Conservation genetics of *Oreochromis mossambicus* across South Africa: Foundational knowledge for management of this vulnerable species

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ABSTRACT

South African freshwater habitats face escalating threats from anthropogenic activities and suboptimal management practices, resulting in a worrisome decline in freshwater taxa. This study addressed a critical knowledge gap concerning the impact of these threats on the genetic diversity and population structure of freshwater fish species in South Africa, focusing on *Oreochromis mossambicus*. Despite its ecological and economic significance, little is known about the genetic diversity and population structure of both farmed and wild *O. mossambicus* in the region. Hybridisation with the introduced *O. niloticus* has further exacerbated the species' vulnerability, leading to its classification as Vulnerable on the IUCN Red List. Establishing baseline genetic data is essential for the effective conservation and management of *O. mossambicus* populations in South Africa. This research pursued five primary objectives: Firstly, this study assessed the genetic diversity and population structure of wild *O. mossambicus* in major river catchments across KwaZulu-Natal, Mpumalanga, and Limpopo provinces. Using 14 microsatellite loci, I established baseline genetic data and evaluated if the current water management practices maintain the species' existing genetic structure. Significant genetic differentiation was found among populations, with STRUCTURE analyses revealing 15 geographically correlated genetic clusters. These findings emphasised the role of anthropogenic activities, changes in catchment use, and water management strategies in shaping the genetic structure of *O. mossambicus*, highlighting the need for conservation-oriented management to preserve existing genetic diversity. Secondly, using 14 microsatellite markers, I determined the genetic diversity and origin of four farmed *O. mossambicus* populations in KwaZulu-Natal and Mpumalanga provinces, comparing them with wild populations from nearby rivers. The results indicated lower genetic diversity in farmed populations compared to surrounding wild populations. In particular, the uMphafa ponds population exhibited distinctive

genetic characteristics, underscoring the need for careful monitoring. This chapter highlighted the potential for using farmed populations from Zini Fish Farm and Fresca Fisheries Farm for selective breeding and broodstock supplementation to maintain genetic diversity in aquaculture practices. Thirdly, this study evaluated the presence of genetic material in farmed and wild *O. mossambicus* as a potential indication of genetic introgression from introduced *O. niloticus* and *O. aureus* using 14 microsatellite loci. Genetic structure analyses revealed evidence of the presence of genetic material and potential introgression primarily between *O. mossambicus* and *O. niloticus* in several wild populations across Limpopo, Mpumalanga, and KwaZulu-Natal. Additionally, shared genotypic frequencies between *O. aureus* and farmed *O. mossambicus* from uMphafa ponds and Fresca Fisheries Farm were detected. This chapter advocated for stringent measures to preserve the genetic integrity of wild *O. mossambicus* populations, particularly those showing no signs of introgression by introduced species, to ensure sustainable management of this vulnerable species. Fourthly, this study reviewed the status of COI and 12S rRNA reference libraries for native and introduced freshwater fish in South Africa. An analysis of DNA records available on GenBank and the Barcode of Life Database (BOLD) revealed significant gaps in the records for both markers. Specifically, 34 species, six genera, and zero families of native South African freshwater fish lack COI barcode records, while 86 species, 22 genera, and eight families lack 12S rRNA records. In contrast, non-native fish had complete barcode records for both COI and 12S rRNA. Establishing comprehensive reference libraries for both markers is a crucial first step in developing an eDNA protocol for the non-invasive monitoring of freshwater fish in South Africa. This eDNA protocol may help enhance the effectiveness of monitoring and conservation efforts for threatened species like *O. mossambicus* and facilitate the early detection and monitoring of invasive species such as *O. niloticus*. Lastly, the study developed and tested the efficacy of environmental DNA (eDNA) metabarcoding as

a non-invasive method for detecting *O. mossambicus* and the introduced *O. niloticus* and *O. aureus* in KwaZulu-Natal. A multi-marker system, incorporating fragments of the cytochrome oxidase I (COI), 12S rRNA, and 16S rRNA gene regions, was employed. The eDNA metabarcoding identified 211 fish-related sequences, including a few sequences of *O. mossambicus* and *O. niloticus*, from 481,913 raw reads, demonstrating the method's effectiveness in detecting diverse fish species. However, achieving species-level identification remained challenging, likely due to incomplete reference databases. This chapter highlighted the potential use of eDNA for detecting and monitoring both native and introduced *Oreochromis* species in South Africa. It emphasised the need to refine the protocol further and design specific primers for more accurate identification. Additionally, this chapter underscored the importance of comprehensive reference libraries and ongoing refinement of eDNA metabarcoding protocols to maximize their potential for freshwater fish monitoring. To enhance conservation efforts, the present study recommends routine genetic monitoring, improved water management practices, expansion of barcode reference libraries, and refinement of eDNA protocols with species-specific primers. Implementing these strategies will aid in better management and preservation of *O. mossambicus* and other freshwater fish, ensuring their long-term viability and ecological health.

PREFACE

The data described in this thesis were collected in KwaZulu-Natal, Mpumalanga, and Limpopo, Republic of South Africa, from February 2017 to September 2022. Experimental work was carried out while registered at the School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg campus, under the supervision of Professors, Sandi Willows-Munro, Gordon O'Brien, and Colleen T. Downs.

This thesis, submitted for the degree of Doctor of Philosophy in the College of Agriculture, Engineering, and Science, University of KwaZulu-Natal, School of Life Sciences, Pietermaritzburg campus, represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others, it is duly acknowledged in the text.

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Mahlatse Fortunate Mashaphu

August 2024

I certify that the above statement is correct, and as the candidate's supervisor, I have approved this thesis for submission.

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Prof. Sandi Willows-Munro
Supervisor
August 2024

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DECLARATION 1 - PLAGIARISM

I, Mahlatse Fortunate Mashaphu, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
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DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis.

PUBLICATION 1- Published: Journal of Global Ecology and Conservation

Genetic diversity and population dynamics of wild Mozambique tilapia (*Oreochromis mossambicus*) in South Africa

MF Mashaphu, G O'Brien, CT Downs & S Willows-Munro

Author contributions:

MFM conceived paper with SWM, GO and CTD. MFM collected and analysed data, and wrote the paper SWM, GO and CTD contributed valuable comments to the manuscript.

PUBLICATION 2- Accepted for publication: Peerj Journal

Genetic assessment of farmed *Oreochromis mossambicus* populations in KwaZulu-Natal and Mpumalanga Provinces, South Africa

MF Mashaphu, G O'Brien, CT Downs & S Willows-Munro

Author contributions:

MFM conceived paper with SWM, GO and CTD. MFM collected and analysed data, and wrote the paper SWM, GO and CTD contributed valuable comments to the manuscript.

PUBLICATION 3- in review: Journal of Biological Invasions

Genetic introgression of farmed and wild *Oreochromis mossambicus* with introduced *Oreochromis* species in Limpopo, Mpumalanga, and KwaZulu-Natal provinces, South Africa

MF Mashaphu, G O'Brien, CT Downs & S Willows-Munro

Author contributions:

MFM conceived paper with SWM, GO and CTD. MFM collected and analysed data, and wrote the paper SWM, GO and CTD contributed valuable comments to the manuscript.

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The status of COI and 12S rRNA DNA barcode reference libraries for freshwater fish in South Africa: Implications for future eDNA projects

MF Mashaphu, G O'Brien, CT Downs & S Willows-Munro

Author contributions:

MFM conceived paper with SWM, GO and CTD. MFM collected and analysed data, and wrote the paper SWM, GO and CTD contributed valuable comments to the manuscript

PUBLICATION 5- in prep

Evaluating the efficacy of eDNA metabarcoding for detecting native *Oreochromis mossambicus* populations in KwaZulu-Natal, South Africa

MF Mashaphu, G O'Brien, CT Downs & S Willows-Munro

Author contributions:

MFM conceived paper with SWM, GO and CTD. MFM collected and analysed data, and wrote the paper SWM, GO and CTD contributed valuable comments to the manuscript

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Mahlatse Fortunate Mashaphu

August 2024

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I would like to express my deepest gratitude to the Omnipotent God of Mount Zion, whose protection and strength sustained me throughout this project. All glory be to God!

Job 42: 2

"I know you can do all things and that no purpose of yours can be restrained."

