure, levels of population subdivision were assessed using the pairwise analyses of Φ_{ST} in Arlequin 3.5 (Excoffier & Lischer, 2010) with 10 000 permutations. Using the same programme, Analyses of Molecular Variance (AMOVA) were conducted to examine the partitioning of mtDNA and S7 intron sequence variation among within-population (Φ_{ST}), within-group (Φ_{SC}) and among-group (Φ_{CT}) components. Two *a priori* AMOVA structures were tested: population subdivision was based on the influence of river systems (the Limpopo, Incomati, Pongola, Kosi and Mhlatuze/Nseleni Rivers) and putative taxonomy. For putative taxonomy (see Chapter 3), populations were grouped according to their membership of a particular major clad. In each AMOVA, 20 000 permutations were performed to determine significance. It was expected that if genetic barriers exist between river systems and inhibit gene flow among populations, then genetic differentiation between populations in different systems should be greater than genetic differentiation between populations in the same system. The parameters of the model of sequence evolution identified in Modeltest were implemented for these analyses.

2.3. RESULTS

2.3.1. DNA sequences and phylogenetic analyses

A total of 194 individuals were sequenced for both the *cyt b* (857 nucleotides) and COI (639 nucleotides) gene fragments. These mtDNA sequences were combined to form a total dataset of 1496 nucleotides. A total of 65 variable sites were detected, of which 47 were parsimony informative and 18 were autapomorphic sites. This resulted in a total of 49 alleles (Table 2.2), with an allelic diversity of 0.925 ± 0.012 , and a nucleotide diversity of 0.0078 ± 0.0039 (SD) for the overall sample.

For the S7 intron data, 380 phased S7 intron sequences, belonging to 190 individuals, with a length of 612 nucleotides were aligned and edited. Despite numerous attempts and the use of different PCR reagents, the Nkomati samples (4 individuals) could not be amplified for the nuclear S7 intron. No indels were observed in the whole dataset. The data yielded only 16 unique alleles (Table 2.3), with 15 polymorphic sites, of which 14 nucleotides were parsimony informative and one was autapomorphic. The overall allelic diversity (0.608 ± 0.021) and nucleotide diversity (0.0012 ± 0.0001) were lower than for the mtDNA genes.

Table 2.2. The occurrence of combined mtDNA cyt b and COI alleles among sampled localities of *Marcusenius* samples collected in South Africa. Sample sizes are given in brackets. See Table 2.1 for locality codes. No asterisk (*) alleles are those of Pongola Lineage, * are Mhlatuse-Nseleni Lineage alleles, and ** are Limpopo Lineage alleles.

	MT (13)	MK (15)	MG (9)	TN (12)	GL (17)	OL (16)	SD (9)	SB (11)	CC (25)	SZNK (4)	PG (21)	NK (13)	SY (11)	NS (7)	MZ (11)	Total
Allele1									1							1
Allele2								1	18			13	11			43
Allele3									1							1
Allele4											3					3
Allele5											1					1
Allele6							6	8	1							15
Allele7								1								1
Allele8								1								1
Allele9					4	12										16
Allele10						2										2
Allele11									1							1
Allele12					4											4
Allele13					1											1
Allele14					3											3
Allele15					1											1
Allele16					1											1
Allele17					3											3
Allele18	6															6
Allele19	1															1
Allele20	2															2
Allele21	1															1
Allele22	1															1
Allele23	1															1
Allele24	1															1
Allele25											14					14
Allele26									1							1

Table 2.2.: continued...

	MT (13)	MK (15)	MG (9)	TN (12)	GL (17)	OL (16)	SD (9)	SB (11)	CC (25)	SZNK (4)	PG (21)	NK (13)	SY (11)	NS (7)	MZ (11)	Total
Allele27									1							1
Allele28											2					2
Allele29							1									1
Allele30							1									1
Allele31							1									1
Allele32										3						3
Allele33										1						1
Allele34											1					1
Allele35									1							1
Allele36*														2	4	6
Allele37*														2	7	9
Allele38**						2										2
Allele39**				5												5
Allele40**				7												7
Allele41**		6														6
Allele42**		5	3													8
Allele43**		2														2
Allele44**		1														1
Allele45**			6													6
Allele46**		1														1
Allele47*														1		1
Allele48*														1		1
Allele49*														1		1

Table 2.3. The occurrence of S7 intron 1 alleles among sampled localities of *Marcusenius pongolensis*. Sample sizes (phased sequences) are given in brackets. See Table 2.1 for locality codes.

	MT (22)	MK (30)	MG (18)	TN (24)	GL (34)	OL (34)	SD (18)	SB (22)	CC (50)	PG (46)	NK (24)	SY (22)	NS (14)	MZ (22)	Total
Allele1							8	12	26	21	20	10			97
Allele2											4	12			16
Allele3													12	17	29
Allele4	22	29	18	19	32	29	8	8	22	23			2	3	215
Allele5														1	1
Allele6														1	1
Allele7		1			2										3
Allele8				2											2
Allele9				3		1				2					6
Allele10						2									2
Allele11								1							1
Allele12								1							1
Allele13									2						2
Allele14						2									2
Allele15							1								1
Allele16							1								1

The topologies from the phylogenetic analyses (ML and BI) of the mtDNA genes and the combined mtDNA and S7 intron data (2108 nucleotides, 57 parsimony informative and 47 parsimony uninformative nucleotides) were identical. The ML tree of the mtDNA genes is presented with Bayesian Posterior Probability (PP) support values ≥ 0.95 in Figure 2.2a. The model of sequence evolution selected for the mtDNA dataset was the TrN+I model of evolution (Tamura & Nei, 1993), with a specified proportion of invariable sites ($i = 0.8152$). Support (PP = 1.00, Figure 2.2a) was provided for the existence of two deeply divergent major clades: clade A is widespread and includes the type locality of *M. pongolensis* (hereafter referred to as Pongola Lineage). This clade was distributed in parts of the Limpopo River system, and was common in the Incomati, Pongola (type locality) and Kosi river systems. Some well-supported geographic structuring was observed among systems within this clade: a well-supported, monophyletic Limpopo group (PP = 0.97) was retrieved, as were the two divergent groups from the Incomati River system, the one clearly restricted to the highland rivers of the Incomati (Sabie and Sand) and the other restricted to the lowland Crocodile River and the Kosi River system. The second major clade, clade B, included two well-supported and divergent lineages, monophyletic alleles that occur in the Limpopo River system (hereafter referred to as Limpopo Lineage), as well as alleles from the southernmost population, the Mhlatuze and Nseleni Rivers (Mhlatuze-Nseleni Lineage).

The estimated divergence time between the major clades corresponds to the Middle Pleistocene, 1.73 Million years (Ma) ago [95% highest posterior density (HPD): 1.10-2.90 Ma] (Figure 2.2b)]. In clade B, the Limpopo and Mhlatuze lineages have split at the end of the Pleistocene, 0.92 Ma (HPD: 0.30-2.3 Ma) ago (Figure 2.2b). This combined mtDNA dataset phylogeny further indicates that the split within the widespread Pongola Lineage occurred very

recently at 0.68 Ma (HPD: 0.40-1.6 Ma) ago between the Limpopo River system and Incomati-Pongola-Kosi river systems.

The estimated model-corrected genetic divergence sequences between the two major clades were 1.22 - 2.18% for the mitochondrial markers (Table 2.4). The divergences among alleles within the Pongola Lineage ranged from 0.00% within the Kosi system to 0.74% between those in the Limpopo and those from other systems. In clade B, the sequence divergences ranged from 0.00-0.13% between alleles in the Mhlatuze-Nseleni Lineage to 1.5% between alleles from the latter lineage (Mhlatuze-Nseleni Lineage) and Limpopo Lineage.

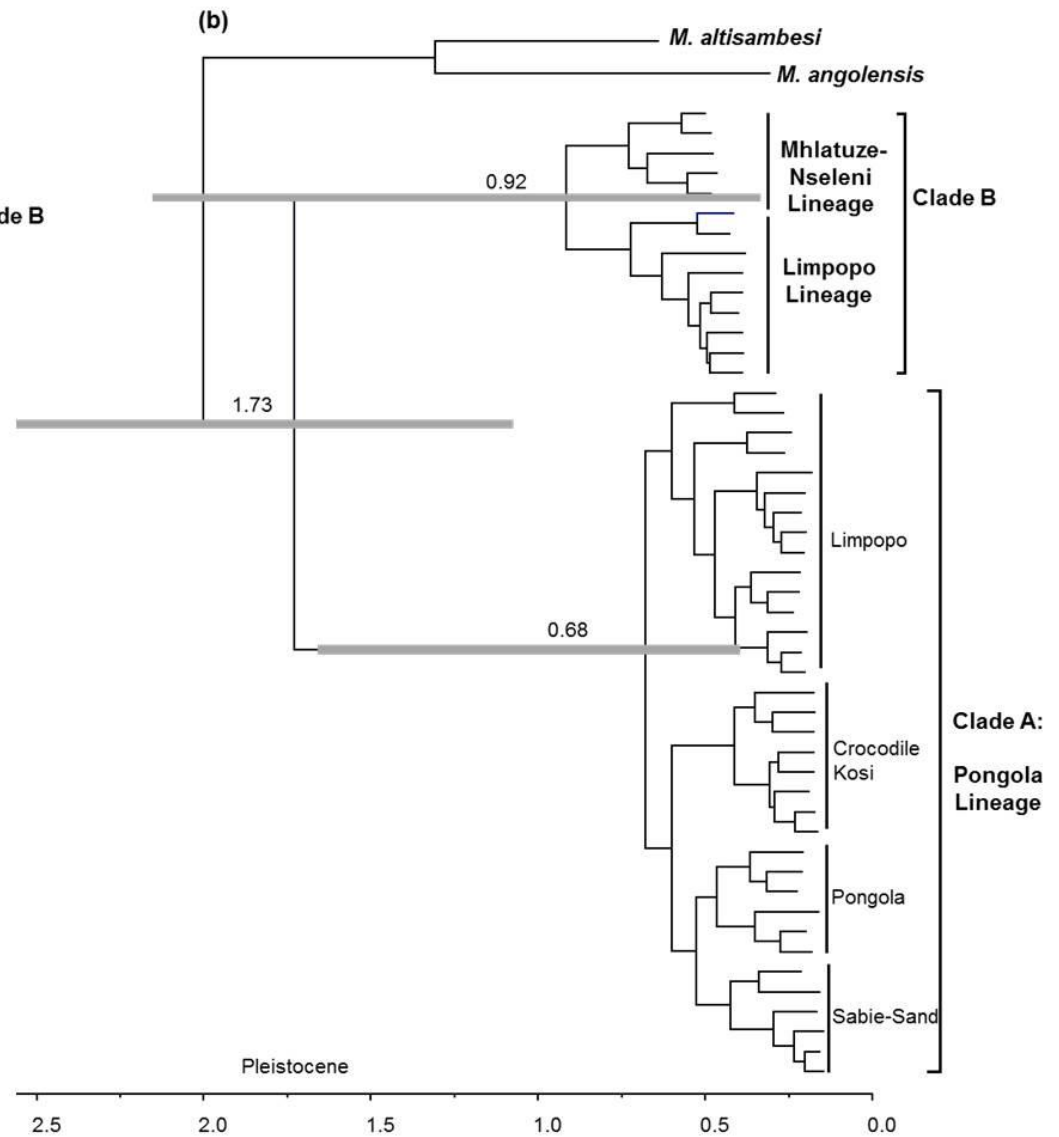
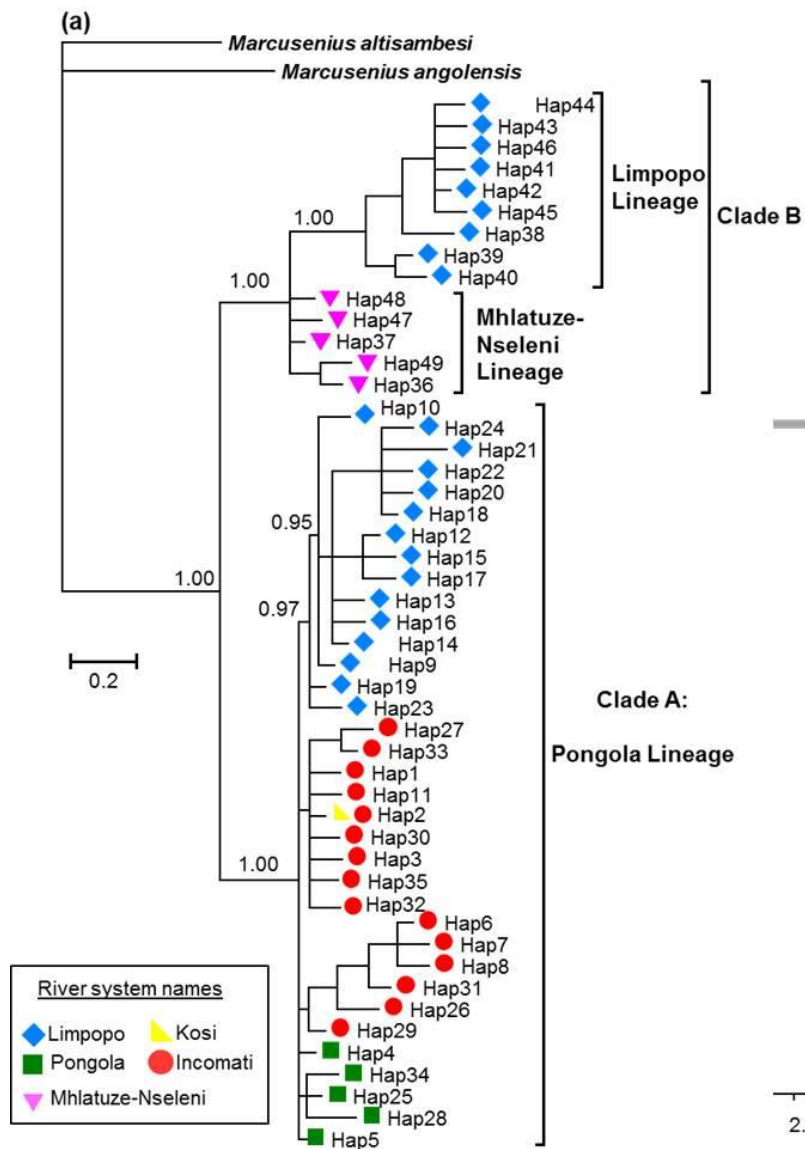


Figure 2.2. (a) Maximum likelihood phylogram (lnL = -2616.09) of relationships among the combined mitochondrial cyt *b* and COI alleles identified in *Marcusenius pongolensis*. Bayesian posterior probabilities are given on the branches. (b) Estimates of divergence times (in Million years ago) for the major lineages inferred using Bayesian coalescent analyses, implemented in BEAST. Values on the nodes represent the estimated mean divergence dates. Bars represent 95% confidence intervals for the divergence estimates.

Table 2.4.: Model-corrected ranges of estimates of evolutionary divergences among the various alleles of *M. pongolensis* based on the combined mtDNA cyt *b* and COI data. No asterisk (*) populations are those of the widespread Pongola lineage, * are Mhlatuse-Nseleni Lineage populations, and ** are Limpopo Lineage populations.

	1	2	3	4	5	6	7	8	9	10	11*	12*	13*	14*	15*
1.Matlabas	-														
2.Grootletaba	0.07-0.53	-													
3.Olifants	0.13-0.60	0.00-0.27	-												
4.Sabie	0.13-0.74	0.20-0.60	0.20-0.47	-											
5.Sand	0.13-0.74	0.20-0.53	0.20-0.33	0.00-0.4	-										
6.Crocodile	0.13-0.67	0.20-0.53	0.20-0.33	0.00-0.27	0.07-0.27	-									
7.Nkomati	0.13-0.74	0.20-0.47	0.20-0.27	0.07-0.27	0.13-0.33	0.07-0.20	-								
8.Pongola	0.13-0.74	0.13-0.53	0.13-0.40	0.07-0.33	0.07-0.27	0.07-0.27	0.07-0.27	-							
9.Nkanini	0.13-0.67	0.20-0.40	0.20-0.27	0.00-0.27	0.07-0.27	0.00-0.07	0.07-0.13	0.07-0.27	-						
10.Siyadla	0.13-0.67	0.20-0.40	0.20-0.27	0.00-0.33	0.07-0.27	0.00-0.07	0.07-0.13	0.07-0.27	0	-					
11.Mhlatuze*	1.22-1.90	1.28-1.56	1.28-1.35	1.29-1.50	1.22-1.42	1.22-1.42	1.22-1.29	1.22-1.35	1.22-1.29	1.22-1.29	-				
12.Olifants**	1.63-2.04	1.63-1.83	1.64	1.70	1.63-1.70	1.56-1.70	1.56-1.63	1.56-1.63	1.56	1.56	0.9-1.5	-			
13.Tshinane**	1.42-2.11	1.49-1.77	1.49-1.63	1.42-1.70	1.49-1.63	1.42-1.63	1.42-1.56	1.42-1.56	1.42-1.49	1.42-1.49	0.9-1.5	0.27-0.34	-		
14.Mokolo**	1.56-2.04	1.63-1.84	1.56-1.71	1.49-1.77	1.49-1.70	1.49-1.70	1.49-1.63	1.49-1.63	1.49-1.56	1.49-1.56	0.9-1.5	0.20-0.27	0.20-0.34	-	
15.Mogalakwena*	1.56-2.18	1.56-1.84	1.56-1.70	1.49-1.77	1.49-1.70	1.49-1.70	1.49-1.63	1.49-1.63	1.49-1.56	1.49-1.56	0.9-1.5	0.20-0.27	0.20-0.34	0.00-0.13	-
16.Nseleni*	1.28-1.90	1.35-1.63	1.35-1.42	1.22-1.49	1.35-1.49	1.29-1.49	1.22-1.35	1.22-1.42	1.22-1.35	1.22-1.35	0.00-0.13	0.9-1.4	0.9-1.4	0.9-1.4	0.9-1.4

2.3.2. Nested clade evolutionary analyses

Of the 49 mtDNA alleles identified, only six (Allele2, 6, 9, 36, 37 and 42) were shared among populations and only one of them was present in more than one river system; Allele2 was present in the Kosi and Incomati River systems (Table 2.2). The nesting designs of the mtDNA alleles presented in Figure 2.3a for the parsimony networks in the nested clade analyses (NCPA) required the removal of two loops. The connection between mitochondrial Allele47 and Allele49 was removed based on the frequency and topological criteria. The second loop was removed based on the topological criterion; Allele46 was connected to a missing allele, but is more likely to be connected to a present rather than a hypothetical allele. The NCPA of the mtDNA indicated significant geographical associations of alleles for eight clades, including the total cladogram (Table 2.5). Allopatric fragmentation was inferred for the geographical distribution of alleles belonging to each of the two major clades identified in the phylogenetic analyses, clade A or the widespread Pongola Lineage (clade 4-1) and clade B for the Limpopo and Mhlatuze lineages (clade 3-3).

The NCPA of the mtDNA evolutionary history of Pongola Lineage (clade 4-1, in Figure 2.3a) revealed at least two distinct geographically isolated minor clades: those from the Limpopo (clade 3-1) and from the Incomati, Pongola and Kosi river systems (clade 3-2) (Figure 2.3a). For the Limpopo clade and among those at the higher nesting level (clade 4-1), there was a strong indication of historical isolation in the form of allopatric fragmentation and/or long distance colonization (Table 2.5). The alleles from the Limpopo River system appear geographically structured and are not found in populations from other systems. Three distinct drainages could be identified in the Limpopo River system: clade 2-1 (the Matlabas drainage), clade 2-2 (the Groot Letaba drainage) and clade 2-3 (consisting of the Olifants [clade 1-5] and Matlabas populations [clade 1-6]). Surprisingly, the network identified genetically-divergent

lineages in the Matlabas drainage (clades 1-6 and 2-1). The NCPA substantiates the results of the phylogeny by also identifying two geographically-distinct clades in the Incomati River system, Sabie-Sand (clade 2-4) and Crocodile-Kosi (clades 2-7). Inadequate geographic sampling in clade 1-9, which included alleles from the Incomati (Nkomati and Sand Rivers) and Pongola river systems precluded any conclusive outcome (Table 2.5).

The mtDNA NCPA network clearly identified and separated the two lineages in clade 3-3 (Figure 2.3a), the Mhlatuze Lineage (clade 2-8) and the Limpopo Lineage (clade 2-9). There were three geographically-distinct drainage systems in the Limpopo Lineage with no shared alleles between them: the Tshinane drainage (clade 1-17), the Olifants drainage (clade 1-18) and the Mokolo-Mogalakwena drainages (clade 1-19). NCPA inferred isolation-type processes and past fragmentation with migration by long-distance colonization to explain the distribution and separation of clades with the Limpopo Lineage (clade 2-9) (Table 2.4).

The two major clades or any other lineages reported for mtDNA dataset could not be identified in the S7 intron 1 network (Figure 2.3b); this is similar to the phylogenetic analyses of this data (figure not shown). There were a high number of shared alleles in the S7 intron dataset (Figure 2.3) with no clear phylogeographic structure. Among the 16 alleles present across South Africa (Table 2.3), only three alleles were shared between systems: Allele1 (shared between the Kosi, Incomati and Pongola), Allele4 (present in the Limpopo and Incomati, as well as the Mhlatuze-Nseleni Rivers) and Allele9 (shared between the Limpopo and Pongola). Six alleles (Alleles1 to 4, and 7 and 9) were shared among populations within systems (Table 2.3). The remaining alleles were unique to a single locality. Three ambiguous branches were broken. The connection between Allele12 and the missing allele connected to Allele11, and the connection between Allele15 and Allele16 were broken under the topography criterion, while the

connection between Allele5 and Allele6 was broken using the frequency criterion (Figure 2.3b). The star-like radiation pattern of unique alleles that are closely related to the high-frequency alleles and the sharing of alleles among geographically-distant river systems are suggestive of ancestral polymorphism within this marker. Only three minor clades, clades 1-2, 2-1 and 3-1 (the total cladogram), showed a significant association with geographic position. The processes inferred from the NCPA for these are shown in Table 2.4. The evolutionary process that best explains the total cladogram was a migration-type process (contagious range expansion), while isolation processes explained the structure and distribution of clades 1-2 and 2-1.

Subsequent to the phylogenetic analyses and NCPA results of the mtDNA dataset, the two major clades (clade A and clade B, Figure 2.2a) were analysed independently for further population genetic structure and demographic histories.

Table 2.5. Evolutionary processes identified through the Nested Clade Analyses using the inference key of Posada & Templeton (2011) for the combined mtDNA *cyt b* and COI data and the nuclear S7 intron data. See Table 2.1 for locality codes and Figure 2.3 for clade information.

Clade	Chi-square Probabilities	Population/s in clade	Inference chain	Migration or Isolation	Inferred evolutionary process
Combined <i>cyt b</i> and COI					
Clade 1-9	0.0136	SD, SZNK, PG	1, 19, 20, 2, 11, 17 (NO)	Inconclusive	Inconclusive outcomes
Clade 2-3	0.0045	MT, OL, GL	1, 19 (NO)	Isolation	Allopatric fragmentation
Clade 2-9	0.0000	MK, TN, OL (all Limpopo populations of Lineage B)	1, 19, 20, 2, 3, 5, 15 (NO)	Isolation	Past fragmentation and/ or long distance colonization
Clade 3-1	0.0000	OL, GL, MT (all Limpopo populations of Lineage A)	1, 2, 3, 5, 15 (NO)	Isolation	Past fragmentation and/or long distance colonization
Clade 3-2	0.0000	SD,SB,CC,SZNK,PG,NK,SY (Incomati, Pongola and Kosi river systems)	1, 2, 3, 5, 15 (NO)	Isolation	Past fragmentation and/or long distance colonization
Clade 3-3	0.0000	Lineage B	1, 19 (NO)	Isolation	Allopatric fragmentation
Clade 4-1	0.0000	Lineage A	1, 19, 20, 2, 3, 5, 15 (NO)	Isolation	Past fragmentation and/ or long distance colonization
Total cladogram	0.0000	All populations	1, 2, 11, 17, 4, 9 (NO)	Isolation	Allopatric fragmentation
S7 intron 1					
Clade 1-2	0.000	All populations, all systems	1,2,3,4(NO)	Isolation	Restricted gene flow with isolation by distance
Clade 2-1	0.000	All populations, all systems	1,2,3,5,15(NO)	Isolation	Past fragmentation and/or long distance colonization
Total cladogram	0.000	All populations, all systems	1,2,11(YES)	Migration	Contiguous range expansion

2.3.3. Phylogeography of the widespread Pongola Lineage

A total of 138 individuals from ten populations across four river systems were analysed for the mtDNA genes. Allelic and nucleotide diversities were greatest within the Limpopo (0.842 ± 0.045) and Incomati river systems (0.763 ± 0.045), and lowest (zero) within the Kosi River system (Table 2.6). Similarly, genetic diversity was also greater for individual populations within the Limpopo River system. Only two alleles were identified in each of the Olifants and Nkomati populations, while the Kosi River system populations (Nkanini and Siyadla) indicated no allelic diversity. Only three alleles were shared among populations and only one of those was found in more than one system: Allele2, which had the highest frequency, occurred in the Incomati (Crocodile and Sabie) and Kosi (Nkanini and Siyadla) river systems; Allele6 was found only in the Incomati River system (Crocodile, Sand and Sabie populations); and Allele9 was shared between the Olifants and Groot Letaba populations in the Limpopo River system. The significant and negative values for the neutrality statistic, Fu's F_s (Table 2.6), indicated the excess of low-frequency alleles expected under a model of sudden population expansion. The unimodal mismatch distributions of the entire widespread Pongola Lineage and that within the Limpopo and the Incomati river systems (results not presented) suggest population expansions. The estimated expansion times for this lineage, and that of river systems are roughly 48 000 – 107 000 years ago, except for the Pongola River system at approximately 16 000 – 28 000 years ago (Table 2.6).

A total of 268 phased S7 intron 1 individual sequences were analysed from the same individuals analysed for mtDNA, and only 12 alleles were identified (Table 2.7). There were low allelic and nucleotide diversities within the lineage, within systems and within populations (Table 2.7). There were a high number of shared alleles within the lineage; only four alleles (allele1 to allele4) were shared across individual populations, river systems and lineages (Table 2.3 and

Figure 2.3b). A significant and negative Fu's F_s for the overall lineage (-6.854, $P < 0.05$) and for the Limpopo system (-4.264, $P < 0.001$) indicated an excess of low-frequency alleles, expected under a model of population expansion.

A Tamura & Nei (1993) model of nucleotide evolution was used to examine population differentiation in Arlequin. This is the most parameter-rich model available in Arlequin and most similar to the TrN+I model of evolution for the mtDNA dataset, identified by Modeltest as the most appropriate for this data set. For the S7 intron data, the same Tamura & Nei (1993) model was used instead of the GTR+I model identified by Modeltest.

Pairwise population Φ_{ST} values for both the mtDNA (range: $\Phi_{ST} = 0.449 - 0.957$) and S7 intron (range: $\Phi_{ST} = 0.016 - 0.710$) data sets revealed significant ($P < 0.05$) genetic heterogeneity among the populations and river systems (Table 2.8). This substantiated the theory that there is strong phylogeographic structure within Lineage A and thus little on-going gene flow. The nuclear S7 intron revealed negative and non-significant Φ_{ST} values for comparisons within the Incomati River system (Crocodile, Sabie and Sand) and between the Incomati and the Kosi populations, while further substantiating significant variation among other systems and strong structure within the Limpopo River system (Table 2.8).

Table 2.6. Genetic diversity measures and neutrality tests based on the mtDNA (cyt *b* and COI) sequences of the Pongola Lineage for the overall lineage, for river systems and for populations. *n* = sample size, *N_A* = number of alleles, *A_D* = allelic diversity, *π_D* = nucleotide diversity.

	<i>n</i>	Polymorphic sites	<i>N_A</i>	<i>A_D</i>	<i>π_D</i>	Fu's <i>F_s</i> (<i>P</i> – value)	Estimated expansion date
Overall Pongola Lineage	138	36	35	0.867 ± 0.021	0.0025 ± 0.0002	-18.519 (< 0.01)	48 000 – 86 000
Limpopo River systems	44	18	15	0.842 ± 0.045	0.1066 ± 0.0607	-4.315 (< 0.05)	60 000 – 107 000
Incomati River systems	49	15	15	0.763 ± 0.045	0.0790 ± 0.0467	-5.409 (< 0.05)	48 000 – 87 000
Pongola River systems	21	5	5	0.547 ± 0.119	0.0265 ± 0.0210	-1.393 (> 0.05)	16 000 – 28 000
Kosi River systems	24	0	1	0	0.000	0	
Populations							
Olifants	13	1	2	0.264 ± 0.136	0.00018 ± 0.00023		
Groot Letaba	17	6	7	0.868 ± 0.045	0.00110 ± 0.00074		
Matlabas	12	12	7	0.795 ± 0.109	0.00190 ± 0.00120		
Crocodile	25	11	8	0.490 ± 0.123	0.00074 ± 0.00056		
Sand	9	5	4	0.583 ± 0.183	0.00100 ± 0.00078		
Sabie	11	6	4	0.490 ± 0.175	0.00073 ± 0.00058		
Nkomati	4	3	2	0.500 ± 0.265	0.00100 ± 0.00089		
Pongola	21	5	5	0.548 ± 0.119	0.00059 ± 0.00048		
Nkanini	13	0	1	0.000	0.0000		
Siyadla	11	0	1	0.000	0.0000		

Table 2.7. Genetic diversity measures and neutrality tests based on the S7 intron sequences of the Pongola Lineage for the overall lineage, for river systems and for populations. n = sample size, N_A = number of alleles, A_D = allelic diversity, π_D = nucleotide diversity.

	Phased n	Polymorphic sites	N_A	A_D	π_D
Overall Pongola Lineage	268	11	12	0.592 $\pm$ 0.019	0.0012 $\pm$ 0.0001
Limpopo River system	88	5	5	0.153 $\pm$ 0.052	0.0003 $\pm$ 0.0005
Incomati River system	90	5	7	0.566 $\pm$ 0.027	0.0001 $\pm$ 0.0009
Pongola River system	44	2	3	0.552 $\pm$ 0.031	0.0009 $\pm$ 0.0009
Kosi River system	46	2	2	0.464 $\pm$ 0.045	0.0015 $\pm$ 0.0012
Populations					
Olifants	32	4	4	0.273 $\pm$ 0.098	0.00074 $\pm$ 0.00075
Groot Letaba	34	1	2	0.114 $\pm$ 0.071	0.00019 $\pm$ 0.00034
Matlabas	22	0	1	0.000	0.0000
Crocodile	50	2	3	0.549 $\pm$ 0.0297	0.00097 $\pm$ 0.00088
Sand	18	2	4	0.634 $\pm$ 0.066	0.00121 $\pm$ 0.00104
Sabie	22	3	4	0.593 $\pm$ 0.070	0.00126 $\pm$ 0.00107
Pongola	46	2	3	0.552 $\pm$ 0.031	0.00097 $\pm$ 0.00088
Nkanini	24	2	2	0.289 $\pm$ 0.103	0.00095 $\pm$ 0.00088
Siyadla	22	2	2	0.519 $\pm$ 0.038	0.00169 $\pm$ 0.00131

Table 2.8. Pairwise Φ_{ST} values among Pongola Lineage populations for the mtDNA (cyt b and COI) sequences (below diagonal), and the S7 intron sequences (above diagonal). Bold values are significant ($P < 0.05$).

	1	2	3	4	5	6	7	8	9	10
1. Matlabas	-	0.016	0.019	0.483	0.440	0.376	*	0.335	0.710	0.493
2. Groot Letaba	0.599	-	0.033	0.486	0.436	0.383	*	0.343	0.701	0.508
3. Olifants	0.710	0.449	-	0.375	0.311	0.319	*	0.278	0.585	0.413
4. Sabie	0.787	0.791	0.878	-	-0.010	-0.002	*	0.013	0.092	0.240
5. Sand	0.746	0.745	0.835	-0.020	-	-0.016	*	-0.013	0.156	0.227
6. Crocodile	0.776	0.736	0.775	0.717	0.637	-	*	-0.012	0.172	0.275
7. Nkomati	0.679	0.679	0.841	0.713	0.590	0.444	-	*	*	*
8. Pongola	0.781	0.745	0.807	0.762	0.690	0.601	0.561	-	0.207	0.272
9. Nkanini	0.809	0.809	0.957	0.869	0.806	-0.015	0.791	0.737	-	0.240
10. Siyadla	0.795	0.796	0.953	0.857	0.788	-0.025	0.765	0.750	0.000	-

* None of the Nkomati individuals could be amplified for the S7 intron marker

The AMOVA of the mtDNA dataset provided additional support for deep genetic structuring across the widespread Pongola Lineage. The river system hierarchy showed populations to be significantly differentiated ($\Phi_{ST} = 0.757$, $P < 0.001$), with 33.17% variation partitioned among the defined system groups ($\Phi_{CT} = 0.332$, $P < 0.05$). The differentiation among populations within systems contributed 42.53% ($\Phi_{SC} = 0.636$, $P < 0.01$) to the total variation. Variation within populations accounted for only 24.30% ($P < 0.001$) of the variation. Using an identical grouping as that in the mtDNA, the S7 intron data revealed low but significant genetic differentiations between populations ($\Phi_{ST} = 0.323$, $P < 0.001$). Only 26.73% of variation was observed between systems ($\Phi_{CT} = 0.267$, $P < 0.001$). Variation among populations within system was much lower at 5.56% ($\Phi_{SC} = 0.076$, $P < 0.05$), due to the sharing of alleles between individuals and populations within systems.

The second analysis, based on higher level clade membership (putative taxonomy) from the mtDNA dataset (i.e. clades 3-1 and 3-2, see Figure 2.3a), substantiated the differentiation of individual populations ($\Phi_{ST} = 0.800$, $P < 0.001$). Much more variation (44.80%; $\Phi_{CT} = 0.448$, $P < 0.001$) was partitioned among clade groups than was partitioned among river system groups. This is also reflected in the decreased percentage of variation attributed to differentiation among populations within these two clades (35.20%; $\Phi_{SC} = 0.638$, $P < 0.001$). Variation among individuals in populations accounted for only 20.00% ($P < 0.001$) of the total variation. A higher level clade membership analysis could not be performed for the S7 intron data. There was no separation between the major lineages and any lower clades identified with the mtDNA data due to the sharing of nuclear alleles.

2.3.4. Phylogeography of clade B (Limpopo and Mhlatuze-Nseleni Lineages)

A total of 58 sequences and 116 phased sequences from six populations were analysed for the mtDNA (cyt *b* and COI, 1496 nucleotides) and the S7 intron (612 nucleotides) datasets, respectively. MtDNA allelic (0.871 ± 0.022) and nucleotide (0.0018 ± 0.0011) diversities were greatest within the Limpopo Lineage and lowest within the Mhlatuze-Nseleni Lineage (0.667 ± 0.083 ; 0.0006 ± 0.0005 , respectively) (Table 2.9). In the mtDNA dataset, 14 alleles were identified (Table 2.2) of which none was shared between the two lineages. Most alleles were confined to a particular population or individual. Only two alleles (Allele36 and Allele37) were shared between two populations, the Mhlatuze and Nseleni Rivers, and a single allele (Allele42) was shared by two populations (Mokolo and Mogalakwena) in the Limpopo Lineage. The significant and negative value of the mtDNA neutrality statistic, Fu's F_s , suggests population expansions (Table 2.9), indicating an excess of low-frequency alleles expected under a model of sudden population expansion. The expansion of the entire clade B is estimated to have happened 46 000 – 82 000 years ago, and only very recently for the Mhlatuze-Nseleni Lineage, 11 000 – 19 000 years ago (Table 2.9).

The overall dataset identified only seven alleles from the 116 analysed phased S7 intron 1 sequences (Table 2.10). The Limpopo Lineage had a low allelic diversity of 0.151 ± 0.055 compared to the Mhlatuze-Nseleni Lineage with 0.310 ± 0.088 . There were a high number of shared alleles between individuals and populations (Table. 2.3).

Table 2.9. Genetic diversity measures and neutrality tests based on the mtDNA (cyt *b* and COI) sequences of clade B, the Limpopo and Mhlatuze-Nseleni Lineages. *n* = sample size, *N_A* = number of alleles, *A_D* = allelic diversity, *π_D* = nucleotide diversity.

River systems	<i>n</i>	Polymorphic sites	<i>N_A</i>	<i>A_D</i>	<i>π_D</i>	Fu's <i>F_s</i> (<i>P</i> -value)	Expansion dates
Overall clade B	58	16	14	0.905±0.014	0.00231±0.00009	-2.093(<0.05)	46 000 - 82 000
Limpopo Lineage	39	9	11	0.871 ± 0.022	0.0018 ± 0.0011	-0.649 (>0.05)	48 000 – 87 000
Mhlatuze-Nseleni Lineage	19	3	5	0.667 ± 0.083	0.0006 ± 0.0005	-1.759 (<0.05)	11 000 – 19 000
Populations							
Mokolo	16	4	5	0.752±0.076	0.00068±0.00055		
Mogalakwena	9	1	2	0.500±0.128	0.00033±0.00035		
Tshinane	12	1	2	0.530±0.076	0.00035±0.00036		
Olifants	2	0	1	0.000	0.000		
Mhlatuze	12	1	2	0.485±0.106	0.00032±0.00034		
Nseleni	7	3	5	0.905±0.103	0.00089±0.00072		

Table 2.10. Genetic diversity measures and neutrality tests based on the S7 intron sequences among *Marcusenius pongolensis* Lineage B populations. *n* = sample size, *N_A* = number of alleles, *A_D* = allelic diversity, *π_D* = nucleotide diversity.