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CHAPTER 1

Introduction

1.1 Freshwater ecosystems

The health and sustainability of freshwater fish populations are intricately tied to the wellbeing of their aquatic ecosystems, influencing critical factors such as survival, growth, and reproductive success (McAllister et al. 1997, Strachan et al. 2015, Darwall and Freyhof 2016, Brown et al. 2019, Lennox et al. 2019, O'Brien et al. 2019, Bănăduc et al. 2022, Declerck and de Senerpont Domis 2023, Moniruzzaman et al. 2023). Unfortunately, despite being the most diverse vertebrate group in freshwater ecosystems these species face substantial threats from various anthropogenic factors, including pollution and habitat loss, posing significant challenges to their conservation (Gibbs 2000, Collares-Peira and Cowx 2004, Ashton 2007, Hewitt et al. 2008, Hermoso et al. 2009, Sarkar et al. 2010, Negi and Mamgain 2013, O'Brien et al. 2019, Barbarossa et al. 2020, Miqueleiz et al. 2020, Costa et al. 2021, Ahmed et al. 2022, Bănăduc et al. 2022, Cooke et al. 2023).

Freshwater ecosystems are integral to human wellbeing because they provide essential services like food, water, and flood control and face escalating pressures from diverse human activities worldwide (Arlinghaus et al. 2015, Miqueleiz et al. 2020, Bănăduc et al. 2022, Dallas and Rivers-Moore 2022, Mishra 2023). The consequences of habitat destruction, pollution, and overfishing extend to fish species reliant on these ecosystems, resulting in a decline in genetic diversity and population viability (Lande 1998, Crook et al. 2015, Machado et al. 2022, Britton 2023, Candolin and Rahman 2023). The vulnerability of freshwater ecosystems to anthropogenic stressors is a global concern (Revenga et al. 2005, Dudgeon 2010, Dudgeon 2019, Belle et al. 2019, Fierro et al. 2019, Reid et al. 2019, Alam et al. 2020, Lima et al. 2023, Thanigaivel et al. 2023), particularly for water-scarce countries such as South Africa (Dallas and Rivers-Moore 2014; Govender et al. 2022). Despite South Africa's freshwater ecosystems boasting high species richness and endemism (Dudgeon 2019, O'Brien et al. 2019, Dallas et al. 2021), they face significant threats from pollution, water extraction, invasive species, and overexploitation of aquatic resources (Dudgeon et al. 2006, Dallas and Rivers-Moore 2014, Dallas et al. 2021, Riddell et al. 2019, Adams et al. 2020, Desai et al. 2021, Evans et al. 2022), posing a substantial risk to the region's freshwater biodiversity (Dallas and Rivers-Moore 2014, O'Brien et al. 2019, Dallas et al. 2021).

Efforts towards effective biodiversity monitoring and identifying vulnerable areas are imperative for conserving freshwater biodiversity. Species identification, discovery, and monitoring have become central to biodiversity conservation and management (Tsoupas et al. 2022, Mashaphu et al. 2023). A comprehensive understanding of the community structure in natural ecosystems is crucial for sustainable conservation efforts and assessing the impact of anthropogenic and natural activities on biodiversity loss (Fierro et al. 2019, Desai et al. 2021, Mashaphu et al. 2023).

1.2 Focus species: *Oreochromis mossambicus*

Oreochromis mossambicus (Peters, 1852), commonly known as Mozambique tilapia (Figure 1.1), is a member of the family Cichlidae. This species is morphologically characterised by a compressed body, with females and juveniles exhibiting a straight head profile, while matured males feature a concave head profile with enlarged jaws and forward-projected teeth (Skelton 2001). The dorsal fins typically possess 10-13 rays and spines (Froese and Pauly 2007). Scales are prominent along the forehead and snout, gradually decreasing in size along the body (Luna 2012, Kuppusamy et al. 2016). The coloration of adult Mozambique tilapia varies from silvery olive to deep blue-grey, with the dorsal and caudal fin exhibiting a distinct red hue (FAO 2012, Luna 2012). In captivity, *O. mossambicus* may display an almost black appearance, potentially influenced by dietary differences (Luna 2012). A versatile feeder, *O. mossambicus* consumes a diverse array of food items (Skelton 2001, FAO 2012). In its natural habitat, the diet is broad and includes plant material, detritus, algae, aquatic insects, crustaceans, earthworms, organic matter, and small fish (Kotze et al. 1999, Skelton 2001, FAO 2012, Luna 2012).

Figure 1.1: Illustration of the Mozambique tilapia (*Oreochromis mossambicus*) (Source: Skelton 2001). This species is listed as Vulnerable (VU) on the IUCN Red List due to threats of hybridisation with the introduced *O. niloticus* (Bills 2019).

1.2.1 Habitat, distribution, and broad environmental tolerance

Oreochromis mossambicus is endemic to southern Africa, with its native range extending into Malawi, Mozambique, South Africa, Eswatini, Zambia, and Zimbabwe (Agustin 1999, Eknath and Huluta 2009, FAO 2012, Luna 2012, Simbine et al. 2014, Zengeya et al. 2015, Wilson et al. 2019, Xiong et al. 2023). In South Africa, the distribution of *O. mossambicus* spans from the Bushmans River in the Eastern Cape, moving southwards to KwaZulu-Natal, and extending northwards within the Limpopo River System, covering the provinces of Gauteng, North West, Mpumalanga, and Limpopo (Skelton 2001, Figure 1.2). However, this species has been introduced to various tropical and warm temperate habitats worldwide (Kazembe 2010, Xiong et al. 2023). Flourishing in standing waters, *O. mossambicus* exhibits adaptability to diverse environments, including brackish water bodies, canals, estuaries, coastal stretches of rivers, and even full marine conditions in the Pacific (Skelton 2001, FAO 2012, Luna 2012). Known for its hardiness and wide-ranging environmental tolerance, the Mozambique tilapia thrives in conditions that would challenge many other species (Skelton 2001, Froese and Pauly 2007, Arumugam et al. 2023). With a remarkable temperature tolerance ranging from 16°C to 35°C, the optimal temperature for growth and reproduction falls between 22°C and 30°C (Trewavas 1983, Kazembe 2010, Luna 2012). *Oreochromis mossambicus* demonstrates resilience to elevated salinity, low oxygen levels, high ammonia concentrations, and both high and low pH levels (Skelton 2001, Jamil et al. 2004, Kamal and Mair 2005). This broad environmental tolerance contributes to its widespread use in the aquaculture industry (Skelton 2001, Luna 2012, Xiong et al. 2023).