Populations	<i>n</i>	Polymorphic sites	<i>N_A</i>	<i>A_D</i>	<i>π_D</i>
Overall clade B	116	6	7	0.504±0.039	0.00096±0.00011
Limpopo River Lineage	76	4	4	0.151 ± 0.055	0.0003 ± 0.0005
Mhlatuze-Nseleni Lineage	40	2	4	0.310 ± 0.088	0.0006 ± 0.0006
Populations					
Mokolo	30	1	2	0.067±0.061	0.00011±0.00026
Mogalakwena	18	0	1	0.000	0.000
Tshinane	24	3	3	0.366±0.115	0.00089±0.00085
Olifants	4	0	1	0.000	0.000
Mhlatuze	20	2	4	0.398±0.122	0.00079±0.00079
Nseleni	20	1	2	0.264±0.136	0.00044±0.00056

The Akaike Information Criterion in Modeltest identified the HKY+I (Hasegawa *et al.*, 1985) model as best describing the mtDNA dataset of clade B. This model is not available in Arlequin and the Tamura & Nei (1993) model was used to examine population differentiation. Pairwise Φ_{ST} values from the mtDNA dataset (range: $\Phi_{ST} = 0.400 - 0.918$) revealed significantly high genetic differentiation between and within systems (Tables 2.11). Pairwise Φ_{ST} values of the S7 intron dataset also revealed significant ($P < 0.05$) genetic variation between systems (range: $\Phi_{ST} = 0.594 - 0.835$) and very low and sometimes negative, non-significant Φ_{ST} values for comparisons within the Limpopo and Mhlatuze-Nseleni Lineages.

Table 2.11. Pairwise Φ_{ST} values for comparisons of Limpopo and Mhlatuze-Nseleni Lineages populations for the mtDNA (cyt *b* and COI) sequences (below diagonal) and the S7 intron sequences (above diagonal). Bold values are significant ($P < 0.05$).

	1	2	3	4	5	6
1. Mokolo	-	-0.018	0.067	-0.140	0.766	0.831
2. Mogalakwena	0.400	-	0.043	0.000	0.749	0.835
3. Tshinane	0.811	0.878	-	-0.093	0.594	0.635
4. Olifants	0.752	0.882	0.896	-	0.630	0.748
5. Mhlatuze	0.843	0.902	0.870	0.918	-	-0.023
6. Nseleni	0.795	0.844	0.813	0.811	0.028	-

AMOVA provided additional support for deep genetic variation between the systems across the lineage's distributional range in the mtDNA dataset. The river system hierarchy showed populations to be significantly differentiated ($\Phi_{ST} = 0.86$, $P < 0.001$) and 45.45% of variation was partitioned among the defined groups ($\Phi_{CT} = 0.45$, $P < 0.05$). The differentiation among populations within systems contributed 40.08% ($\Phi_{SC} = 0.73$, $P < 0.001$) to the total variation. Variation within populations accounted for a low 14.47% ($P < 0.001$) of the variation. A higher-level clade membership arrangement for the mtDNA would have been similar to that of the river system hierarchy. The S7 intron data also substantiated high genetic differentiation with 73.55% ($\Phi_{CT} = 0.74$, $P < 0.05$) of the variation found between the Limpopo and Mhlatuze-

Nseleni Lineages. Individual populations were also significantly differentiated ($\Phi_{ST} = 0.75$, $P < 0.001$). Differentiation among populations within systems was only 0.25% ($\Phi_{SC} = 0.009$, $P > 0.05$) of the total variation. There was no separation between the major lineages and any lower clades identified using the mtDNA data due to the sharing of nuclear alleles.

2.4. DISCUSSION

2.4.1. Taxonomic implications

This study, together with Kramer *et al.*'s (2007), confirms the presence of deeply divergent lineages of *Marcusenius* in South Africa. In addition to the distribution identified by Kramer *et al.* (2007), the Pongola Lineage, which represents “true” *M. pongolensis* based on morphology (type locality Pongola River) (see Chapter 3), is distributed in parts of the Limpopo (populations are known from Matlabas, Groot-Letaba and Olifants) and is widely distributed in the Incomati and Pongola river systems. The Kosi River system represents the southernmost population of the Pongola Lineage. Both the widespread Pongola Lineage and the Limpopo Lineage occur in sympatry, at least in the Olifants River, where the former was more abundant than the Limpopo Lineage. Strikingly, the distinctness of the Mhlatuze-Nseleni Lineage from the Limpopo Lineage reflects their separation by the vast distance which is likely to represent an important dispersal barrier. The percentage of the mitochondrial sequence divergence between these lineages is 1.4%. Limpopo and Mhlatuze-Nseleni Lineages are distinguished from the widespread Pongola Lineage by significant mitochondrial genetic divergences (mean 2.3%; range 1.7-2.9% *cyt b*).

The oldest split between the three lineages was estimated to have happened in the Early Pleistocene (1.73 Ma), splitting the widespread Pongola Lineage from the group of the

Limpopo and Mhlatuze-Nseleni Lineages. The divergence time estimates suggest that the split of the Limpopo Lineage and Mhlatuze-Nseleni Lineage occurred during the Late Pleistocene, 0.92 Ma (0.30-2.3 Ma) ago. If one considers both the genetic divergence and the divergence times in mitochondrial genes, the average divergence between the Pongola, Limpopo and Mhlatuze-Nseleni Lineages is too high to be consistent with intraspecific variation in mormyrids, especially since the Limpopo and Pongola Lineages were also found in sympatry. These are comparable to species-level divergences of other *Marcusenius* species assessed by Kramer *et al.* (2007) (1.5 – 8.5% cyt *b*), Kramer & Wink (2013) (1.6 - 5.2% cyt *b*) and in Chapter 3 (1.5 - 8.5%).

Morphology shows that Pongola Lineage individuals are more streamlined and have a fusiform shape for active swimming in the presence of strong water currents (Kramer *et al.* 2007). Individuals of the Limpopo and Mhlatuze-Nseleni Lineages have their dorsal and anal fins set well forward which decreases the trunk length and increases body depth. This allows for a longer posterior section of the body, probably to augment speed over short distances, power and manoeuvrability; a similar pattern also seen in deep-bodied *M. altisambesi* of the Upper Zambezi and Okavango systems (Kramer *et al.*, 2007). The individuals from the Mhlatuze-Nseleni Lineage and Limpopo Lineage appeared to be morphologically different, with the dorsal profile of the head of Mhlatuze-Nseleni specimens having a steep, nearly vertical hump in front of the eye. It is slightly concave behind the eye as compared to being straight in the Limpopo Lineage, which also clearly have a short and deep body.

With the exception of the Olifants River, individuals of Limpopo Lineage and Mhlatuze-Nseleni Lineage were only found in the shallower and slower-flowing reaches of rivers, with sandy bottoms and dense vegetation cover, while those of the widespread Pongola Lineage

were found in deep rapids and fast-flowing sections with rocky bottoms and less vegetation cover. These differences in habitat preference could have driven the separation between these lineages, similar to the *Marcusenius* species described by Kramer *et al.* (2007) and *Petrocephalus* (Kramer *et al.*, 2012). The present results are in agreement with previous studies that found strong genetic divergences and morphological differentiation of freshwater fishes among the sub-tropical river systems of South Africa (Engelbrecht & Mulder, 1999; Oosthuizen *et al.*, 2009; Matlala *et al.*, 2010; Dorn *et al.*, 2011). Therefore, results of this study require that the taxonomic status of the three lineages, Pongola, Limpopo and Mhlaluze-Nseleni be investigated.

In contrast to the mitochondrial data and the above preliminary morphological observations, the S7 intron did not resolve the distinction between the lineages. This is probably due to the slow mutation rate of the nuclear marker (Hoelzer, 1997). The S7 intron results showed that either very few mutations exist to differentiate between the two *Marcusenius* lineages or that their separation is too recent to be detected by this nuclear marker. The S7 intron provided limited insight for *M. pongolensis* and the majority of the discussion will, therefore, be based on the mitochondrial dataset, unless indicated otherwise.

2.4.2. Comparative phylogeography of the three *Marcusenius* Lineages

The surveyed populations show a clear phylogeographic pattern. Genetically distinct clades, within all three lineages, have evolved within respective drainage basins. The divergence times between the Limpopo and Mhlaluze-Nseleni Lineages (0.92 Ma ago) and those between river systems within the widespread Pongola Lineage (0.68 Ma ago) coincide with a period characterised by elevated sea levels and the dramatic climatic changes (Trauth *et al.*, 2005) around southern Africa. These extrinsic factors are documented to have significantly affected

the population history of both terrestrial and inland aquatic taxa inhabiting these regions. During the Pleistocene, especially in inter-pluvial periods, a significantly reduced summer rainfall was proposed for the interior plateau of South Africa (Partridge *et al.*, 1997; Trauth *et al.*, 2005; Finch & Hill, 2008), where river systems became isolated, reducing the possibility of gene flow. The divergence times also suggested that the uplift of the Escarpment Axis and the formation of the Ciskei-Swaziland Axis resulted in deep incisions of South African rivers to the east of these axes which is shown to separate the coastal rivers, such as the Pongola, Kosi, Mhlatuze and Nseleni from those far inland (e.g. Limpopo) (Partridge & Maud, 1987; Moore, 1999; Moore *et al.*, 2009).

This was also confirmed by the AMOVA and NCPA showing that the total genetic variance was strongly explained by the existence of two clades within the widespread Pongola Lineage, i.e. the Limpopo vs Incomati-Pongola-Kosi river systems, and the divergences between the Limpopo Lineage vs the Mhlatuze-Nseleni Lineage. Similarly, short-lived killifish, *Nothobranchius furzeri*, populations in the savannah regions of the Incomati and Limpopo river systems are known to have diverged in the Late Pleistocene (0.9 Ma) (Dorn *et al.*, 2011). Although the divergence dates were not available in Matlala *et al.* (2010), this pattern was consistent with those observed in suckermouth fish species of the genus *Chiloglanis* distributed across the Limpopo and the Incomati river systems analysed using mitochondrial genetic markers. These studies suggest that primary freshwater fishes across the sub-tropical river systems of South Africa have been isolated for a long period and allopatric populations in these isolated systems have differentiated from each other.

There was strong evidence for colonization followed by population expansion in the present study, as seen in the NCPA inferences of migration-type processes from the S7 intron data and

the significant and negative values for the neutrality statistics of the mtDNA. This hypothesis is reinforced by the fact that signals of population expansion during the Holocene (48 000 – 107 000 years ago) were detected within the Limpopo and the Incomati River systems. While the population expansion in the Mhlatuze-Nseleni Lineage (11 000 – 19 000 years ago) had occurred well after those of the other lineages, they represent the most recent demographic events that have masked the signal of the earlier expansion estimated. This may imply that the widespread Pongola Lineage, Limpopo Lineage and the Mhlatuze-Nseleni Lineage have concurrently experienced large demographic expansions, following the onset of considerably wetter Holocene climatic conditions in southern Africa (Trauth *et al.*, 2005; Finch & Hill, 2008).

Inter-basin dispersal has been shown to be an important mechanism of facilitating range expansions of freshwater-restricted taxa (Bermingham & Martin, 1998; BurrIDGE *et al.*, 2006; Chakona *et al.*, 2013a, b). While there is no information to suggest that a distinct “Limpopo-Incomati-Pongola” paleoriver system existed, Moore and Larkin (2001) have shown that the Paleo-Zambezi and Limpopo systems drained the majority of the lowland Limpopo Province across a large flat plain in southern Mozambique, before discharging into the Indian Ocean. It is probable that this paleo-system was connected to the Incomati River system across low relief terrain (Jubb, 1971), facilitating the observed distribution of *M. pongolensis* across these river systems. Similarly, *Chiloglanis swierstrai* had several unique mitochondrial alleles that were shared between the Limpopo and Incomati River systems (Matlala *et al.*, 2010), evidence of the possible confluence of these river systems or of inter-basin dispersal. While the present study suggested that this may be true to explain the phylogeography of the widespread Pongola Lineage across different river systems, the Mhlatuze and Nseleni Rivers flow in a different direction, draining the central KwaZulu-Natal region. These two systems, the Limpopo and

Mhlatuze-Nseleni, would not have had a common confluence or shared low relief terrain during a time frame relevant to the evolution of the Mhlatuze-Nseleni Lineage. The occurrence of this lineage in the Mhlatuze and Nseleni rivers cannot be explained by the hypothesis of confluence of rivers, and remains unclear. Regardless, the monophyly of the two lineages, Limpopo and Mhlatuze-Nseleni, and the general level of sequence divergence (1.4% sequence divergence) between them suggest relatively long periods of historical isolation that require further investigation.

The geographic clades observed within the Limpopo River system for both the Pongola Lineage (the Matlabas, Groot Letaba and Olifants-Matlabas drainages) and the Limpopo Lineage (the Tshinane and Mokolo-Mogalakwena drainages) showed remarkable differences in genetic diversity from those observed within the Incomati, Kosi and the Mhlatuze-Nseleni river system (Figure 2.3). Under current climatic conditions, which have remained slightly similar to those of the Late Quaternary (Cockcroft *et al.*, 1987), the Limpopo's drainages are clearly disconnected from the main system by large riverine distances and channels that are often dry (personal observation). According to Matlala *et al.* (2010), isolation of these drainages within the Limpopo River system seems to be determined by many factors, including ecological and physical barriers. The extent to which such factors isolated populations was not clear during their study. In this study, the existence of two distinct lineages within the Matlabas drainage is interpreted as strong evidence for local ecological adaptation during allopatric isolation that was then brought into sympatry as a result of range expansion or secondary contact between drainages or rivers.

The present study also indicated that gene flow between the currently isolated Incomati and Kosi river systems in the widespread Pongola Lineage was very recent. The lack of genetic

differentiation between these systems is inferred to be as a result of recent inter-drainage range expansions, following recent episodic drainage connections, especially across the flood prone areas of southern Mozambique (Skelton, 2001). The Pongola Lineage is common across the entire Kosi River system, especially in the shallow low floodplains and persistent wetlands areas, such that episodic dispersal is possible during flooding or over-topping of rivers and lakes. In other mormyrid studies, it has been shown that *Petrocephalus catostoma* in similar habitats has the ability to lower its metabolic rate and maintain a low oxygen intake to withstand extreme hypoxia in lakes or refugia (Chapman & Chapman, 1998). The species was observed to exhibit an inverted swimming posture during aquatic surface respiration to efficiently pump water from the oxygen-rich surface level, which could be an important intrinsic character for their survival and persistence in swamp refugia. An extended survival period of this species in refugia and sometimes low oxygen pools is known to have played an important role in allowing the current pattern of *P. catostoma* distribution (Chapman & Chapman, 1998). While the present study does not confirm the role of any intrinsic ecological attributes of *Marcusenius* individuals or confirm its possible persistence in refugia or small isolated pools between currently isolated river systems, it could well be a survival strategy that would explain the widespread distribution, especially within the Pongola Lineage.

An important conclusion which can be drawn from the present study is that the divergences in the mitochondrial *cyt b* and COI, and differences in morphology indicate that there is significant genetic and morphological variation to recognise these three *Marcusenius* lineages as different taxa. There is also ecological evidence that supports this view and could provide strong support to the recognition of different species. These lineages could represent different species with similar evolutionary histories, which show that lineages in different river systems were historically fragmented approximately in the same time frame and, to a large degree, have

remained isolated since then. The role of vicariance (e.g. uplifting of the Escarpment Axis and Ciskei-Swaziland Axis) and dispersal in the evolution of the sub-tropical freshwater fish fauna will be completed through intensive phylogeographic investigations that adequately represent the extant diversity of species in this biogeographically unique region of South Africa.

CHAPTER 3: DESCRIPTION OF THREE NEW SPECIES OF *MARCUSENIUS* Gill, 1862
(TELEOSTEI: MORMYRIDAE) FROM SOUTH AFRICA AND MOZAMBIQUE*

ABSTRACT

Morphological and genetic studies of mormyrid fishes belonging to the genus *Marcusenius* from South Africa and Mozambique revealed four species of which three are described as new. *Marcusenius pongolensis* is widespread throughout the Incomati, Pongola and Kosi river systems, and sparsely represented in the Limpopo River system in South Africa. *Marcusenius krameri* sp. nov. is endemic to the Limpopo River system, and is the sister taxon of *M. caudisquamatus* sp. nov. from the Nseleni and Mhlatuze river systems in the KwaZulu-Natal Province of South Africa. The Ruvuma specimens were well differentiated from all southern and eastern African species of *Marcusenius* and are therefore recognised as *M. luombesi* sp. nov. Phylogenetic analysis of the mitochondrial cytochrome *b* gene showed that the three new species and *M. altisambesi* form a monophyletic lineage that is sister to a group containing *M. pongolensis* and *M. macrolepidotus*. *Marcusenius krameri* and *M. luombesi* seem to prefer slow-flowing reaches of rivers, whereas *M. pongolensis* and *M. caudisquamatus* seem to mainly inhabit rapid-flowing sections of rivers with a rocky substrate. A key to the southern African species of *Marcusenius* is provided.

*This work has been published as: Maake, P.A., Gon, O. & Swartz, E.R. (2014). Descriptions of three new species of *Marcusenius* Gill 1862 (Teleostei: Mormyridae) from South Africa and Mozambique. *Zootaxa*, 3780: 455–480

3.1. INTRODUCTION

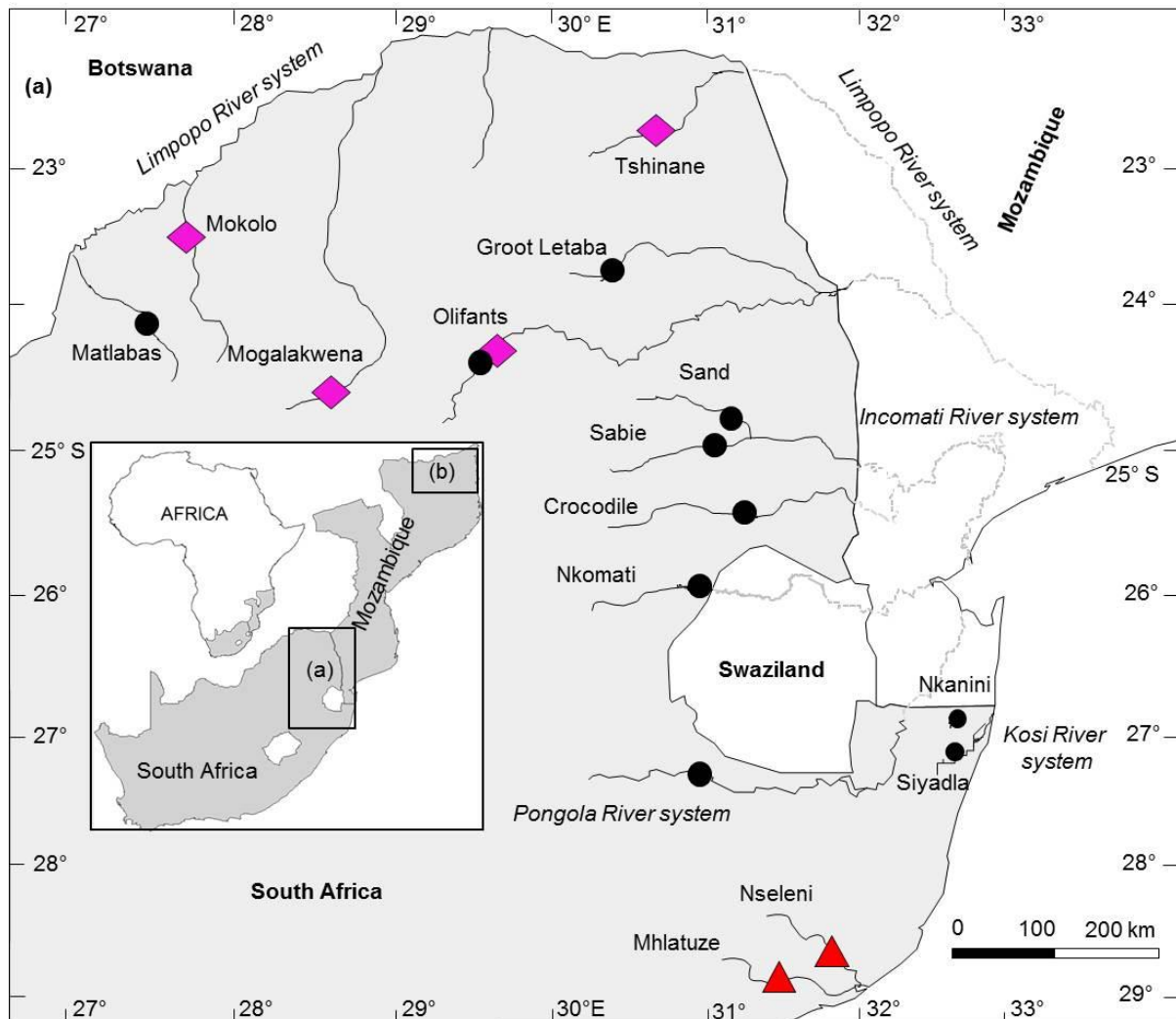
Mormyrids have been of widespread research interest ever since Lissmann (1951) discovered that they produce and receive weak electric signals for the purposes of mating, communication and object-location (e.g. Lissmann & Machin, 1958; Bennett, 1971; Kirschbaum, 1975; Szabo & Fessard, 1974; Hopkins, 1981, 1986, 2009; Kramer & Westby, 1985; Bastian, 1986; Hanika & Kramer, 2000; Werneyer & Kramer, 2005; Carlson *et al.*, 2011). The electric organs of these fishes, located in the caudal peduncle, generate an electric signal, referred to as the electric organ discharge (EOD) that is often species-specific and has been used as a taxonomic character in systematic studies of this group (Alves-Gomes & Hopkins, 1997; Boden *et al.*, 1997; Moritz *et al.*, 2009, Kramer *et al.*, 2012, 2013; Kramer, 2013a).

The genus *Marcusenius* is the largest genus of the Mormyridae, currently with 38 valid species widely distributed from the Nilo-Sudan (Boden *et al.*, 1997) to South Africa (Kramer *et al.*, 2007). Since the systematic revision by Taverne (1971b, 1972), many new species have been described (e.g. Bigorne & Paugy, 1990; Boden *et al.*, 1997; Kramer *et al.*, 2007). Kramer (2013b) added a new species from West Africa, while Kramer and Wink (2013) described another from the Cunene River on the border between Angola and Namibia. Kramer *et al.* (2007) discovered that there are at least five species in southern Africa rather than a single widespread species. These were *M. macrolepidotus* from the Lower Zambezi River, *M. angolensis* from Cuanza River in Angola and *M. pongolensis* from the Incomati, Pongola, Kosi and Mhlatuze rivers in South Africa; they also described *M. devosi* from Kenya's Tana River and *M. altisambesi* from the Upper-Zambezi and Okavango river systems.

The earliest record of the genus in South Africa was a single specimen from the Waterberg District in the Limpopo River system identified as *Marcusenius macrolepidotus* by Gilchrist and Thompson (1913) (Figure 3.1a). The next specimen, collected in the upper Pongola River was described as *M. pongolensis*, the southernmost species of the genus, by Fowler (1934). Crass (1960) considered Fowler's species as *M. macrolepidotus* because it differed from typical *M. macrolepidotus* only in having a more slender body. He justified the synonymy by arguing that other species with a similar distribution are commonly more slender in headwaters than further downstream. No other work on *Marcusenius* was done in South Africa until Kramer *et al.* (2007) demonstrated that *Marcusenius* from southern and eastern Africa has more diversity than previously thought and emphasized the importance of a critical re-evaluation of the populations from these regions. For example, they showed that Limpopo River specimens (Figure 3.2b) differed from other South African samples in at least five morphological characters and cannot be referred to as *M. pongolensis* (Figure 3.2a and c). Their findings implied that species-level differentiation may exist in South African *Marcusenius*, but Kramer *et al.* (2007) suggested that more information was required before further descriptions could be made.

Kramer *et al.* (2007) also showed that some populations of *M. macrolepidotus*, such as those from the Ruvuma River system in northern Mozambique (Figure 3.1b), were morphologically different from the South African ones, indicating that *M. macrolepidotus* might contain more unrecognized taxa (Figure 3.2d). In this study we provide morphological and genetic evidence to show that there are three species of *Marcusenius* that occur in South Africa and that specimens from the Ruvuma are different from these and other species from elsewhere in southern and eastern Africa. Three new species are described (*M. krameri* sp. nov., *M. caudisquamatus* sp. nov., and *M. luombesi* sp. nov.) and we provide a key to species of the

genus *Marcusenius* that occur in southern and eastern African river systems, including the Tana, Ruvuma, Zambezi, Cuanza, Cunene, Limpopo, Pongola and Mhlatuze river systems.



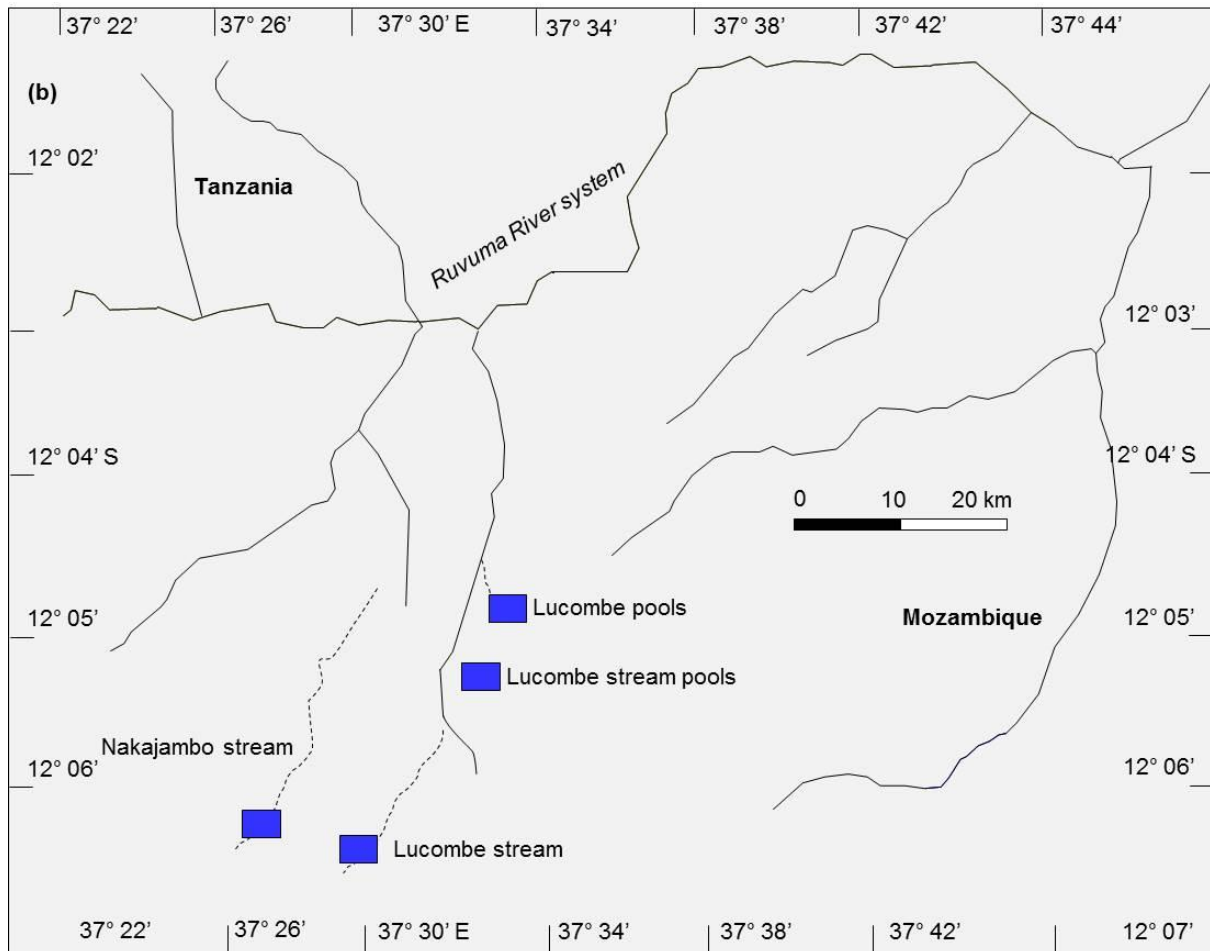


Figure 3.1. (a) Map of South Africa showing collection sites of *Marcusenius pongolensis* (black dots), *M. krameri* sp. nov. (pink diamonds) and *M. caudisquamatus* sp. nov. (red triangles), and (b) northern Mozambique showing the distribution of *M. luombesi* sp. nov. (blue squares) with streams indicated by broken lines. Names of major river systems are indicated in italics.

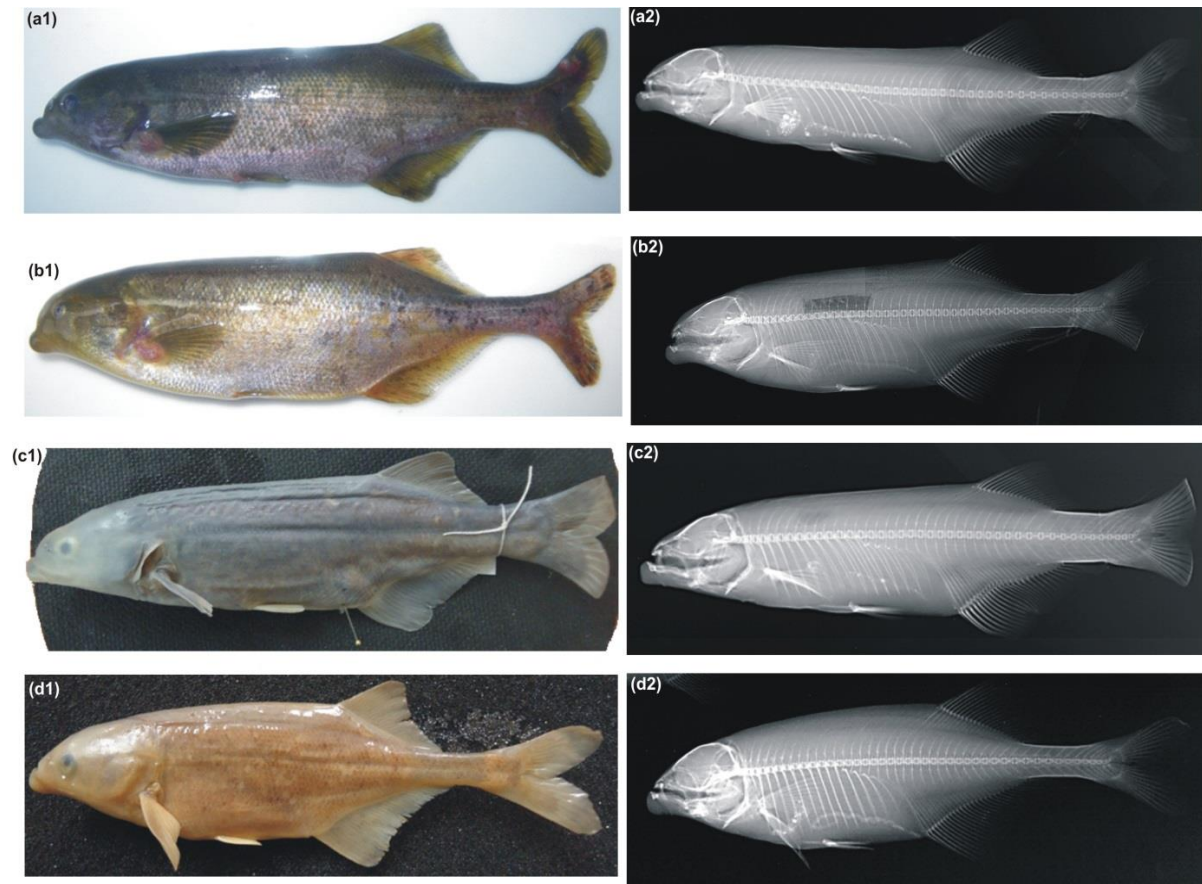


Figure 3.2. Photographs and radiographs of preserved specimens of four species of *Marcusenius*: (a1 and a2) *M. pongolensis*, SAIAB 188296, 165 mm SL, male, Pongola River, (b1 and b2) *M. krameri* sp. nov., SAIAB 188295, 116.21 mm, male, holotype, Mogalakwena River, (c1 and c2) *M. caudisquamatus* sp. nov., SAIAB 88684, 166 mm SL, male, holotype, Mhlatuze River, and (d1 and d2) *M. luombesi* sp. nov., SAIAB 73884, 74.0 mm SL, male, holotype, Lucombe River.

3.2. MATERIALS AND METHODS

3.2.1. Sample collection

Marcusenius specimens were caught using electro-fishing and the fish were euthanized with an overdose of 2-phenoxy-ethanol. Muscle tissue samples were placed in 99% ethanol and deposited in the Biomaterial Bank of the South African Institute for Aquatic Biodiversity (SAIAB, Grahamstown, South Africa). Voucher specimens collected during the course of this study were accessioned with SAIAB, BMNH (Natural History Museum) [formerly British Museum (Natural History), London], MRAC (Musée Royal de l'Afrique Centrale, Tervuren), and USNM (National Museum of Natural History, Smithsonian Institution) [formerly United States National Museum, Washington D.C]. Comparative materials from the Ruvuma System, Mozambique, and of other southern African species were borrowed from SAIAB (Appendix 3.1). Abbreviations used to represent institutions and collections cited follow Sabaj Pérez (2013), with the exception of RUSI which has been replaced by SAIAB.

3.2.2. DNA sequencing and phylogenetic methods

Genomic DNA was isolated from the ethanol-preserved tissues using a purification kit (Promega). Amplification of a partial section of the mitochondrial cytochrome *b* gene (cyt *b*) was achieved with the primers GluF (Machordom & Doadrio, 2001) and H15917 (Kramer *et al.*, 2003) for comparison to published data. A partial region near the 5' end of the cytochrome *c* oxidase subunit I gene (COI) was amplified with the primers VF2-T1 and VR1-T1 (Ivanova *et al.*, 2007). COI sequences were generated to aid in future species identification using DNA barcoding (Hebert *et al.*, 2003) and the assignment of genotypes (see Chakrabarty, 2010). Amplification was performed in 25 µl volumes containing ~1-3 µl (depending on the DNA

concentration) template DNA, 1 x reaction buffer, 25 mM MgCl₂, 0.125 mM of dNTPs (Promega), 0.2 units of Super-therm® Taq DNA polymerase and 25 pmol of the forward and reverse primers. Conditions for the amplification of COI were 3 minutes at 94 °C, then 38 cycles of 30 seconds at 94 °C, 30 seconds at 54 °C and 50 seconds at 72 °C, finishing with 7 minutes at 72 °C. Cyt *b* was amplified under the following conditions: 4 minutes at 94 °C, 31 cycles of 45 seconds at 94 °C, 1 minute at 52 °C and 2 minutes at 72 °C, finishing with 5 minutes at 72 °C. Prior to sequencing, PCR products were purified with the QIAquick PCR purification kit (QIAGEN, Valencia, California), using the BigDye Terminator 3.1 cycle-sequencing ready reaction kit (Applied Biosystems, Foster City, California). Cycle-sequencing products were precipitated using an ethanol precipitation procedure (Sambrook & Russell, 2001). Final products were sequenced at Macrogen Inc. (Seoul, South Korea) and at Rhodes University (Grahamstown, South Africa).

Additional cyt *b* sequences for *M. macrolepidotus*, *M. altisambesi* and *M. devosi*, published by Kramer *et al.* (2007), were available for comparison on GenBank. Cyt *b* sequences of *Campylomormyrus compressirostris* (Genbank no. DQ630644) (Feulner *et al.*, 2006) and *M. stanleyanus* (SAIAB 77713) (from SAIAB fish database) of the subfamily Mormyrinae were used as outgroups to root the phylogenetic tree. Both COI and cyt *b* sequences of *Marcusenius* were aligned using ClustalX2.1 (Larkin *et al.*, 2007). A model of nucleotide substitution that best fitted the cyt *b* sequence data was selected from 56 such models with the Akaike Information Criterion test in Modeltest 3.7 (Posada & Crandall, 1998). These parameters were used in a maximum likelihood (Felsenstein, 1981) estimation of phylogenetic relationships among the *Marcusenius* sequences in the program PAUP* (Swofford, 2002). A parsimony analysis was used to assess branch support with 10 000 non-parametric bootstrap replicates. Each bootstrap replicate was performed with 10 replicates of random taxon addition. The

parsimony analysis was performed using heuristic searches, tree bisection–reconnection branch swapping and 1 000 random additions of taxa. COI sequence data are only presented to aid in future species identification using DNA barcoding and the assignment of genotypes (Chakrabarty, 2010).

3.2.3. Morphology and statistical analyses

Morphological measurements were done on 113 specimens using the landmarks shown in Figure 3.3 and summarised in Table 3.1. All measurements were made to the nearest 0.1 mm using a digital vernier caliper. Vertebrae were counted from radiographs taken by a digital x-ray system at SAIAB and do not include the urostyle. Gill rakers (GR) were counted on both the anterior and posterior sides of the first gill arch. This character is not commonly used in mormyrid identification, due to the reduced opening of the operculum. As this required dissection, these were counted in only a small number of specimens to minimise damage to museum specimens. GR counts include all rakers, developed and rudimentary, and are presented using the following formula: GR (a-b) + (c-d) = (x-y): where (a-b) is the range of the counts of the upper limb; (c-d) is the range of the counts on the lower limb; and (x-y) is the observed total range. GR_t is the total gill raker count. In the descriptions below, data for the holotypes are given first followed by the range for paratypes in brackets. Character information required for the identification key of species outside South Africa was collected from published literature, except for the total vertebrae and gill raker counts, which were based on material loaned from SAIAB (Appendix 3.1).