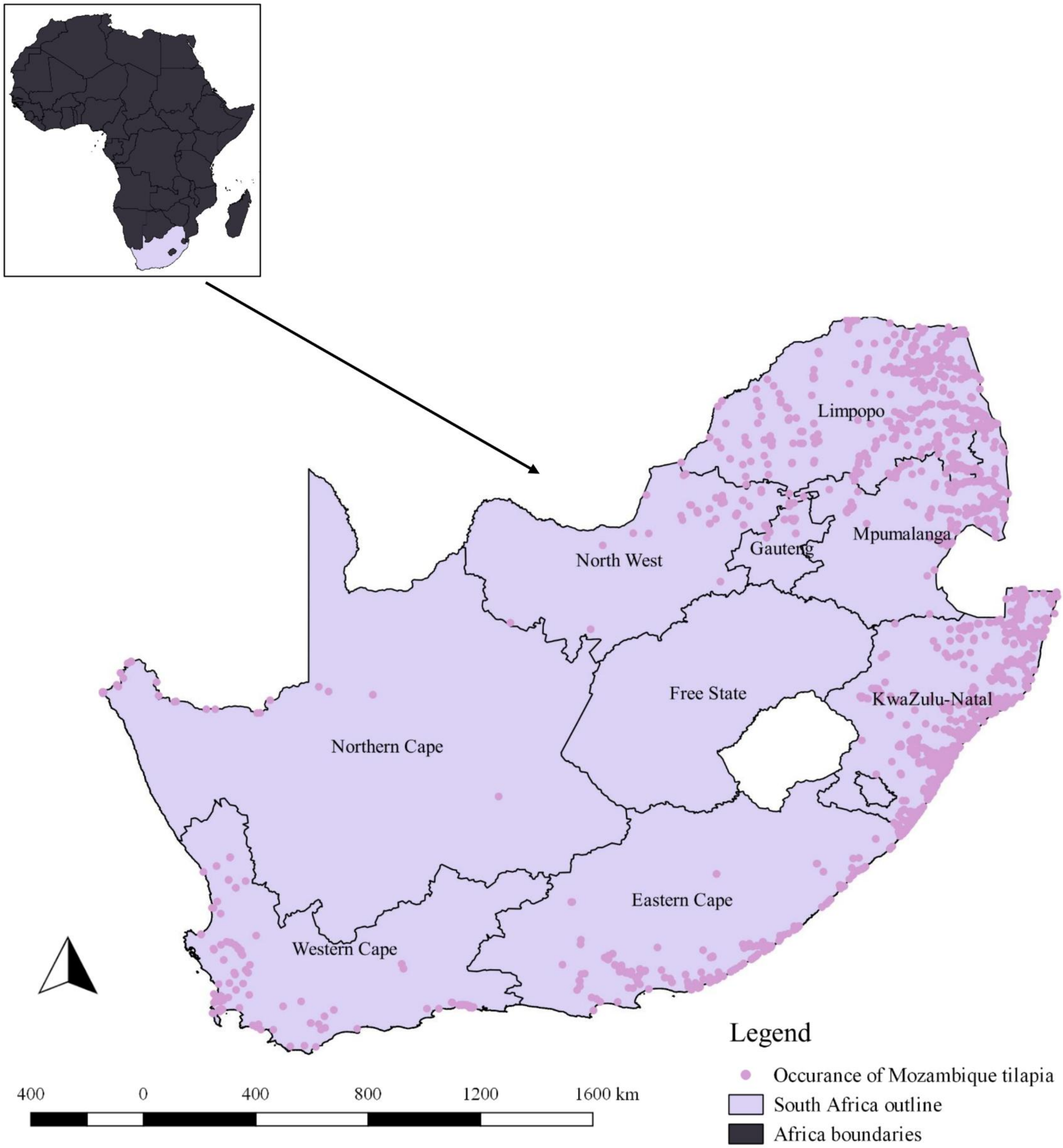

Figure 1.2: Distribution of *O. mossambicus* in South Africa. (Source: Cambray and Swartz 2007, GBIF 2017).

Oreochromis mossambicus is a polygamous maternal mouth brooder (Luna 2012). In sandy and muddy habitats, *O. mossambicus* usually establish breeding territories where they dig spawning pits (Oliveira and Almeda 1998, Froese and Pauly 2015). The spawning pits are aggressively defended, and males assume a dark colouration to attract receptive females (Allen et al. 2002). Their spawning ratio is usually one male to three females (Skelton 2001). To initiate spawning, males will lead females to the territory for courtship (Froese and Pauly 2015). A female lays eggs, which she then scoops up, followed by the scooping of the male's sperm. Fertilisation then occurs in the buccal cavity of the female (Trewavas 1983, Oliveira and Almeda 1998, Froese and Pauly 2015). The female incubates the fertilised eggs and depending on temperature, hatching occurs within 3 to 5 days (Allen et al. 2002, Lamboj 2004). Within 10 to 14 days of hatching, the females will release the fry into the water but will continue to maintain a close protective relationship with the fry for up to weeks (Froese and Pauly 2007, Luna 2012). Juveniles are generally more sensitive to environmental fluctuations than adults (Luna 2012). Mozambique tilapia juveniles reach sexual maturity at 2 months and adult size is reached within 5 to 6 months after hatching (Luna 2012).

1.2.2 Ecological and economic importance

Oreochromis mossambicus is utilised extensively in aquaculture and commercial and subsistence fisheries (Skelton 2001, Changadeya et al. 2003, D'Amato et al. 2007, Firmat et al. 2013, Froese and Pauly 2015, Prabu et al. 2019, Wilson et al. 2019, Xiong et al. 2023). This species is important for aquaculture due to its fast growth, ability to tolerate a wide range of environmental conditions, capacity to feed on various food sources, suitability for high-density culture, and adaptability to changes in oxygen availability and salinity production (Mckaye et al. 1995, Courtenay 1997, Coward and Little 2001, Canonico et al. 2005). However, it is not as preferred, especially for commercial aquaculture purposes, because it is prone to stunting, where energy is redirected towards reproduction at a relatively small size, thus limiting its suitability for commercial. (Mckaye et al. 1995, Courtenay 1997, Coward and Little 2001, Canonico et al. 2005). This fish species is also regarded as a valuable angling fish and plays a vital role in biological, physiological, and behavioural research (Weber 2010, Skelton 2001, Cavallino et al. 2023).

1.3 Importance of wild fish resources for aquaculture

The global aquaculture industry has witnessed remarkable growth, playing a pivotal role in primary food production and poverty alleviation worldwide (Nadarajah and Flaaten 2017, Novaes et al. 2019, Boyd et al. 2022, FAO 2022). As a source of employment and income, the aquaculture and fisheries sector supports the livelihoods of approximately 12% of the global population, emphasising its socio-economic significance (FAO 2018, 2022). Despite this global surge, aquaculture production in Africa, while steadily increasing, lags behind other continents (de Graaf and Garibaldi 2014, Oyeleke 2017, Hounmanou et al. 2018, Chan et al. 2019, Akegbejo-Samsons 2022, Hinrichsen et al. 2022). Africa contributed only ~1.9% to global aquaculture production in 2019-2020, indicating slower growth than in previous years (FAO 2020, 2022). Nevertheless, fish production in Africa remains crucial, supplying approximately 19% of animal protein and essential nutrients for human consumption (Kassebaum et al. 2014, Béné et al. 2015, Chan et al. 2019, Olaifa et al. 2022).

While aquaculture significantly contributes to African economies, research efforts have predominantly focused on production improvement, overshadowing the importance of managing natural resources for sustainable aquaculture (Béné and Heck 2005, Brummett et al. 2009, Subasinghe et al. 2009, FAO 2020, Jolly et al. 2023). This oversight includes neglecting fish genetic resources, both in farmed and wild populations (Lind et al. 2012, Sonesson et al. 2023). Effective management of fish genetic resources is imperative for achieving sustainable aquaculture production, a facet sometimes overlooked in the broader context of aquaculture research and development (FAO 2020, Sonesson et al. 2023).

In the South African context, despite being prioritised for development in the Ocean Economy Planning, the aquaculture sector is still in a developing phase, with an annual production rate of 6.6%, contributing less than 1% to global aquaculture production (Changadeya et al. 2003, DAFF 2016, Ngarava et al. 2023). However, South Africa holds immense potential for aquaculture growth, given its diverse fish production, a relatively high population growth rate of 11.7% per year, and persistent food security concerns in the southern African region (FAO 2014, FAO 2017, Jolly et al. 2023). Presently, freshwater species utilised for aquaculture in South Africa include trout (*Oncorhynchus mykiss and Salmo trutta*), tilapia (*O. mossambicus, O. niloticus and O. rendalli*), catfish (*Clarias gariepinus*), carp (*Cyprinus carpio*), and marron crayfish (*Cherax tenuimanus*) (DAFF 2016, Adeleke et al. 2020). In South African freshwater aquaculture, trout and tilapia (specifically, *O. mossambicus* and *O. niloticus*)emerge as the predominant cultured species, with trout leading the way. This contrasts with the global pattern where tilapia typically dominates aquaculture (Dinesh et al. 2017, Hounmanou et al. 2018, Moyo and Rapatsa 2021, El-Sayed and Fitzsimmons 2023). There is substantial potential for the development of tilapia farming in South Africa, particularly in provinces such as KwaZulu-Natal, Mpumalanga, and Limpopo, where favourable climates and legal constraints against farming invasive species present opportunities for native species, such as *O. mossambicus*, contributing to both aquaculture and conservation efforts in the region (Oyeleke, 2017).

1.4 Constraints to the development of aquaculture

The aquaculture industry holds significant promise as a primary source of food production and employment, particularly in the African context (Miller et al. 2004, D'Amato et al. 2007, Firmat et al. 2013, Chan et al. 2019, FAO 2022). Despite its potential to contribute positively to the economies of many African countries, including South Africa, the industry faces various challenges that presently impede its growth. These challenges include poor conservation practices for native species used in farming, anthropogenic activities, natural drivers such as climate change, loss of genetic diversity in both farmed and wild populations, and contamination of indigenous and wild gene pools by invasive species (Pullin and Capilli 1988, Appleyard and Mather 2000, Kumar 2000, Changadeya et al. 2003, Gupta and Acosta 2004, D'Amato et al. 2007, Brummett 2008, Bezault et al. 2012, Firmat et al. 2013, Nadarajah and Flaaten 2017, Gcebe et al. 2018, Shechonge et al. 2018, Chan et al. 2019, Tolley et al. 2019, Popoola 2022, Sonesson et al. 2023). This project aimed to address some of these critical factors, with a primary focus on providing a comprehensive genetic assessment of both farmed and natural populations of the Mozambique tilapia (*Oreochromis mossambicus*) in the provinces of KwaZulu-Natal, Mpumalanga, and Limpopo, South Africa. By doing so, the research endeavoured to propose improved management practices that contribute to the conservation and sustainable use of this species within the context of South African aquaculture. Through a genetic lens, the project sought to enhance our understanding of the present state of *Oreochromis mossambicus* populations, offering valuable insights for informed decision-making and the development of effective strategies for the industry's long-term viability and ecological sustainability.

1.5 Main threats to Mozambique tilapia in the wild

Despite the adaptability of Mozambique tilapia to various habitats, they remain sensitive to significant alterations in aquatic environments. Anthropogenic activities and natural environmental factors pose formidable threats to the survival of these freshwater fish (D'Amato et al. 2007, Crispo et al. 2011, Firmat et al. 2013, Shechonge et al. 2018). Designated as Vulnerable by the International Union for Conservation of Nature (IUCN), Mozambique tilapia face the primary threat of genetic contamination through hybridisation with the introduced invasive species *O. niloticus*, contributing to the decline in wild stocks (Cambray and Swartz 2007, Firmat et al. 2013, Bills 2019).

Beyond hybridisation, Mozambique tilapia confront a myriad of challenges, highlighting their vulnerability in the wild. Habitat degradation resulting from urbanisation, agricultural expansion, and infrastructure development contributes to the loss and fragmentation of critical habitats (Steffy and Kilham 2006, de Mello et al. 2020, Comte et al. 2021, Paredes del Puerto et al. 2022). Water pollution from various sources, including agricultural runoff, industrial discharges, and urban effluents, poses a significant risk to the health and reproductive success of Mozambique tilapia populations (Malik et al. 2020, Dar et al. 2021, Duarte et al. 2022, Duncan et al. 2023). The introduction of invasive species, beyond hybridisation with *O. niloticus*, creates competition for resources and habitat, compounding the threats faced by the Mozambique tilapia (Leung et al. 2002, Canonico et al. 2005. Ricciardi et al. 2017, Madibana et al. 2020, Moyo and Rapatsa 2021, Nobinraja et al. 2023, Omweno et al. 2023). Climate change-related factors, including temperature fluctuations, altered precipitation patterns, and extreme weather events, contribute to the instability of ecosystems supporting Mozambique tilapia (Comte and Olden 2017, Bellard et al. 2019). Unsustainable fishing practices, both commercial and subsistence, can lead to overexploitation and population decline

(Brown et al. 2019, Hilborn et al. 2020, Pham et al. 2023). Additionally, disease outbreaks, whether natural or introduced, pose a significant threat to the health and stability of Mozambique tilapia populations (Madanire-Moyo et al. 2012, Shinn et al. 2023, Smit et al. 2023). The Vulnerable conservation status highlights the need for effective management strategies to address the impact of these challenges and ensure the continued survival of *O. mossambicus* in its natural habitat.

1.6 Importance of genetic diversity in natural populations

Measuring genetic diversity is essential for implementing effective conservation strategies, particularly for threatened species (Jamieson 2007, Johnson et al. 2009, Hermoso et al. 2016, Beddek et al. 2018, Watanabe et al. 2018, Schmidt et al. 2023). Genetic diversity within populations is critical for their adaptive capacity and potential for speciation (Johnson et al. 2009, Dieleman et al. 2019, Hoban et al. 2023, Schmidt et al. 2023). Identifying populations with unique genetic, physiological, and behavioural traits is crucial for targeted conservation interventions (Firmat et al. 2013, Garg et al. 2014, Watanabe et al. 2018, Cooke et al. 2023). While some species may tolerate reduced genetic diversity without any apparent fitness reduction (Brodie 2007, Mable 2019, Andersson and Purugganan 2022), severe population size reductions often result in significant genetic diversity loss because of genetic drift and inbreeding, leading to reduced fitness and increased susceptibility to inbreeding depression (Hale and Briskie 2007, Leberg and Firmin 2008, Thomson 2022, Robinson et al. 2023).

Research on tilapia populations, specifically *O. mossambicus*, highlights the consequences of reduced genetic diversity. A study of South African wild populations, encompassing only eight locations, identified 26 distinct mitochondrial DNA (mtDNA) haplotypes. This finding suggests a higher level of genetic diversity compared to the feral *O.*

mossambicus stocks examined in the Australian Pacific region (Agustin, 1999). However, it is important to consider that the number of haplotypes indicative of high diversity can vary depending on the species and the mtDNA marker used. Further research with a larger sample size in both regions and potentially using additional genetic markers, could provide a more comprehensive picture. Recent literature emphasises the importance of considering both neutral and adaptive genetic variation in conservation assessments to capture a comprehensive understanding of a species' genetic diversity (Frankham 2015, Shafer et al. 2015, Hoban et al. 2020, 2023). Incorporating recent advances in genomics, such as high-throughput sequencing technologies, can enable a more thorough exploration of genetic diversity, aiding conservation practitioners in developing targeted and informed management strategies (Shafer et al. 2015, Hoban et al. 2020, Segelbacher et al. 2022). In this study, I also aimed to assess the utility of high-throughput sequencing technologies by designing and testing an environmental DNA method for detecting *Oreochromis* species in South African rivers.

1.7 Factors that impact genetic diversity and population structure.

The genetic diversity and population structure of natural freshwater fish populations are intricately influenced by various factors, including demographic dynamics, historical gene flow, barriers to movement, species dispersal capacities, and both natural and anthropogenic flow regulation (Hurwood and Hughes 1998, Riginos et al. 2014, Grummer et al. 2019, Qin et al. 2022, Hagen 2023, Lloyd-Jones et al. 2023). Anthropogenic activities, such as pollution, landscape modifications (artificial waterfalls, weirs, altered flow regimes, and impoundments), catchment transformation, overexploitation of biological resources, introduction of invasive species, and climate change, also play a significant role in shaping these populations (Hurwood and Hughes 1998, Hewitt 2000, Crispo et al. 2011, Dudgeon 2014, Crookes and Shaw 2016, Ellegren and Galtier 2016, Jaisuk and Sananan 2018, Mather et al. 2018, Amoussou et al. 2019, Koblmüller et al. 2019, Dudgeon 2019, Dallas and Rivers-Moore 2022). Collectively, these factors contribute to the dynamic genetic landscape of freshwater fish populations, highlighting the necessity for comprehensive studies to understand and manage their genetic diversity effectively.

To comprehend the genetic population structure of freshwater fish, particularly tilapia species, an exhaustive exploration of factors influencing their genetic diversity is essential. As illustrated by Bezault et al. (2011) in their study on Nile tilapia (*Oreochromis niloticus*) populations across Africa, geographical barriers play a pivotal role. Additionally, habitat fragmentation has been shown to significantly influence the genetic diversity and structure of Nile tilapia in Ethiopia (Tesfaye et al. 2021). The introduction of invasive species, selective breeding practices in aquaculture (Guo 2009, Janssen et al. 2017, Wyban 2019, Kang et al. 2023, Sonesson et al. 2023), and hybridisation between native and non-native strains (Weigel et al. 2003, D'Amato et al. 2007, Firmat et al. 2013, Blackwell et al. 2021, Castagné et al. 2023) further shapes the genetic landscape. Additionally, local adaptation to environmental conditions, as investigated by Arnaud-Haond et al. (2007), underscores the dynamic nature of genetic responses to contaminants. Collectively, these studies emphasise the multifaceted influences on the genetic population structure of tilapia species, providing valuable insights for effective conservation and management strategies amidst both natural and anthropogenic pressures.

1.7.1 Alteration and modification of habitats

The escalating impact of anthropogenic activities on the natural environment is driving biodiversity loss at an alarming rate (Crispo et al. 2011, Crook et al. 2015, Terrado et al. 2016, Borgwardt et al. 2019, Prakash and Verma 2022, Bănăduc et al. 2023, Wang et al. 2023).