The sex of each preserved specimen was determined by examining the body profile along the base of the anal fin (following Pezzanite & Moller, 1998): mature males have a strongly indented anal fin base and juveniles or mature females have a nearly straight anal fin base. No

significant sexual dimorphism was observed in the selected morphometric characters; therefore, the data analysis was performed without taking the sex of the individuals into consideration.

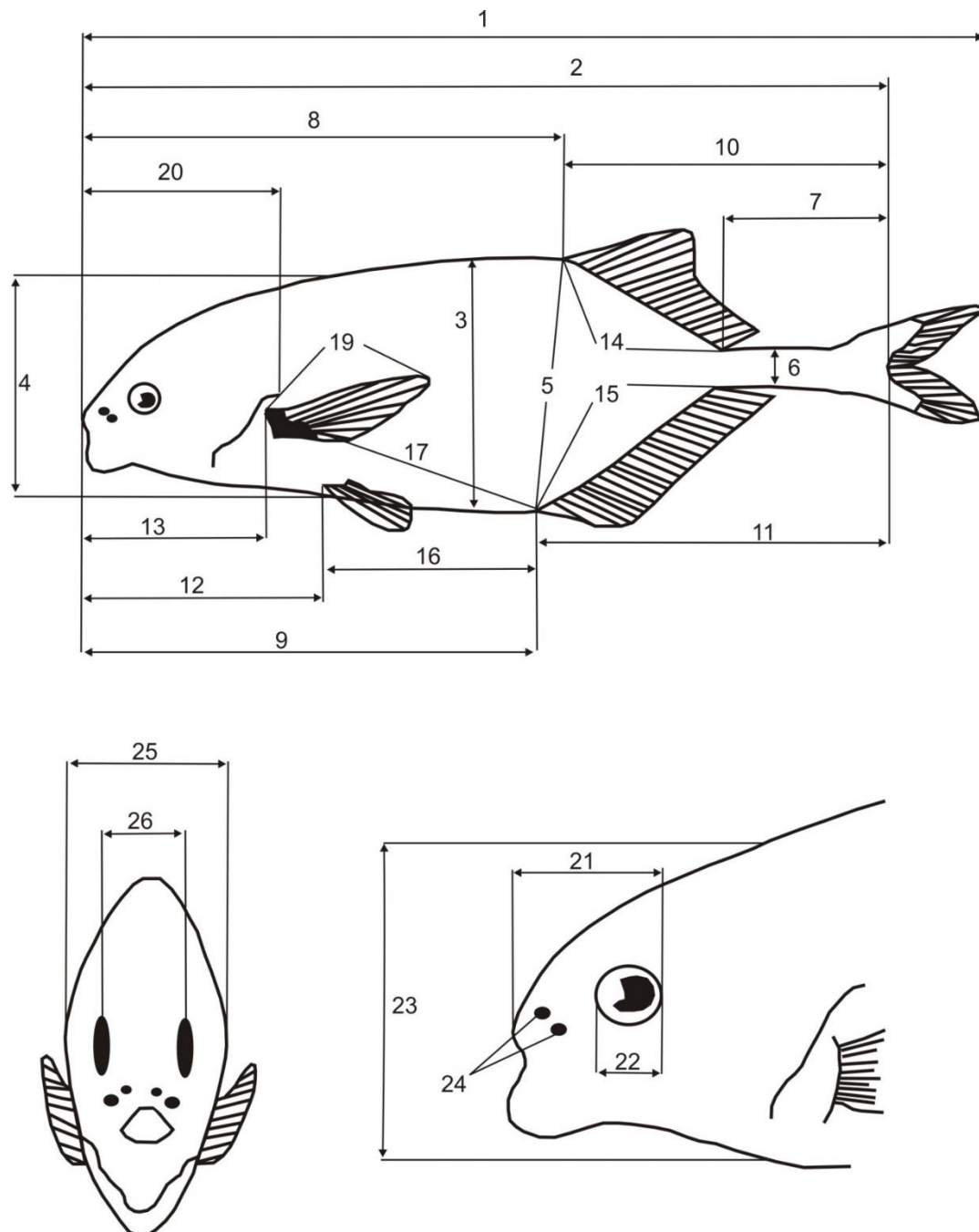


Figure 3.3. Morphological measurements for *Marcusenius* species used in the present study. Character definitions are given in Table 3.1. Illustration adopted from Boden *et al.* (1997).

Table 3.1. Definitions of the morphometric and meristic characters of *Marcusenius* (see also Figure 3.3).

No. in Fig. 3	Abbreviation	Full Name	Definition
1	TL	Total Length	Distance from tip of upper lip to end of caudal fin
2	SL	Standard Length	Distance from tip of upper lip to the end of the hypural plate
3	mBD	Middle Body Depth	The greatest vertical distance across body
4	vBD	Body Depth	Vertical depth of the body from the origin base of the pelvic fin
5	dAD		Vertical depth from the origin base of anal fin to origin base of dorsal fin
6	CPD	Caudal Peduncle Depth	The least vertical distance across the caudal peduncle
7	CPL	Caudal Peduncle Length	End of anal fin base to midbase caudal fin
8	PDL	Predorsal Length	Distance from tip of upper lip to dorsal fin origin
9	PAL	Preanal Length	Distance from tip of upper lip to anal fin origin
10, 11	pD and pA		Distance from dorsal and anal fin origin to end of caudal peduncle
12	pVL	Prepelvic Length	Distance from anterior tip of upper lip to anterior base of pelvic fin
13	pPL	Prepectoral Length	Distance from anterior tip of upper lip to anterior base of pectoral fin
14	LD	Length of Dorsal Fin	Distance from origin to end dorsal fin base
15	LA	Length of Anal Fin	Distance from origin to end anal fin base
16	dVA	Distance Pelvic to Anal Fin	Distance from origin of pelvic fin to origin of anal fin
17	dPA	Distance Pectoral to Anal Fin	Distance from origin of pectoral fin to origin of anal fin
18	dPV	Distance Pectoral to Pelvic fin	Distance from origin of pectoral fin to origin of pelvic fin
19	LP	Length of Pectoral fin	Distance from anterior to posterior end of the pectoral fin
20	HL	Head Length	Distance from tip of the upper lip to furthest bony edge of the operculum
21	LSN	Length of Snout	Distance from tip of upper lip to posterior border of eye
22	ED	Eye Diameter	Horizontal diameter of the eye defined by orbital rims
23	HD	Head Depth	Greatest vertical distance across the head, usually taken below base of gill opening
24	LN	Length between nares/ nostrils	Smallest distance between the pair of nares of one side (from centre to centre)
25	HW	Head Width	Distance between the posterior ends of the opercula
26	IOW	Interorbital Width	Distance between centre of eyes
	Counts		
	LSS	Scales on Lateral Line	Number of scales in longitudinal series along the lateral line; level of accuracy, ± 2 scales
	nD and nA		Numbers of dorsal and anal fin rays
	SPc		Number of circumpenducular scales counted at the narrowest place
	nTU and nTL		Numbers of teeth on the upper and lower jaws
	TV	Total vertebrae	
	GRt	Total gill raker count	

Principal component analyses (PCA) were conducted using measurement data standardised as a ratio of the standard length (SL), or as a ratio of head length (HL) for all head measurements. PCA was used to test differences in body shape among groups and populations, because it does not require *a priori* assumptions about taxonomic groups. Statistical analyses were performed using PAST v. 2.17c (Hammer *et al.*, 2001) and JMP v. 10.0.2 (SAS Institute Inc., 2007) software. The component loadings, i.e. the principal component structure, were used for interpreting the principal components in terms of a character's significance (McGarigal *et al.*, 2000). The following levels of significance were used to assess the significance of component loadings (McGarigal *et al.*, 2000; Kramer *et al.* 2007): loadings >0.32 or <-0.32 are poor, >0.45 or <-0.45 fair, >0.55 or <-0.55 good, >0.63 or <-0.63 very good, and >0.71 or <-0.71 are excellent. These account for 10%, 20%, 30%, 40% and 50% of the variance in the component, respectively. Multivariate analysis of variance (MANOVA) was used to test for significant differences between samples from different systems or groups. The MANOVA discrimination power among groups or species was compared using Wilks' λ , with smaller values indicating higher discrimination power. Analyses of variance (ANOVA) were performed to test hypotheses of no difference between groups or species for each character individually.

3.3. RESULTS

3.3.1. DNA sequence analyses

For the *cyt b* dataset, the transversional model (TVM) with a gamma distribution (α value of 0.4383) found in Modeltest fitted the data best and was used in the maximum likelihood analysis and estimation of genetic distances. The resulting phylogenetic reconstruction shows that *M. krameri* sp. nov. and *M. caudisquamatus* sp. nov. are sister taxa that are each clearly separated from other species (Figure 3.4). *Marcusenius lucombesi* sp. nov. is only moderately supported (72% bootstrap) as being related to the lineage that includes *M. krameri* and *M.*

caudisquamatus. *Marcusenius altisambesi* is the closest related species to the three newly described species, forming with them a lineage that is separate from a group that includes *M. macrolepidotus* and *M. pongolensis* (97% bootstrap support). *Marcusenius devosi* was basal to all the other taxa. Sequence divergences between *M. krameri* and *M. caudisquamatus*, and between the latter two species and *M. lucombesi* were 0.9-2.4% and 1.0-2.9%, respectively (Table 3.2). All the new species differ by at least 2.0-6.8% divergence from all other species in the phylogeny. This level of divergence was much larger than observed within the new species, *M. krameri* (0.1-0.5%), *M. caudisquamatus* (0.0-0.5%) and *M. lucombesi* (0.2-0.5%).

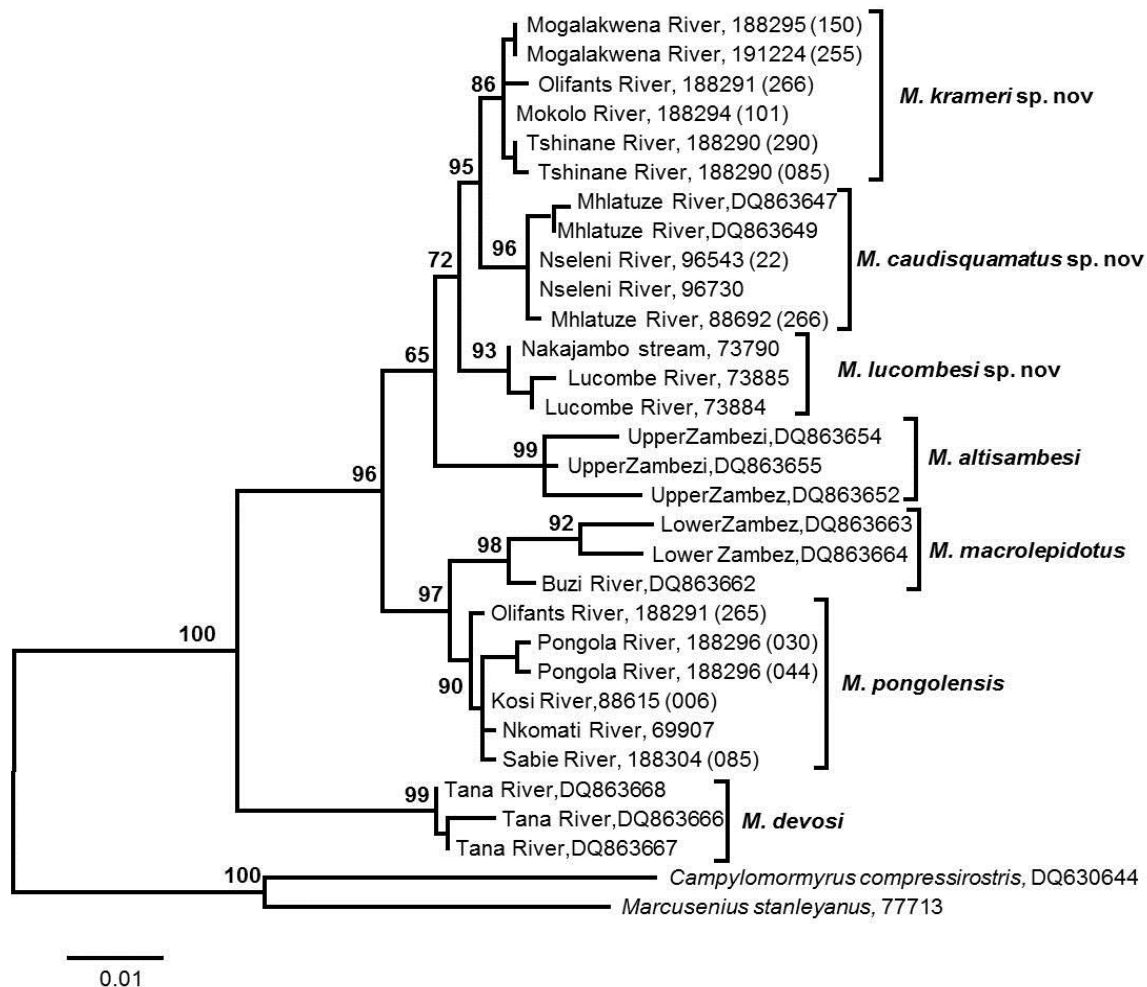


Figure 3.4. Maximum likelihood phylogram (lnL = 2537.87) based on mitochondrial cytochrome *b* sequences from the present study and GenBank using *Marcusenius stanleyanus* and *Campylomormyrus compressirostris* as outgroups. Values above the branches are bootstrap support based on 10 000 parsimony bootstrap replicates. Locality names, SAIAB and Genbank accession numbers are indicated for each species individually.

Table 3.2. Range of genetic divergences within and among nine mormyrid species based on mitochondrial cytochrome *b*.

	Within	1	2	3	4	5	6	7	8
1. <i>M. devosi</i> (3)	0.1-0.7								
2. <i>M. macrolepidotus</i> (3)	1.5-2.4	4.8-6.6							
3. <i>M. pongolensis</i> (6)	0.1-0.8	5.4-5.5	1.3-3.2						
4. <i>M. altisambesi</i> (3)	0.9-1.7	4.8-6.2	3.3-5.6	2.8-4.2					
5. <i>M. caudisquamatus</i> (5)	0.0-0.5	5.3-6.8	2.8-5.8	2.3-4.3	2.2-3.5				
6. <i>M. krameri</i> (6)	0.1-0.5	5.0-5.8	2.7-4.8	2.0-2.7	2.0-3.1	0.9-2.4			
7. <i>M. lucombesi</i> (3)	0.2-0.5	5.8-5.6	2.8-4.8	2.8-3.3	2.0-3.1	1.2-2.9	1.0-1.7		
8. <i>M. stanleyanus</i>	-	9.3-9.8	10-11.4	9.5-10.0	10.0-10.6	9.7-10.4	9.7-10	9.4-9.7	
9. <i>Campylomormyrus compressirostris</i>	-	9.4-9.9	10-11.5	9.7-10.2	10.1-10.8	10.4-10.6	10.0-10.4	10.2-10.4	7.9

3.3.2. Morphological comparisons

The PCA showed that the first seven components of 24 variables were significant (i.e. eigenvalues greater than the Jolliffe cut off = 0.7) and captured 80.13% of the variance in the dataset; hence, they were very successful in reducing redundancy (Appendix 3.2). Scatter plots of PC1-PC2 explained most of the variation present and showed four non-overlapping groups (Figure 3.5a) referred to as: *M. krameri* ($N = 35$), endemic to the Limpopo, *M. caudisquamatus* ($N = 15$) from the Mhlatuze and Nseleni rivers, *M. luombesi* ($N = 13$) for the specimens from Luombesi River, tributary of the Ruvuma River system, and the widespread *M. pongolensis* which includes the holotype ($N = 49$). None of the four groups encompassed an outlier from any other group. The holotype of *M. pongolensis* was within all the other “*pongolensis*” specimens.

Principal component 1 (PC1, Appendix 3.2) for the whole data set explained much of the variance (eigenvalue = 7.06; % variance = 29.44) and was positively loaded by pD, pA, nD, nA (levels of significance: “excellent”), mBD (“very good”), dAD, LD, LA (all “good”), vBD (“fair”), CPL and HL (both “poor”). It was negatively loaded by dPA, (“excellent”), CPD/CPL and dVA (“very good”), PAL and SPc (all “good”), and PDL, dPV and LSN/HL (all “poor”). PC1, thus, represents a morphological gradient for a long LD and LA and a deep body, together with a short anterior body and a reduced head and snout lengths. These characters loaded heavily for both *M. krameri* and *M. luombesi* on the PCA biplot (not shown). PC2 (eigenvalue = 4.78, % variance = 19.92) was positively loaded by pVL (“excellent”), PAL (“very good”), vBD, dAD, mDB, CPD/CPL, PDL, pPL (all “good”), SPc, dPV, LA (“fair”), and negatively by IOW/HL (“fair”), and ED/HL and LSN/HL (“poor”). These characters loaded well for *M. pongolensis* and *M. caudisquamatus* on the PCA biplot. PC2 therefore represents a gradient for a long anterior part of the body with a moderately deep body, together with a deeper caudal

peduncle and higher SPc counts. PC3 captured an additional 8.78% of the variance (eigenvalue = 2.11) and was positively loaded by LD, dVA (“fair”) and several “poor” characters (dPA, nD, nA, HW/HL); negatively by HL (“excellent”) and CPL (“good”).

A MANOVA (Wilks’ $\lambda = 0.0009704$; $F_{78, 249.1} = 29.3$, $p < 0.0001$) on all morphometric and meristic characters also rejected the hypothesis of no morphological difference among the four groups (Table 3.3). The subsequent ANOVA post hoc tests using Tukey-Kramer’s pairwise comparisons (Tukey, 1953; Kramer, 1956) showed that all but six characters (CPL, LP/HL, HD/HL, LN, nTL and nTU) contributed to this result. *Marcusenius pongolensis* differed significantly from *M. krameri* in ten characters, and from *M. caudisquamatus* in 14 characters. The head measurements and those between fins (pVL, dVA, dPA, dPV) are not significantly different between *M. pongolensis* and *M. krameri*. Dorsal and anal fin rays (nD and nA), pPL, LD and LA were not significantly different between *M. krameri* and *M. caudisquamatus*, indicating their close relationship and similarity. *Marcusenius lucombesi* differed significantly in all characters from *M. pongolensis* except pVL and from *M. caudisquamatus* for all except LA and HL. Ten different characters separated *M. lucombesi* and *M. krameri* (Table 3.3).

When comparing the present samples of *M. pongolensis* from different systems, noticeable intraspecific variation emerged (Table 3.4; Figure 3.5b). As demonstrated by PCA of the morphometric data of these samples [from the Pongola River system ($N = 7$), Incomati River system ($N = 13$), Kosi River system ($N = 10$), Nkomati River system ($N = 4$) and Limpopo River system ($N = 15$)], PC1 captured 24.57% of the variation in the data set, PC2 10.65%, PC3 9.01% and PC4 8.35%. The overall MANOVA result rejected the hypothesis of no differentiation among systems (Wilks’ $\lambda = 0.0003659$; $F_{108, 74.02} = 4.346$, $p < 0.0001$), and this

was supported by significant univariate ANOVA results for characters that were significantly different only between the Kosi and other river systems (Table 3.4).

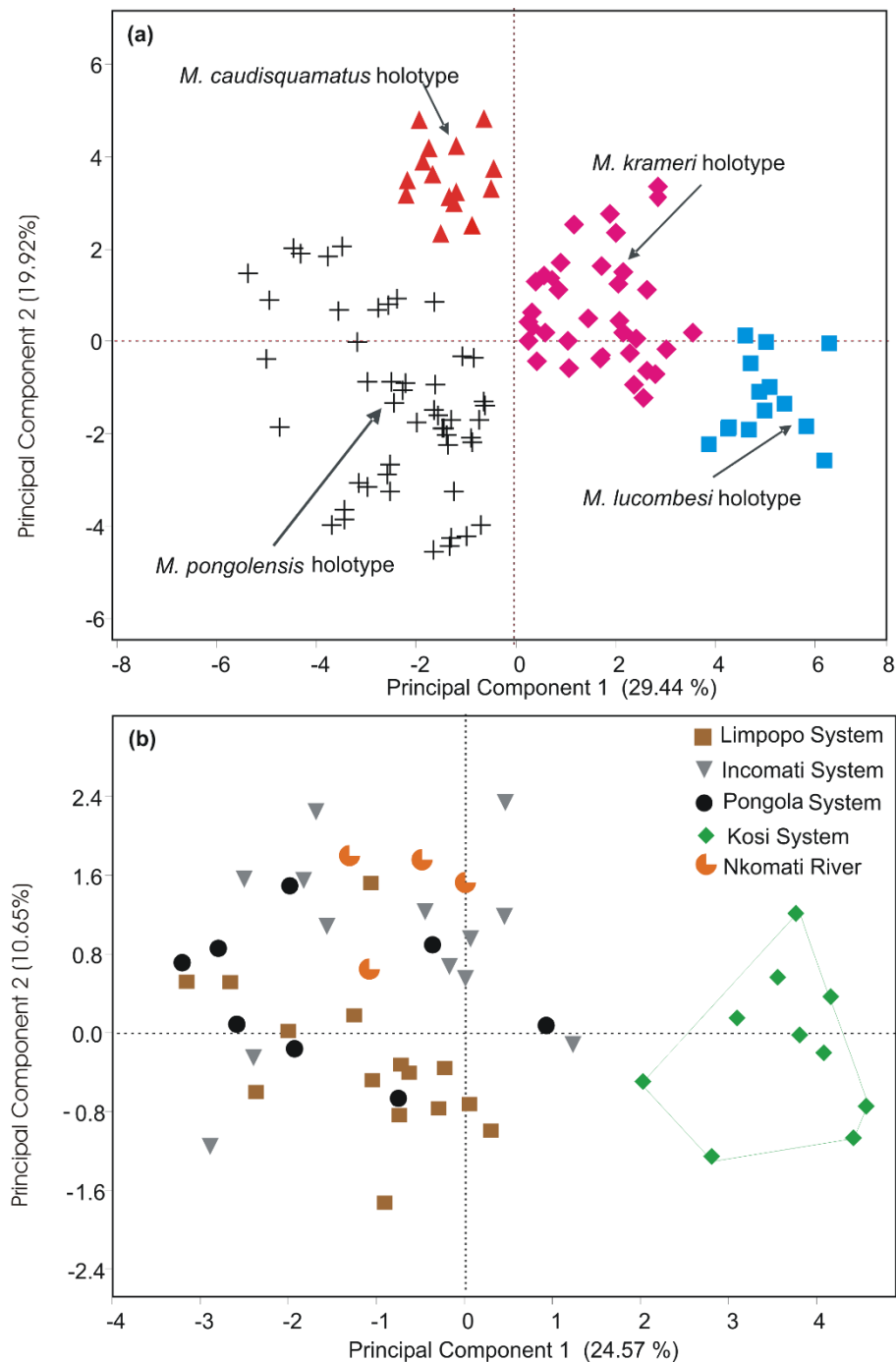


Figure 3.5. Principal components analysis showing differentiation: (a) between species *Marcusenius pongolensis* (black dot), *M. krameri* (Pink diamond), *M. caudisquamatus* (Red triangles), *M. luombesi* (Blue squares); (b) within *M. pongolensis* of different South African systems.

Table 3.3. Multivariate analysis of variance (MANOVA), followed by analyses of variance (ANOVAs) for individual morphological characters, and pairwise post-hoc tests comparison among four species of *Marcusenius*: *M. caudisquamatus*, *M. krameri*, *M. lucombesi* and *M. pongolensis*, *p* values > 0.05 are not shown. See Table 3.1 for characters definitions.

	nD	nA	SCP	vBD	mDB	dAD	CPD/ CPL	PDL	PAL	pD	pA	pPL	pVL	dVA	dPA	dPV	LD	LA	HW	HL
MANOVA	<0.0001																			
ANOVA	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	0.000 5
<i>pongolensis</i> vs <i>krameri</i>	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01				<0.01	<0.01	<0.01						<0.01		
<i>pongolensis</i> vs <i>caudisquamatus</i>	<0.01		<0.01	<0.05	<0.01	<0.01	<0.01		<0.01	<0.01	<0.01	<0.01	<0.01			<0.05		<0.01	<0.01	
<i>pongolensis</i> vs <i>lucombesi</i>	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.05		<0.01	<0.01	<0.01	<0.01	<0.01	<0.05	<0.01
<i>krameri</i> vs <i>lucombesi</i>	<0.01	<0.01	<0.01				<0.01		<0.01				<0.01		<0.01	<0.05	<0.01		<0.01	
<i>krameri</i> vs <i>caudisquamatus</i>			<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01		<0.05	<0.01	<0.01	<0.01			<0.05	<0.05
<i>caudisquamatus</i> vs <i>lucombesi</i>	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01		<0.01	

Table 3.4. Multivariate analysis of variance (MANOVA) followed by analyses of variance (ANOVAs) for individual morphological characters, and pairwise post-hoc test comparisons among *Marcusenius pongolensis* from different river systems in South Africa, *p* values > 0.05 are not shown. See Table 3.1 for characters definitions.

	vBD	mDB	dAD	CPD/ CPL	PDL	PAL	pD	pA	pPL	pVL
MANOVA	<0.0001									
ANOVA	<0.0001	<0.0001	<0.0001	<0.0001	0.000683	<0.0001	0.0375	0.0419	0.00129	0.000126
Kosi, Pongola	<0.001	<0.001	<0.001	<0.001	<0.05	<0.01			<0.01	<0.01
Kosi, Incomati	<0.001			<0.001	<0.05	<0.01				<0.01
Kosi, Nkomati	<0.001	<0.05	<0.001	<0.001	<0.01	<0.01	<0.05	<0.05		
Kosi, Limpopo	<0.001	<0.05	<0.05	<0.001	<0.01					<0.05

3.4. SYSTEMATICS

3.4.1. *Marcusenius caudisquamatus*, sp. nov.

Figure 3.2c, Table 3.5

Lineage B, Mhlatuze-Nseleni clade, Figure 2.2: Chapter 2

Holotype: SAIAB 88684, 166 mm SL, male, Mhlatuze River, under the bridge on road R102, 28° 50' 44.2" S, 31° 51' 59.6" E, South Africa, P. Maake, B. Kramer and B. Mackenzie, 8 November 2009. Hologenotype COI (GenBank number KJ174311), Hologenotype *cyt b* (GenBank number KJ174301).

Paratypes: SAIAB 191225, 6: 87.6-175 mm SL, Paragenotypes COI (GenBank KJ174309 and KJ174310), Paragenotypes *cyt b* (GenBank KJ174299 and KJ174300), same collection data as holotype. MRAC, B3-16-P-3, 113.07 mm SL, Paragenotype COI (GenBank KJ174312), Paragenotype *cyt b* (GenBank KJ174302), same collection data as holotype. SAIAB 79149, 4: 60.3-114.4 mm SL, KwaMaZulu stream close to where it flows into Goedertrouw Dam, part of the Mhlatuze River system in KwaZulu-Natal, 28° 25' 30" S, 31° 1' 30" E, J. Engelbrecht and B. Kramer, 12 August 1999. SAIAB 96607, 2: 200-218 mm SL, Nseleni River - Nsezi Lake, 28° 44.766' S, 31° 58.728' E, O. Weyl, R. Karsing and B. Ellender, 27 May 2010. BMNH 2013 9.4.74, 215 mm SL, same collection data as previous lot. SAIAB 88692, 2: 242-255 mm SL, Nseleni River, 28° 42' 57.8" S, 31° 59' 34.4" E, P. Maake and O. Weyl, 10 November 2009. SAIAB 96543, 2: 192-193 mm SL, Nseleni River - Nature Reserve - Richards Bay, 28° 41.965' S, 32° 00.077' E, O. Weyl, R. Karsing, R. Jones and B. Ellender, 23 May 2010. USNM 410775, 184 mm SL, same collection data as previous lot. SAIAB 96603, 196 mm SL, Lower Nseleni

River opposite Davidson's Farm, 28° 41.965' S, 32° 00.077' E, O. Weyl, R. Karsing, R. Jones and B. Ellender, 27 May 2010.

Diagnosis: Middle body depth 23.8-25.9% SL; dorsal fin length 15.4-19.2% SL; anal fin length 23.0-24.9% SL; pre-dorsal length 61.7-66.7% SL; pre-anal length 60.7-62.8% SL; caudal peduncle depth 40.4-51.7% of its length (greater than and non-overlapping with *M. krameri* and *M. lucombesi*); dorsal fin rays 20-22; anal fin rays 27-28; circumpenduncular scales 19; lateral line scales 72-73; anterior GR (4) + (5-6) = (9-10); posterior GR (6) + (9) = 15; conical teeth on upper/lower jaws 5-5.

Description: Measurements and meristic counts are given in Table 3.5. Head with terminal mouth well in front of eye; mental lobe on lower jaw protruding in front of upper jaw; snout rounded and blunt; pre-anal distance shorter than pre-dorsal distance; distance from origin of anal fin to origin of dorsal fin greater than middle body depth; pre-pelvic distance twice as long as distance between pelvic and anal fin; dorsal and anal fins set well back on the body, dorsal fin situated about three fourths of standard length from snout and opposite anal fin; dorsal fin shorter, originating on vertical at 4th or 5th anal fin ray and ending before anal fin base; distal margin of dorsal and anal fins obliquely orientated, with rays becoming gradually shorter posteriorly; dorsal fin rays 20 (20-22), its anterior rays highest, with distal margin rounded and slightly concave; anal fin rays 27 (27-28); males at sexual maturity have kink in the base of the anal fin distinctly curving inward; anterior anal fin rays of sexually mature males strong and sometimes darker, 3rd - 5th rays longer than the first two rays, crescentic and rounded or curved backwards, but anteriorly sharp or pointy in females and juveniles; pectoral fin rays 10; pectoral fin length 73.4-96.9% of head length; middle body depth 25.1% (23.7-25.9%) of SL; caudal peduncle thicker and stronger, subcylindrical across its entire length, 19.7% (18.6–21.6%) in

SL; scales along the caudal peduncle circumference 19 ($N=15$); caudal peduncle depth 45.2% (40.4-51.7%) into its length; lateral line scales 70 (72-73); lateral line scales cycloid with reticulate striae; jaws with 5-5 conical teeth; 44 vertebrae (excluding urostyle); anterior GRt = 9 (9-10); on the first gill arch (Table 3.6), gill rakers of the anterior row on first arch conicals, thin and more developed (longer and smooth); posterior GRt = 15 on the first gill arch; rakers on the posterior row shorter, thicker and with flattened tips.

Table 3.5. Basic statistics for morphological characters of *Marcusenius caudisquamatus*. See Table 3.1 for characters definitions. Asterisks indicate non-overlapping characters with other *Marcusenius* species examined in the present study.

Character	Holotype	N	Mean/Median	Min	Max	Std. Dev	
SL, mm	166 mm	15	157.19	84.64	250	49.23	
HL, mm	35.34 mm	15	32.77	19.13	51.3	8.89	
Ratio of SL							
HL	0.2129	15	0.2114	0.1868	0.2273	0.0117	
vBD	0.2404	15	0.2399	0.2305	0.2481	0.0050	
mBD	0.2512	15	0.2495	0.2376	0.2588	0.0065	
dAD	0.2519	15	0.2556	0.2443	0.2727	0.0081	
CPL	0.1966	15	0.2017	0.1864	0.2168	0.0083	
PDL	0.6527	15	0.6414	0.6171	0.6668	0.0138	
PAL	0.6165	15	0.6186	0.6078	0.6285	0.0052	
pD	0.4047	15	0.4056	0.4001	0.4092	0.0028	* MP
pA	0.4313	15	0.4339	0.4301	0.4379	0.0026	* MP
pPL	0.2301	15	0.2365	0.2301	0.2464	0.0056	
pVL	0.4119	15	0.4089	0.3912	0.4234	0.0090	
dVA	0.2134	15	0.2127	0.1927	0.2336	0.0110	
dPA	0.4010	15	0.3951	0.3696	0.4160	0.0096	
dPV	0.1954	15	0.1916	0.1773	0.2059	0.0085	
LD	0.1616	15	0.1676	0.1541	0.1920	0.0111	
LA	0.2361	15	0.2391	0.2302	0.2487	0.0058	* MP
CPD/CPL	0.4516	15	0.4486	0.4038	0.5166	0.0300	* ML, MK
Ratio of HL							
HW	0.5948	15	0.6307	0.5942	0.6581	0.0212	
LSN	0.4680	15	0.4768	0.4442	0.5150	0.0182	
IOW	0.4044	15	0.4140	0.3763	0.4443	0.0239	
ED	0.2083	15	0.2004	0.1810	0.2233	0.0136	
LP		15	0.8297	0.7339	0.9696	0.0714	
Counts							
nD	20	15	21	20	22	0.6172	
nA	27	15	28	27	28	0.5164	
SPc	19	15	19	19	19	0	*MP, MK, ML
nTL	5	15	5	5	5	0	
nTU	5	15	5	5	5	0	
TV	44	7	44	44	44	0	* MP
LSS	70	6	72	72	73	0.5477	

MP = *M. pongolensis*, ML = *M. luombesi*, MK = *M. krameri*

Electric organ discharge: Kramer *et al.* (2007) concluded that the EODs of seven individuals of this species from the Mhlatuze River (SAIAB 79149, 4, and ZSM 35090, 3) cannot be differentiated from those of *M. pongolensis* collected from the Incomati and Pongola river systems.

Remarks: *Marcusenius caudisquamatus* can easily be distinguished by its shorter and thicker caudal peduncle, (40.4-51.37% of peduncle length as opposed to 26.0-40.6% in *M. krameri* and 28.7-45.66% in *M. pongolensis*). The deeper caudal peduncle has more circumpeduncular scales than in other species (19 vs 16 for *M. krameri* and 16-17 for *M. pongolensis*, Table 3.7). The dorsal profile of the head of *M. caudisquamatus* specimens from the Mhlatuze River has a steep, nearly vertical hump in front of the eye. It is slightly concave behind the eye (Figure 3.2c) as compared to being convex in *M. pongolensis* and *M. luombesi* (Figures 3.2a and 3.2d, respectively) and straight in *M. krameri* (Figure 3.2b). The number of GR on the posterior side of the first arch is constant and a reliable character to distinguish this species from both *M. pongolensis* and *M. krameri* (Table 3.6). Compared to *M. krameri* and *M. pongolensis* in which the scales series across their body are easily visible, *M. caudisquamatus* appears smooth and the scales are almost fully embedded in the skin (observed both in live and preserved specimens). The species is genetically closely related to *M. krameri* (Figure 3.4). Sequence divergences between *M. krameri* and *M. caudisquamatus* are 0.9-2.4%, and that is much larger than within the latter species (0.0-0.5%).

Colouration: in 70% alcohol preservation, specimens are grey to blackish. The upper back and belly are covered with a milky-grey mucus layer, especially on the head where opaque ‘mormyrid skin’ (electroreceptor organ area) is present. Live colouration of specimens not recorded.

Distribution and ecology: Currently this species is known only from the Nseleni and the Mhlatuze rivers in KwaZulu-Natal, South Africa (Figure 3.1). The Nseleni River was generally deep with strong currents and all the specimens were collected with gill nets. The Mhlatuze River was wide and shallow, about 1.5 m deep, with a sandy or rocky bottom with dense reed beds on the periphery. Water current was moderate to fast in sections where *M. caudisquamatus* was collected. The water conductivity was relatively high (503 $\mu\text{S}/\text{cm}$ to 707 $\mu\text{S}/\text{cm}$) in summer (November 2009) as compared to the 134 $\mu\text{S}/\text{cm}$ recorded by Kramer *et al.* (2007) in winter (12 August 1999). The water temperature at noon was around 25 to 26 °C in November 2009, the water was clear and specimens were caught at various depths depending on the sampling station. The Mhlatuze River system has the southernmost record of mormyrids in Africa (Kramer *et al.*, 2007). The biology and ecology of the species is unknown, but the characteristic anal fin notch of sexually mature males was observed from about 96.7 mm SL. The largest male was 250 mm and the largest female was 215 mm SL.

Etymology: A combination of the Latin words *cauda*, for tail, and *squama* for scale. This name refers to the high number of circumpeduncular scales in this species relative to the other members of the genus in South Africa.

Table 3.6. Frequency distribution of gill raker counts for *Marcusenius caudisquamatus*, *M. krameri*, *M. luombesi* and *M. pongolensis*. An asterisk indicates counts for the holotype of each species. Body of the Table gives number of specimens.

	Gill arch, anterior row						Gill arch, posterior row					
	Upper limb			Lower limb			Upper Limb			Lower limb		
Number of GR	4	5	6	4	5	6	6	7	8	7	8	9
<i>M. caudisquamatus</i> (n =10)	10*				9*	1	10					10
<i>M. krameri</i> (n = 15)		13*	2		15*				15*			15*
<i>M. luombesi</i> (n = 5)	4*	1		4*	1		5*			5*		
<i>M. pongolensis</i> (n = 21)	17	4			21		18	3		18	3	

Total Gill Rakers												
	Gill arch, anterior row					Gill arch, posterior row						
	8	9	10	11	13	14	15	16	17			
<i>M. caudisquamatus</i> (n =10)		9	1				10*	-				
<i>M. krameri</i> (n = 15)			13*	2				-	15*			
<i>M. luombesi</i> (n = 5)	3*	2			5*			-				
<i>M. pongolensis</i> (n = 21)		17	4		18	3		-				

Table 3.7. Frequency distribution of dorsal fin ray counts of dorsal and anal fin rays, and circumpeduncular scales in *Marcusenius caudisquamatus*, *M. krameri*, *M. luombesi* and *M. pongolensis*. An asterisk indicates the counts for the holotype of each species. Body of the Table gives number of specimens.