Anthropogenic-induced historical and ecological processes significantly influence freshwater biodiversity, particularly through inter-basin water transfer (IBWT) projects (Berbel-Filho et al. 2016, Gallardo and Aldridge 2018, Rollason et al. 2022, Bourguignon 2023). Despite being conducted to improve water supply for human use, IBWT projects can conflict with biodiversity conservation, leading to changes in riverine biodiversity, loss of native species, disruption of gene flow, and introductions of invasive species (Grant et al. 2012, Woodford et al. 2013, Berbel-Filho et al. 2016, Davies et al. 1992, Kadye and Booth 2013, Rollason et al. 2022, Bourguignon 2023). Understanding the implications and impacts of such activities is crucial for positively contributing to the conservation of freshwater biodiversity (Davies et al. 1992, Grant et al. 2012, Berbel-Filho et al. 2016, Qin et al. 2022).

Anthropogenic activities have been traced to drastic modifications of ecosystems, loss of genetic diversity, and complete species loss (Sarkar et al. 2010, Crispo et al. 2011, Negi and Mamgain 2013, Wang and Gu 2021, Dornelas et al. 2023). Alterations to the physical landscape and changes in species distribution resulting from these activities can influence gene flow and introgression, impacting the integrity of reproductive barriers and leading to a loss of local adaptation (Crispo et al. 2011, Bourret et al. 2011, Ottenburghs 2021, Ålund et al. 2023). Conversely, population fragmentation may occur because of decreased population connectivity resulting from anthropogenic habitat alteration (Debinski and Holt 2000, Crispo et al. 2011, Brauer and Beheregaray 2020, Stoffels et al. 2022), leading to genetic drift and eventual loss of genetic diversity in small populations (Reed 2004, Keyghobadi 2007, Crispo et al. 2011, Hoban et al. 2022, Kovach et al. 2022).

The impacts of landscape changes vary among species, as certain alterations may facilitate colonisation and enhance gene flow among populations. However, individual behaviour, dispersal ability, and survival probability in different habitats significantly influence dispersal (Hellberg 1996, Baguette and Van Dyck 2007, Hoehn et al. 2007, Knowlton and Graham 2010, Cosentino et al. 2011, Watanabe et al. 2018). River capture events have been identified as contributors to gene flow among populations of disjunct freshwater fish (Waters et al. 1994, Hurwood and Hughes 1998, Echelle and Echelle 1998, Swartz et al. 2009, Crispo et al. 2011, Chakona et al. 2013, Waters et al. 2020, Oliveira-Silva et al. 2023), suggesting that natural gene flow across different river systems may occur. However, Bishop (1995) indicated that drastic natural changes in drainage systems occur at a relatively low frequency and should not substantially impact the population structure of freshwater fish species. Conducting fine-scale phylogeographic studies of freshwater fish is crucial for clarifying this subject.

Additionally, various water and catchment management strategies may influence the genetic diversity and population structure of freshwater fish (Prunier et al. 2018, González-Ferreras et al. 2022). River connectivity, guided by hydrological connectivity, plays a crucial role in shaping the genetic variation of freshwater taxa (Standford and Ward 1992, Brauer et al. 2013, Silva et al. 2018, Oliveira-Silva et al. 2023). Generally, genetic patterns follow the hierarchy of river connectivity, with populations in close proximity experiencing similar ecological pressures (Hurwood and Hughes 1998, Nicol et al. 2017, Harrison 2022, Reyne et al. 2023). However, in water-scarce countries, mitigating water insecurity through dam construction and inter-basin transfer schemes may disrupt genetic patterns in freshwater taxa (Snaddon et al. 1999, Lynch et al. 2011, Matchaya et al. 2019, Purvis and Dinar 2020, Harris et al. 2022, Qin et al. 2023).

In South Africa, where water scarcity and rising demand for water resources are significant challenges, there may be implications for both water availability and quality, thereby affecting freshwater ecosystems (Dallas and Rivers-Moore 2014, Du Plessis 2023). Coupled with anthropogenic activities and intensive land modification, the country's water resources are deteriorating, posing threats to many river systems and their biodiversity (Nel et al. 2011, Driver et al. 2012, Lemley et al. 2015, O'Brien et al. 2019, Dudgeon 2019, Dallas and Rivers-Moore 2022, Evans et al. 2022). This emphasises the need to evaluate the genetic population structure of freshwater taxa residing in these systems and refine management strategies for the long-term viability of threatened fish species such as *O. mossambicus*.

1.7.2 Introduction of invasive species and hybridisation

The introduction of invasive species poses a significant threat to freshwater biodiversity, impacting native fish populations through resource exploitation, disease spread, and hybridisation (Rahel 2002, Collares-Peira and Cowx 2004, Lind et al. 2012, Genner et al. 2013, Deines et al. 2014, Ellender and Weyl 2014, Ndiwa et al. 2014, Dieleman et al. 2019, Weyl et al. 2020, Ahmed et al. 2022, Britton et al. 2023). This threat is particularly pronounced in tilapiine fishes, such as *O. mossambicus*, where recent divergence and hybridisation pose risks to genetic integrity (Appleyard and Mather 2000, Kumar 2000, Gupta and Acosta 2004, Canonico et al. 2005, D'Amato et al. 2007, Hughes et al. 2008, Saju et al. 2010, Angienda et al. 2011, Simbine et al. 2014, Zengeya et al. 2015, McAndrew et al. 2016, Meier et al. 2019, Chuhila et al. 2023).

While economically and socially beneficial, the intentional introduction of non-native fish species worldwide for fisheries and aquaculture purposes remains a threat to native freshwater fish populations (Gozlan et al. 2010, Zengeya et al. 2015, Yongo et al., 2023). The consequences of invasive species introductions may not be immediately clear, and negative impacts on native fish populations often manifest years later (Barel et al. 1985, Genner et al. 2013, Mayfield et al. 2021, Muñoz-Mas et al. 2023). The introduction of *O. niloticus* and *Lates niloticus* to Lake Victoria, aimed at enhancing fisheries, led to the loss of many endemic cichlid species (Barel et al. 1985). Conservation strategies for natural fish populations used in aquaculture are often slow and inadequate, resulting in the rapid decline of many freshwater fish species (Canonico et al. 2005, Angienda et al. 2011, Xie et al. 2019, Troell et al. 2023). In particular, while hybridisation may offer benefits for aquaculture, it can be unpredictable and result in the loss of genetically pure strains, leading to the eventual loss of the entire gene pool (Lind et al. 2012, Deines et al. 2014, Bradbeer et al. 2018, Dieleman et al. 2019, Setyawan et al. 2022, Troell et al. 2023). For instance, the extinction of *O. variabilis* was recorded in Lake Victoria after *O. niloticus* and *O. mossambicus* were introduced to the system (Welcomme 1967). Although *O. niloticus* and *O. mossambicus* were eventually also lost from the system, their genes have been detected in other species in the region, highlighting the long-term impacts of invasive species (Agnẽse et al. 1998, Moralee et al. 2000). Hybrids produced by *O. mossambicus* and *O. niloticus* exhibit reduced tolerance to cold water temperatures compared to their parental strains, despite generally showing better growth, survival rates, and food conversion ratios (Trewavas 1983, Esterhuyse 2002). According to Moralee et al. (2000), the introduction of *O. niloticus* to natural populations of *O. mossambicus* would lead to the extinction of genetically pure lineages as hybrids could outcompete the parental stock over time (Deines et al. 2014).

While considerable research has been conducted on the introduction of invasive *Oreochromis* species in South Africa, particularly *O. niloticus* (D'Amato et al., 2007; Lind, Brumett & Ponzoni, 2012; Firmat et al., 2013; Zengeya, Booth & Chimimba, 2015; Marr et al., 2018; Mboweni et al., 2020; Weyl et al., 2020), a comprehensive understanding of the extent of these introductions and their impact on the genetic diversity and population structure of native *Oreochromis* populations in South Africa remains elusive. Consequently, there is a pressing need for a comprehensive assessment of the present status of wild tilapia genetic resources to inform effective management strategies and enhance sustainable aquaculture practices (Falk et al. 2004, Hallerman and Hilsdorf 2014, Abwao et al. 2023, Sonesson et al. 2023).

1.8 Use of molecular markers in population and conservation genetics

Various genetic markers, such as mitochondrial regions, nuclear sequences, microsatellites, Restriction Fragment Length Polymorphisms (RFLP), and allozymes, have been extensively employed to assess genetic variation within and among populations of different species (Avise et al. 1987, Parker et al. 1998, Grover and Sharma 2016, Reshma and Das 2021, Danish et al. 2021, Ramya and Behera 2023). Microsatellites, also known as Simple Sequence Repeats (SSRs), stand out as one of the most popular genetic markers in conservation and population genetics (Christiakov 2006, Abdelkrim et al. 2009, Zimmerman et al. 2020, Hohenlohe et al. 2021). They are highly polymorphic markers abundantly distributed throughout the genome, making them particularly valuable for examining relationships among closely related populations or species (Litt and Luty 1989, Tautz 1989, Li et al. 2004, Poke et al. 2005, Christiakov 2006, Barbosa et al. 2008, Tóth et al. 2000, Barroca et al. 2012, Lei et al. 2021, Ramya and Behera 2023). Microsatellites offer advantages such as high variability due to their repetitive nature, ease of detection, and ability to provide information on genetic diversity, population structure, and hybridisation events (Sanz et al. 2009, Dubut et al. 2010, Harrison et al. 2014, Wenne 2023)

In aquaculture and fisheries, microsatellites have been pivotal in characterising genetic stocks, aiding in broodstock selection for marker-assisted breeding programs, and mapping economically important quantitative traits or identifying genes responsible for desirable traits (Liu and Cordes et al. 2004, Christiakov 2006, Abdelkrim et al. 2009, Kumar et al. 2019, Olubunmi 2019, Danish et al. 2021, Kariuki et al. 2021, Ramya and Behera 2023, Wenne 2023).

Autosomal microsatellites are particularly effective in identifying hybridisation events (Svåsand et al. 2007, Dubut et al. 2010, Bezault et al. 2012, Kariuki et al. 2021, Ramya and Behera 2023) and reconstructing demographic history, assessing impacts of inbreeding, bottlenecks, social structure, dispersal, and reproductive behaviour on genetic structure (Goldstein and Schlotterer 1999, Bezault et al. 2012, Mather et al. 2018, Danish et al. 2021, Kariuki et al. 2021, Ramya and Behera 2023).

Studies employing microsatellites in aquaculture have contributed valuable insights into how anthropogenic activities and aquaculture affect wild fish populations (Hall 2001, Crispo et al. 2011, Naz and Abbas 2022, Wenne 2023). The disruption of fish stocks, habitat destruction, overfishing, and the introduction of invasive species pose significant threats to fisheries' sustainability, underscoring the importance of robust resource management practices (Ryman et al. 1995, Ryman 1997, Yokota and Watanabe 1997, Utter 1998, D'Amato et al. 2007, Eknath and Huluta 2009, Firmat et al. 2013, Zengeya et al. 2015, Shechonge et al. 2018, Dieleman et al. 2019, Wang and Gu 2021, Dornelas et al. 2023). Microsatellites are instrumental in monitoring genetic variation, estimating migration rates, and elucidating geographic structuring in fish populations (Hall 2001, Svåsand et al. 2007, Bezault et al. 2012, Mather et al. 2018, Hohenlohe et al. 2021, Kariuki et al. 2021, Ramya and Behera 2023). Examples of microsatellite applications include assessing fish stocks in species such as *Dicentrarchus labrax* (Garcia de Leon et al. 1997), *Thunnus obesus* (Grewe and Hampton 1998), *Clarias gariepinus* (Agnèse et al. 1997), various Salmon species (McConnell et al. 1995, Adkison 1996, McConnell et al. 1995, Small et al. 1998), *Sperata seenghala* (Garg et al. 2014), and numerous tilapia species (Hall 2001, Esterhuyse 2002, Hassanien and Gilbey 2005, D'Amato 2007, Saju et al. 2010, Firmat 2013, Simbine et al. 2014, Dieleman et al. 2019, Kariuki et al. 2021, Ahmed et al. 2023, Kwikiriza et al. 2023).

Previous studies using microsatellites have assessed genetic variation within populations of *Oreochromis* spp. (Padmaja 1995, Hall 2001, Esterhuyse 2002, Bhassu et al. 2004, Hassanien and Gilbey 2005, D'Amato 2007, Saju et al. 2010, Angienda et al. 2011, Firmat 2013, Simbine et al. 2014, Dieleman et al. 2019, Makeche et al. 2022, Ahmed et al. 2023, Kwikiriza et al. 2023). Primer development for several *Oreochromis* species, including *O. mossambicus* and *O. niloticus*, has facilitated their widespread use in genetic studies and conservation efforts (Lee and Kocher 1996, Bhassu et al. 2004, Saju et al. 2010, Simbine et al. 2014, Tibihika et al. 2019).

1.9 Use of environmental DNA (eDNA) metabarcoding techniques for monitoring and conservation of freshwater fish

Fish populations are typically assessed through capture methods such as electrofishing and gill netting, as well as morphological identification (Hill et al. 2005, Shaw et al. 2016, Hering et al. 2018, Belle et al. 2019, King et al. 2022). However, these invasive techniques can compromise animal health, increasing stress and predation risk (Shaw et al. 2016, Belle et al. 2019, Rourke et al. 2022). Additionally, these methods are selective, potentially excluding certain species during surveys (Pidgeon 2004, Shaw et al. 2016, Rourke et al. 2022, Sahu et al. 2023). In contrast, DNA sequencing of environmental samples, such as water, sediments, soil, or air, offers a non-invasive means to rapidly identify multiple taxa without the need for morphological identification (Thomsen et al. 2012, Taberlet et al. 2012, Shaw et al. 2016, Miya et al. 2020, Carvalho et al. 2022). Environmental DNA (eDNA) sequencing provides advantages by enabling the identification of cryptic and juvenile taxa, offering a more comprehensive view of biodiversity (Belle et al. 2019, Rishan et al. 2023, Sahu et al. 2023). The rapid collection and processing of eDNA samples, coupled with decreasing sequencing costs, make this method suitable for enhancing species detection, monitoring, and conservation (Mardis 2011, Shaw et al. 2016, Sahu et al. 2023).

Environmental DNA metabarcoding, which uses the power of high-throughput sequencing, has proven to be a valuable tool for monitoring freshwater fish communities, detecting invasive species, and mapping the distribution of both rare and abundant species in freshwater ecosystems (Shaw et al. 2016, Belle et al. 2019, Alam et al. 2020, Rishan et al. 2023). The eDNA metabarcoding approach allows for the simultaneous detection of multiple species through the amplification of a short fragment of eDNA from the target taxa using universal primers in polymerase chain reaction (PCR) (Alam et al. 2020, Miya et al. 2020). Incorporating the eDNA approach into freshwater conservation efforts will contribute to the development of more efficient and accurate monitoring and conservation frameworks (Belle et al. 2019, Bernos et al. 2023, Clarke et al. 2023).

In African countries such as South Africa, the application of such methods is still in its nascent stage but holds great potential for monitoring threatened fish species, including cichlids such as *O. mossambicus* in the region. These innovative, robust, and effective eDNA methods are contributing to sustainable water resource management and conservation globally, and South Africa can benefit similarly from their application. The presence of tilapia species in DNA barcode reference databases, especially for markers such as COI and 12S rRNA commonly used in eDNA methods, presents an opportunity to complement monitoring and conservation efforts with eDNA alongside microsatellites (Mashaphu et al. 2023). Despite the challenges posed by their rapid evolutionary radiation, which can diminish the accuracy of species-level identification for cichlids, recent eDNA studies have successfully identified various tilapia species (Robson et al. 2016, Edmunds and Burrows 2019, Doble et al. 2020, Villacorta-Rath and Burrows 2020). These findings highlight the potential of eDNA as a monitoring tool for cichlid populations, although further development for species-level accuracy might be necessary.

1.10 Problem statement

Oreochromis mossambicus is one of the important species for aquaculture and other non-commercial purposes globally. Despite this, the species is classified as Vulnerable by the IUCN, largely attributed to the adverse effects of hybridisation with the invasive *O. niloticus*. Naturally inhabiting the major river catchments in South Africa, particularly within warmer regions, *O. mossambicus* plays an important role in both aquaculture and natural ecosystems. However, there is a notable lack of comprehensive information regarding these populations' genetic diversity and structure, hindering effective management strategies for monitoring and conserving this valuable species.

This research project aimed to bridge this knowledge gap by conducting a comprehensive genetic assessment of *O. mossambicus* across three South African provinces (Limpopo, Mpumalanga, and KwaZulu-Natal). The overarching objective was to provide essential insights that can guide improved regulatory frameworks and management practices, ultimately contributing to the conservation of *O. mossambicus* and fostering sustainable use within the context of South African aquaculture.