	Dorsal fin rays (nD)						Anal fin rays (nA)						Circumpeduncular scales (SPc)					
	20	21	22	23	24	25	26	27	28	29	30	31	14	15	16	17	18	19
<i>M. caudisquamatus</i>	6*	8	1					7*	8								-	19*
<i>M. krameri</i>			4	26*	3	2			20	11*	3	1			35*		-	
<i>M. luombesi</i>			1	7*	5				4	9*			5*	5	3		-	
<i>M. pongolensis</i>	5	15*	25	5			4	33*	13						25*	25	-	

3.4.2. *Marcusenius krameri*, sp. nov.

Figure 3.2b; Table 3.8

Marcusenius Limpopo population: Kramer *et al.* (2007): p. 661; Lineage B, Limpopo clade, Figure 2.2: Chapter 2

Holotype: SAIAB 188295, 116.21 mm SL, male, Mogalakwena River (also known as Nyl), Modimolle town, Pretoria Street, above a drift, 24° 42' 04.2" S, 28° 24' 40.8" E, Limpopo River system, South Africa, P. Maake and O. Gon, 14 October 2010. Hologenotype COI (GenBank KJ174307), Hologenotype *cyt b* (GenBank KJ174297).

Paratypes: SAIAB 191224, 5: 92-117 mm SL, Paragenotypes COI (GenBank KJ174305 and KJ174306), Paragenotypes *cyt b* (GenBank KJ174295 and KJ174296), same collection data as holotype. MRAC B3-16-P-1-2, 2: 100-107.77 mm SL, Paragenotype COI (GenBank KJ174308), paragenotypes *cyt b* (GenBank KJ174298), same collection data as holotype. SAIAB 188294, 11: 50-147 mm SL, Mokolo River, above dam wall (opposite bank) of dam at intersection of roads R518 and R510, 23° 22' 01.7" S, 27° 41' 09.6" E, P. Maake and O. Gon, 12 October 2010. USNM 410774, 2: 119.73-130.8 mm SL, same collection data as previous lot. SAIAB 188290, 20: 57-197 mm SL, Tshinane River, R523 below bridge at Ngudza, 22° 53' 56.3" S, 30° 31' 28.7" E, P. Maake and O. Gon, 10 October 2010. BMNH 2013.9.4.72-73, 2: 110-142 mm SL, same collection data as previous lot.

Non-type specimens: SAIAB 79147, 6: 37.2-122 mm SL, Blyde River. SAIAB 188301, 2: 62.2-140.92 mm SL, Cross River, Danda village, below bridge, tributary from Nwanedi Dam. SAIAB 84510: 22 mm SL, Damani Dam. SAIAB 84485, 5: 35-48mm SL, Damani Dam.

SAIAB 84439, 2: 52-79 mm SL, Sambandou Junction. SAIAB 84527, 7: 32-56 mm SL, Mbwedi Pumphouse.

Diagnosis: Middle body depth 21.9-28.1% SL; distance from dorsal fin origin to end of caudal peduncle 39.9-41.7% SL, distance from anal fin origin to end of caudal peduncle 42.8-44.9% SL; dorsal fin length 15.5%-20.0% SL; anal fin length 23.2-25.5% SL; depth of caudal peduncle 26.0-40.6% of its length; circumpenduncular scales 16; total vertebrae 44; anterior GR (5-6) + (5) = (10-11); posterior GR (8) + (9) = 17; dorsal fin rays 22-25; anal fin 28-31 rays; scales along lateral line 69-72. Conical teeth on upper/lower jaws 4-6.

Description: Measurements and meristic counts are given in Table 3.8. Head with terminal mouth well in front of eye; mental lobe on lower jaw protruding in front of upper jaw; head and body compressed; snout rounded and blunt; pre-anal distance shorter than pre-dorsal distance; distance from origin of anal fin to origin of dorsal fin slightly greater or equal to middle body depth; pre-pelvic distance twice as long as the distance between pelvic and anal fins; dorsal and anal fins set well back on the body, situated about two thirds of standard length from the snout and opposite each other; dorsal fin shorter, originating on vertical at 4th or 5th anal fin ray and ending before anal fin base; distal margin of dorsal and anal fins obliquely orientated, with rays becoming gradually shorter posteriorly; dorsal fin rays 23 (22-25), its anterior rays highest; anal fin rays 29 (28-31); males at sexual maturity have kink in anal fin base, distinctly curving inward; anterior anal fin rays of sexually mature males strong and sometimes darker, 3rd - 5th rays longer than first two rays, crescentic and rounded or curved backwards, but anteriorly sharp or pointy in females and juveniles; pectoral fin rays 10, fin length 53.7-82.3% of head length; body depth 26.6% (21.9- 28.1%) SL; caudal peduncle slender, subcylindrical across its entire length, 19.2% (17.6-24.5%) SL; 16 scales (N=35)

around the caudal peduncle; caudal peduncle depth 34.9% (26.0-40.6%) of its length; lateral line scales 70 (69-72), cycloid and with reticulate striae; jaws with 4-6 (usually 4-5, $N = 27$) conical teeth; 44 vertebrae (excluding urostyle); gill rakers on anterior side more developed (longer and smooth, i.e. no spines), anterior GRt = 10 (10-11) on the first gill arch (Table 3.6), gill raker of anterior side conicals with thin tips; posterior GRt 17 on the posterior side of first gill arch; gill rakers on posterior side shorter, thicker and with flattened tips.

Coloration: Immediately after death specimens are medium brown, speckled with darker, irregular blotches on the sides, fins yellowish (Figure 3.2b). In 70% alcohol, the head (including eye), fins and upper back sometimes covered by milky-grey mucus layer. Specimens from Tshinane River are similar to those from type locality, but clearly darker from belly to back, including the fins.

Electric organ discharge: According to Kramer *et al.* (2007), the EODs of ten individuals (of which six were examined morphologically in the present study: SAIAB 79147) of this species (referred to as *Marcusenius* Limpopo population) were distinct from those of *M. pongolensis*, *M. macrolepidotus*, *M. altisambesi* and *M. devosi*. Kramer *et al.* (2007) concluded that, when comparing EODs from South African systems: (1) the durations of the N phase or *Ndur* variable of female and juvenile EODs from *M. krameri* increased with size, while not a single EOD variable correlated with size in *M. pongolensis*, (2) the *M. krameri* EODs differed significantly from those of *M. pongolensis* in three variables, i.e. *Ndur*, Parea and Narea (see Table VIII in Kramer *et al.*, 2007).

Table 3.8. Basic statistics for morphological characters of *Marcusenius krameri* sp. nov. See Table 3.1 for characters definitions. Asterisks indicate non-overlapping characters with other *Marcusenius* species examined in the present study.

Character	Holotype	N	Mean/Median	Min	Max	Std.	
SL, mm	116.21 mm	35	113.59	48.2	207	34.65	
HL, mm	24.07 mm	35	23.37	11.63	42.07	6.44	
Ratio of SL							
HL	0.2071	35	0.2083	0.1860	0.2427	0.0129	
vBD	0.2573	35	0.2434	0.2188	0.2711	0.0136	
mBD	0.2662	35	0.2511	0.2190	0.2808	0.0145	
dAD	0.2730	35	0.2586	0.2432	0.2802	0.0089	
CPL	0.1915	35	0.2053	0.1762	0.2448	0.0139	
PDL	0.6298	35	0.6343	0.6178	0.6538	0.0109	
PAL	0.5935	35	0.5971	0.5581	0.6172	0.0129	
pD	0.3989	35	0.4047	0.3989	0.4168	0.0038	*MP
pA	0.4319	35	0.4360	0.4280	0.4490	0.0051	*MP
pPL	0.2311	35	0.2317	0.2123	0.2467	0.0080	
pVL	0.3848	35	0.3951	0.3805	0.4140	0.0077	
dVA	0.2193	35	0.2090	0.1846	0.2350	0.0133	
dPA	0.3823	35	0.3786	0.3450	0.3994	0.0150	
dPV	0.1740	35	0.1840	0.1580	0.2074	0.0104	
LD	0.1867	35	0.1813	0.1554	0.2002	0.0115	
LA	0.2472	35	0.2404	0.2319	0.2547	0.0063	* MP
CPD/CPL	0.3486	35	0.3393	0.2602	0.4058	0.0327	* ML, MC
Ratio of HL							
HW	0.5991	35	0.5893	0.4985	0.6362	0.0267	
LSN	0.4936	35	0.4765	0.4383	0.5197	0.0223	
IOW	0.4591	35	0.4475	0.3483	0.5094	0.0310	
ED	0.1683	35	0.1897	0.1368	0.2267	0.0183	
LP		35	0.7029	0.5370	0.8227	0.0657	
Counts			Median				
nD	23	35	23	22	25	0.6585	
nA	29	35	28	28	31	0.7778	* MP
SPc	16	35	16	16	16	0	* MC
nTL	6	35	5	4	6	0.5314	
nTU	5	35	5	4	5	0.4710	
TV	44	22	44	44	44	0	* MP
LSS	70	23	70	69	72	0.9639	

NB: MP = *M. pongolensis*, ML = *M. lucombesi*, MC = *M. caudisquamatus*.

Remarks: Compared with *M. pongolensis*, *M. krameri* specimens are best distinguished by their moderately short, laterally compressed deep body. *Marcusenius krameri* also has the greater distance from dorsal fin origin to end of caudal peduncle (39.9-41.7% vs 37.1-39.8% SL) and from anal fin origin to end of caudal peduncle (42.8-44.9% vs 40.8-42.1% SL), and its anal-fin base is longer (23.2-25.5% vs 19.5-21.9 % of SL), all of which do not overlap with *M. pongolensis*. *Marcusenius krameri* differed clearly from *M. macrolepidotus* with the dorsal and fin length, caudal peduncle depth and body depth, all of which are lower and caudal peduncle length and number of scales around caudal peduncle which are higher in the latter species. *Marcusenius krameri* also has the highest number of posterior gill rakers and anal fin rays among the South African species (Tables 3.6 and 3.7, respectively). Caudal peduncle depth of *M. krameri* increased significantly with size as compared to other species, resulting in the unusually large range of this character. *Marcusenius krameri* is closely related to *M. caudisquamatus*, but the latter species has a deeper caudal peduncle into its length (40.4-51.7% vs 26.0-40.5) and 19 circumpeduncular scales (see also Table 3.6). *Marcusenius krameri* species can also be distinguished based on the phylogenetic relationships forming a sister species to *M. caudisquamatus* and its genetic divergence in the in the cyt *b* dataset (0.9-2.4%) (Figure 3.4).

Distribution and ecology: *Marcusenius krameri* occurs in the mainstem of the Limpopo River and several of its tributaries, ranging from the Matlabas River in the west to the Olifants River in the east. Individuals of this species were found in groups. During the day they were commonly encountered below undercut river banks, especially in dense networks of tree roots or reed beds along the stream margins. Places with shallow water and slow-flowing reaches of rivers and streams are especially attractive to them. Large numbers of small juveniles were found in bushes of aquatic reeds and floating grass on the periphery of the river or in side

channels. The conductivity in most rivers where it occurred ranged from 55 to 106 $\mu\text{S}/\text{cm}$ (type locality), and a pH from 7.41 to 8.5 units in October 2010 (pH = 7.86, in type locality). In summer, water temperature was 24-27°C between 10 am and 6 pm. Kramer *et al.* (2007) reported a higher water conductivity of 154 $\mu\text{S}/\text{cm}$ and a much colder temperature, 16.7°C, between the 25th and 26th September 1998. The turbidity where specimens were collected was moderate to high at a depth not exceeding 1.5 m.

The biology of the species is unknown, but males mature from an estimated length of 84 mm SL (the characteristic notched anal fin of males was observed at this size), but females probably mature at a larger size. The largest specimens were a 207 mm SL female and a 192 mm SL male.

Etymology: *Marcusenius krameri* is named for Prof. Bernd Kramer from the Zoological Institute of the University of Regensburg, Germany, in recognition of his contribution to the systematics of southern African mormyrids.

3.4.3. *Marcusenius luombesi*, sp. nov.

Figure 3.2d, Table 3.9

Marcusenius macrolepidotus: Kramer *et al.* (2007): p. 664, fig. 9 (*M. macrolepidotus* Ruvuma 25650 and 25651); p. 695, SAIAB 73790, SAIAB 73884.

Holotype: SAIAB 73884, 74.0 mm SL, male, Lucombe River, Lucombe stream pools, Nyati Road, Niassa Game Reserve, 12° 5' 15" S, 37° 33' 38" E, Ruvuma River system, R. Bills, 22 August 2003. Hologenotype COI (GenBank KJ174304), Hologenotype cyt *b* (GenBank KJ174293).

Paratypes: SAIAB 191226, 6: 52-72 mm SL, Paragenotype COI (GenBank KJ174303), Paragenotype *cyt b* (GenBank KJ174294), same collection as the holotype. MRAC, B3-16-P-4, 61.85 mm SL, same collection data as holotype. SAIAB 73885, 58.3 mm SL, same collection data as holotype. SAIAB 73886, 2: 48.8-55.5 mm SL, upper Lucombe River, isolated swamp pools in forest, Mbatamila-Nyati road, 12° 5' 2" S, 37° 33' 43" E, R. Bills, 26 August 2003. SAIAB 73816, 73 mm SL, Lucombe River, fourth stream, pools in bed, Mbatamila-Matondovela Road, 12° 7' 49" S, 37° 26' 10" E, R. Bills, 26 August 2003. SAIAB 73790, 4: 73.0-121.5 mm SL, Nakanjambo River, Nakajambo stream, pools in bed, Mbatamila-Matondovela Road, 12° 7' 45" S, 37° 21' 41" E, R. Bills, 14 August 2003. BMNH 2013.9.4.75, 71mm SL, same collection data as previous lot.

Diagnosis: distance dorsal fin origin to end of caudal peduncle 40.0-43.2% of SL; distance anal fin origin to end of caudal peduncle 43.6-45.9% of SL; shortest pre-anal length 56.6–59.2% of SL; pre-dorsal length 59.5-63.3% of SL; low caudal peduncle depth 25.0-30.8% into its length; circumpenduncular scales 14-16; anal fin rays 28-29; dorsal fin rays 22-24; total vertebrae 44; lateral line scales 55-56; anterior GR (4-5) + (4-5) = (8-10); posterior GR (6) + (7) = 13; jaws with 5-5 conical teeth.

Description: Measurements and meristic counts are given in Table 3.9. Head with terminal mouth well in front of eye, mental lobe on lower jaw protruding in front of upper jaw; head and body compressed; snout very pointy; pre-anal distance shorter than pre-dorsal distance; distance from origin of anal fin to origin of dorsal fin equal to the middle body depth; pre-pelvic distance twice as long as the distance between pelvic and anal fins; dorsal and anal fins set well back on the body, situated about two thirds of standard length from the snout and opposite each other; dorsal fin shorter, originating on vertical at 3rd anal fin ray and ending

before anal fin base; distal margins of dorsal and anal fins obliquely orientated, with rays becoming gradually shorter posteriorly; dorsal fin rays 23 (22-24), its anterior rays highest, with distal margins rounded and slightly concave; anal fin rays 29 (28-29); males at sexual maturity have kink in the base of the anal fin distinctly curving in ward; anterior anal fin rays of sexually mature males longer, appear stronger, are crescentic and rounded, but anteriorly sharp or pointy in females and juveniles; pectoral fins rays 10; pectoral fins distinctly very long, reaching origin of pelvic fin (when pressed against body); middle body depth 28.4% (25.0-28.4%) of SL; caudal peduncle thinner, sub-cylindrical across its entire length 20.3% (19.7-21.7%) in SL; 14 (14-16) scales along the caudal peduncle circumference; caudal peduncle depth 26.7% (25.0-30.8%) into its length; jaws with 5-5 conical teeth; lateral line scales 55 (55-56); lateral line scales cycloid with reticulate striae; 44 vertebrae (excluding urostyle). gill rakers on anterior side covered with many minute spines, anterior GRt = 8 (8-9) on the first gill arch (Table 3.6); posterior GRt 13 on the posterior side of first gill arch; gill rakers on posterior side shorter, thicker and with covered by minute spines.

Electric organ discharge: no data

Coloration: all specimens light brown when preserved. Homogeneous coloration without any blotches, increasingly lighter on the belly.

Distribution: This species is currently known only from the Lucombe River and its small tributary streams, as well as the Nakajambo River, both tributaries of the Ruvuma River system within the Niassa Game Reserve, Mozambique. It was collected with *Petrocephalus catostoma* (Günther, 1866) in aquatic weed beds, marginal vegetation and root-stocks of bank vegetation in headwater streams through to floodplain margins (Kramer *et al.*, 2007; R. Bills, *pers.*

comm.). Water conductivity was 104-268 $\mu\text{S}/\text{cm}$ in August 2003, probably reflecting human impact on this aquatic system (Kramer *et al.*, 2012).

Table 3.9. Basic statistics for morphological characters of *Marcusenius lucombesi*. See Table 3.1 for characters definitions. Asterisks indicate non-overlapping characters with other *Marcusenius* species examined in the present study.

Character	Holotype	N	Mean/Median	Min	Max	Std. Dev	
SL, mm	77.0 mm	13	64.92	53.00	74.00	7.54	
HL, mm	15.0 mm	13	14.23	12.00	16.00	1.30	
Ratio of SL							
HL	0.2027	13	0.2199	0.2027	0.2333	0.0101	
vBD	0.2703	13	0.2576	0.2429	0.2703	0.0084	
mDB	0.2838	13	0.2648	0.2500	0.2838	0.0104	* MP
dAD	0.2838	13	0.2689	0.2545	0.2838	0.0103	
CPL	0.2027	13	0.2086	0.1972	0.2167	0.0067	
PDL	0.6216	13	0.6167	0.5946	0.6333	0.0110	
PAL	0.5811	13	0.5767	0.5660	0.5915	0.0092	
pD	0.4189	13	0.4155	0.4000	0.4324	0.0096	* MP
pA	0.4595	13	0.4479	0.4364	0.4595	0.0084	* MP
pPL	0.2203	13	0.2271	0.2143	0.2394	0.0075	
pVL	0.3919	13	0.3861	0.3731	0.3944	0.0075	
dVA	0.2027	13	0.1926	0.1786	0.2083	0.0093	
dPA	0.3649	13	0.3500	0.3378	0.3662	0.0100	* MP
dPV	0.1757	13	0.1709	0.1607	0.1833	0.0067	
LD	0.1946	13	0.1893	0.1803	0.2000	0.0067	* MP
LA	0.2487	13	0.2384	0.2264	0.2537	0.0091	
CPD/CPL	0.2667	13	0.2794	0.2500	0.3077	0.0198	
Ratio of HL							
HW	0.6000	13	0.5611	0.5000	0.6000	0.0343	
LSN	0.5333	13	0.4601	0.4167	0.5333	0.0405	
IOW	0.4667	13	0.4421	0.4000	0.5000	0.0279	
ED	0.2667	13	0.2382	0.1875	0.2757	0.0302	
Counts			Median				
nD	23	13	23	22	24	0.6304	
nA	29	13	29	28	29	0.4804	
SPc	14	13	15	14	16	0.4385	*MP, MK
nTL	5	13	5	4	6	0.5547	
nTU	5	13	5	5	5	0	
Total vertebrae	44	13	44	44	44	0	* MP
LSS	55	8	55	55	56	0.5470	*MP, MK, MC

NB: MP = *M. pongolensis*, MK = *M. krameri*, MC = *M. caudisquamatus*

Remarks: *Marcusenius lucombesi* may be the smallest species of *Marcusenius* in southern Africa reaching sexual maturity at only 56 mm SL and with none of the males in the SAIAB collection exceeding 75 mm SL. The largest female was 120.50 mm SL. In comparison to other

species examined in this study, *M. luombesi* has the lowest number of scales along the lateral line (55-56), and the anal fin has a more anterior origin than the dorsal fin by only three rays as compared to 4-5 rays in *M. krameri* and *M. caudisquamatus*. It should be emphasised that *M. luombesi* also differs significantly from *M. macrolepidotus* of the Lower Zambezi in several characters, i.e. distance from dorsal fin origin to end of caudal peduncle, caudal peduncle depth, pre-anal and dorsal lengths, all of which were greater in *M. macrolepidotus* (Kramer *et al.*, 2007). *Marcusenius luombesi* is also genetically divergent, and morphologically distinct in having significantly lower caudal peduncle depth, higher dorsal fin and head lengths, and one anal and dorsal fin ray more than in *M. devosi* (Kramer *et al.*, 2007). Another bulldog species, *Marcusenius livingstonii*, occurs in the Ruvuma but it differs from *M. luombesi* in having a dark uniform bar on the body below the origin of the dorsal fin and bicuspid teeth. These two characters also distinguish *M. livingstonii* from all other known species of *Marcusenius* in southern and eastern African rivers. *Marcusenius luombesi* is therefore distinct from *M. livingstonii* and *M. macrolepidotus*.

Etymology: The specific name refers to the type locality of this species, Lucombe River, a tributary of the Ruvuma system in the Niassa Game Reserve.

3.4.4. *Marcusenius pongolensis* (Fowler 1934)

Figure 3.2a, Table 3.10

Gnathonemus macrolepidotus: Gilchrist & Thompson 1913: 330–331, Tete, Lower Zambezi, Mozambique.

Gnathonemus pongolensis Fowler 1934: 419, fig. 6.

Gnathonemus macrolepidotus: Crass 1960: 416.

Marcusenius pongolensis Taverne 1971b: 103; Kramer *et al.* (2007): 684, figs. 1D, 1M.

Holotype: ANSP 54950, 172.5 mm SL; Pongola River at Paulpietersburg district, South Africa, KwaZulu-Natal; not examined.

Material examined:

Limpopo River system: SAIAB 188291, 11: 111-193 mm SL, Olifants River, below iron bridge, after Mogologolo village. SAIAB 188303, 4: 115-147 mm SL, Olifants River, above Loskop dam in Aventura, bridge on dirt road. SAIAB 188302, 20: 55-150 mm SL, Groot Letaba River, Broederstroom drift about 10 km from (south) of Tzaneen. SAIAB 188289, 24: 59-183 mm SL, Matlabas River, on road R510 bridge towards Thabazimbi.

Incomati River system: SAIAB 188297, 13: 46-136 mm SL, Crocodile River, below N4 bridge at Matsulu village. SAIAB 188300, 4: 129-189 mm SL, Omnia farm dam, Kopmeiden. SAIAB 54448, 12: 49-118 mm SL, Sabie River, above Hazyview. SAIAB 188280, 2: 77-107 mm SL, Sabie River at Hoxani area below dam. SAIAB 188279, 5: 61-158 mm SL, Sabie River, Sabana tributary at the back of the Sabie Adventures. SAIAB 188293, 2: 52-53 mm SL, Sabie River, Marite. SAIAB 188304, 11: 82-136 mm SL, Sand River.

Nkomati River system: SAIAB 69893, 2: 65.8-78.8 mm SL, Phophonyane River, Ntfontjeni Road. SAIAB 69907, 3: 113-252 mm SL, Mlumati River, Driekoppies Dam. SAIAB 69949, 7: 39.0-73.5 mm SL, Nyonyane River, Sisa Ranch.

Pongola River system: SAIAB 79148, 5: 115.6-176.5 mm SL, Pongola River, bridge at road connecting Ndumo with Kosi Bay. SAIAB 188277, 2: 129-140 mm SL, Sitalo River, below bridge on N2 road to Jozini. SAIAB 188296, 16: 79-165 mm SL, Pongola River on R33 road to Paulpietersburg.

Kosi River system: SAIAB 88637, 2, Nkanini stream, below bridge just outside Manguzi on road to Mozambique. SAIAB 88633, 11: 98.8-147.7 mm SL, Mahlampane River, at Manguze village below bridge. SAIAB 88615, 11: 61.7-178.3 mm SL, Siyadla River, below the bridge just about one metre down and above the bridge.

Remarks: The measurements and meristic data of Kramer *et al.*'s (2007) *M. pongolensis* were not always in agreement with our data (Table 3.10), probably due to the inclusion of the Mhlathuze and Nseleni rivers as a southern population of *M. pongolensis*. Since these populations are now recognised as a new species, *M. caudisquamatus*, a new diagnosis for *M. pongolensis* is necessary.

Diagnosis: *Marcusenius pongolensis* is distinguished from other species in its more slender body (elongated, lower body depth), with pD (37.1-39.8% SL), pA (40.8-42.2% SL) and LA (19.5-21.9% SL) (Table 3.10), all shorter and non-overlapping with *M. krameri* (Table 3.8), *M. caudisquamatus* (Table 3.5) and *M. luombesi* (Table 3.9). Total vertebrae ranges between 42-43 vs 44 in other species; posterior total gill rakers (GRt) 13-14 vs 15 and 17 in *M. caudisquamatus* and *M. krameri*, respectively (Table 3.6). Lateral line scales 70-73. Table 3.7 provides the range for counts of dorsal and anal fin rays, and circumpeduncular scales in *M. pongolensis*, *M. krameri*, *M. caudisquamatus* and *M. luombesi*. Dorsal fin situated about three fourths of standard length from snout. Colour in life of *M. pongolensis* ranges from a

homogeneous medium brown to grey-brown with yellowish to golden sheen, underside lighter, body has darker blotches on sides with yellowish fins (Figure 3.2a). These diagnostic characters agree with those mentioned in Kramer *et al.* (2007) for the holotype of *M. pongolensis*. *Marcusenius pongolensis* is considered closest to *M. macrolepidotus* based on phylogenetic analyses of the mitochondrial *cyt b* dataset (Figure 3.4), and significant divergence from *M. krameri*, *M. caudisquamatus* and *M. luombesi* (Table 3.2).

Electric organ discharge: Detailed in Kramer *et al.* (2007).

Distribution: Kramer *et al.* (2007) presumed that *M. pongolensis* was absent from the Limpopo River system. However, with increased sampling *M. pongolensis* was found in small numbers in the Limpopo system, specifically the Olifants, Groot Letaba and Matlabas Rivers (Figure 3.1). *Marcusenius pongolensis* therefore occurs from the tropical sections of the Limpopo River system in the north to the Kosi River system in the south.

Biology and ecology: Mostly, *M. pongolensis* was collected in deep rapids and fast-flowing river sections with rocky bottoms and less vegetation cover. Studies on behavioural characteristics revealed that male *M. pongolensis* did not construct a nest, and neither provided parental care. The male shows very little aggression towards the female during spawning near the male's hiding place (Wayner & Kramer, 2005; Machnik & Kramer, 2008). *Marcusenius pongolensis* populations from an oligotrophic impoundment in Swaziland mature within the first year, at an estimated minimum length of 134 and 119 mm SL for males and females, respectively (Booth & Khumalo, 2010).

Table 3.10. Basic statistics for morphological characters of *Marcusenius pongolensis*. See Table 3.1 for characters definitions.

Character	Holotype*	N	Mean/ Median	Min	Max	Std. Dev
SL, mm	172.5 mm	49	125.54	62.27	197	31.72
HL, mm	32.99 mm	49	25.5	12	36.6	5.92
Ratio of SL						
HL	0.1913	49	0.2044	0.1797	0.2312	0.0118
vBD		49	0.2266	0.1942	0.2595	0.0163
mDB	0.1971	49	0.2298	0.2027	0.2496	0.0139
dAD		49	0.2421	0.2169	0.2628	0.0123
CPL	0.2029	49	0.2007	0.1660	0.2187	0.0100
PDL	0.6336	49	0.6330	0.6081	0.6646	0.0126
PAL	0.5936	49	0.6008	0.5600	0.6265	0.0154
pD	0.4017	49	0.3864	0.3706	0.3983	0.0067
pA		49	0.4152	0.4082	0.4216	0.0035
pPL		49	0.2207	0.2020	0.2325	0.0062
pVL		49	0.3917	0.3717	0.4123	0.0105
dVA		49	0.2169	0.1970	0.2422	0.0112
dPA		49	0.3886	0.3623	0.4213	0.0154
dPV		49	0.1830	0.1629	0.1994	0.0091
LD	0.1693	49	0.1748	0.1607	0.1925	0.0080
LA	0.2058	49	0.2152	0.1945	0.2198	0.0046
CPD/CPL	0.3800	49	0.3650	0.2868	0.4566	0.0392
Ratio of HL						
HW		49	0.5854	0.5355	0.6758	0.0325
LSN	0.4667	49	0.4922	0.4513	0.5858	0.0260
IOW		49	0.4451	0.3801	0.5513	0.0369
ED		49	0.2052	0.1707	0.2525	0.0190
LP		49	0.7879	0.6161	0.9458	0.0786
Counts			Median			
nD	21	49	22	20	23	0.8118
nA	27	49	27	26	28	0.5654
SPc	16	49	17	16	17	0.5051
nTL		49	6	5	6	0.4657
nTU		49	5	4	5	0.3734
Total vertebrae		21	43	42	43	0.4629
LSS		30	71	70	73	0.9595

* Measurements taken from Kramer *et al.* (2007)

Key to eastern and southern African species of the genus *Marcusenius* (the river system and country name where the holotype was collected is given for each species in brackets).

1. Dorsal fin rays 26-28; anal fin rays 32-33; vertebrae 45-46 (Cuanza River system, Angola) *Marcusenius angolensis*
 - Dorsal fin rays 20-26; anal fin rays 26-31; vertebrae 41-44 2
2. Teeth on both jaws bicuspid; dark brown uniform bar on the body between origins of dorsal and anal fins; total vertebrae 42 (Ruvuma River system, Mozambique) *Marcusenius livingstonii*
 - Teeth on both jaws unicuspid, conical; dark brown bar between origins of dorsal and anal fins absent 3
3. Lateral scales series 69-73 4
 - Lateral scale series 52-66 6
4. Dorsal fin rays 22-25; anal fin rays 28-31; gill rakers on inner side of first gill arch 17 (Limpopo River system, South Africa) *Marcusenius krameri* sp. nov
 - Dorsal fin rays 20-23; anal fin rays 26-28; gill rakers on inner side of first gill arch 13-15 5
5. Circumpeduncular scales 19; caudal peduncle depth 8.4-10.2% SL; gill rakers on inner side of first gill arch 15; vertebrae 44 (Mhlatuze River, South Africa) *Marcusenius caudisquamatus* sp. nov
 - Circumpeduncular scales 16-17; caudal peduncle depth 6.10-8.3% SL; gill rakers on inner side of first gill arch 13-14; vertebrae 42-43 (Pongola River system, South Africa) *Marcusenius pongolensis*
6. Gill rakers on inner side of first gill arch 18-19; Circumpeduncular scales 14-18 7
 - gill rakers on inner side of first gill arch 13-17; Circumpeduncular scales 12-16 8
7. Vertebrae 41 (Tana River, Kenya) *Marcusenius devosi*
 - Vertebrae 43-44 (Lower Zambezi River system, Mozambique) *Marcusenius macrolepidotus*
8. Preanal distance 56.6-59.2% SL; gill rakers on inner side of first gill arch 13 (Ruvuma River system, Mozambique) *Marcusenius lucombesi*
 - Preanal distance 59.3-65.9% SL; gill rakers on inner side of first gill arch 15-17 9
9. Vertebrae 45-46; gill rakers on inner side of first gill arch 15-17 (rarely 17); gill rakers on outer side of first gill arch 9 (Cunene River, Angolan/Namibian border) *Marcusenius multisquamatus*
 - Vertebrae 43-44; gill rakers on inner side of first gill arch 17; gill rakers on outer side of first gill arch 10 (Upper Zambezi River system, Namibia) *Marcusenius altisambesi*

3.5. DISCUSSION

We recognize three species of *Marcusenius* in South Africa and *M. luombesi* sp. nov. from Mozambique based on the study of EODs by Kramer *et al.* (2007) and the morphological and genetic datasets of the present study. Two of the three South African species are also new, namely *M. krameri* sp. nov. that occurs in the Limpopo River system and *M. caudisquamatus* sp. nov. from the Mhlatuze and Nseleni rivers. In a PCA of all the morphological characters studied, the specimens of *M. pongolensis* form a coherent cluster on a plot of the first and second components that is non-overlapping with *M. krameri*, *M. caudisquamatus* and *M. luombesi* (Figure 3.5a). As shown by the DNA sequence analysis (Figure 3.4 and Table 3.2), *M. krameri*, *M. caudisquamatus*, *M. luombesi* and *M. altisambesi* are the sister group of a lineage made up of topotypical “*pongolensis*” and “*macrolepidotus*”. We, therefore, conclude that the significant morphological differences and genetic divergences between the *Marcusenius* lineages found in the present study warrant their recognition as separate species.

The morphological differentiation of the studied species may reflect adaptations to different geographical areas and life history strategies. When *M. krameri* and *M. luombesi* are compared to *M. pongolensis* and *M. caudisquamatus*, their dorsal and anal fins are set well forward which decreases the trunk length and increases body depth. This allows for a longer posterior section of the body, probably to augment rapid swimming thrust over short distances, power and manoeuvrability (Kramer *et al.*, 2007, 2012). Both these species inhabit rivers with weaker flowing water and ample reed cover, typical habitat where speed over short distances and manoeuvrability may be important. A similar pattern is also seen in *M. altisambesi* of the Upper Zambezi and Okavango systems (Kramer *et al.*, 2007) and in *Petrocephalus longianalis* Kramer, Bills, Skelton & Wink, 2012 from the tropical Luapula River. The converse of this is

the increased anterior body length with the dorsal and anal fins positioned backwards, an increased trunk length, decreased body depth and increased caudal peduncle depth. This type of body shape is characteristic of active swimmers and serves to increase speed and acceleration for long periods, an adaptation for survival in the strong currents found in the rivers inhabited by *M. pongolensis* and *M. caudisquamatus*.

The morphometric analyses of the present study demonstrated a degree of differentiation within *M. pongolensis* between different South African river systems (Figure 3.5b). Particularly the Kosi population showed a decreased trunk length and increased body and caudal peduncle depth (Table 3.4). Compared to the differentiation between other *Marcusenius* species, the differentiation within *M. pongolensis* is minor and may be as a result of local adaptation to the currently isolated Kosi River system.

The gill raker counts proved to be important in the differentiation of the South African species (Table 3.6). The patterns and counts of gill rakers in the posterior series do not overlap between *M. pongolensis*, *M. krameri* and *M. caudisquamatus*. Gill rakers of fishes have evolved in accordance with food and feeding habits and are an important character in identifying different species and their feeding preferences in other families (e.g. Abuzinadah, 1995). Gill raker counts and differences in the vertebral and teeth counts may be informative in mormyrid systematics, but need to be explored further. Sullivan and Hopkins (2005) have already shown that differences in vertebral counts, amongst other characters, proved important in identifying species of the mormyrid genus *Stomatorhinus* Boulenger 1898. Many questions regarding the EODs of the new species also remain, all of which are potential areas for more research. In studies of other southern African mormyrids, morphology and genetics yielded congruent

results with EODs, confirming its usefulness as a systematic character (Kramer *et al.*, 2007; 2012; Kramer & Swartz, 2010; Kramer & Van der Bank, 2011).

Sequence analysis of the mitochondrial *cyt b* gene confirmed that specimens of *M. krameri*, *M. caudisquamatus* and *M. lucombesi* were distinct from each other (0.9-3.5% divergence, see Table 3.2) and all three species are distinct from *M. pongolensis* and *M. macrolepidotus* (2.0-5.8% divergence) (Figure 3.4). The level of *cyt b* genetic divergence between all the analysed species (0.9-6.8%) are comparable with species-level differences in other mormyrids, such as *Marcusenius* species assessed by Kramer *et al.* (2007) (1.5-8.5%), and *Petrocephalus* species assessed by Lavoué *et al.* (2008) (1.5-14.5%) and by Kramer *et al.* (2012) (0.8-4.2%). Based on a crude molecular clock of about 2% sequence divergence/Ma (Brown *et al.*, 1979) and a molecular clock of 0.0058 % sequence divergence/Ma (Burridge *et al.*, 2008), the divergence of the new species from their most recent ancestor (*M. altisambesi* of the Upper Zambezi) possibly occurred between 1.0 and 2.5 or 3.4 to 6.0 million years ago, respectively. The basal position of *M. altisambesi* within this group is consistent with the hypothesis that the Upper Zambezi discharged to the Indian Ocean through the Limpopo valley until about 2 million years ago (Moore & Larkin, 2001; Skelton, 2001). The geological reviews in Moore and Larkin (2001) and Stankiewicz and de Wit (2006) show that the headwaters of the lower Zambezi captured the middle Zambezi via the Kariba Gorge, where the middle Zambezi captured the Upper Zambezi via the Batoka Gorge, creating the present course of the main Zambezi River. This could explain the isolation of the three species in South Africa, but further research is required to understand relationships among all the *Marcusenius* species.