1.11 Aims

The project had four aims. Firstly, to examine the population structure and genetic diversity of wild *O. mossambicus* populations in major river catchments in Limpopo, Mpumalanga, and KwaZulu-Natal. Secondly, to evaluate genetic diversity and genetic differentiation of farmed *O. mossambicus* populations in KwaZulu-Natal and Mpumalanga. Thirdly, to determine if there has been genetic contamination of farmed and wild populations through hybridisation of *O. mossambicus* with the introduced *O. niloticus* and *O. aureus* in Limpopo, Mpumalanga, and KwaZulu-Natal. Fourthly, to develop an environmental DNA (eDNA) protocol, which would allow for non-invasive monitoring and detection of tilapia species in South Africa. In addition, this present study aimed to identify crucial conservation units or important populations that will contribute to sustainable aquaculture in South Africa.

1.12 Objectives

The following research objectives were proposed:

- Explore how South African water management and catchment strategies impact the preservation and management of existing genetic diversity and population structure in wild *O. mossambicus* populations across the Limpopo, Mpumalanga, and KwaZulu-Natal regions.
- Identify distinct genetic units within wild *O. mossambicus* populations that may require separate management strategies.
- Investigate the potential for translocations by assessing the origins of farmed *O. mossambicus*.
- Examine the genetic differentiation between farmed populations and wild populations of *O. mossambicus* in KwaZulu-Natal and Mpumalanga, with a focus on determining if farmed populations accurately represent the genetic diversity of their wild counterparts for potential release programs.
- Assess the extent of contamination with *Oreochromis niloticus* and *Oreochromis aureus* in various aquatic systems, prioritising genetically pure *O. mossambicus* populations for conservation efforts.

- Evaluate the status of DNA barcode reference databases for all freshwater fish species, including tilapia species in South Africa.
- Investigate the suitability of the eDNA approach for detecting and monitoring tilapia species in South African water bodies.

1.13 Study outline

The main body of this thesis has the chapters prepared as manuscripts for submission to international peer-reviewed journal articles. The first chapter (Chapter 1) is an introduction to the study, which provides a literature review of the concepts covered in this study. The next five chapters (Chapters 2, 3, 4, 5, and 6) are experimental chapters, with each one covering a specific objective. Each chapter is formatted according to the journal it is intended to be submitted to. Because of this format, a certain degree of repetition, especially in the methods section, was unavoidable. However, this is deemed to be of little concern as this format allows the reader to read each chapter separately without losing the overall context of the thesis. The chapters are arranged in the following order:

Chapter 2: Genetic diversity and population dynamics of wild Mozambique tilapia (*Oreochromis mossambicus*) in South Africa

Chapter 3: Genetic assessment of farmed *O. mossambicus* populations in KwaZulu-Natal and Mpumalanga Provinces, South Africa.

Chapter 4: Genetic introgression of farmed and wild *O. mossambicus* with introduced *Oreochromis* species in Limpopo, Mpumalanga, and KwaZulu-Natal provinces, South Africa.

Chapter 5: The status of COI and 12S rRNA DNA barcode reference libraries for freshwater fish in South Africa: Implications for future eDNA projects.

Chapter 6: Evaluating the efficacy of eDNA metabarcoding for detecting native *O. mossambicus* populations in KwaZulu-Natal, South Africa.

Chapter 7: General discussions and recommendations

1.14 References

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CHAPTER 2

Genetic diversity and population dynamics of wild Mozambique tilapia (*Oreochromis mossambicus*) in South Africa

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Running header: Genetic diversity of *Oreochromis mossambicus* in South Africa

2.1 Abstract

South Africa is a water-scarce region of the world, and through urbanisation and water resource development, this region now faces multiple water resource management challenges.

Anthropogenic activities and poor management have affected freshwater ecosystems, especially aquatic biodiversity. The impact of water management practices on the region's freshwater fish populations, in particular, is poorly understood. *Oreochromis mossambicus* is endemic to southern Africa but has become established in the global aquaculture industry. *Oreochromis mossambicus* naturally occurs in the east-flowing rivers of the sub-tropical and tropical regions of South Africa. Although important and potentially threatened by alien *Oreochromis* spp., resulting in the "Vulnerable" listing on the IUCN Red List, the genetic diversity and population structure of *O. mossambicus* remains poorly understood. Our study aimed to assess the genetic diversity and population structure of wild *O. mossambicus* in the major river catchments in three South African provinces (KwaZulu-Natal, Mpumalanga, and Limpopo). To evaluate the genetic diversity of these populations, we conducted a comprehensive analysis using 14 microsatellite loci to establish essential baseline data for the determination, conservation, and monitoring of critical genetic units within *O. mossambicus* distribution in the region. Furthermore, we investigated the influence of present water management practices on the species' genetic structure. We assessed the genetic diversity across various spatial scales, namely different water catchment management areas, primary to quaternary catchment scales in a river, local to provincial administrations, and eco-regions. Genetic diversity was found to be relatively low within sampling localities, with significant genetic differentiation evident among *O. mossambicus* populations. STRUCTURE analyses revealed 15 geographically correlated genetic clusters, highlighting substantial differentiation in *O. mossambicus* from various sampling localities, such as Mtamvuna River in KwaZulu-Natal, Pieter Vorster Dam in Mpumalanga, and the Sand River, Shingwedzi, and Letaba rivers in Limpopo. Our study highlighted the role of anthropogenic activities, change in catchment use/management over time, water resource management strategies, and connectivity in shaping *O. mossambicus'* genetic population structure in Limpopo, Mpumalanga, and KwaZulu-Natal. Given the well-defined genetic patterns within wild *O. mossambicus* in these regions, conservation-oriented management must prioritise maintaining existing genetic diversity, ensuring the long-term viability of this vulnerable fish species.

Keywords: conservation, genetic diversity, water resource management, *Oreochromis mossambicus*, population structure, river connectivity.

2.2 Introduction

The genetic diversity and population structure of natural freshwater fish populations are influenced by a multitude of factors (Hurwood and Hughes 1998, Hewitt 2000, Crispo et al. 2011, Crookes and Shaw 2016, Ellegren and Galtier 2016, Mather et al. 2018, Amoussou et al. 2019). These factors include demographic dynamics, historical gene flow, barriers to movement, dispersal capacities of species, flow regulation, and various anthropogenic activities (Crispo et al. 2011, Dudgeon 2014, Crookes and Shaw 2016, Ellegren and Galtier 2016, Jaisuk and Sananan 2018, Amoussou et al. 2019, Koblmüller et al. 2019). Pollution, landscape modifications (such as artificial waterfalls, weirs, altered flow regimes, and impoundments), catchment transformation, overexploitation of biological resources, the introduction of invasive species, and climate change are the main human activities influencing fish populations (Hewitt 2000, Crispo et al. 2011, Dudgeon 2014. Crookes and Shaw 2016, Mather et al. 2018, Koblmüller et al. 2019, Dudgeon 2019, Dallas and Rivers-Moore 2022). In freshwater rivers the lack of connectivity between populations of aquatic taxa can drive processes such as genetic drift and inbreeding in small, isolated populations leading to reduced genetic diversity, survival, and environmental adaptation of taxa (Avise 2000, Reed 2004, Keyghobadi 2007, Mcdowall 2008, Nikolic et al. 2009, Crispo et al. 2011, Dudgeon 2014, Watanabe et al. 2018, Amoussou et al. 2019). Even in rivers that do not have physical barriers affecting connectivity, changes in water temperature and chemistry can produce chemical barriers, impeding gene flow and further influencing genetic diversity and population structure (Rivers-Moore et al. 2007, Watanabe et al. 2018, O'Brien et al. 2019, Thieme et al. 2023).

River connectivity plays a crucial role in shaping the genetic variation of freshwater taxa (Standford and Ward 1992, Brauer et al. 2013, Silva et al. 2018, Oliveira-Silva et al. 2023), and generally, the genetic patterns of species follow the hierarchy of river connectivity, with populations living in proximity experiencing similar ecological pressures and showing similar genetic patterns (Hurwood and Hughes 1998, Nicol et al. 2017). Management strategies are often guided by the hydrological connectivity of rivers, and so does, in part, consider historical connectivity (O'Brien et al. 2019, Szaboics et al. 2022, Thieme et al. 2023). In such cases, genetic patterns in freshwater taxa are maintained (Darwall et al. 2018, Davis et al. 2019, Blanchet et al. 2020, Manel et al. 2020, Hvilsom et al. 2022, Hoban et al. 2023). This may not be the case in water-scarce countries, where water insecurity is mitigated by constructing dams and moving water through inter-basin transfer schemes (Snaddon et al. 1999, Lynch et al. 2011, Matchaya et al. 2019, Purvis and Dinar 2020, Harris et al. 2022, Qin et al. 2023).