CHAPTER 4: PHYLOGENY AND BIOGEOGRAPHY OF MORMYRID FISHES OF THE
GENERA *MARCUSENIUS* AND *PETROCEPHALUS* (TELEOSTEI: MORMYRIDAE)
IN SOUTHERN AND EASTERN AFRICA

ABSTRACT

Evolutionary relationships among species of *Marcusenius* and *Petrocephalus* genera in southern and eastern Africa were estimated based on mitochondrial *cyt b* and nuclear S7 intron 1 DNA markers. This chapter presents a total evidence phylogeny for all ten southern and eastern African *Marcusenius* species and one Congo Basin lineage. The results of all phylogenetic approaches are highly congruent and provide very strong support for recognizing *Marcusenius* as a monophyletic assemblage that evolved from a single common ancestor. A deeply divergent *M. livingstonii* appeared more basal and ancestral to the remaining species. The southern and eastern African *Petrocephalus* is divided into two deeply mitochondrial and nuclear divergent species groups that are separated by the Victoria Falls. The Western group evolved from the Congolese species and the Eastern group from the species north of the East Coast systems. The phylogenetic relationships within each group were not supported and future studies incorporating more molecular characters and species will likely help to resolve the relationships. Congruence found between the available geological history and the divergence times ranging between 0.8-5 Ma ago made it possible to infer the biogeographical history of both genera in southern and eastern Africa, which suggested that vicariant events associated with the formation of the major river systems played a major role in the evolution and distribution of *Marcusenius* and *Petrocephalus* species in southern and eastern Africa. Interestingly, this pattern was shared with other members of the freshwater fish fauna of the same region. The high degree of genetic differentiation and the discovery of at least three previously unidentified lineages in this study suggest that mormyrid diversity within southern and eastern Africa may be underestimated and requires further systematic study.

4.1. INTRODUCTION

Freshwater fishes are informative subjects in biogeographical studies (Lundberg, 1993; Bermingham & Martin, 1998; Avise, 2000; Arbogast & Kenagy, 2001), because their evolution is tightly linked to the geological history of the drainage basins they inhabit, as they do not disperse easily through salt water or across land (Posadas *et al.*, 2006; Brown *et al.*, 2010; Pinton *et al.*, 2013). The biogeographical patterns of freshwater species provide an historical record which can resolve how key events in the geological history of drainage basins occurred (Cotterill & De Wit, 2011).

A combination of climatic and geological changes fundamentally modified the Southern and Eastern African Basins through the recent geological past (Figure 4.1), and is now recognised as having dominated the evolution of their biodiversity (Cotterill, 2003a). Molecular studies of freshwater fishes in these basins have revealed concordant patterns of historical biogeography and regional genetic structuring, although the degree of structuring within taxa differed between studies (Koblmüller *et al.*, 2006; Katongo *et al.*, 2007; Dorn *et al.*, 2011). The historical biogeography proposed for the Southern and Eastern African Basins shows that speciation and the principal divergence events of primary freshwater fishes have interfaced closely with evolving drainage systems across tropical Africa (Cotterill, 2003a; Goodier *et al.*, 2011; Pinton *et al.*, 2013). The geological timing proposed for the biogeography agrees with the formation and the activities of the East African Rift System (EARS), geologically dated to have started between 4 and 12 million years (Ma) ago (Moore & Larkin, 2001; Stankiewicz & de Wit, 2006). Brown *et al.* (2010) indicated that the ancestor of the southern and eastern African mastacembelids (spiny-eels) appeared ~11.9 Ma, but did not diversify until ~5 Ma, after a relatively long lag-time. This pattern was further identified in a large-scale phylogeny

of *Synodontis* catfishes in tropical Africa, where the divergence between the eastern African clade in Lake Tanganyika (Figure 4.1) and the Zambezi/Congo clade was dated at 8.8 Ma (6-13 Ma) (Pinton *et al.*, 2013). The congruence of these studies indicates that Miocene-Pliocene divergences isolated the Congo and the Eastern African species from each other, and this concurs with known events in the western branch of the active EARS, with an apparent focus around 5 Ma ago (Goodier *et al.*, 2011). In addition to river fishes, these geomorphological changes had an important impact on the region's mammalian biodiversity and evolution, as indicated by the biogeography of *Damaliscus* and *Kobus* antelopes (Cotterill, 2000, 2003b). A Pliocene evolutionary event is also known to have split mole rats of the genus *Fukomy* (Van Daele *et al.*, 2007) and *Heliophobius* (Faulkes *et al.*, 2011) that are widely distributed on both sides of the EARS.

The observed biogeographic patterns suggest that freshwater taxa in these regions have an older established association with the hydrogeological changes and have attained a common pattern of geographical subdivision as a result of being subjected to the same environmental history. All subsequent divergences in the Pleistocene, ~2 Ma ago, correspond to periods of several recent drainage rearrangements in the Lower Congo and the Zambezi drainage systems, i.e. in the Southern African Basin (Moore & Larkin, 2001; Cotterill & De Wit, 2011). The affiliation of the Upper Zambezi fish fauna with that of the Congo River was long recognised and there is evidence of existing discharges between streams and swamps in the Congo-Zambezi watershed (Bell-Cross, 1965). These can be attributed to the rearrangements of Upper Zambezi and Paleo-Chambeshi headwaters during the Pleistocene (Cotterill & De Wit, 2011).

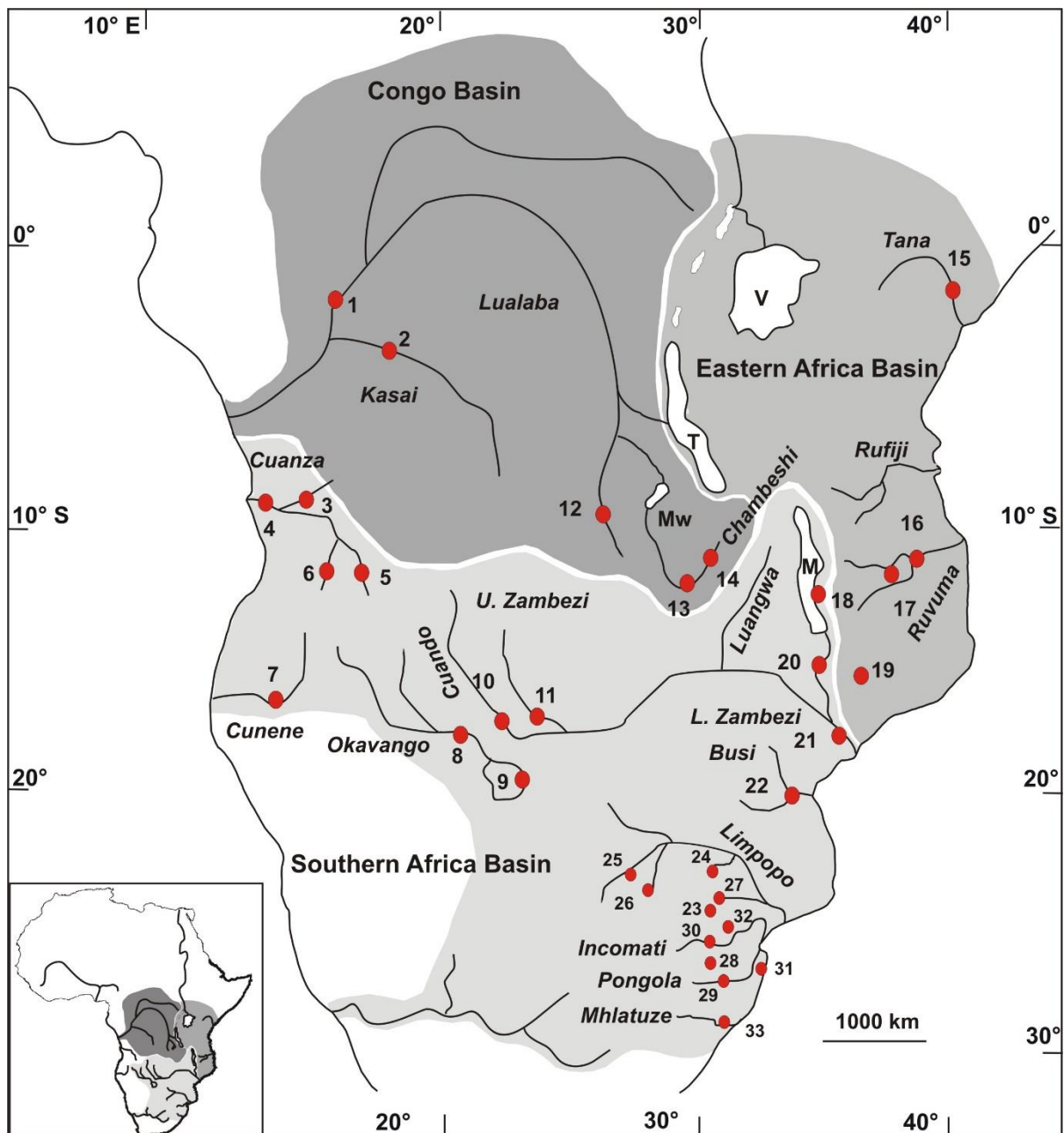


Figure 4.1. The origin of the studied samples of *Marcusenius* and *Petrocephalus* from the Congo, Southern and Eastern African Basins. Only the larger rivers are shown. Numeric codes for origin of samples are detailed in Table 4.1. Mw = Mweru Lake, M = Lake Malawi, T = Lake Tanganyika, and V = Lake Victoria.

Phylogenetic species relationships and the processes of speciation within the species-rich mormyrid genera, *Petrocephalus* Marcusen, 1854 and *Marcusenius* Gill, 1862 in the Southern and Eastern Africa Basins are poorly understood. *Petrocephalus* is the only genus of the sub-family Petrocephalinae and currently comprises 30 species (Kramer *et al.*, 2012; Lavoué, 2012) that are distributed across tropical Africa to subtropical freshwater regions of South Africa (Kramer & Van der Bank, 2000). Kramer *et al.* (2007) recognised 38 valid species in the genus *Marcusenius*, distributed from tropical Nilo-Sudan Basin (Boden *et al.*, 1997) to sub-tropical regions of South Africa (Kramer *et al.*, 2007; Chapter 3). Since then, Kramer (2013b) added a new species from the Comoé River, Côte d'Ivoire; Kramer and Wink (2013) described another from the Cunene River on the border between Angola and Namibia, and three more species were described in Chapter 3, raising the number of species to 43 across tropical and sub-tropical Africa.

According to various studies (Whitehead & Greenwood, 1959; Gosse, 1984; Seegers, 1996; Lavoué, 2012; Kramer *et al.*, 2013), *Petrocephalus* in the Southern and Eastern African Basins is distributed from the Katonga River, Lake Victoria, in the north to the Pongola River in South Africa in the south, and from the Congo, Cuanza and Cunene Rivers in the west to the central-southern drainage systems such as the Okavango, Cuando and Zambezi-Luangwa Rivers (Figure 4.1). After comprehensively reviewing southern and eastern African *Petrocephalus*, Kramer *et al.* (2012) recognized 15 valid species in these regions, including *P. catostoma*, now only known from the Ruvuma River (Figure 4.1, locality no. 16). They reinstated species previously synonymised with *P. catostoma*: *P. degeni* from Lake Victoria, *P. stuhlmanni* from Kingani River, in Tanzania, *P. haullevillii* from Lower Congo, *P. congicus* and *P. steindachneri* from Rufiji drainage, and elevated *P. squalostoma* from Lukinda River, a tributary of Mweru Lake and *P. tanensis* from the Tana River to a species rank. They also described *P. okavangensis*

and *P. magnitrunci* from the Okavango Delta, *P. magnoculis* from the Cunene River, *P. longicapitis* from the Upper Zambezi, *P. petersi* from the Lower Zambezi River, and *P. longianalis* from the Lufubu River in Northern Zambia. Lavoué (2012) added another species, *P. frieli*, from the Luapula River system, (Congo Basin). For the South African taxon, interspecific differentiation was reported in *P. wesselsi* (Kramer & Van der Bank, 2000).

The distribution of *Marcusenius* in the Southern and Eastern Africa Basins is that described for *Petrocephalus* (Figure 4.1). In a revision of the widespread *M. macrolepidotus* species complex, Kramer *et al.* (2007) discovered that there are at least six *Marcusenius* species in southern and eastern Africa, five of those species were found within the *M. macrolepidotus* species complex. They described *M. devosi* from Kenya's Tana River and *M. altisambesi* from the Upper-Zambezi and Okavango river systems, and resurrected *M. angolensis* of the Cuanza River and *M. pongolensis* of the Pongola River from the synonymy of *M. macrolepidotus*, now only known from the Lower Zambezi River and its tributaries. The sixth species, *M. livingstonii* is clearly different from those recognised in Kramer *et al.* (2007) and occurs in parts of the Malawi-Shire and Ruvuma river systems. *Marcusenius multisquamatus* was described from the Lower Cunene River, Namibia (Kramer & Wink, 2013). *Marcusenius luombesi* was recently described from the Ruvuma River, northern Mozambique (Chapter 3), together with two new species from South Africa: *M. krameri*, which occurs in the Limpopo River system, and *M. caudisquamatus* from the Mhlathuze and Nseleni Rivers (Chapter 3).

Additional *Marcusenius* diversity is still suggested by reports of substantial morphological differentiation. The morphological divergence or differentiation of *Marcusenius* specimens from the Luapula drainage has long been noted, with Worthington (1933) reporting that they differed from topotypical *M. macrolepidotus*. Worthington (1933) clearly suggested that his

form of *Marcusenius* represented a different species, but he never described it, citing that it should only be done when more material was available for study. Worthington (1933) made no mention of *M. moeruensis*, described from Lake Mweru, Luapula drainage. Kramer *et al.* (2007) indicated that the synonymy of *M. moeruensis* and *M. graeverti* from Ruaha River, Tanzania, was unjustified, concluding that more taxonomic studies were required to resurrect this species.

Based on a phylogeny of only four *Marcusenius* species (*M. devosi*, *M. macrolepidotus*, *M. altisambesi*, *M. pongolensis*), Kramer *et al.* (2007) indicated that southern African *Marcusenius* may have originated from an eastern African ancestor, with the Tana River's *M. devosi* rooted at the base of phylogeny. Furthermore, it has been proposed that *M. altisambesi* of the Upper Zambezi and the South African *M. pongolensis* are the descendants of the same *Marcusenius* species which previously inhabited the proto-Zambezi-Limpopo System that drained into the Indian Ocean through the Limpopo Valley until about 2 Ma ago. Based on a limited number of species from the Okavango, Upper Zambezi, Ruvuma and Limpopo river systems, Kramer *et al.*'s (2012) genetic results, confirmed by morphology, showed that the Victoria Falls in the Zambezi River system was a strong barrier that divided *Petrocephalus* into two major clades: the species-rich western group (from the Cuanza, Cunene, Okavango and the Upper Zambezi rivers) and the eastern group (from the Tana, Ruvuma and Lower Zambezi rivers systems, and the Limpopo River in South African), which generally holds fewer fish species.

Given the large number of *Petrocephalus* and *Marcusenius* species described lately (Kramer *et al.*, 2007, 2012; Lavoué, 2012, Kramer, 2013b; Kramer & Wink, 2013; Chapter 3), further analyses with more extensive taxon representation from the Southern and Eastern African Basins are required to reconstruct any evolutionary history in adequate detail. To this end,

Waters and Wallis (2000) and Swartz *et al.* (2009) agree that phylogenetic and biogeographic analyses should be inclusive of extant, historically- and/or evolutionarily-isolated lineages across the taxon's distribution, as these are more informative than currently described species and are the appropriate units for understanding the biogeography of any widespread taxa. Because *Marcusenius* and *Petrocephalus* have overlapping distributions in all major southern and eastern African river systems, they are expected to exhibit comparable biogeographic patterns and these are expected to be comparable to those shown by other freshwater fishes, as they may have been exposed to the same historical events. Any congruent patterns across these taxonomically- and ecologically-diverse genera will allow for more powerful inferences of the major events that have influenced the evolutionary history of this regional biota. Conversely, dissimilar patterns may reflect the influence of species-specific responses to a shared history due to differences in ecology, life history or dispersal capabilities among the taxa (Bermingham & Martin, 1998).

In the present study, the phylogenetic relationships and biogeographical scenarios for the majority of the species of *Marcusenius* and *Petrocephalus* in the Southern and Eastern African Basins, from the Tana River in Kenya to South Africa's Mhlatuze River in the south, were investigated by the analyses of sequence variation in one mitochondrial cytochrome *b* (cyt *b*) marker and an unlinked ribosomal nuclear S7 intron 1 marker (S7 intron 1). Cytochrome *b* is useful for elucidating both deep-level and shallow time relationships (Hillis, 1987), but in order to validate phylogenetic hypotheses and determine deeper relationships, the S7 intron 1 was also used. The purpose is to understand the speciation of *Marcusenius* and *Petrocephalus* within the context of the broader drainage basin patterns and processes that have led to the present-day biodiversity in the Southern and Eastern African Basins. In addition, an improved understanding of the timing of the above-mentioned geological events, combined with

biogeographic data, should help elucidate divergence times within *Marcusenius* and *Petrocephalus*, and, further, indicate how dispersal may also have modified their contemporary ranges.

4.2. MATERIALS AND METHODS

4.2.1. Sample collection and DNA extractions

Using electro-fishing, specimens of *M. krameri*, *M. caudisquamatus* (see Chapter 3 for species descriptions), *M. pongolensis* and *Petrocephalus wesselsi* were collected in rivers or tributaries of the Limpopo, Incomati, Pongola, Kosi, Nseleni and Mhlatuze River systems in South Africa (Table 4.1; Figure 4.1). Muscle tissue samples were placed in 99% EtOH in the field and transferred to a -20°C freezer upon returning to the laboratory. In addition, whole fish specimens from which muscle tissue was dissected were fixed in formalin and deposited in the National Fish Collection of the South African Institute for Aquatic Biodiversity (SAIAB), Grahamstown, as voucher specimens. Representatives of species of *Marcusenius* and *Petrocephalus* from outside South Africa were sourced from SAIAB and the Institute of Pharmacy and Molecular Biotechnology (IPMB), University of Heidelberg, Germany (Table 4.1; Figure 4.1).

Table 4.1. *Petrocephalus* and *Marcusenius* specimens included in the present study. Numbers in brackets indicate the sampling localities depicted in Figure 4.1. Multiple accession numbers represent different collections at the same site. SAIAB accession numbers are presented without a prefix. Institute of Pharmacy and Molecular Biotechnology accession numbers are prefaced by IPMB. GenBank accession numbers for the *cyt b* data are given.

Species name	Locality name (no. on Figure 4.1)	River System	Latitude/Longitude	SAIAB / IPMB numbers	Cyt b GenBank numbers
<i>Petrocephalus</i>					
<i>P. catostoma</i> Günther, 1866	Lake Chilwa (20)	Lake Malawi	15°48'35"S, 35°38'47"E	182194	KJ409663
	Ruo River (19)	Lake Malawi	16°03'16"S, 35°40'16"E	119063	KJ409664, KJ409665
	Lumbo River (18)	Lake Malawi	16°06'16"S, 35°28'33"E	119005; 87187	KJ409668 KJ409666, KJ409667
	Wami River	Rufiji			GU982923, GU982924
	Divue River	Rufiji			GU982925
	Kalimbero River	Rufiji			GU982926
<i>Petrocephalus</i> "Cuanza"	Kutato River (6)	Cuanza River	12°16'38"S, 16°21'3"E	85038	
	Lucala River (3)	Cuanza River	09°4'37"S, 15°59'59"E	85135	
	Kuquema River (5)	Cuanza River	12°1'39"S, 17°37'51"E	85013	
<i>P. haullevillii</i> Boulenger, 1912	Oubangui River (1)	Congo-Kasai		77773, 77609	
<i>Petrocephalus</i> "Lualaba"	Kisanfu River (12)	Congo-Lualaba	10.8003°S, 25.98129°E	82854	
<i>P. longicapitis</i> Kramer, Bills, Skelton & Wink, 2012	East Caprivi, Katima Mulilo (11)	Upper Zambezi	17°29'11"S, 24°15'25"E	83809	KJ409669 KJ409674, KJ409676, KJ409677
<i>P. magnoculis</i> Kramer, Bills, Skelton & Wink, 2012	Cunene River (7)	Cunene	17°24'24"S, 14°13'1"E	78788	KJ409675
				78790; 83893	KJ409671
<i>P. okavangensis</i> Kramer, Bills, Skelton & Wink, 2012	Okavango river, Popa rapids (8)	Okavango River	18°7'19"S, 21°34'59"E	83866	KJ409672, KJ409670
	Cuando River (10)	Okavango River	17°46'52"S, 23°20'26"E	83809	KJ409673
	Katima Mulilo (11)	Upper Zambezi	17°29'11"S, 24°15'25"E	188270	KJ409658, KJ409659
<i>P. wesselsi</i> Kramer & Van der Bank, 2000	Mokolo River (25)	Limpopo	23°22'01.7"S, 27°41'09.6"E	188239	KJ409660, KJ409661
	Olifants River (23)	Limpopo	24°31'41.6" S, 29°32'47.2"E	70080	KJ409662
	Nkomati River (28)	Incomati	25°46'5"S, 31°28'29"E		

Table 4.1: continued...

Species name	Locality name (no. on map)	River System	Latitude/Longitude	SAIAB / IPMB numbers (individual no.)	GenBank numbers
<i>Marcusenius</i>					
<i>M. angolensis</i> (Boulenger, 1905)	Kawa River (3)	Cuanza	09°10'14"S, 13°21'59"E,	84768	KJ409681
	Cuanza River (4)	Cuanza	09°10'17"S, 13°22'5"E	84735	KJ409680
	Kuquema River (5)	Cuanza	09°10'3"S, 13°22'12"E	84751	KJ409679
	Lucala River (6)	Cuanza	09°31'21"S, 14°23'10"E	84707	KJ409678
<i>M. altisambesi</i> Kramer, Skelton, Van der Bank & Wink, 2007	Upper Zambezi (11)	Upper Zambezi	18°04'53"S, 24°02'35"E	IPMB: Nam1, Nam 148	DQ863654, DQ863655
<i>M. caudisquamatus</i> sp. nov.	Mhlatuze River (33)	Mhlatuze	28°50'44.2" S, 31°51'59.6"E,	88692	KJ409683
	Nseleni River, (not shown)	Nseleni	28°44.766"S, 31°58.728"E	96730	KJ409684
<i>M. devosi</i> Kramer, Skelton, Van der Bank & Wink, 2007	Tana River (15)	Tana River	-1.87725000, 40.13958333	96543	KJ409682
				IPMB: 22668.	DQ863666,
				TanaR 22669.	DQ863667,
				TanaR 22665.	DQ863668
<i>Marcusenius krameri</i> sp. nov.	Olifants River (23)	Limpopo	24°31'41.6" S, 29°32'47.2"E	188291	KJ409695
	Tshinane River (24)	Limpopo	22°53'56.3" S, 30°31'28.7"E	188290	
	Mokolo River (25)	Limpopo	23°22'01.7" S, 27°41'09.6"E	188294	
	Mogalakwena River (26)	Limpopo	24°42'04.2" S, 28°24'40.8"E	188295	KJ174297
<i>Marcusenius luombesi</i> sp. nov.	Lucombe stream, Niassa Reserve (17)	Ruvuma	12°5'15"S, 37°33'38 "E	73884	KJ174293
				73885	
<i>M. livingstonii</i> (Boulenger 1989)	Ruvuma River (16)	Ruvuma	11°25'30"S, 38°28'48 "E	73882	KJ409686
				87178	KJ409685
<i>Marcusenius</i> "Luapula"	Chambeshi River, Kapita bridge (13)	Luapula-Congo	12°01'55.4" S, 30°25'52.2"E	99026	
	Mushilashe River (14)	Luapula-Congo	11°00'56" S, 31°05'21"E	96131	
<i>M. macrolepidotus</i> (Peters, 1852)	Marromeu Lagoon (21)	Lower Zambezi	18°19"S, 35°55"E	61603	DQ863664;
				61592	DQ863663
	Lucite River, Nyangapwa Stream (22)	Buzi	19°58'S, 33°24'52 "E	67369	DQ863662
<i>M. multisquamatus</i> Kramer and Wink, 2013	Cunene River waterfall (7)	Cunene	08°45.58'S, 31°6.73"E,	79879;	KJ409689
				78785;	KJ409687
				87287	KJ409688

Table 4.1: Continued...

Species name	Locality name (no. on map)	River System	Latitude/Longitude	SAIAB / IPMB numbers (individual no.)	GenBank numbers
<i>M. pongolensis</i> (Fowler, 1934)	Pongola River (29)	Pongola	27°18'33.7"S, 30°53'51.7"E	188296	KJ409693; KJ409694
	Siyadla River (31)	Kosi	27°04'53"S, 32°47'11"E	88615	KJ409690
	Nkomati River (28)	Incomati	25°46'04"S, 31°28'28"E	69907	KJ409691
	Sand River (32)	Incomati	24°42'53"S, 31°12'14"E	188304	KJ409692
Outgroups					
<i>Campylomormyrus compressirostris</i>					DQ630644
<i>M. stanleyanus</i> (Boulenger 1897)	Baidou River (2)	Congo-Kasai		77713	

IPMB (University of Heidelberg, Institute of Pharmacy and Molecular Biotechnology, Im Neuenheimer Feld 364, D-69120 Heidelberg)

Total DNA extractions were performed following the protocol of the Promega DNA purification kit (Madison, New York). Amplification of a partial section of the mitochondrial *cyt b* gene of the *Marcusenius* species was achieved with the primers GluF (Machordom & Doadrio, 2001) and H15917 (Kramer *et al.*, 2003). Conditions for the amplification of *cyt b* in *Marcusenius* are as detailed in Chapter 2. For *Petrocephalus* *cyt b*, the primer combination of M172F 5' AAC CAC CGT TGT TAT TCA ACT A 3' and ThrR 5' ACC TCC GAT CTT CGG ATT ACA AGA CCG 3' (Machordom & Doadrio, 2001) was used to amplify the DNA under the following PCR conditions: 2 minutes at 94°C; 35 cycles of 45 seconds at 94°C, 45 seconds at 56°C and 90 seconds at 72°C; and extension of 10 minutes at 72°C. The amplification of the S7 intron 1 for both *Marcusenius* and *Petrocephalus* was as detailed in Chapter 2. Amplification of all the genetic markers were performed in separate 25 µl reaction and each contained: ~1-3 µl template DNA (depending on the DNA concentration) template DNA, 1 x reaction buffer, 25 mM MgCl₂, 0.125 mM of dNTPs (Promega), 0.2 units of Super-therm® Taq DNA polymerase and 25 pmol of each of the forward and reverse primers. All successful PCR products were purified with the QIAquick PCR purification kit (QIAGEN, Valencia, California) before sequencing, using the BigDye Terminator 3.1 cycle-sequencing ready reaction kit (Applied Biosystems, Foster City, California). The end products of cycle-sequencing were precipitated using an ethanol precipitation procedure (Sambrook & Russell, 2001). Sequences were screened on an ABI 3730xl DNA Genetic Analyzer (Applied Biosystems) at Macrogen Inc. (Seoul, South Korea) or on an ABI 3100 at Rhodes University (Grahamstown, South Africa).

Original sequence chromatograms were checked manually and edited for ambiguities using Lasergene v. 9.0.5 (DNA Star, Inc., Madison, WI). Multiple alignments were performed using

ClustalX 2.1 (Larkin *et al.*, 2007). The program DnaSP 5 (Librado & Rozas, 2009) was used to translate nucleotide sequences to amino acid sequences to test and check for stop codons to determine whether the sequences were of mitochondrial origin and not pseudogenes. Nuclear S7 intron sequences were subjected to subsequent phylogenetic analyses with standard one-letter ambiguity symbols.

4.2.2. Phylogenetic analyses

Sequences of *Campylomormyrus compressirostris* isolate K05 (Genbank numbers DQ630644 for cyt *b* and DQ630552 for S7 intron 1) (Feulner *et al.*, 2006) and *M. stanleyanus* (SAIAB 77713) of the subfamily Mormyrinae were used as outgroups to root the *Marcusenius* phylogenetic trees. Both genera, *Marcusenius* and *Campylomormyrus*, have long been recognised as sister genera using both cyt *b* and S7 intron analyses (Lavoué *et al.*, 2000, 2003; Sullivan *et al.*, 2000). *Marcusenius stanleyanus* (SAIAB 77713) and *Petrocephalus microphthalmus* (cyt *b* Genbank no. AP009609) were used as outgroups for the phylogenetic analyses of southern and eastern African *Petrocephalus* species. *Marcusenius stanleyanus* in the *Marcusenius* analysis and *P. microphthalmus* in the *Petrocephalus* analysis were included as additional out-groups and representatives of the species that occur outside the Southern and Eastern African Basins. Additional cyt *b* sequences for *M. macrolepidotus*, *M. altisambesi* and *M. devosi* published by Kramer *et al.* (2007) were available on GenBank and included in the analyses, while the same individuals accessioned in SAIAB and/or IPMB were amplified for the S7 intron marker (Table 4.1). Four additional cyt *b* sequences of *Petrocephalus catostoma* from Lavoué *et al.* (2010) were downloaded from the GenBank. A model of evolution for each dataset was chosen using the Akaike Information Criterion (AIC) in Modeltest version 3.7 (Posada & Crandall, 1998).

Lavoué *et al.* (2003) have indicated that the *cyt b* and S7 intron datasets of various mormyrid genera are incongruent with each other when performing the incongruence length difference test (Bull *et al.*, 1993). Due to this, a partition homogeneity test was not conducted before this study. Even though Bull *et al.* (1993) suggested that incongruent data should not be combined for phylogenetic analyses, the combination of such data can increase the resolution and the support within phylogenetic trees, revealing ‘hidden signals’ present in the separate datasets (Gatesy *et al.*, 1999; Lavoué *et al.*, 2003). Thus, partition homogeneity tests were not performed and incongruence length difference was not used as a criterion to determine the congruency of the *cyt b* and S7 intron datasets prior to combining these for analysis.

Phylogenetic analyses were performed on each genetic marker separately and combined using maximum likelihood (ML) and parsimony (MP) implemented in PAUP* (Swofford, 2002), and by Bayesian methods using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). For ML, the parameters of the chosen model were implemented in a heuristic tree search, which employed TBR-branch-swapping and 100 random stepwise taxon-addition replicates. Nodal support was not determined for the ML phylogeny. The parsimony analysis was performed using heuristic searches, tree bisection–reconnection branch swapping and 1000 random additions of taxa. A parsimony analysis was used to assess branch support with 1000 non-parametric bootstrap replicates. Each bootstrap replicate was performed with 100 random taxon addition replicates. Two independent BI analyses were performed for each dataset. The Markov chain Monte Carlo process was set so that four chains (three heated and one cold) ran simultaneously for ten million generations. Parameter values and trees were sampled every 2000 generations. Stationarity was determined by examining

graphical plots of sampled likelihood scores from each of the analyses. For each dataset, the first 20% of sampled generations in each run were discarded as ‘burn-in’. Majority-rule consensus trees were calculated from the remaining 4000 trees pooled from the posterior probability distributions for each analysis. Posterior probability values greater than or equal to 95% were considered to represent significant support for the relationships reflected in the trees (Ronquist & Huelsenbeck, 2003). Model-corrected sequence divergences among sequences were determined using PAUP and the parameters of the respective model.

4.2.3. Estimates of divergence times

The mitochondrial *cyt b* datasets were used to estimate approximate divergence times for both genera as these datasets contained more species, including some sourced from GenBank, representing a broader distribution of the genera in southern and eastern Africa. The S7 intron 1 data could not be used for the dating analyses as it failed to resolve phylogenetic relationships within both genera, due to possible incomplete lineage sorting or low sequence variation (see below). A Bayesian Markov chain Monte Carlo algorithm, implemented in the program BEAST 1.7.1 (Drummond *et al.*, 2012), was used to estimate the age of the divergence of lineage. A relaxed molecular clock method that allows branch-specific rate variation, drawn from a log-normal distribution, was employed to co-estimate the tree and date the divergences of the lineages, under the chosen model. Due to a lack of a calibrated mutation rate for both *Petrocephalus* and *Marcusenius*, a general conservative *cyt b* sequence divergence of 1.4 - 2.5% per million years, as used in other mormyrid studies (Sullivan *et al.*, 2002), was used here. This range includes the 2% mean rate for vertebrate mtDNA that has been used in a number of studies for fishes that have unknown rates (Brown *et al.*, 2010; Goodier *et al.*, 2011). A maximum age of appearance 12 Ma

ago, determined for the southern and eastern African freshwater fauna (Brown *et al.*, 2010; Goodier *et al.*, 2011), was used as a calibration point for the root of the tree with a log-normal distribution. Tree priors were modelled according to a Yule speciation process and all other priors were set to their default values. The MCMC analyses were run for 60 million generations and sampled every 2000th generation, with the first 10% of samples discarded as burn-in. TRACER v1.5 (Rambaut & Drummond, 2007) was used to inspect and confirm the stationarity and that effective sample sizes were adequate for all parameters.

4.3. RESULTS

4.3.1. *Marcusenius*

Cytochrome b

A total of 818 nucleotides of a partial *cyt b* fragment were analysed, yielding 136 parsimony informative, 81 parsimony uninformative and 601 constant characters. Two equally parsimonious trees (RI = 0.883; CI = 0.777; tree length = 319 steps) were identified for the *cyt b* dataset. The resulting strict consensus phylogenetic tree obtained using PAUP* is represented in Appendix 4.1 with bootstrap values > 50%. A GTR+G model (Rodriguez *et al.*, 1990) with gamma distribution of rate variation and a proportion of invariant sites was identified for the dataset. The *cyt b* sequence divergence estimates among all the species or lineages varied from 0.9 to 11.4%, while divergences among individuals within species or lineages were between 0.0 and 2.4% (Table 4.2). Sequence divergences among individuals within species were higher for *M. macrolepidotus* (1.5-2.4%) than for *M. angolensis*, *M. caudisquamatus*, *M. multisquamatus* and *Marcusenius* “Luapula” (0.0-0.05%).

Table 4.2. Estimates of model-corrected sequence divergences (shown as percentages) among and within *Marcusenius* taxa. The Cyt *b* genetic distances are below the diagonal, while the S7 intron 1 divergences are above the diagonal.

	Within cyt <i>b</i>	1	2	3	4	5	6	7	8	9	10	11	12
1. <i>M. devosi</i>	0.1-07	-	0.69-1.0	0.69-1.0	0.69-1.0	0.7-1.10	0	0.69-1.0	0.69-1.0	2.7-3.8	0.69-1.0	3.9-4.3	3.8-4.1
2. <i>M. macrolepidotus</i>	1.5-2.4	4.8-6.6	-	0	0	0	0	0	0	2.1-2.8	0	3.3	3.1
3. <i>M. pongolensis</i>	0.1-0.8	5.4-5.5	1.3-3.2	-	0	0	0	0	0	2.1-2.8	0	3.3	3.1
4. <i>M. altisambesi</i>	0.9-1.7	4.8-6.2	3.3-5.6	2.8-4.2	-		0	0	0	2.1-2.8	0	3.3	3.1
5. <i>M. multisquamatus</i>	0.00	4.6-6.0	3.4-6.3	2.5-4.0	1.0-1.6	-	0	0	0	2.1-2.8	0	3.3	
6. <i>M. caudisquamatus</i>	0.0-0.5	5.3-6.8	2.8-5.8	2.3-4.3	2.2-4.5	2.3-4.6		0	0	2.1-2.8	0	0	0
7. <i>M. krameri</i>	0.1-0.5	5.0-5.8	2.7-4.8	2.0-2.7	2.0-3.1	2.0-3.0	0.9-2.4	-	0	2.1-2.8	0	3.3	3.1
8. <i>M. lucombesi</i>	0.2-0.5	5.8-5.6	2.8-4.8	2.8-3.3	2.0-3.1	1.9-3.0	1.2-2.9	1.0-1.7	-	2.1-2.8	0	3.3	3.1
9. <i>Marcusenius</i> "Luapula"	0.00	5.3-5.9	4.6-6.1	3.9-4.5	3.4-4.3	3.6-4.5	3.5-3.8	3.5-3.8	3.4-3.9	-	2.1-2.8	5.2-5.8	4.9-5.7
10. <i>M. angolensis</i>	0.0-0.5	3.8-4.8	2.9-4.8	2.3-3.2	2.0-2.6	2.5-3.0	1.9-2.60	1.8-2.6	2.0-2.6	2.9-3.3	-	3.3	3.1
11. <i>M. livingstonii</i>	0.9	8.6-9.9	8.5-10.5	8.0-9.4	8.4-10.1	7.9-10.0	8.3-9.5	8.3-9.5	8.1-9.2	8.4-9.5	7.7-8.8	-	1.2-1.6
12. <i>M. stanleyanus</i>	-	9.3-9.8	10-11.4	9.5-10.0	10.0-10.6	10.0-9.5	9.7-10.4	9.7-10	9.4-9.7	10.4	9.0-9.4	7.3-8.4	-

The *cyt b* MP, ML and BI analyses generated largely the same tree topology, and produced well resolved phylogenies. Both parsimony bootstrap (>80% BP, Appendix 4.1) and Bayesian PP (≥ 0.95 Figure 4.2) analyses indicated strong support for many species or lineages and relationships, while *M. multisquamatus* and the relationships between *M. lucombesi*, *M. caudisquamatus* and *M. krameri* were not supported ($\leq 70\%$ BP; < 0.95 PP).

The phylogenies consistently retained *M. livingstonii* from the Ruvuma River in a basal position, while the branch length indicated that this species is deeply divergent from all the analysed species included. Supported by strong nodal support (0.98 PP), *M. devosi* from the Eastern African Tana River appeared basal to the remaining species. This is similar to the phylogeny presented by Kramer *et al.* (2007), except that they did not have *M. livingstonii* in their phylogeny. *Marcusenius* “Luapula” from the Chambeshi River in the Congo Basin assumed an intermediate position between *M. angolensis* from the Cuanza River and the *M. altisambesi-multisquamatus* clade in the present study. *Marcusenius* “Luapula” was genetically divergent from the other species and may represent a new taxon, important to understanding the origin and evolution of the species inhabiting the Zambezi Basin.

Marcusenius multisquamatus and *M. altisambesi* were strongly supported as sister species (98% BP, 1.00 PP); however, the monophyly of the former is weakly supported by the parsimony (62% BP) analyses (Appendix 4.1) and not supported by the Bayesian (< 0.95 BP) analyses (Figure 4.2). Together, *M. altisambesi* and *M. multisquamatus* formed a well-supported sister clade to the species east of the Victoria Falls (0.99 PP), which was composed of two sister groups: *M. macrolepidotus* and *M. pongolensis* (99% BP, 1.00 PP), and *M. lucombesi*, *M. caudisquamatus* and *M. krameri* (70% BP, < 0.95 PP). Surprisingly, *M.*

lucombesi was a sister species to the clade formed by South African species, *M. krameri* and *M. caudisquamatus* (79% BP).

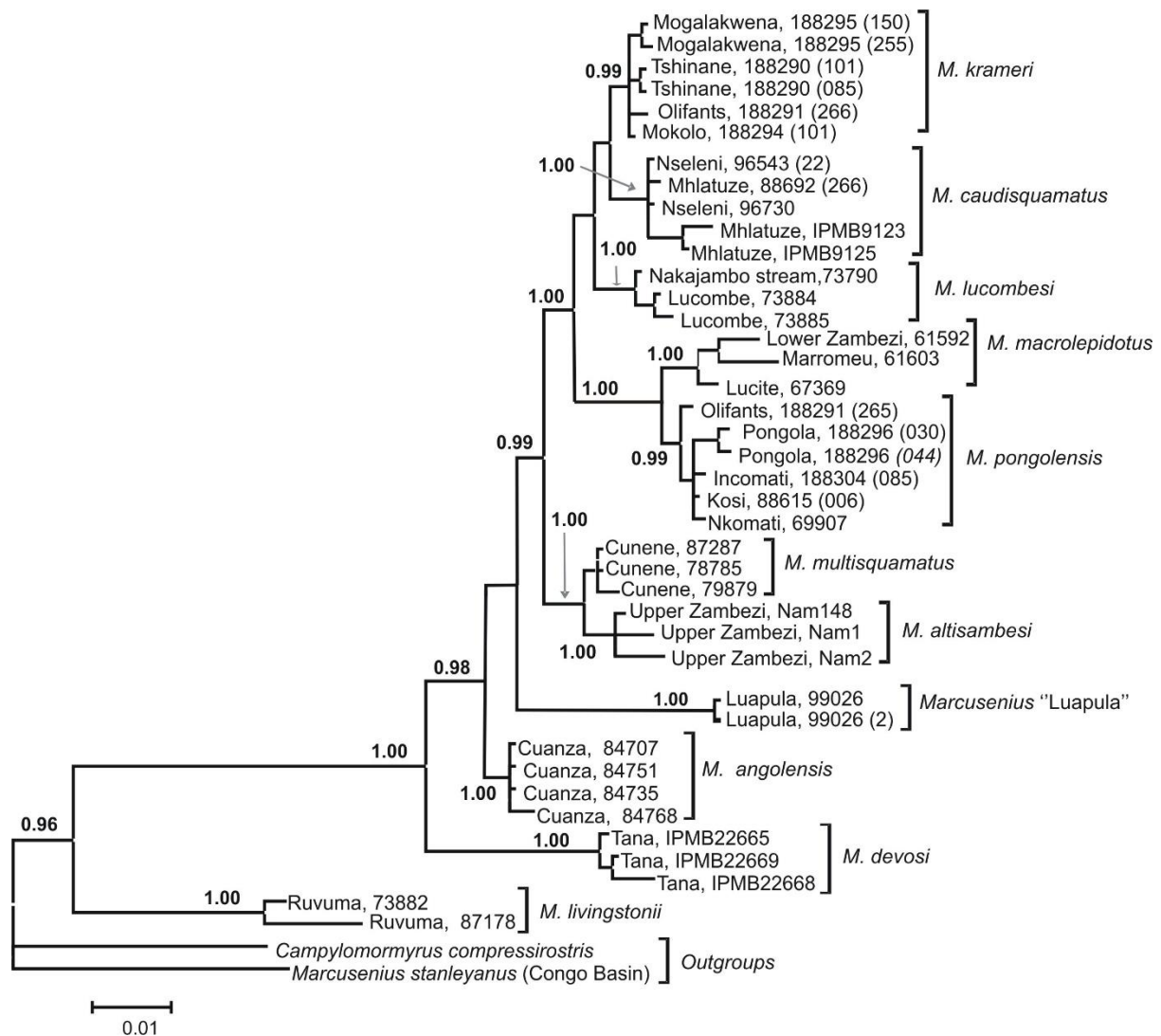


Figure 4.2. Maximum Likelihood ($-\ln L = 2352.48$) phylogram of relationships among *Marcusenius* species as reflected by the cytochrome *b* data, with Bayesian posterior probabilities > 0.95 indicated on the branches. For each species or lineage, individual localities, SAIAB and IPMB numbers are indicated. Individual numbers are indicated in brackets for accession numbers where more than one individual was sequenced.

S7 intron 1

In the 588 nucleotide characters of the S7 intron 1 data, only two non-overlapping gaps, nine and four nucleotides long, were identified for *M. devosi* and *M. stanleyanus*, respectively, and were treated as missing data. A total of 545 characters were constant, 36 were parsimony informative and 7 were uninformative. The parsimony analyses identified six equally-parsimonious trees (tree length = 45 steps, CI = 0.978, and RI = 0.986). The model that best described the S7 intron 1 data was a TVM (Posada, 2003) model. There were no sequence divergences between species, except for the divergence between both *M. devosi* individuals and between the two sequences of *Marcusenius* “Luapula” (Table 4.2). Furthermore, the MP, ML and BI analyses of the S7 intron dataset did not resolve the phylogeny (Appendix 4.2).

Combined cyt b and S7 intron 1 (cyt b-S7)

A total of 1406 nucleotides were analysed for the combined cyt *b* (818 nucleotides) and S7 intron 1 (588 nucleotides) dataset. Only 176 characters were parsimony informative, while 1143 were constant. Two equally parsimonious trees were identified for the cyt *b*-S7 data (tree length = 365 steps, CI = 0.789 and RI = 0.888) (Appendix 4.3). An unequal-frequency Kimura 3-parameter plus Gamma model (K81uf+I+G) (Kimura, 1981) (proportion of invariant sites = 0.4415, and gamma distribution of rate variation = 0.9572) was selected for the cyt *b*-S7 dataset.

Three phylogenetic analyses (MP, ML and BI) of the combined cyt *b*-S7 data yielded trees that were identical to those of the cyt *b* data alone. There was no strong support for the monophyly of *M. altisambesi* in the cyt *b*-S7 intron data (<0.95 PP and 66% BP, Figure 4.3 and Appendix 4.3, respectively), compared to the 1.00 PP (Figure 4.2) and 86% BP (Appendix 4.1) from the

cyt *b* data. Furthermore, the sister species relationship of *M. luombesi* with *M. caudisquamatus-krameri* was only supported by the combined cyt *b*-S7 dataset. This clade did not have significant support in the cyt *b* analyses (<0.95 PP; 70% BP), but was well-supported (0.95 PP, Figure 4.3; 70% BP, Appendix 4.3) by the cyt *b*-S7 analyses. All the other major nodes defining supported clades and species relationships in the cyt *b* analyses were also significantly supported by the cyt *b*-S7 bootstrap and posterior probability estimates.

Divergence time estimates

According to the cyt *b* data and divergence dating, the Ruvuma River's *M. livingstonii* diverged from the other *Marcusenius* species in the Southern and Eastern African Basins in the Pliocene, around 5.1 Ma (HPD: 2.86-8.23 Ma) ago (Figure 4.4). Another eastern African species, *M. devosi*, was split 3.2 Ma (HPD: 1.9-4.8 Ma) ago from the remaining species. *Marcusenius angolensis* and *Marcusenius* "Luapula" were retrieved as a monophyletic clade by the Bayesian Markov Chain Monte Carlo algorithm implemented in the BEAST, contrary to the previous analyses. This monophyletic clade was split from all the species of the Zambezi and Cunene drainages in the Southern African Basin in the Early Pleistocene, 2.4 Ma (HPD: 1.5-3.6 Ma) ago. Subsequently, around 2.0 Ma (HPD: 1.2-2.9 Ma) ago *M. altisambesi* and *M. multisquamatus* were isolated from those species east of the Victoria Falls. These two closely related species were separated 0.9 Ma (HPD: 0.3-1.7 Ma) ago. The two monophyletic species groups east of the Victoria Falls diverged 1.6 Ma (HPD: 1.0-2.5 Ma) ago and seem to have evolved concurrently. The split between *M. pongolensis* and *M. macrolepidotus* was very recent, around 1.2 Ma (HPD: 0.6-1.950 Ma) ago, as was the split between *M. luombesi*, from the tributary of the Ruvuma, and *M. caudisquamatus* and *M. krameri*, both from South Africa, around 1.3 Ma (HPD: 0.7-1.9 Ma) ago.

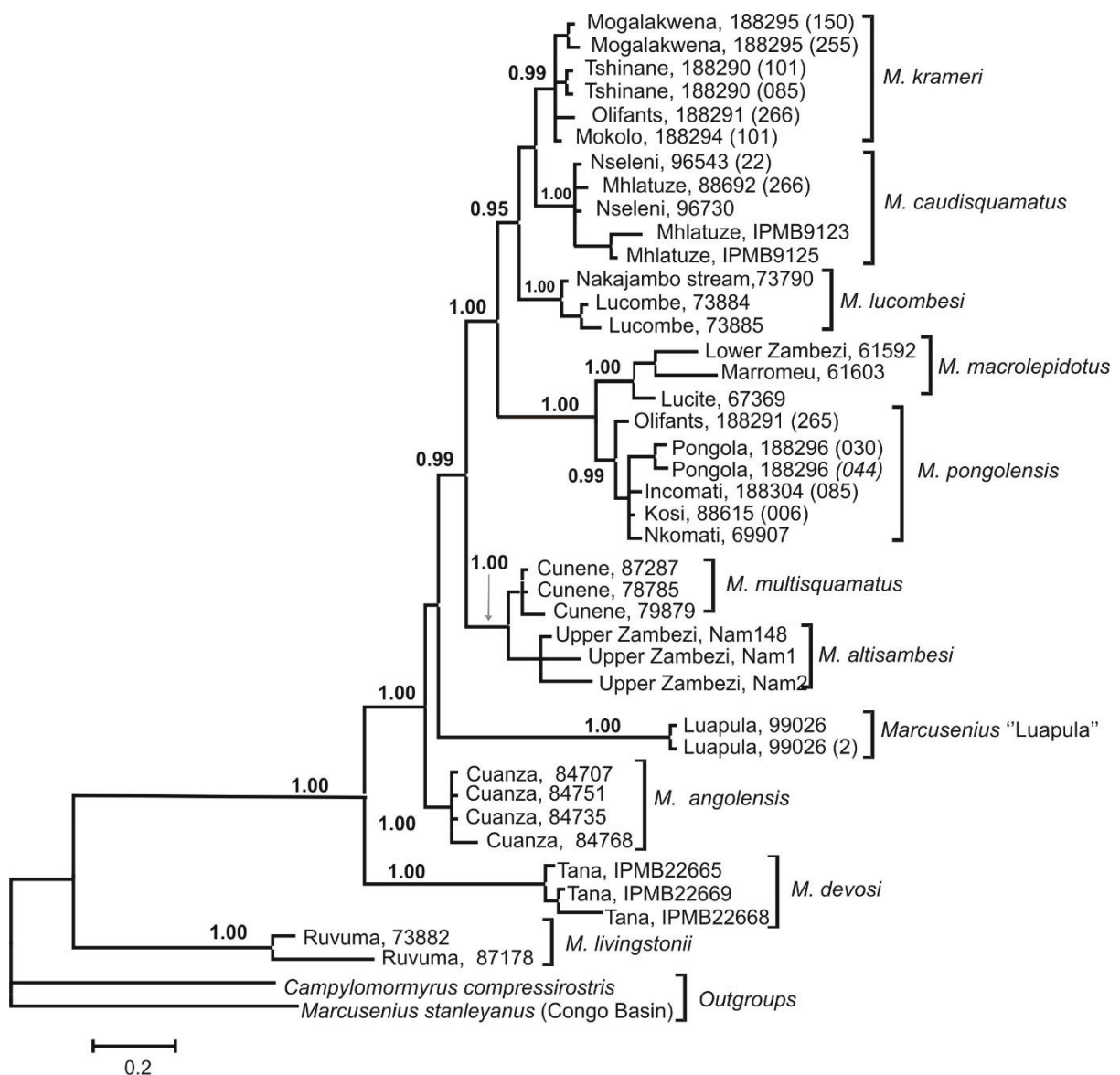


Figure 4.3. Maximum Likelihood ($-\ln L = 4149.29$) phylogram of relationships among *Marcusenius* as reflected by the combined cytochrome *b* and the S7 intron 1 with Bayesian posterior probabilities > 0.95 indicated on the branches. For each species or lineage, individual localities, SAIAB and IPMB numbers are indicated. Individual numbers are indicated in brackets for accession numbers where more than one individual was sequenced.

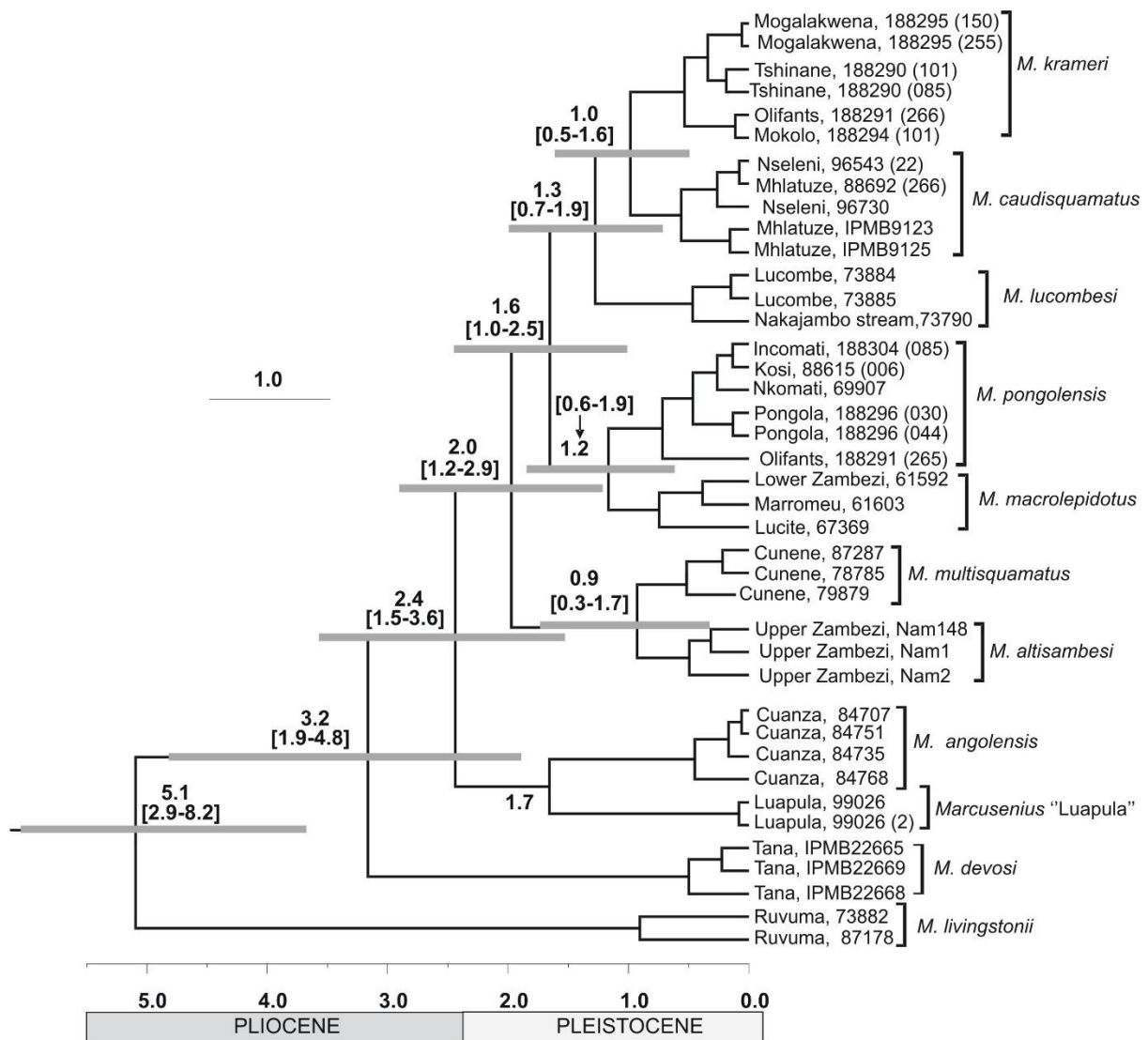


Figure 4.4. Estimates of divergence times (Millions of years ago) from the *cyt b* sequence data for southern and eastern African *Marcusenius* species. Values on the nodes represent the estimated mean divergence times and ranges (in square brackets) of species, inferred using Bayesian coalescent analyses implemented in BEAST. Bars represent 95% confidence intervals for divergence estimates. For each species or lineage, SAIAB and IPMB numbers are indicated. Individual numbers are indicated in brackets for accession numbers where more than one individual was sequenced.

4.3.2. *Petrocephalus*

Cytochrome b

A 720 nucleotide alignment of the partial *cyt b* fragment was analysed for *Petrocephalus* from the Southern and Eastern African Basins. The *cyt b* dataset contained 125 parsimony informative characters, 77 parsimony uninformative characters and 518 constant characters. Parsimony analyses yielded 5 equally parsimonious trees (tree length = 300 steps, RI = 0.633, and CI = 0.712). The nucleotide substitution model selected by the AIC for the *cyt b* dataset was TrN+I+G (Tamura & Nei, 1993). The phylogenies from the *cyt b* data (MP: Appendix 4.4; Bayesian posterior probabilities (PP): Figure 4.5) provided high support ($\geq 99\%$ BP and ≥ 0.97 PP) for the separation of species into two groups. Firstly, the Western group consisting of sequences from the Cuanza River (*Petrocephalus* “Cuanza”), Lualaba River (*Petrocephalus* “Lualaba”), *P. longicapitis*, *P. magnoculis*, *P. okavangensis* and *P. haullevillii*. All these species occur to the west of the Victoria Falls in the Middle Zambezi System, hence the Western group. The second group (henceforth referred to as the Eastern group) includes species east of the Victoria Falls, including that in South Africa, *P. catostoma* sequences from the Wami, Divue and Kalimbero Rivers (all from the Rufiji System), *P. catostoma* from the Lake Malawi system and *P. wesselsi* of South Africa. All monophyletic clades identifying species were strongly supported, at least in the MP analyses ($\geq 75\%$ BP, Appendix 4.4), but not all of these were supported by the Bayesian posterior probabilities (Figure 4.5).

According to the *cyt b* dataset, the Congolese species, *P. haullevillii* and *Petrocephalus* “Lualaba”, are well supported within the Western group (99% BP, Appendix 4.4 and 1.00 PP, Figure 4.5), as was expected given the close relationships between the Zambezian and Congolese ichthyofauna. *Petrocephalus* “Lualaba” was a weakly supported sister species to *P. okavangensis* in the MP analysis (57% BP, Appendix 4.4). In the ML (Figure 4.5) analysis, the *P. okavangensis* specimens were not monophyletic, while they retained their monophyly but with no support in the BI analysis (Appendix 4.5). The monophyly of the Cuanza specimens, henceforth referred to as *Petrocephalus* “Cuanza”, was well-supported in the parsimony analyses (76% BP) and this clade was closely related to *P. longicapitis* with 77% BP. These relationships between species from distant, isolated systems indicate recent connections. For example, one individual of *P. okavangensis* [Katima, 83809 (220)] was collected in sympatry with *P. longicapitis* in the Upper Zambezi River. In both the MP (Appendix 4.1) and BI (Figure 4.5) *cyt b* phylogenies, the relationships among the species in the Western group were unsupported polytomies, presumably due to the possibility of recent species divergences or lack of resolution due to retained ancestral polymorphism in the mitochondrial genome to delineate lineage boundaries.

The monophyly of the Eastern group was also well supported in all analyses, with 100% BP and 0.97 PP (Appendix 4.4 and Figure 4.5, respectively). The Eastern group is distributed from the northeast coast of Tanzania down into South Africa, with all the lineages from Rufiji River, appearing at the base of this group, especially in the parsimony phylogeny (Appendix 4.4). The monophyly of *P. wesselsi* and *P. catostoma* (Lake Malawi system) was only weakly supported in the *cyt b* parsimony analysis (57% BP) and the relationship was broken in the ML (Figure 4.5) and BI (Appendix 4.5), forming polytomies with the Rufiji River specimens.

The *cyt b* genetic distances between the Western and the Eastern groups were high, ranging between 7.4 and 9.4%, (Table 4.3). The minimum *cyt b* divergences within the Western group (1.2 to 1.5%) were observed between *P. okavangensis* and *Petrocephalus* “Lualaba”, and the highest (4.5 to 5.2%) when other species were compared to *P. haullevillii*. The divergences between species in the Eastern group ranged from 1.5 to 1.8% between *P. catostoma* and *P. wesselsi*, to between 2.0 and 2.5% when *P. wesselsi* was compared to the Rufiji specimens.

S7 intron 1

The S7 intron 1 dataset did not include sequences from Rufiji system as these were retrieved from GenBank and, as such, the Eastern group was represented only by *P. wesselsi* and *P. catostoma* (Lake Malawi system). Analyses of the S7 intron 1 data included 471 nucleotides, of which 299 were constant, 108 were parsimony uninformative and 64 were parsimony informative. Only 16 equally-parsimonious trees were identified (tree length = 194, RI = 0.984 and CI = 0.953). The HKY+G model (Hasegawa *et al.*, 1985) was identified as the best model fit for the S7 intron 1 data. The S7 intron 1 did not resolve the phylogenetic relationships between species, presumably due to the low sequence variability among them (Table 4.3). However, this marker confirmed the separation of species into two groups (Appendix 4.6), the Western and the Eastern groups, with the divergence among them ranging from 8.5 to 11.3% (Table 4.3). The MP, ML and BI tree topologies were similar, such that only the likelihood tree with Bayesian posterior probabilities is presented in Appendix 4.6.

Table 4.3. Estimates of evolutionary corrected sequence divergences (shown as percentages) among and within *Petrocephalus* taxa. Below diagonal is the cyt *b* genetic distances whiles above diagonal are the S7 intron 1. * indicates S7 intron divergences that were not calculated due to missing taxa.

.	Within	1	2	3	4	5	6	7	8	9	10
1. <i>P. haullevillii</i>	0.0	-	4.0 - 4.5	4.4	4.2	4.0 - 4.2	4.0 - 4.2	*	*	10.6 - 11.5	11.0 - 11.3
2. <i>Petrocephalus</i> "Cuanza"	0.0-0.5	4.5 - 5.2	-	0.42 - 0.64	1.6 - 1.9	1.4 - 1.9	1.4 - 2.1	*	*	8.5 - 9.5	8.9 - 9.3
3. <i>P. longicapitis</i>	-	4.8	1.7-2.2	-	1.7	1.4-1.9	1.9 - 2.1	*	*	8.9 - 9.6	9.3
4. <i>Petrocephalus</i> "Lualaba"	-	4.4	2.1	2.6	-	0 - 0.02	0.64 - 0.85	*	*	8.7 - 9.3	9.1
5. <i>P. okavangensis</i>	0.2-0.5	4.1-4.8	2.5-2.8	2.6-3.1	1.2-1.5	-	0.4 - 0.11	*	*	8.4 - 9.1	8.9 - 9.1
6. <i>P. magnoculis</i>	0.2-0.6	4.1-4.3	2.6-2.8	2.9-3.0	2.1-2.2	2.5-3.1	-	*	*	8.9 - 9.1	8.7 - 9.3
7. Kalimbero lineage	-	8.6	7.5-7.8	7.6	8.1	7.6	7.6	-	*	*	*
8. Divue-Wami lineage	0.0	9.3	7.4-7.7	8.1	8.2	7.5-7.8	7.9-8.2	2.5	-	*	*
9. <i>P. catostoma</i>	0.2-0.8	9.1-9.4	7.5-8.1	7.8-8.1	8.3-8.5	7.8-8.2	8.1-8.8	1.8-2.1	2.0-2.4	-	0.2 - 0.8
10. <i>P. wesselsi</i>	0.0	8.9	7.4-7.8	8.1	8.5	7.6-8.2	8.3-8.6	2.2	2.3-2.5	1.5-1.8	-

Combined cyt b and S7 intron 1 (cyt b-S7)

A combined dataset of 1191 nucleotides (cyt *b* = 720; S7 intron = 471) was analysed, of which 870 nucleotides were constant and only 155 were parsimony informative. Four equally-parsimonious trees were identified for the cyt *b*-S7 data (tree length = 421 steps, CI = 0.853 and RI = 0.946). The unequal-frequency Kimura 3-parameter plus gamma (K81uf+G) model of evolution and $\alpha = 0.3473$ was identified for the combined dataset (Kimura, 1981).

Three phylogenetic analyses (MP, ML and BI) of the combined cyt *b*-S7 data yielded identical trees with strong nodal support (100% BP, 1.00 BPP) for the Eastern and Western groups (Figure 4.6 and Appendix 4.7). Most included species-formed well-supported monophyletic clades (>75% BP, >0.95 PP) in both MP and BI analyses. In the MP phylogeny, specimens of *P. catostoma* from the Lake Malawi system formed a well-supported monophyletic clade (88% BP), contrary to the ML and BI analyses where these did not form a monophyletic clade. In the Western group, *P. haullevillii* appeared basal to the remaining species in the MP analysis with moderate support (70% BP). However, this position was not supported by the Bayesian posterior probabilities (Figure 4.6).

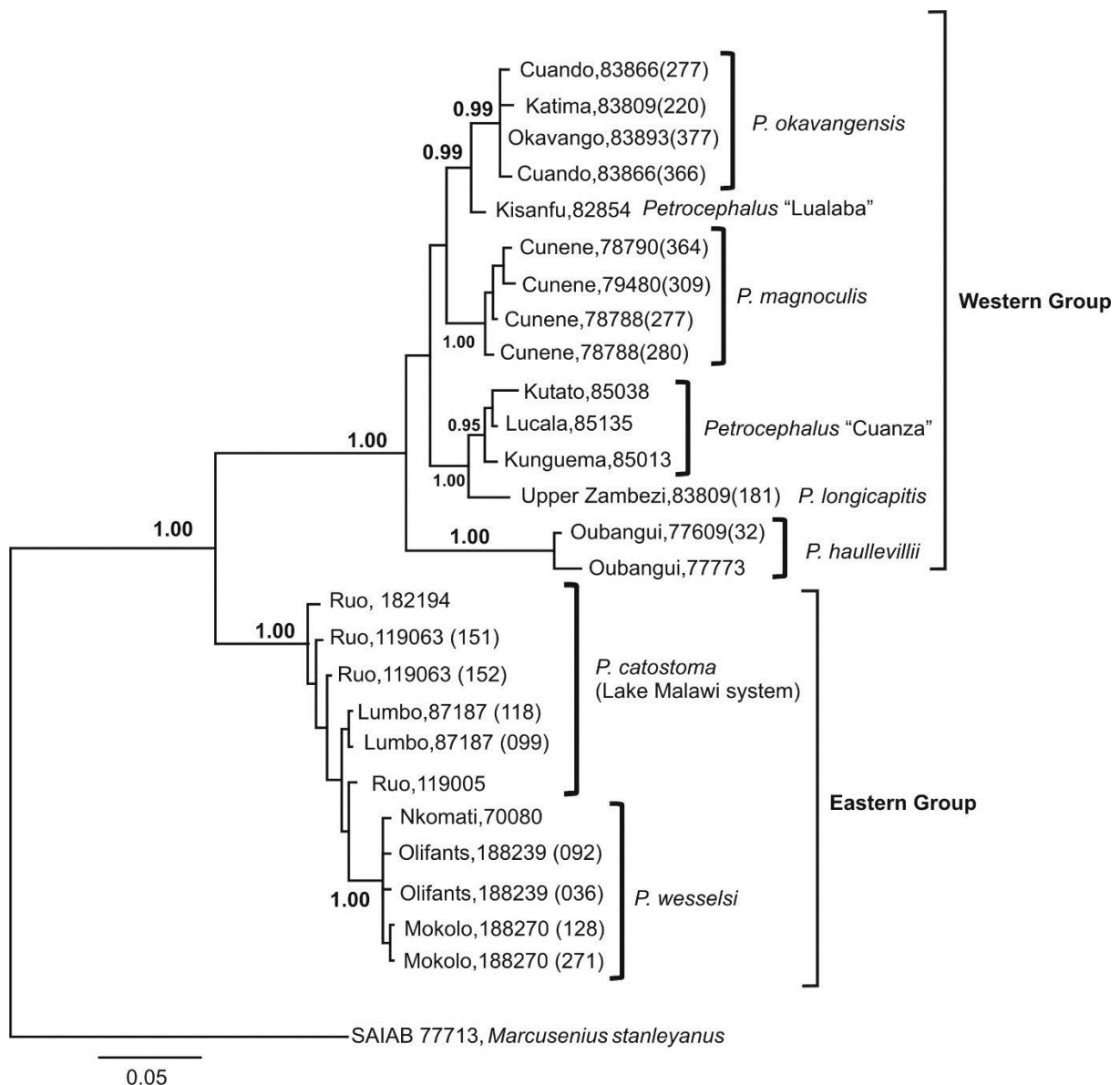


Figure 4.6. Maximum Likelihood ($-\ln L = 3676.90$) phylogram of relationships among *Petrocephalus* species as reflected by the combined cytochrome *b* and the S7 intron 1 with Bayesian posterior probabilities ≥ 0.95 indicated on the branches. For each species or lineage, individual localities and SAIAB numbers (with individual numbers in brackets for accession numbers where more than one individual was sequenced) are indicated.

Divergence time estimates

According to the dating of divergences based on the *cyt b* dataset, the Western and Eastern groups diverged in the Mid-Pliocene, 4.5 Ma (95% highest posterior density, HPD: 2.8-6.5 Ma) ago (Figure 4.7). It appears that the Western and Eastern groups evolved concurrently. Within the Western group, the oldest divergence was during the Early Pleistocene at 2.0 Ma (HPD: 1.1-3.2 Ma) ago, when *P. haullevillii* was split from the other species. During the mid-Pleistocene, 1.4 Ma (HPD: 0.9-2.2 Ma) ago, a lineage of *Petrocephalus* diverged from the ancestor of those species currently in the Upper Zambezi, Cunene and Congo Rivers, and further split into *P. longicapitis* and *Petrocephalus* “Cuanza” around 0.80 Ma (0.30-1.5 Ma) ago. The monophyletic group of *P. longicapitis* and *Petrocephalus* “Cuanza” is ancestral to a clade that split into *P. magnoculis* ~1.1 Ma and clade of *P. okavangensis* and *Petrocephalus* “Lualaba”. *Petrocephalus* “Lualaba”, from the Congo Basin, diverged very recently from *P. okavangensis* at 0.8 Ma (HPD: 0.3-1.5 Ma) ago. The oldest divergence in the Eastern group was that of *P. catostoma* from the Rufiji River system during the mid-Pleistocene around 1.3 Ma (HPD: 1.5-2.5 Ma) ago. Although not strongly supported, another lineage from the Rufiji River system (the Kalimbero lineage) split from *P. wesselsi* and *P. catostoma* about ~1.0 Ma ago, followed by the divergence between the latter species around ~0.8 Ma ago.

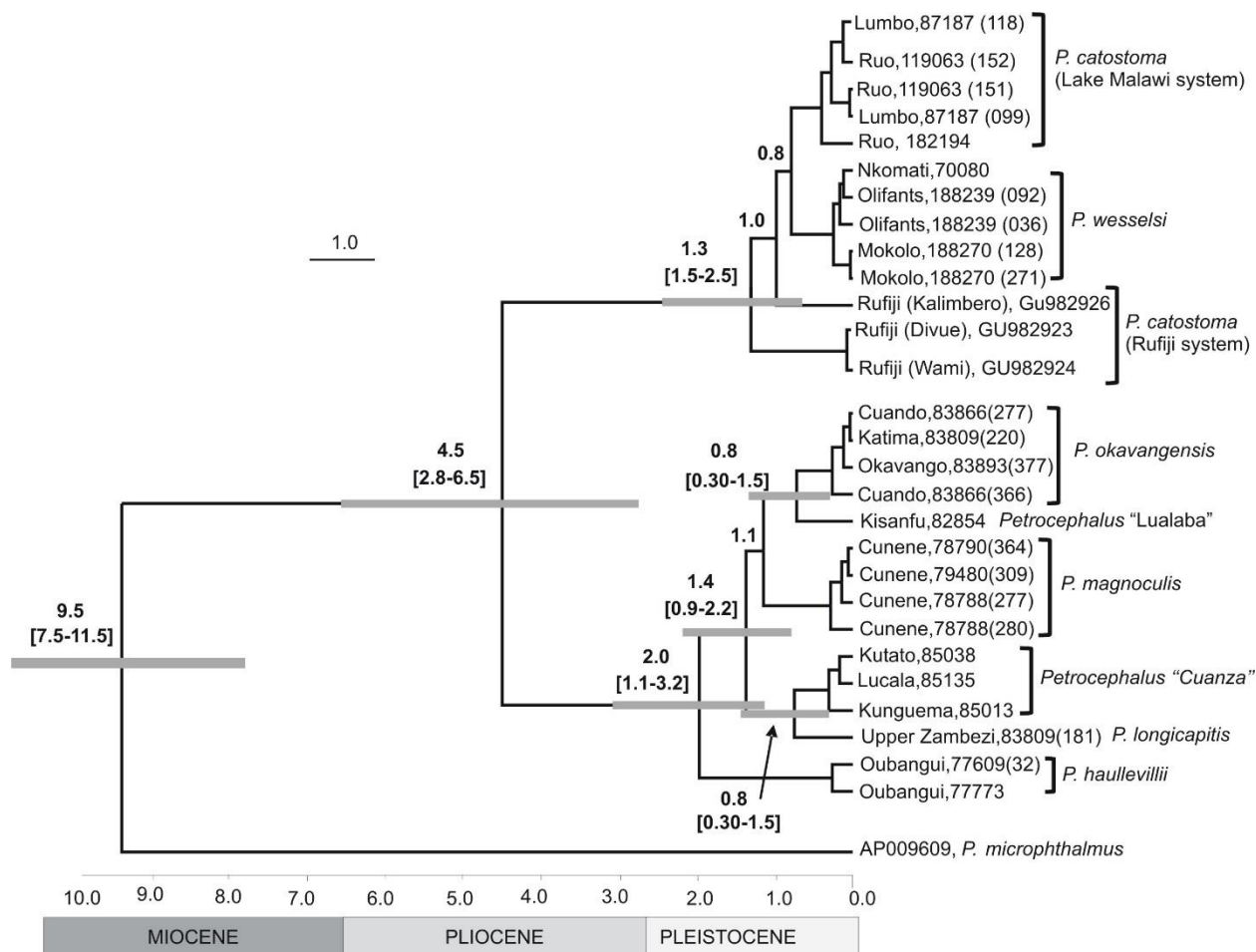


Figure 4.7. Estimates of divergence times (Millions of years ago) among southern and eastern African *Petrocephalus* species based on cytochrome *b* sequence data. Values on the nodes represent the estimated mean divergence time and ranges are presented in square brackets for divergence among species, as inferred using Bayesian coalescent analyses implemented in BEAST. Bars represent 95% confidence interval for divergence estimates. For each species or lineage, SAIAB numbers are indicated, with individual numbers in brackets for accession numbers where more than one individual was sequenced.

4.4. DISCUSSION

4.4.1. Phylogenetic relationships and comparative biogeography

In all genetic analyses, except the S7 intron 1 analysis, the recovery of congruent evolutionary relationships lends confidence in the southern and eastern African *Marcusenius* phylogenies obtained in the present study. Even though the present study clearly had more taxonomic coverage than the earlier studies (Kramer *et al.*, 2007; Kramer & Wink, 2013; Chapter 3), the *cyt b* analyses recovered the same overall topologies. All these molecular phylogenetic studies showed that southern and eastern African *Marcusenius* constitute a monophyletic assemblage that evolved from a single common ancestor (e.g. see Figure 4.2). The majority of species relationships were supported. For example, an earlier study of Kramer *et al.* (2007) showed that *M. devosi* from the Tana River appeared at the base of the phylogenetic trees. The position of this species has not changed considerably in the present analyses; however, *M. livingstonii*, collected from the Malawi-Shire system, included in the present study appeared more basal. Kramer *et al.* (2007) showed that *M. altisambesi* was basal to the *M. pongolensis-macrolepidotus* clade, and are descendants of the same *Marcusenius* ancestor that inhabited the Proto-Zambezi-Limpopo river system, and the present study further revealed *M. multisquamatus* and *M. macrolepidotus* as descendants of the same ancestral *Marcusenius* lineage. The phylogenies produced in this study, as well as those of earlier studies (Kramer *et al.*, 2007; Kramer & Wink, 2013; Chapter 3), significantly improve our knowledge of the diversity of *Marcusenius* (see below) and the evolutionary relationships among its species.

While the southern and eastern African *Marcusenius* was a monophyletic lineage that possibly evolved from a single Congolese ancestral species, *Petrocephalus* of southern and eastern Africa apparently evolved from two different ancestral lineages. The genetic divergences between the Eastern and Western species groups of *Petrocephalus* in southern and eastern Africa are so great that they are typical of generic differences (Kramer & Swartz, 2010; Kramer & Van Der Bank, 2011). The sequence divergence between the two groups from the mitochondrial *cyt b* gene ranged from 7.6 to 9.3% divergence. The slow-evolving nuclear S7 intron data also revealed substantial genetic divergences (8.5 to 11.5%) between these groups. These values contrast with the *cyt b* divergences of 1.2 to 4.8% within the Western group, and 1.5 to 2.5% within the Eastern group, which are typical of congeneric-species divergences observed within other southern and eastern African mormyrid genera [e.g. *Hippopotamyrus* species (2.4 to 2.6%) (Kramer & Swartz, 2010), *Pollimyrus* (2.2 to 3.6%) (Kramer *et al.*, 2013) and *Marcusenius* (0.4 to 5.2%) (Kramer & Wink, 2013)]. Until such time as the entire genus is reviewed, this study does not make any recommendations regarding the deeper mitochondrial and nuclear divergences of the southern and eastern African *Petrocephalus* genus, as this was beyond the scope of this study.

In spite of the extended taxonomic coverage of the present study of southern and eastern African *Petrocephalus* species as compared to that of Kramer *et al.* (2012), the relationships within the Western and the Eastern species groups were not strongly supported. The *Petrocephalus* species studied in these regions have been suggested to represent a recent adaptive radiation, where rapid speciation was fuelled by population fragmentation brought on by vicariant events and possibly divergent sexual selection (Kramer *et al.*, 2012). Despite this, short branches in other southern and eastern African taxa such as cichlids (Joyce *et al.*, 2005; Salzburger *et al.*, 2005; Day *et al.*, 2009),

Synodontis catfishes (Koblmüller *et al.*, 2006; Pinton *et al.*, 2013) and *Mastacembelus* eels (Brown *et al.*, 2010), provided enough phylogenetic signal to evaluate evolutionary hypotheses. The dating of divergences in the present phylogenetic analyses is congruent with several consecutive evolutionary events in the Southern and Eastern African Basins (e.g. Katongo *et al.*, 2007; Brown *et al.*, 2010; Goodier *et al.*, 2011; Pinton *et al.*, 2013), which seemed to have started in the Early Pliocene (see below).

The differences in the phylogenies of the southern and eastern African *Marcusenius* and *Petrocephalus* species reveal that the diversity and distribution of their species reflect a complex history in which older Pliocene incidents of vicariance have affected each genus differently. An Early Pliocene divergence event at 5.1 Ma separated *M. livingstonii*, which occurs in parts of the Malawi-Shire and Ruvuma river systems, from all other *Marcusenius* species in the present study. According to Tweddle (2010), Lake Malawi and its associated rivers are of special significance within the Southern African Basin. Its fauna, like *M. livingstonii*, is so distinct that, biologically and morphologically (see Chapter 3), its fauna is distantly related to other drainage systems of southern and central Africa; most of its fauna is derived from the Congo River system and not from East Coast Systems (Tweddle, 2010). The divergence times in the present study are congruent to those found in *Mastacembelus* eels (2.5-6.0 Ma) (Brown *et al.*, 2010) and in *Synodontis* catfishes (1.9-9.6 Ma) (Pinton *et al.*, 2013), where the sequence of events coincided with the rifting and uplifting activities of the East African Rift System (EARS), causing the formation of Lake Malawi and the Lake Tanganyika Plateau. The divergence between the north-eastern African *M. devosi* and the remaining *Marcusenius* species in the Pliocene, 3.2 Ma ago, slightly postdates events in the region north of the Tanzanian Plateau and can be attributed to such rifting. The above

evolutionary signatures are also congruent with events that split molarats of the genus *Heliophobius* across the Malawi Rift in the Late Pliocene (Faulkes *et al.*, 2011). These events are all geologically-dated to have started 4-12 Ma ago (Cohen *et al.*, 1993; Ebinger *et al.*, 1993; Johnson *et al.*, 1996; Stankiewicz & de Wit, 2006) and have long been known to be synonymous with the evolution of African biota (Cotterill, 2000, 2003a), with species known to have invaded the eastern African systems via the Upper Congo-East Coast route (Bell-Cross, 1972). Many changes took place during various rifting and uplifting episodes in East Africa, but by the Pliocene the Rufiji River system in northeast Tanzania, which used to drain the Upper-Congo-East system into the Indian Ocean was “beheaded”, isolating what was earlier a continuous major river system (Moore & Larkin, 2001; Stankiewicz & de Wit, 2006). It is plausible that the events that formed Lake Malawi and the Lake Tanganyika Plateau, for example the EARS, also disrupted a former Upper Congo-East Coast route and consequently isolated *M. livingstonii* and *M. devosi* from other southern and eastern African *Marcusenius* species.

According to the present *Petrocephalus* dataset, it is possible that a different or parallel Pliocene event 4.5 Ma ago possibly split the southern and eastern African *Petrocephalus*, causing deeper divergences between the Eastern group and the Western group, which together did not diversify until ~2 Ma, after a relatively long lag-time. The initiation of the drainage re-organization within the Zambezi Basin, as a result of the propagations of the EARS, formed the Victoria Falls, which is now known to demarcate two sections of the Zambezi River with marked differences in geomorphic characters (Moore & Larkin, 2001; Moore *et al.*, 2012) and biological composition (Tweddle, 2010; Kramer *et al.*, 2012). These river rearrangements have not only been important in facilitating species dispersions in *Petrocephalus* and other taxa but, particularly in the Plio-

Pleistocene, have also led to the disruption of formerly continuous populations of *Cyphomyrus* above and below the falls (Kramer & Van Der Bank, 2011), providing a driving force for speciation. The EARS biogeographic signature and the one discussed above are also congruent with a similar Pliocene divergence at 3.9 Ma, that split tigerfishes into clades represented by the *Hydrocynus tanzaniae* complex, distributed east of the Victoria Falls and in the eastward flowing rivers of Tanzania, and the *H. vittatus* complex, which is well represented throughout the Zambezi system west of the Victoria Falls, excluding the Upper Kafue and Upper Shire rivers (Goodier *et al.*, 2010).

All subsequent, divergences within both *Marcusenius* and *Petrocephalus* groups are a signature of the southward propagations of the EARS and a period of several drainage rearrangements in the Zambian-Congo and the Upper Zambezi drainage systems with the timing collectively constrained to the Early to Late Pleistocene (Stewart, 2001; Goudie, 2005; Stankiewicz & de Wit, 2006; Moore *et al.*, 2007; Cotterill & De Wit, 2011; Goodier *et al.*, 2011). A Pleistocene divergence around 2.4 Ma ago split the monophyletic clade of *Marcusenius* “Luapula” (Luapula-Chambeshi drainage) and *M. angolensis* (Cuanza River) from those distributed from the Cunene and the Zambezi Drainage system, including those in South Africa. It has been argued that freshwater fishes have first invaded the Upper Zambezi via a Congo-Angola route (Bell-Cross, 1965). This scenario is evident in the present study as shown by the ancestral or older divergence of *Petrocephalus haullevillii* of the Congo-Kasai drainage from the Zambezian *Petrocephalus* species at 2.0 Ma ago. Stankiewicz & de Wit (2006) described historical changes in river directions in this area based on geological evidence. They suggested that the north-east-flowing Chambeshi River was reversed in the Miocene and became landlocked, possibly originating from Lake Bangwuelu. This system was

captured first by the Kafue River to become part of the Zambezi River network that mixed the Congolian and Zambezian fish faunas (Cotterill, 2003a). In the Lower Pleistocene, this river was captured again by the Luapula River to become part of the new, west-flowing Congo River network (Moore & Larkin, 2001; Stankiewicz & de Wit, 2006). Thus, there have been opportunities for the transfer of fish species in the area, as proposed by Worthington (1933) and Genner *et al.* (2007), explaining the sympatric distribution and low divergences among *Petrocephalus* species in the Upper Zambezi River. Based on the ranges of freshwater fishes, Seegers (1996) concluded that fishes migrated continuously across watersheds between the Luapula drainage and other neighbouring systems. Further evidence from haplochromine cichlid fishes indicated that another recent invasion of Congo taxa into southern river systems occurred 2 Ma ago, most likely over relatively shallow watersheds between the upper branches of the Congo River (from Lake Mweru in the Luapula drainage) and the Upper Zambezi River (Salzburger *et al.*, 2005). This could possibly explain the recent biogeographic patterns observed.

At a similar time to the invasion of the Congo ichthyofauna into the southern Zambezi River system, *M. altisambesi-multisquamatus* diverged from other species east of the Victoria Falls, around 2.0 Ma ago. The species east of the Victoria Falls then diverged into the *M. pongolensis-macrolepidotus* group and *M. luombesi-krameri-caudisquamatis* group 1.6 Ma ago. According to the biogeographic scenario considered at present, the Okavango and Upper Zambezi previously drained into the Indian Ocean via the Limpopo Valley (Cotterill, 2000; Moore & Larkin, 2001; Stankiewicz & de Wit, 2006). The headwaters of the Luangwa and Middle Zambezi Rivers changed course from their previous north-easterly flow towards an eastward-flowing Paleo-Congo River and may have become landlocked until captured by the Lower Zambezi River. This suggests

that some species of the Upper Zambezi and Limpopo river systems should form a monophyletic group, similar to those from the Lower Zambezi and Ruvuma river systems. Further sampling along the eastern African coast, especially between the Tana River and the Limpopo River system, including the Luangwa River of the Middle Zambezi, is required to shed more light on these possible links and divergences in *Marcusenius*, as well as to lead to the full understanding of the evolutionary history of *Petrocephalus*.

According to the proposed sequence of events, the divergence of *M. luombesi* and *Petrocephalus catostoma* from the Rufiji River 1.3 Ma ago is suggestive of dispersal between adjacent headwaters along the east coast systems. Skelton (2001) suggested that opportunities for the transfer of fish species across the low relief areas of Mozambique could arise by advancing watersheds or by coastal drainage links that capture headwaters of opposing drainage systems. Phylogeographic studies of *M. pongolensis* (Chapter 2) have indicated that river systems across southern Mozambique are likely to have overland connections and thus dispersal. Any connections are therefore likely to have facilitated gene flow between currently isolated systems and were followed by the isolation of populations and divergence into species.

The remaining divergence dates within *Petrocephalus*'s Western group represent relatively recent and complicated Late Pleistocene divergence events that created significant phylogeographic structure between the Cunene, Okavango, and Upper Zambezi river systems. The first divergence event, dated at 1.4 Ma ago, separated a lineage comprising the *Petrocephalus* "Cuanza" and *P. longicapitis* from the remaining species from Cunene, *P. magnoculis*, and from Okavango, *P. okavangensis*, as well as *Petrocephalus* "Lualaba". There is no precise, geochronological evidence

for the sequence of these recent events but it is known that the upper reaches of the Okavango River formed the headwaters of the upper Cunene and Cuanza rivers, such that the dispersal of fauna between these rivers was possible (Moore & Larkin, 2001). This is well substantiated by the relationship between *P. longicapitis* from the Upper Zambezi system and *Petrocephalus* “Cuanza” of the Cuanza River.

This study, which included all *Marcusenius* species known in southern and eastern Africa, confirmed the monophyly of the genus in this region, which receives high support in all analyses that were conducted. Within *Petrocephalus*, the present analyses also confirm the divergences of the latter genus into two species groups in southern and eastern Africa (Kramer *et al.*, 2012). The phylogenetic relationships within the analysed southern and eastern African *Petrocephalus* species varied across analyses, and where a sister relationship was consistent it was not well supported. Future studies incorporating more molecular characters and species will likely help to resolve the relationships between these *Petrocephalus* species. The biogeographic pattern observed for *Marcusenius* here reinforces the hypothesis that most southern and eastern African freshwater fishes are of Congo origin and the Miocene-Pliocene events in the western branch of the active EARS disrupted the formerly continuous populations, while the Pleistocene divergences correspond to the recent arrangements between the Zambezi-Congo drainages. Improved representation of *Petrocephalus* taxa will, however, be required to fully resolve the biogeographic history in the studied region, especially from areas under-represented in the current analyses (the Eastern African Basin).

4.4.2. Taxonomic implications

Beyond corroborating the morphological evidence, the current phylogenies have shown that both *Marcusenius* and *Petrocephalus* genera in the Southern and Eastern African Basins exhibit considerable genetic variation between species. The present discovery of previously unrecognised lineages indicated that the diversity in both genera has been underestimated and more species will be discovered. This includes a possible new species in *Marcusenius*, two in *Petrocephalus* (see below) and possible additional diversity within *P. catostoma* (i.e., separation of Lake Malawi specimens from the Rufiji specimens). As shown by DNA sequence analysis (Figure 4.5 – 4.7 and Table 4.3), the present study identified two genetically divergent *Petrocephalus* lineages that occurred in areas where no other *Petrocephalus* species were recorded. According to the estimates of mtDNA cyt *b* sequences divergence, *Petrocephalus* “Cuanza” from the Cuanza River in Angola is 1.7 to 5.2% divergent from other species in the Western group, whilst *Petrocephalus* “Lualaba” from the Lualaba drainage in the Congo Basin is 1.2 to 4.4% divergent. It is plausible to argue that these lineages have been isolated for a considerable time (0.80 Ma ago, Figure 4.7) from their congeneric species and await formal taxonomic characterization.

The Congo Basin lineage, *Marcusenius* “Luapula”, was greatly divergent at mitochondrial (2.9 to 10.4% sequence divergence) and nuclear (2.1 to 5.8%) markers from other *Marcusenius* species in the Southern and Eastern African Basins. Worthington (1933) and Kramer *et al.* (2007) have indicated that the Luapula-Chambeshi drainage where *Marcusenius* “Luapula” was collected is inhabited by a *Marcusenius* lineage different from those in the Southern and Eastern African Basins. Kramer *et al.* (2007) showed with morphology that the synonymised *M. moeruensis* species, only known from inside Lake Mweru (synonym of *M. macrolepidotus*), is well

differentiated from all *Marcusenius* species they were able to study, including the *M. macrolepidotus*. Although the samples of *Marcusenius* “Luapula” in the present study were collected from the Chambeshi River, which drains into Lake Mweru, they could not be regarded as representative of the synonymised *M. moeruensis* as the latter species was described from a single “unique” specimen from inside the lake, and it is also not known if it occurs in the Chambeshi River or other tributaries of Lake Mweru. In order to describe the marked degree of differentiation of *Marcusenius* “Luapula” and its placement within the genus *Marcusenius*, many more specimens need to be sampled from inside Lake Mweru and its tributaries, which will improve taxonomy of the genus.

The present molecular study of *Petrocephalus* and that of Kramer *et al.* (2012) and morphological systematics (Whitehead & Greenwood, 1959; Kramer *et al.*, 2012) showed that the Southern and Eastern African Basins, together, include species that are not each other’s closest relatives, with one of the two species groups occurring exclusively to the east of the Victoria Falls (such as *P. catostoma* from the Rufiji and Lake Malawi river systems, and *P. wesselsi*) and the other to the west of the Victoria Falls, including the Lower Congo (such as *P. okavangensis*, *P. magnoculis* and *P. longicapitis*, *P. haullevillii*). The traditional explanation for the split in the genus is that the Victoria Falls divides the fish fauna of the Zambezi and East Coast Africa into a species-rich upper or western section and a lower or eastern section that holds different fish species (Balon, 1974; Skelton, 2001; Kramer & Van Der Bank, 2011; Kramer *et al.*, 2012). A systematic investigation by Whitehead & Greenwood (1959) firstly noted size differences between the Western and Eastern *Petrocephalus* species groups. However, due to paucity of material they could not elaborate further on this observation. Therefore, they united all the species they studied, including those from the

Congo Basin as members of a single *P. catostoma*. Only recently was it shown by Kramer *et al.* (2012) that the Eastern group has an increased trunk length and body depth, as well as caudal peduncle depth, which reflects a trend of increased swimming speed to overcome strong currents, such as in the Incomati river system inhabited by *P. wesselsi* in South Africa. Conversely, the Western group have a more slender trunk depth, have a shorter body length, and clearly have increased fin lengths, probably for increased manoeuvrability and skilful swimming (Kramer *et al.*, 2012). A full understanding of the taxonomic implications of the morphological and genetic differences between the Western and the Eastern groups requires a phylogeny that includes the species of the entire genus across Africa, to determine the origin of both groups. However, this was beyond the scope of the present research.

This study has unambiguously identified evolutionary lineages in both *Marcusenius* and *Petrocephalus*, whose deep divergences and allopatric distributions corroborate available species descriptions. Molecular divergence reveals that these lineages have been allopatric for a very long time, but interestingly, the distribution of other lineages appears to be potentially due to secondary contact between river systems, e.g. *P. okavangensis* was collected in sympatry with *P. longicapitis* in the Upper Zambezi River. Further work with more sampling is especially needed in the Lower Congo Basin and across the Southern and Eastern African Basins, together with a comprehensive analysis of morphological variation where more species could still be discovered.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS

This thesis investigated the systematics, evolutionary history, biogeography and phylogeography of *Marcusenius* and *Petrocephalus* in southern and eastern Africa. It integrated several techniques, through the collection of complementary but separate data sets, thereby providing multiple lines of evidence of species separation and for the phylogeography and biogeography of the studied taxa. Prior to this thesis, no study had used a large nucleotide dataset, or combined both mtDNA *cyt b* and nuclear S7 intron 1 data to study *Marcusenius* and *Petrocephalus* in the Southern and Eastern African Basins. In this regard, the thesis has documented the phylogeography and population genetic structure of *Marcusenius* populations in South Africa using mitochondrial (*cyt b* and COI) and nuclear (S7 intron 1) genetic markers (Chapter 2). Chapter 2 demonstrated that the evolution of *Marcusenius* in South Africa has followed a complex pattern of colonisation, range expansions and contractions, and secondary contact between presently distant and isolated river systems. Three clearly genetically-divergent and morphologically-different lineages were observed, *M. pongolensis* and two unidentified lineages. The one unidentified lineage was endemic to the Limpopo River system and the other endemic to the Mhlatuze and Nseleni Rivers. Applying both the Phylogenetic Species Concept and Evolutionary Species Concept, Chapter 3 provided information on the morphological variation within South African *Marcusenius*, describing *M. krameri*, endemic to the Limpopo River system, and *M. caudisquamatus*, endemic to the Mhlatuze and Nseleni rivers. *Marcusenius lucombesi* from the Lucombe River, tributary of the Ruvuma River system, northern Mozambique, was also described. Chapter 4 analysed the phylogenetic relationships among species of *Marcusenius* and *Petrocephalus*, presenting phylogenetic hypotheses that differed among the genera, and refined their taxonomy. In this chapter, the divergence times among clades at different hierarchical levels (i.e. lineages and species) were estimated and

related to major events in the geological history of the southern and eastern African region. The results were consistent with the hydrogeological hypothesis (Goodier *et al.*, 2011; Pinton *et al.*, 2013), which proposes that changes of the drainage basins, linked with the geological context, have been the cause of diversification of most freshwater fishes in southern and eastern Africa.

5.1. DIVERSITY

In terms of the diversity identified in the present thesis, the three new *Marcusenius* species increase the total known diversity of the genus in southern and eastern Africa to ten described species, and the genus to 41 species across Africa; however, the outcomes from this study showed that this number of species is an underestimation. There are several undescribed taxa that require formal description in the future. *Marcusenius* “Luapula”, *Petrocephalus* “Lualaba” and *Petrocephalus* “Cuanza” lineages warrant further examination, as they appear to be different taxa with species-level genetic divergences (Chapter 4). Additional diversity within *P. catostoma* was also identified in this study, with the deeper divergence between the Malawi river system and the Rufiji River system specimens. When described, these lineages would further increase the species diversity of both genera and, therefore, the southern and eastern African ichthyofauna.

Marcusenius “Luapula”, from the Luapula drainage, Congo Basin, a population still commonly thought to be *M. macrolepidotus* (Huchzermeyer, 2012), was genetically divergent at mitochondrial and nuclear DNA and does not form a monophyletic clade with the topotypical *M. macrolepidotus* population. Populations from the Luapula drainage did show morphological variation or differences according to Worthington (1933) and Kramer *et al.* (2007). Referring

to this undescribed taxon as *M. macrolepidotus* would be unjustified because the taxonomic status of the former species is firmly established and the species is restricted to the Lower Zambezi and its tributaries (Kramer *et al.*, 2007). Taxonomic re-assessment is justified for this lineage and the investigation should also include the synonymised *M. moeruensis* species (Kramer *et al.*, 2007).

Petrocephalus “Lualaba” and *Petrocephalus* “Cuanza” clades are not monophyletic and do not belong to the same species group as *P. catostoma* (Figure 4.4 & 4.5), a species that was formerly regarded as being widespread across the Southern and Eastern African Basins, including the parts of the Congo Basin. The regions where *Petrocephalus* “Lualaba” and *Petrocephalus* “Cuanza” were collected have been known to harbour unknown mormyrid diversity (Worthington, 1933; Kramer *et al.*, 2007, 2012; Kramer & Swartz, 2010; Lavoué, 2012), and these lineages represent an undescribed species. Future investigations should also make use of the existing type material of other species from isolated systems to more accurately place divergent lineages in a taxonomic context and to determine whether these taxa are indeed novel.

5.2. MOLECULAR SYSTEMATICS AND TAXONOMY

Molecular identification and genetic tools have been suggested as a way of complementing traditional morphology-based identification of species (Hebert *et al.*, 2003), but genetics alone is not enough to identify species. It does not compete with traditional taxonomy (Gregory, 2005). Integration of genetic and morphological evidence confers a more robust taxonomy (Vilaça *et al.*, 2006). For example, Kramer *et al.* (2007) suggested that the southern-most population of *M. pongolensis* was in the Mhlathuze River. In the present study, this population

was distinguished as a distinct lineage using DNA sequence data (Chapter 2) and this was used as an *a priori* hypothesis for detecting statistically-significant morphological differences and for identifying the diagnostic characters of this lineage. Indeed, this lineage was described as a new species, *M. caudisquamatus*, together with *M. krameri* and *M. luombesi*, which were previously recognised as *M. pongolensis* and *M. macrolepidotus*, respectively (Chapter 3).

Following on previous studies of southern and eastern African *Marcusenius* and *Petrocephalus*, this thesis demonstrates that species which were morphologically-identifiable and were also shown to possess unique electric organ discharges (Kramer & Van der Bank, 2000; Kramer *et al.*, 2007, 2012; Kramer & Wink, 2013), form coherent, monophyletic clades with the genetic data (Figures 3.5, 4.2 & 4.4) and showed significant sequence divergences among them (Table 4.2 & 4.3). In all three chapters (Chapters 2, 3 & 4), genetic divergence among species ranged between 0.90-10.6% for mtDNA and 0.69-5.7% for the nuclear S7 intron 1, which are well within the range of intrageneric variation in mormyrids fishes (Lavoué *et al.*, 2003). These led to one of the primary conclusions of this study: that by incorporating the strengths and resolution provided by molecular tools into the rich history of morphology-based systematics and taxonomy, a robust approach for describing biodiversity was established.

Prior studies on *Marcusenius* and *Petrocephalus* in southern and eastern Africa were primarily descriptive in nature (Kramer & Van der Bank, 2000; Kramer *et al.*, 2007, 2012) and only used genetics as supporting evidence for results from morphology and EODs. Kramer *et al.* (2007) presented a phylogeny of southern and eastern African *Marcusenius* with only four species, and did not comment on the taxonomic implications of those relationships, except that the South African *M. pongolensis* and *M. altisambesi* of Upper Zambezi are the descendants of the same *Marcusenius* ancestor that previously inhabited the Proto-Zambezi-Limpopo River

system. The results of the present work supported the conclusion of Kramer *et al.* (2007) and added *M. multisquamatus* and *M. macrolepidotus* as descendants of the same ancestral *Marcusenius* lineage. While *Marcusenius* was a monophyletic lineage that possibly evolved from a single Congolese ancestor, *Petrocephalus* of southern and eastern Africa apparently evolved from two different ancestral lineages. The Western group clearly evolved from a Congolese ancestor that entered the Zambezi River system through the Congo-Angola Route (Bell-Cross, 1965). The phylogenetic position of *Petrocephalus catostoma* (from the Rufiji River system) suggests that the Eastern group evolved concurrently with the Western group from an ancestor that inhabited eastern African river systems. The most important, previously-unseen, factor is the importance of the Congolese species and their relationships with the remaining southern and eastern African species in both genera.

Boden *et al.* (1997) and Hopkins *et al.* (2007) have shown that the continuous discovery of mormyrid diversity requires practical identification keys based on diagnostic characteristics from external morphology. Lavoué *et al.* (2004, 2010) also indicated that some *Petrocephalus* specimens, collected across Gabon and the Odzala National Park, Republic of the Congo, did not belong to any of the described species and the lack of taxonomic revisions and keys made these difficult to identify. To overcome this problem for the southern and eastern African *Marcusenius*, this study sourced original descriptions of the valid southern and eastern African species to compile a comprehensive, practical species identification key (Chapter 3). Several potential morphological characters were compared (Tables 3.6 & 3.7) and the use of gill raker counts proved to be important for the identification of the South African species. Similarly, vertebral counts were very useful and were used together with the anal and dorsal fin rays to successfully identify different species. The use of gill raker counts and accurate anal and dorsal fin ray and vertebral counts, using either light microscopy or X-rays (see Chapter 3) in this

study, highlights the importance of these characters for future mormyrid studies, as these lead to the identification of more variation between species. Furthermore, Sullivan & Hopkins (2005) and the present study showed that sample size is not an issue when using these meristic characters because they were usually different between species, overlapping only rarely compared to most morphological measurements. Without any doubt, the incorporation of these characters in the species descriptions improved the taxonomy of this genus in terms of clearer species diagnoses and less overlap of characters. In the past, these morphological characters were not explored and, even when differences were observed, were considered insufficient to warrant species distinction (Worthington, 1933; Seegers, 1996; Kramer *et al.*, 2007). The present study provided the first practical identification key to the known *Marcusenius* species of southern and eastern Africa, as there is now relatively good knowledge of their diversity and important characters for separating the species have been found (Chapter 3).

The morphometric analyses (Chapter 3) revealed that other morphological differences are related to changes in the body shape, body depth and caudal peduncle size, as well as the length of the unpaired dorsal and anal fins (Appendix 3.1). In Chapter 3, for example, two groups could be identified: firstly, the *M. pongolensis* and *M. caudisquamatus* group have a much longer and slimmer anterior body together with a shorter and thicker posterior section of the body or tail (Figure 3.2). This is converse to the *M. krameri* and *M. lucumbesi* species group, which have distinctly shorter and deeper anterior body sections and longer tails, but thinner caudal peduncles. The variation in the shape of the anterior body and the posterior body observed in the present study could be an indication of the presence of different ecological and survival characteristics specific to each species (Chapter 3), such as those of *Marcusenius* species described by Kramer *et al.* (2007), *Hippopotamyrus* by Kramer *et al.* (2004) and Kramer & Swartz (2010), and *Petrocephalus* by Kramer *et al.* (2012).

The combination of genetics, morphometrics and meristic characters (all in Chapter 3) reliably separated the four species and was sufficient to support the recognition of these species, regardless of the size of the particular individuals being compared. Either this is a common characteristic of mormyrid taxa or not; allometric growth has not been reported in systematic studies of this family (e.g. Boden *et al.*, 1997; Kramer & Van der Bank, 2000; Kramer *et al.*, 2003, 2004, 2007, 2012; Sullivan & Hopkins, 2005; Kramer & Swartz, 2010; Kramer & Van der Bank, 2011; Kramer, 2013a, b). This is despite previous studies that suggested that size may bias analyses of morphological data if not statistically size-corrected, because most fish species exhibit allometric growth (Reist, 1985; Elliott *et al.*, 1995; Lleonart *et al.*, 2000; Gunawickrama, 2007).

5.3. HISTORICAL BIOGEOGRAPHY AND PHYLOGEOGRAPHY

According to the hydrogeological hypothesis developed by Lundberg *et al.* (1993) and Montoya-Burgos (2003), much diversity of freshwater fishes might be the outcome of paleohydrological changes that promoted dispersal followed by vicariant divergences. The historical comparative biogeography of *Marcusenius* and *Petrocephalus* in the present study reveals that the many documented hydrogeological changes are most probably at the origin of all main cladogenetic events observed in the phylogeny of these genera. Comparisons with published distribution patterns of unrelated fish groups indicate that several of the reconstructed and dated hydrogeological–cladogenetic events may have acted at a large scale on the diversification of tropical African freshwater fish fauna (Katongo *et al.*, 2007; Brown *et al.*, 2010; Goodier *et al.*, 2011; Pinton *et al.*, 2013). Estimates of divergence times (Chapter 2

& 4) for various nodes within the phylogenies generally corresponded to major events in the geological history of the Southern and Eastern African Basins.

The separation of *Marcusenius livingstonii* and *M. devosi* from the other southern and eastern African *Marcusenius* species approximately 2.8-8.6 million years ago (Ma) coincides with the rifting and uplifting activities of the East African Rift System (EARS), as well as the formation of Lake Malawi and the Lake Tanganyika Plateau (Figure 4.2) (Moore & Larkin, 2001; Stankiewicz & de Wit, 2006). These were major activities that disrupted the formerly eastward-draining Paleo-Congo River system that ended at the present Rufiji Delta, Tanzania (Stankiewicz & de Wit, 2006). The older divergence of *M. livingstonii* from the other species is also clearly evident in the morphological comparisons of this species with the other species from which it differed by its bicuspid teeth and the dark brown uniform bar on the body between the origins of the dorsal and anal fins, none of which was found in the remaining species studied in this thesis (Chapter 3). *Marcusenius* species distributed in the Congo, such as *M. moorii*, are characterised by the dark grey-brown band between the dorsal and anal fins and noticeable bicuspid teeth on the upper jaw (Boden *et al.*, 1997). The divergence dates of *M. livingstonii* and *M. devosi* discussed above are similar to those estimated for the divergence of the Western and Eastern *Petrocephalus* species groups (Figure 4.6). These groups of species all seemingly represented a common pattern: the common ancestor of each group was widespread across southern and eastern Africa during the Pliocene, subsequently splitting and speciating during the Pleistocene and Holocene periods, such that the genera are currently represented by individual species occurring in allopatry and, rarely, in sympatry.

This pattern of genetic divergence in interplay with landscape evolution is not unique to these mormyrid fishes. Multiple genetic studies of other freshwater taxa, including *Synodontis*

squeaker catfishes (Koblmüller *et al.*, 2006; Pinton *et al.*, 2013), serranochromine cichlids (Katongo *et al.*, 2007), *Mastacembelus* eels (Brown *et al.*, 2010) and *Hydrocynus* tigerfishes (Goodier *et al.*, 2011)) have all identified divergences among genetic lineages broadly coinciding with major events in the evolution of the Southern and Eastern African Basins. The occurrence and timing of these events is corroborated by molecular studies of various taxa, such as antelopes (Cotterill, 2000, 2003a, b) and molerats (Van Daele *et al.*, 2007; Faulkes *et al.*, 2011), which have revealed concordant patterns of historical biogeography and regional genetic structuring. From these diverse taxa, as well as the present study, two major periods of divergence can be observed: 1) a Miocene-Pliocene divergence about 5 million years ago, generally corresponding to the period of activities and uplifts in the EARS (Stankiewicz & de Wit, 2006; Moore & Larkin, 2001), and (2) Pleistocene divergences about 2 million years ago, corresponding to the time periods of several recent drainage rearrangements in the Lower Congo and Southern African Basin systems (Moore & Larkin, 2001; Goodier *et al.*, 2011). In addition to the geological context, climate changes in the history of *Marcusenius* and *Petrocephalus*, though difficult to elucidate, probably also played a significant role. It is possible that fluctuating environmental conditions throughout these periods would have affected individual lineages differently, according to their ecological tolerances and adaptive potential. A possible consequence of this is that divergence progressed at different rates over time creating a complex mixture of phylogenetic and phylogeographic histories.

Significantly reduced rainfall was proposed for the interior plateau of South Africa during the Pleistocene (Finch & Hill, 2008), where river systems became isolated, reducing the possibility of gene flow and, thus, producing the strong population genetic structure observed (Chapter 2). Physical factors, such as the uplift of the Great Escarpment and Ciskei-Swaziland Axis in South Africa (Partridge & Maud, 1987; Moore, 1999; Moore *et al.*, 2009), were

proposed to have separated the inland rivers (e.g. Limpopo) and coastal rivers (e.g. Kosi, Mhlatuze) from each other. This has been shown to be the case in several studies of other freshwater fishes distributed across the Limpopo and Incomati River systems (Oosthuizen *et al.*, 2009; Matlala *et al.*, 2010; Dorn *et al.*, 2011; Bartáková *et al.*, 2013). These studies, together with Chapter 2, in particular suggest that taxa distributed across the Limpopo and Incomati River systems are highly fragmented and geographically-structured.

5.4. CONSERVATION CONCERNS

Ecological theory and conservation genetics have traditionally been put forth to predict species responses to pressures (Moritz, 1995; Loarie *et al.*, 2009). According to Loarie *et al.* (2009), ecological theory suggests that species with high dispersal ability are more likely to escape extinction, while Moritz (1995) indicated that conservation genetics suggests that the loss of genetic variation may increase the probability of population extinction or reduce opportunities for future adaptation of a species or populations to environmental changes (Moritz, 1995). Given the current widespread distribution, as well as high genetic diversity within *M. pongolensis*, there is enough variation within and differentiation among *M. pongolensis* populations and, therefore, no threat to the natural evolutionary processes of the species. The present study has highlighted the morphological variation of *M. pongolensis* samples from the currently-isolated and closed Kosi River system. The results of the present and previous studies (Teske *et al.*, 2009, 2011; Maake *et al.*, 2013) highlight the need for ongoing protection of this system and its biodiversity, currently under conservation and management by the iSimangaliso Wetland Parks Authority.

During the course of the field expeditions conducted for this study (September-November 2009, October-November 2010 and October-November 2011), ecological and flow regime data indicated that the Limpopo River, inhabited by *M. krameri*, could be a very harsh environment compared with the perennial rivers and streams inhabited by *M. pongolensis* and *M. caudisquamatus*. Extreme seasonal changes in water volume, a reduction to a series of pools in the dry season and violent flooding in the few months of the wet season, are characteristic of the Limpopo River system (Boroto, 2000; FAO, 2004). In some pools where *M. krameri* was collected, water temperature was high, and this could have also caused oxygen levels to drop sharply. Mormyrids cannot breathe atmospheric air like certain galaxiids (Chakona *et al.*, 2011), thus, these harsh conditions require *M. krameri* to have ecological adaptations in life history and reproductive strategy that are different from *M. pongolensis* and *M. caudisquamatus*, which inhabit cooler and oxygen-rich water all year round. How *M. krameri* survives these pressures is a key question that requires continued investigation if we are to plan for pre-emptive conservation actions. Although the genetic diversity of *M. krameri* is high, its distribution is restricted. *Marcusenius caudisquamatus* is also restricted, known only from two populations, the Mhlatuze and Nseleni Rivers, and not enough data are available to assess the conservation status of the two species. However, possible threats to the studied South African mormyrid fishes (*M. pongolensis*, *M. krameri* and *M. caudisquamatus*) and other freshwater-dependent species were identified during the course of this study. These include: continued excessive water extraction, sedimentation and pollution from various developments, agricultural and industrial sources. Matlala *et al.* (2010) indicated that the disappearance of freshwater catfish *Chiloglanis* species from areas where they were previously recorded in the Limpopo and Incomati River systems may be attributed to the factors mentioned above, as well as flow regime fluctuations, changes in system connectivity, physico-chemical conditions and a lack of vegetation cover. Conservation assessments are urgent, because if any of the known

populations of *M. krameri* and *M. caudisquamatus* were to be lost, the genetic information contained within them would not be found in any other river systems in South Africa.

5.5. CHALLENGES AND FUTURE DIRECTIONS

Despite considerable effort during fish surveys in South Africa from 2009-2011, collecting of *Petrocephalus* fish was unsuccessful at all localities listed in the literature (e.g. Kramer & Van der Bank, 2000; Skelton, 2001). Only two populations were collected in the Limpopo River system, on the Mokolo and Olifants Rivers (Figure 2.1). These findings are alarming, given that many *Petrocephalus* species are known to live in large schools and can be very common where they are found (Hopkins, 1986; Lavoué *et al.*, 2004). It is not clear at this stage whether previously reported *Petrocephalus* populations have become extinct at their respective localities or perhaps moved, due to human interference. Regardless, they were very rare and difficult to find during the course of this study.

The relationships among many *Petrocephalus* species were poorly resolved in the present phylogenetic analyses (Figure 4.4 & 4.6, Chapter 4), with short branch lengths and low support values. Phylogenetic analyses of all current *Petrocephalus* taxa would further enhance our knowledge of the systematics and evolution of this enigmatic genus as the current study only included the southern and eastern African species. The resulting phylogenies will provide a framework for investigating the morphological evolution in this group, its biogeography and for understanding drainage evolution.

Electric organ signal diversity in many mormyrids is a direct consequence of morphological variation in electric organs (Sullivan *et al.*, 2002; Moritz *et al.*, 2009). Studies of other southern

African mormyrids have shown congruency between morphology, genetics and EODs, confirming EODs as useful systematic characters (Kramer *et al.*, 2007; 2012; Kramer & Swartz, 2010; Kramer & Van der Bank, 2011). This still needs to be demonstrated for the EODs of the newly described species and undescribed lineages. Future studies of South African mormyrids should explore this, focusing on the connection between morphological diversity and EOD waveform, especially using *M. pongolensis* and *M. caudisquamatus*, given that Kramer *et al.* (2007) reported similarity in their EOD signals. Such a study was critical in describing patterns of geographic variation in electric signal waveforms among populations of the mormyrid electric fish species *Paramormyrops kingsleyae* (Günther 1896) in Gabon, West-Central Africa (Gallant *et al.*, 2011). Furthermore, EODs can be used to discover geographically-distinct populations, isolated from each other by barriers to migration, as was shown for *Paramormyrops kingsleyae*. It is recommended that future research evaluates the importance of EOD signals and morphological diversity in the reproductive isolation of the South African species. The aim should be to determine whether the para-, allo- and/or sympatric populations of different *Marcusenius* species are reproductively isolated, by amplifying microsatellite loci in order to genotype individual species and quantitatively assess their reproductive isolation (Feulner *et al.*, 2008).

The phylogeographic analysis of *Marcusenius* raised a number of issues that will require investigation in the future. To understand the phylogeographic structure in *M. pongolensis*, more samples from additional localities will need to be included. Increased sampling will help to characterise the geographical distribution of this species in the Limpopo River system and establish the extent of sympatry with the newly described *M. krameri*. Additional sampling of rivers in southern Mozambique, such as the Save, Buzi and Phungwe Rivers, and in Swaziland, may help to establish a better understanding of the low phylogeographic structure observed

between the Incomati River system and the distant and isolated Kosi River system. Such an investigation will help to characterise the distributional boundary of *M. pongolensis* and, in doing so, identify environmental factors that may limit or enhance dispersal of this species. Some mormyrids have been shown to have ecophysiological adaptations, such as the lowering of metabolism and oxygen intake that increase survival and persistence in swamp refugia or low-water areas (Chapman & Chapman, 1998). Laboratory experiments could be designed or used to investigate whether *M. pongolensis* individuals adjust their behaviour, tolerance and physiological responses to emersion and reverse to a normal state after re-immersion and hypoxia. These intrinsic characteristics, if studied, could explain how a species occurring in fast-flowing water, such as *M. pongolensis*, could have attained its known range across different river systems (see Chapter 2).

Several phylogeographic studies revealed significant levels of intron variability and, thus, patterns of genetic structure that parallel and complement results based on mtDNA markers (Chow & Hazama, 1998; Cooke *et al.*, 2009; Maake *et al.*, 2013). However, the present study (see Chapter 2) and others (Gay *et al.*, 2004; Guo & Chen, 2010) have detected low levels of intron variability which precluded phylogeographic interpretation or the effective use of this marker for population level analyses. This is probably due to the slow mutation rate of the nuclear marker in comparison to the faster-evolving mtDNA (Hoelzer, 1997). The phylogenetic analyses of the S7 intron also showed that very few mutations exist to differentiate species within *Marcusenius* and *Petrocephalus* (Chapter 4). For *Petrocephalus*, the results of this marker were only congruent with those from the mtDNA in identifying deeper or older divergences, such as the separation of the Eastern and Western species groups within the Southern and Eastern African Basins. Although mtDNA was informative in the phylogeography and phylogenetic studies in this research and other studies (e.g. Waters &

Cambrey, 1997; Bermingham & Martin, 1998; Hewitt, 2000; Qi *et al.*, 2007; Matlala *et al.*, 2010; Bartáková *et al.*, 2013; Maake *et al.*, 2013), other procedures such as Double Digest Restriction Associated DNA (ddRAD) sequencing for detecting SNPs (Single Nucleotide Polymorphisms) (Peterson *et al.*, 2012), and use of microsatellites (Hey *et al.*, 2004; Arnegard *et al.*, 2005; D'Amato *et al.*, 2007; Baird *et al.*, 2008; Feulner *et al.*, 2008) have demonstrated high levels of polymorphism and have recently been applied to a range of biological problems in a diversity of organisms. To determine any finer-scale or recent phylogeographic patterns that could not be identified in the mtDNA studies, it is important that such markers be used in future studies of gene flow patterns in *M. pongolensis* and *M. krameri* and other freshwater species in South Africa.

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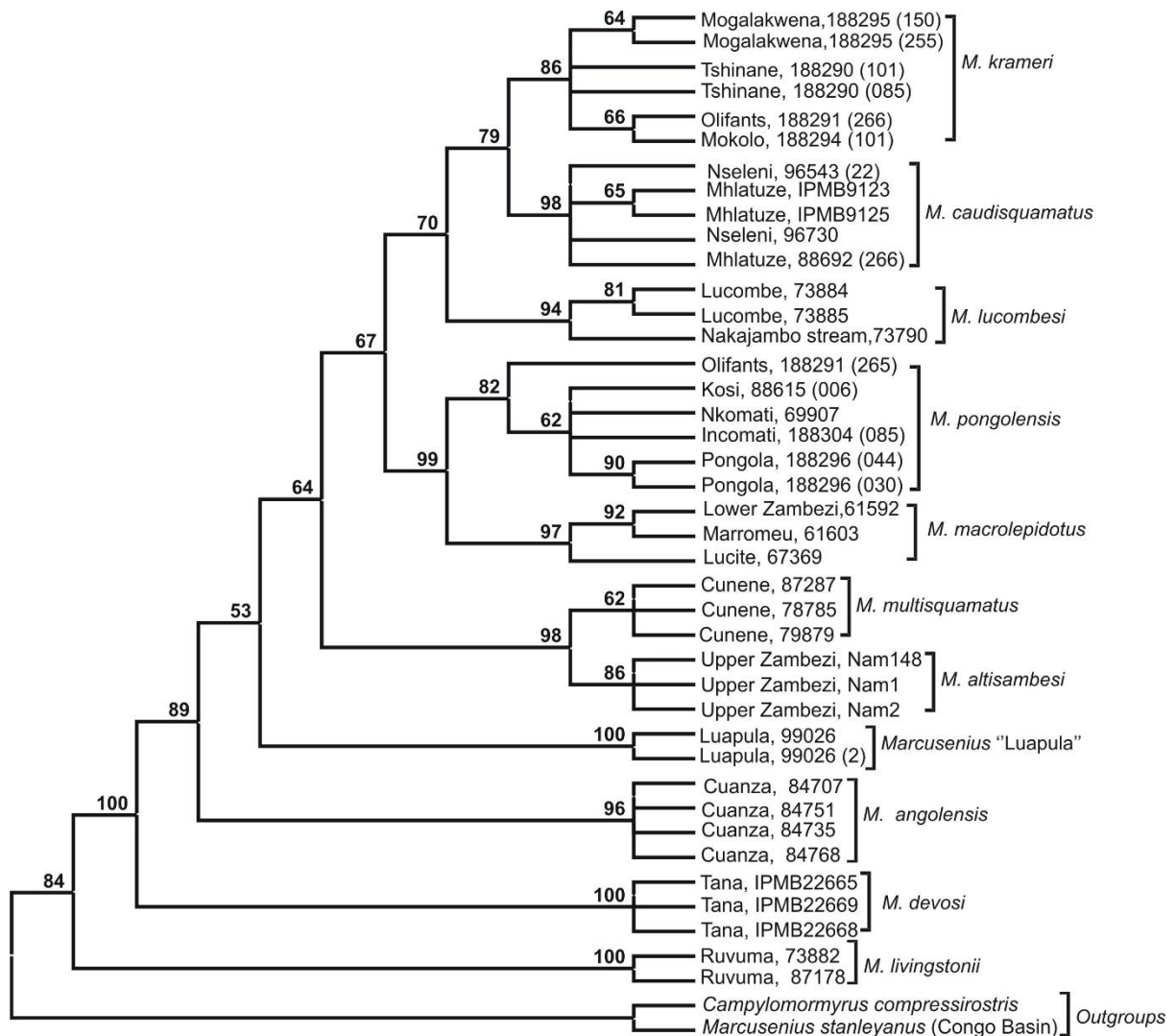
LIST OF APPENDICES

Appendix 3.1. Additional materials of the genus *Marcusenius* occurring in southern and eastern African river systems observed for vertebral and gill raker counts.

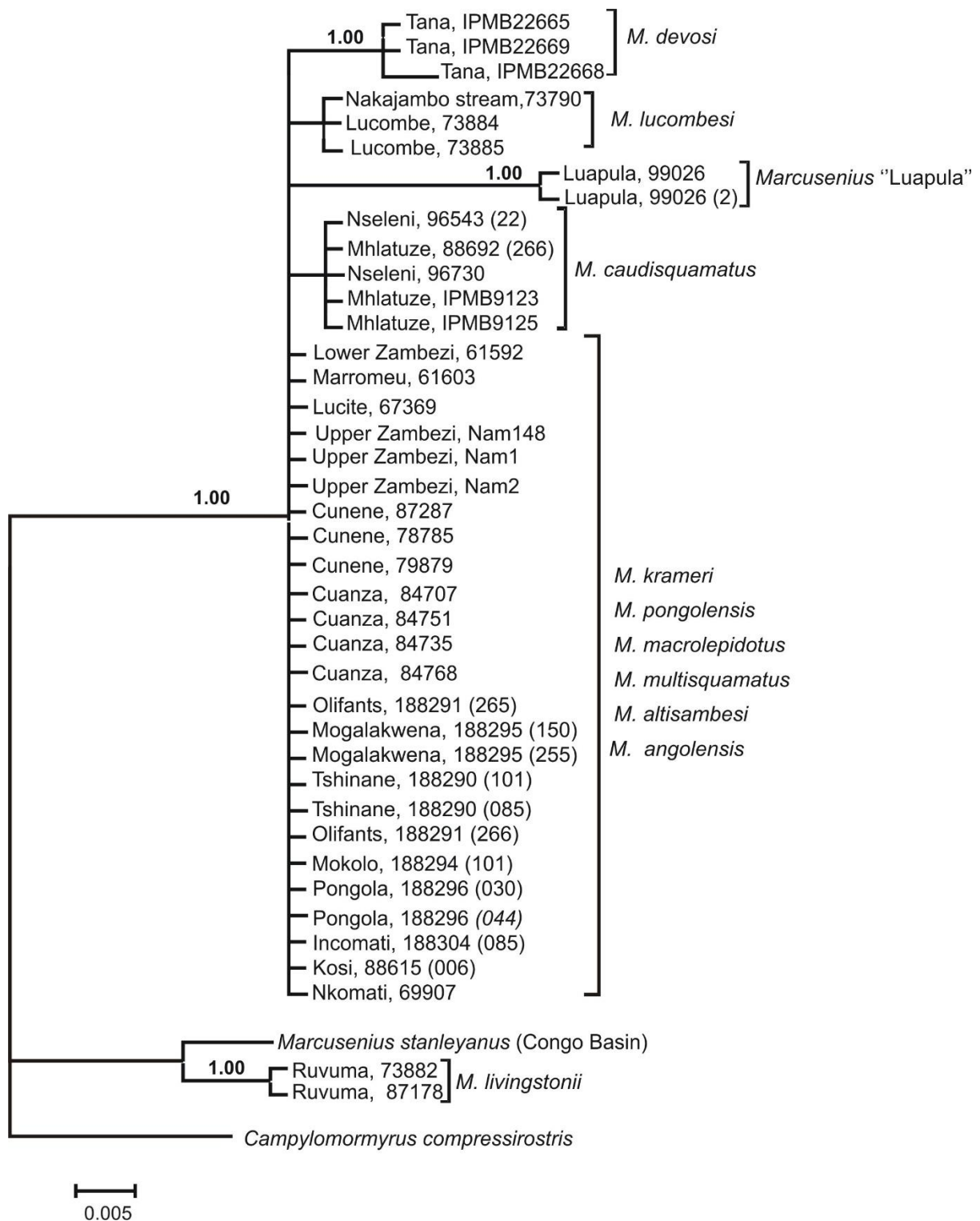
1. *M. angolensis* (Boulenger, 1905): SAIAB 84751, 11: 83-143 mm SL, Cuanza River, above confluence with Kawa River. SAIAB 84768: 105 mm SL, same collection data as previous lot. SAIAB 84707, 3: 107-124 mm SL, Lucala River. SAIAB 84735: 120 mm SL, Kawa River.
2. *M. altisambesi* Kramer, Skelton, Van der Bank & Wink, 2007: SAIAB 79136, 7: 129.3-180.0 mm SL, Lisiliki River, Upper Zambezi system. SAIAB 98584, 21, Kalimbeza channel backwater, Upper Zambezi system.
3. *M. devosi* Kramer, Skelton, Van der Bank & Wink, 2007: SAIAB 79138 (holotype), 102.5 mm SL, Tana River. SAIAB 79139 (Paratypes), 9: 96.6-116.7 mm SL, Tana River.
4. *M. livingstonii* (Boulenger, 1899): SAIAB 35825, 6. Lifune River, Lake Chiuta System. SAIAB 50153, 7: 49.1-60.8 mm SL, Pool in stream bed above Lake Chikukutu. SAIAB 73882, 116.6mm SL, Ruvuma River. SAIAB 73883, 3: 82.4-105.7 mm SL, Ruvuma River. SAIAB 35797, 4, Njerwa Beach, Lake Chiuta System. SAIAB 73853, 3: 44.1-64.6 mm SL, Nandanda River, Lugenda System.
5. *M. macrolepidotus* (Peters, 1852): SAIAB 61603, 4, Lagoon behind sugar fields, Lower Zambezi system. SAIAB 67369, 25: 69.4-148.1 mm SL, Lucite River, Lower Zambezi system. SAIAB 64835, 2: 135-140 mm SL, Maputo River, Maputo System.
6. *M. multisquamatus* Kramer, 2013: SAIAB 59455, 5: 123.6-156.4 mm SL, Ruacana River, Cunene system. SAIAB 186772, 7: 80.0-124.0 mm SL, Cunene system. SAIAB 59458, 2: 113.9-137.9 mm SL, Ruacana gorge, Cunene system. SAIAB 28065, 3, Canal at Mahanene, Cunene system. SAIAB 87287, 180 mm SL, Cunene lagoon, Cunene system.

Appendix 3.2. Components loadings and principal components from the correlations of morphological characters of the four *Marcusenius* species in the present study ($N = 113$). For abbreviations of morphological characters, see Material and Methods.

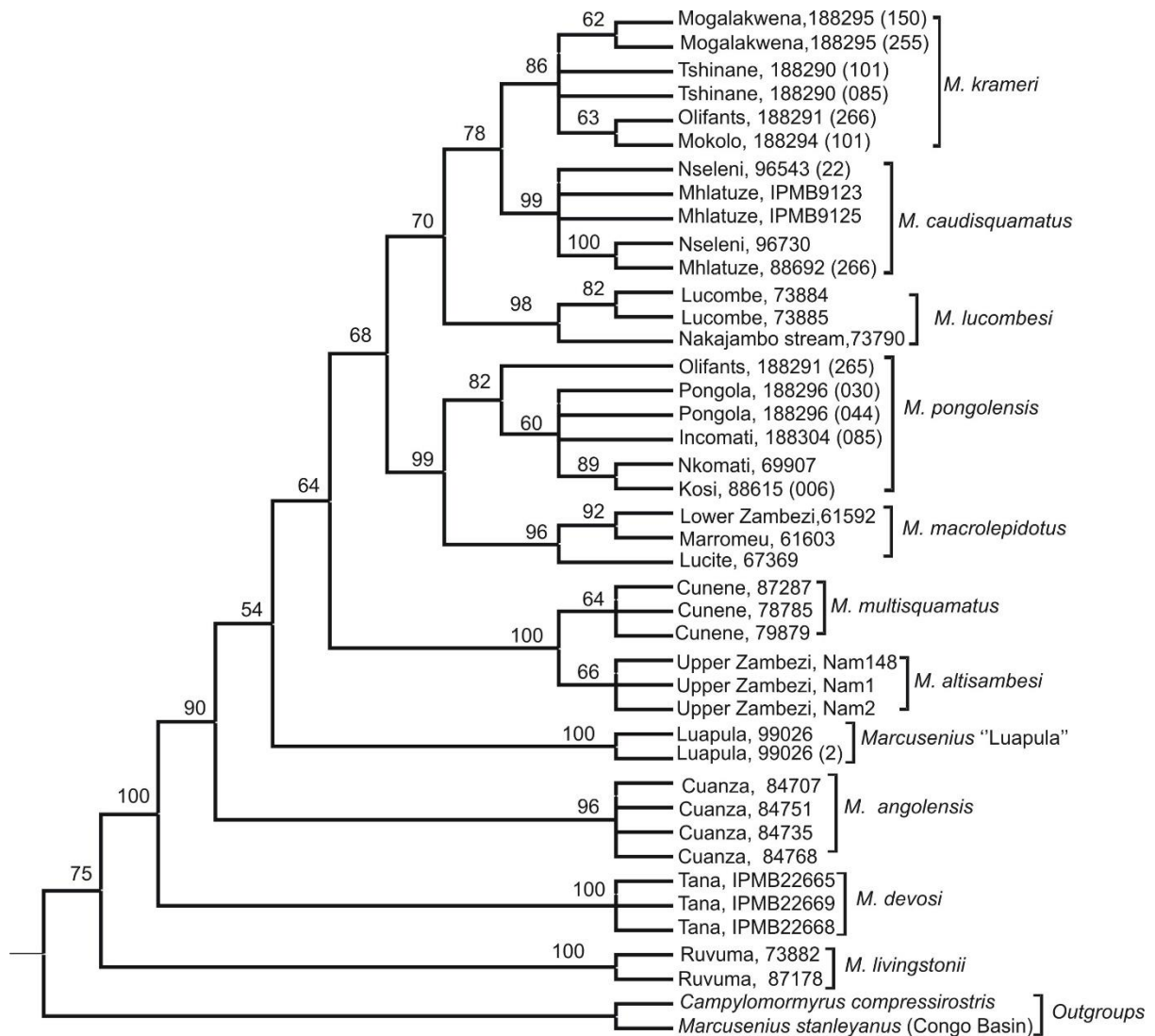
	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7
Eigenvalue	7.06	4.78	2.11	1.81	1.40	1.14	0.93
% variation	29.44	19.92	8.78	7.54	5.82	4.75	3.88
Cum percentage	29.44	49.52	58.35	65.91	71.76	76.51	80.41
Components loadings on PCA axis							
vBD	0.5256	0.5651	0.0451	0.0074	0.4810	-0.1057	-0.2517
mDB	0.6098	0.5721	0.2116	0.0342	0.2572	-0.0404	-0.2730
dAD	0.5947	0.5538	0.1266	0.0725	0.3048	-0.1117	-0.2052
CPD/CPL	-0.6445	0.5618	0.0958	0.0103	-0.2633	-0.0473	-0.2260
CPL	0.3479	-0.0541	-0.5810	0.2116	0.1358	-0.1663	0.0438
PDL	-0.4079	0.5756	-0.1410	-0.0980	0.3946	-0.0432	0.1446
PAL	-0.5834	0.6557	-0.0116	-0.0797	0.1262	-0.0911	0.0413
pD	0.7712	0.3337	-0.0291	0.1757	-0.2333	0.2555	-0.0189
pA	0.8160	0.3397	0.0791	0.2061	-0.1872	0.2067	-0.0581
pPL	0.2771	0.6253	-0.2680	-0.0295	-0.3270	-0.0943	0.1420
pVL	-0.2253	0.7239	-0.3066	0.1350	0.1287	0.1116	0.2755
dVA	-0.6531	0.1801	0.4720	-0.2307	0.1204	-0.0415	-0.1995
dPA	-0.7698	0.3612	0.3701	-0.0340	0.1116	0.1206	0.0839
dPV	-0.4198	0.4467	0.1612	0.2001	0.2296	0.4181	0.4754
LD	0.5603	-0.1723	0.4803	-0.1485	0.0803	0.1734	0.0895
LA	0.6072	0.5096	0.1365	0.2112	-0.2648	0.0983	0.0578
HL	0.3644	0.3059	-0.7208	-0.2749	0.1264	-0.0841	-0.0034
HW/HL	-0.2538	0.2985	0.2508	0.7102	-0.1234	-0.1956	-0.1106
LSN/HL	-0.3560	-0.3514	-0.0547	0.6003	0.1413	-0.2313	-0.0951
IOW/HL	0.0542	-0.5022	0.0065	0.6438	0.3187	-0.1234	0.1942
ED/HL	0.1695	-0.3499	-0.1858	0.1974	0.2318	0.7046	-0.2868
nD	0.7400	-0.0724	0.3460	-0.1604	0.0606	-0.2806	0.2226
nA	0.7250	0.1512	0.3253	0.0879	-0.1307	-0.1563	0.2725
SPc	-0.5703	0.4943	-0.1571	0.2515	-0.3892	0.0348	-0.1601



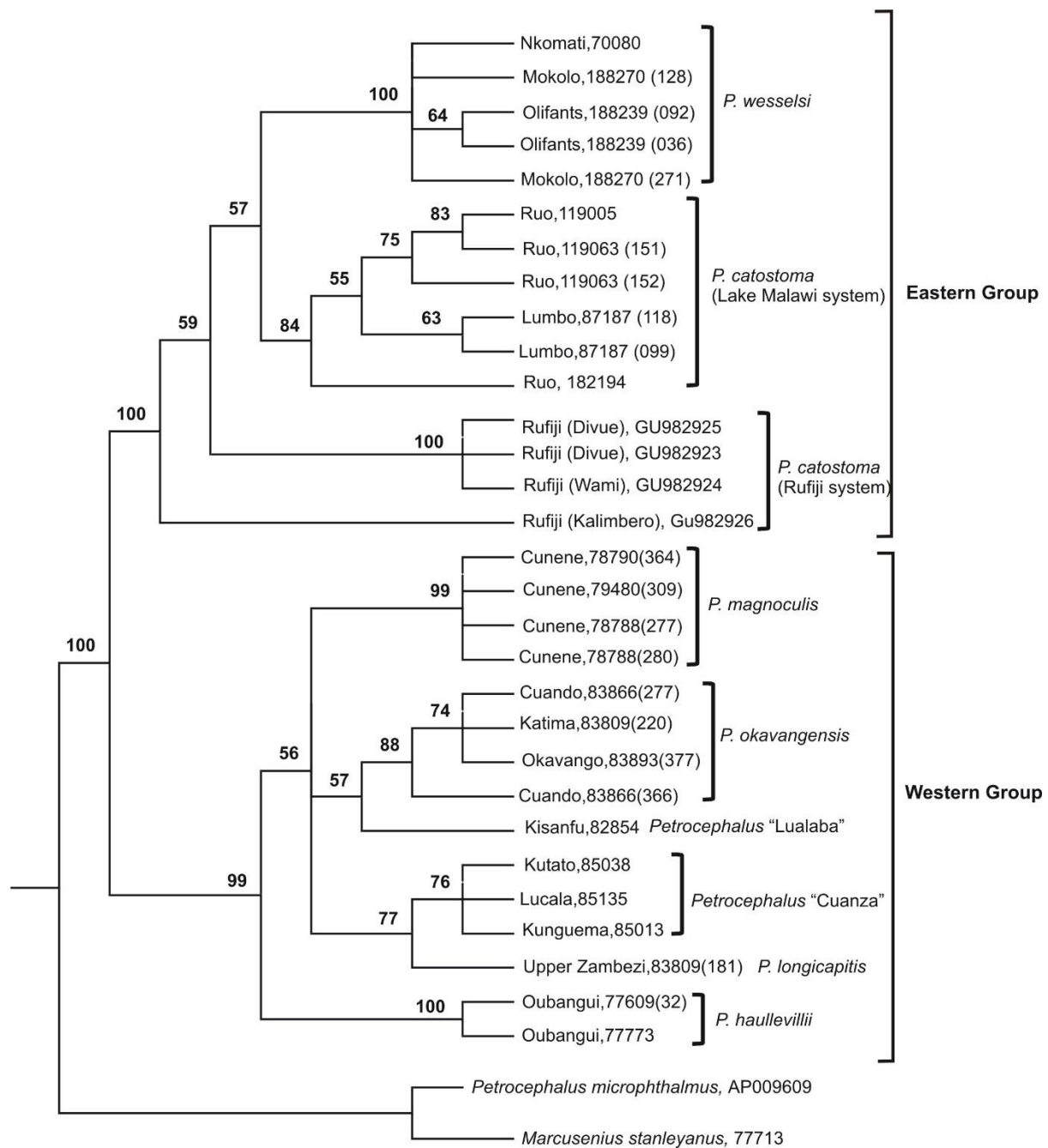
Appendix 4.1. Parsimony analyses of *Marcusenius* as reflected by cytochrome *b* data with bootstrap values. For each species or lineage, SAIAB and IPMB numbers are indicated. Individual numbers are indicated in brackets for accession numbers where more than one individual was sequenced.



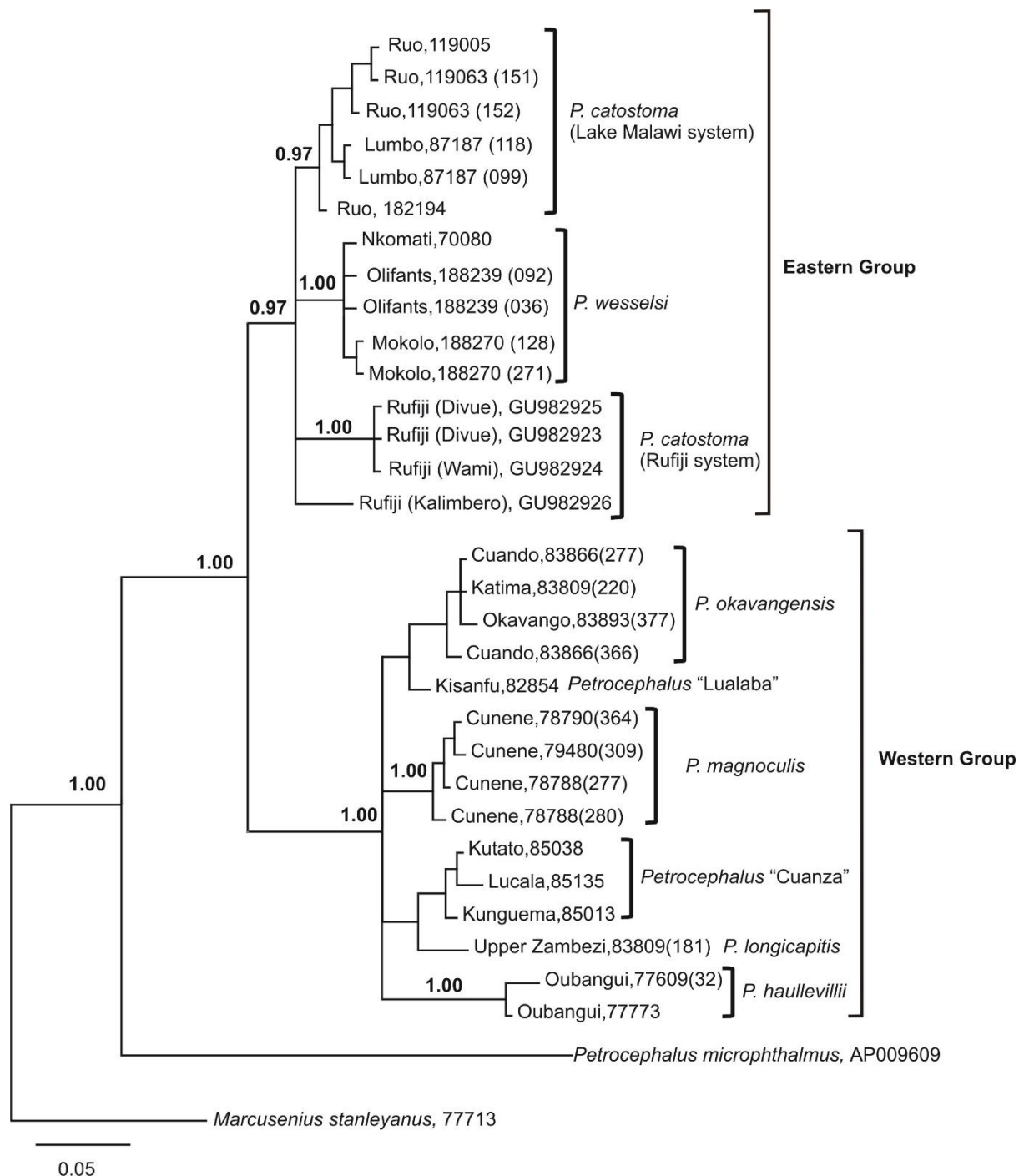
Appendix 4.2. Maximum Likelihood ($-\ln L = 1106.12$) phylogram of relationships among of *Marcusenius* species as reflected by S7 intron 1 data, with Bayesian posterior probabilities > 0.95 indicated on the branches. For each species or lineage, SAIAB and IPMB numbers are indicated. Individual numbers are indicated in brackets for accession numbers where more than one individual was sequenced.



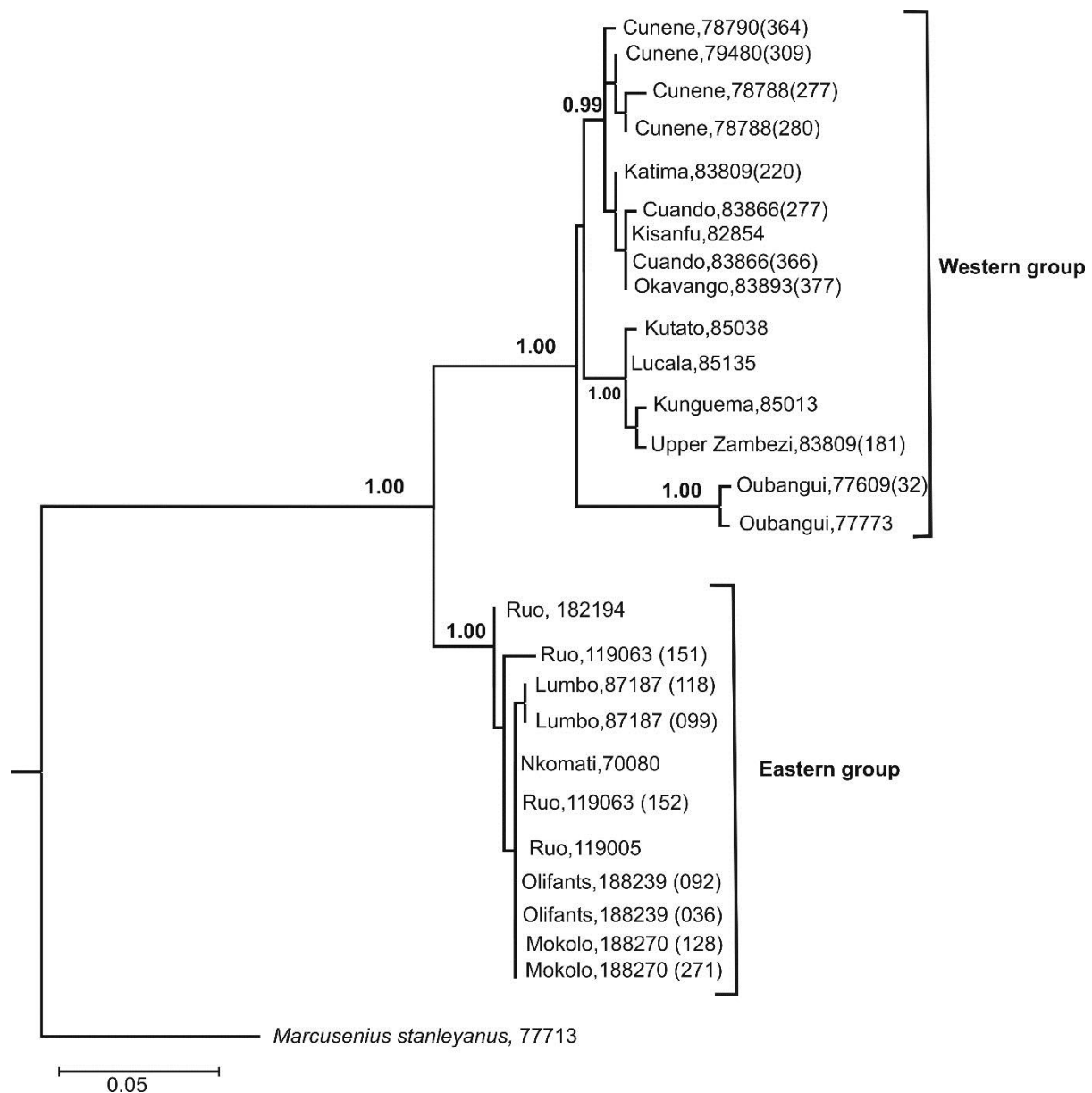
Appendix 4.3. Parsimony analyses of *Marcusenius* as reflected by combined cytochrome *b* and S7 intron 1 data with bootstrap values on branches. For each species or lineage, SAIAB and IPMB numbers are indicated. Individual numbers are indicated in brackets for accession numbers where more than one individual was sequenced.



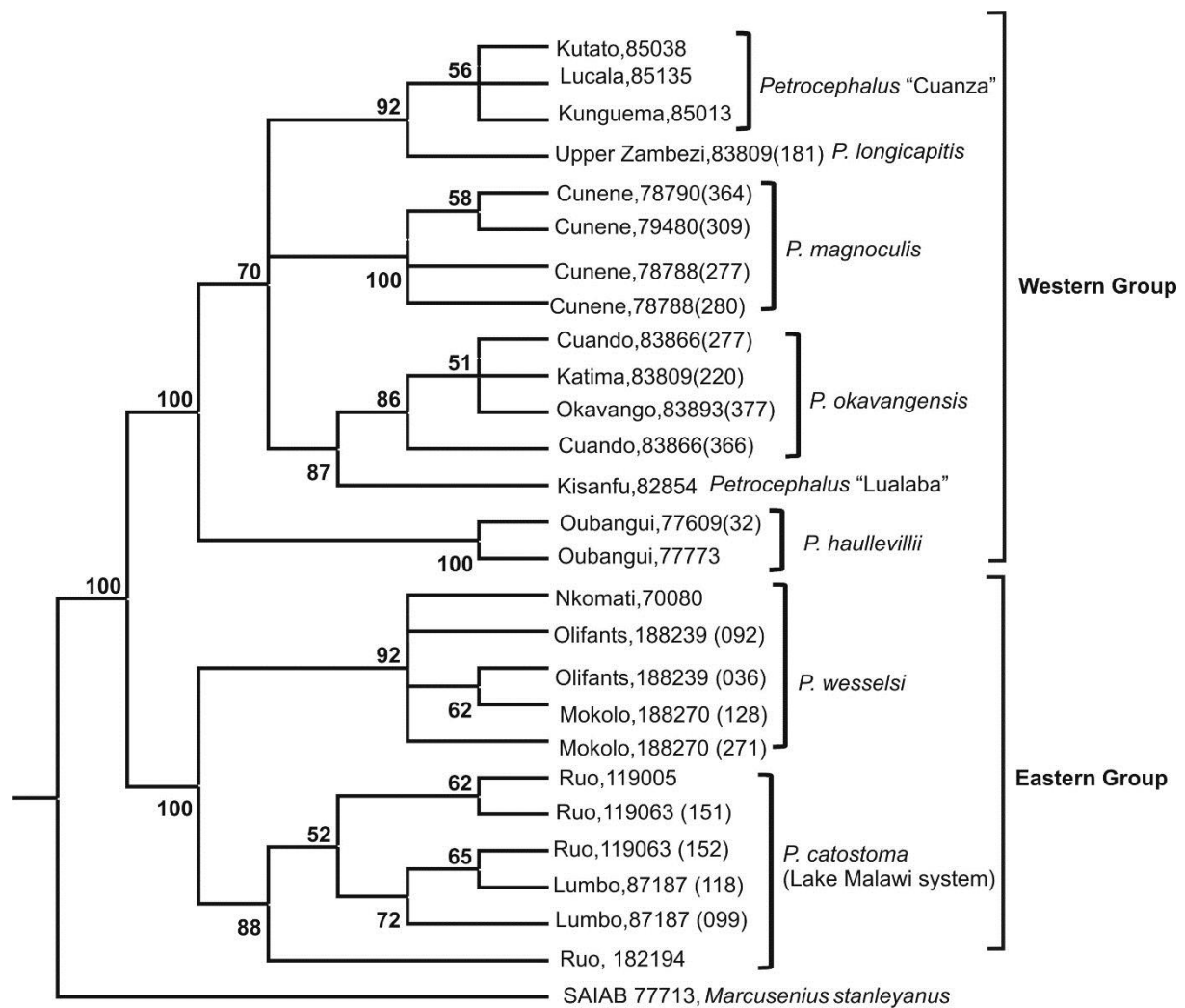
Appendix 4.4. Parsimony analyses of *Petrocephalus* as reflected by cytochrome *b* data with bootstrap values >50% on branches. For each species or lineage, GenBank and SAIAB numbers are indicated with individual numbers in brackets for SAIAB accession numbers where more than one individual was sequenced.



Appendix 4.5. Bayesian phylogram of *Petrocephalus* as reflected by cytochrome *b* data with posterior probabilities >0.95 values indicated on the branches. For each species or lineage, individual localities, Genbank and SAIAB numbers are indicated with individual numbers in brackets for SAIAB accession numbers where more than one individual was sequenced.



Appendix 4.6. Maximum Likelihood ($-\ln L = 1459.16$) phylogeny of *Petrocephalus* as reflected by S7 intron 1, with Bayesian posterior probabilities > 0.95 indicated on the branches. For each species or lineage, individual localities and SAIAB numbers are indicated with individual numbers in brackets for accession numbers where more than one individual was sequenced.



Appendix 4.7. Parsimony analyses of *Petrocephalus* as reflected by combined cytochrome *b* and S7 intron 1 data with bootstrap values > 50% indicated on the branches. For each species or lineage, individual localities and SAIAB numbers are indicated with individual numbers in brackets for accession numbers where more than one individual was sequenced.