South Africa is a water-scarce country, with increased demand for water resources impacting water availability and water quality so affecting the state of freshwater ecosystems (Binns et al. 2001, Dallas and Rivers-Moore, 2014, Du Plessis, 2023). Coupled with anthropogenic activities and intensive land modification, the country's water resources continue to deteriorate, and many river systems and their biodiversity are now threatened and unsustainable (Nel et al. 2011, Driver et al. 2012, Lemley et al. 2015, O'Brien et al. 2019, Dudgeon 2019, Dallas and Rivers-Moore 2022, Evans et al. 2022).

There are presently six major transnational river basins in South Africa, including the Incomati, Limpopo, Maputo, Orange-Senqu, Thukela, and uMbeluzi systems (Ashton et al. 2008, Maree et al. 2016). These rivers are shared between South Africa and neighbouring countries Lesotho, Eswatini, Mozambique, Zimbabwe, Botswana, and Namibia (Ashton et al., 2008). The trans-boundary nature of these systems with differences in management legislation between countries makes managing the genetic diversity of populations that move between countries difficult (Maree et al. 2016, Huusko et al. 2023). The socioeconomic growth in the region and the associated development of water resources result in multiple stressors that affect the condition of these rivers (Nel and Drivers 2015). In South Africa, there is presently a debate on how water resources should be managed (O'Brien et al. 2019). Historically, river management and water resource management were guided by a nested hierarchy of hydrological catchments (Midgley and Pitman 1969, Midgley et al. 1981, Midgley et al. 1994), from primary (1^{st} order, based on main rivers) catchment, and levels of sub-catchment division for different scales - secondary (2^{nd} order), tertiary (3^{rd} order), quaternary (4^{th} order) and quinary (5^{th} order) (Nel and Drivers 2015). This nested hierarchical catchment management was employed for a wide range of applications, including water resource management, conservation planning, environmental impact assessments, monitoring climate change, and hydrological modelling (Maherry et al. 2013, Adom and Simatele 2022, Pringle et al. 2023). Additionally, river management was guided by ecoregion classifications, which categorised rivers based on ecological similarities, considering the nested rivers hierarchy in the landscape (Nel and Drivers 2015). South Africa is classified into 31 ecoregions (Kleynhans et al. 2005, Nel and Drivers 2015).

Presently, water resources and river catchments are managed based on designated Water Management Areas (WMAs). Initially, 19 Water Management Areas (WMAs) were described in 2004 (DWAF 2004, King and Pienaar 2011, DWA 2013). These were reduced to nine in 2012 by the National Water Resource Strategy in response to limited human and financial resources and for ease of management (DWA 2013). All these WMAs have sustainable water resource challenges, and water resources of the Incomati to uSuthu, Olifants, Limpopo, and Pongola to Mtamvuna WMAs are heavily used and, as a result, are presently highly threatened (Driver et al. 2012, O'Brien et al., 2019).

The overexploitation of freshwater ecosystems in South Africa poses a significant threat to fish populations (O'Brien et al. 2013, Du Plessis 2019, O'Brien et al. 2019, Evans et al. 2022). Government policies, such as the South African Draft White Paper on Conservation and Sustainable Use of Biodiversity (July 2022) and The National Environmental Management Biodiversity Act (NEMBA), 2004 (Act No. 10 of 2004), emphasise the importance of protecting and sustainably using biodiversity. However, achieving these objectives requires a thorough understanding of the genetic variability of populations and patterns to maintain the country's biodiversity (as required by NEMBA, Act No. 10 of 2004). This is particularly important in the face of mounting anthropogenic pressures on these river catchments. There is, therefore, an urgent need to assess genetic patterns and redefine the management of freshwater fish populations in South Africa with this information to ensure their long-term conservation.

Studies on the population genetics of freshwater taxa can shed light on the various factors that have influenced their genetic diversity and structure (Ricklefs and Schluter 1993, Avise et al. 1998, Bermingham and Moritz 1998, Avise 2000, Knowles and Maddison 2002, Rocha et al. 2007, Crispo et al. 2011, Van Schaik et al. 2018, Petrosino et al. 2022, Sanchez-Bernal et al. 2023). Genetic data can also clarify how freshwater fish populations are connected and identify areas of high conservation value to assist in the protection and effective management of natural populations (Finn et al. 2007, Hughes et al. 2009. Moser et al. 2019, Manel et al. 2020, Ali and Siva 2022).

Among South African freshwater fish species, the Mozambique tilapia *Oreochromis mossambicus* (Peters, 1852) holds significant importance in global aquaculture (Li et al. 2006, Toguyeni et al. 2009, FAO 2010, Wu et al. 2012, Ansah et al. 2014). Endemic to southern Africa, this species inhabits rivers flowing south-eastward, from the Ligonha River in northern Mozambique to the Boesmans River in South Africa (Scott et al. 2006). Despite its critical role in the South African aquaculture industry, little is known about the genetic diversity, population structure, and connectivity of native *O. mossambicus* populations (Hall 2001, D’Amato 2007). Furthermore, the species faces a threat from hybridisation with the introduced *O. niloticus*, leading to its vulnerable status on the IUCN Red List (Bills 2019, Mojekwu et al. 2021). Comprehensive baseline data is essential to our understanding, conservation, and monitoring of important genetic units of *O. mossambicus* populations in South Africa. Additionally, limited information exists regarding the relationship between river system management and the conservation of fish genetic diversity in South Africa (Impson et al. 2008). These river systems are also threatened by anthropogenic activities that, when combined with shifts in water resource management strategies, exacerbate the threat to the genetic integrity of *O. mossambicus* populations. Using microsatellite data, our study aimed to provide important baseline information for future monitoring of natural *O. mossambicus* populations, provide information on which populations require prioritisation for conservation and management plans, and assess the impact of different water resource management strategies on the genetic patterns of *O. mossambicus* populations in Limpopo, Mpumalanga, and KwaZulu-Natal. We have formulated and tested twelve scenarios (Supplementary Table 2.1) based on historical and present river and water resource management strategies in South Africa. Our study identified the most effective river and water resource management scenario that was the best fit for preserving the existing distinct genetic patterns of wild *O. mossambicus* populations in South Africa.

2.3 Methods

2.3.1 Sampling

We collected samples of *Oreochromis mossambicus* from three provinces (Limpopo, Mpumalanga, and KwaZulu-Natal) in South Africa from 2017 to 2021. Before the commencement of the study, we obtained all necessary permits for sample collection from the relevant authorities, specifically Ezemvelo KwaZulu-Natal Wildlife (permit numbers: OP1583/2017, OP1432/2018, and OP871/2021) and the Mpumalanga Tourism and Parks

Agency (permit number: MPB. 56932) and from the Department of Economic Development, Environment and Tourism Limpopo. Ethical clearance for conducting research involving animals was granted by the University of KwaZulu-Natal Animal Ethics Subcommittee (Reference: AREC/023/020).

Our sampling efforts were guided by historical distribution records of *O. mossambicus* in Limpopo, Mpumalanga, and KwaZulu-Natal (Cambray and Swartz 2007, GBIF 2017). We collected a total of 370 samples from 29 sampling localities. Seven localities were sampled in Limpopo, nine in Mpumalanga, and 13 in KwaZulu-Natal (Supplementary Table 2.2 and Figure 2.1). To investigate the population structuring at different spatial scales, surveys targeted multiple populations within catchments, including multiple localities along these rivers whenever feasible (Supplementary Table 2.2).

Fish collection methods included standard passive techniques, such as the use of fyke and gill nets, and active techniques, including electro-fishing, seine nets, and cast net throws. After capture the *O. mossambicus* were identified to species level (Skelton 2001) and anesthetised using clove oil (0.5 ml/L) (Bennett et al. 2016). Each fish was weighed (g), measured (SL and TL in mm), and photographed for reference purposes. A non-lethal fin clip of approximately 5mm^2 to 10mm^2 was taken from each anesthetised fish and immediately preserved in 99% ethanol, stored in a refrigerator, and transported to the laboratories of the University of KwaZulu-Natal for subsequent genetic analyses. The captured fish were put in a recovery bucket and then released back to the place of capture.

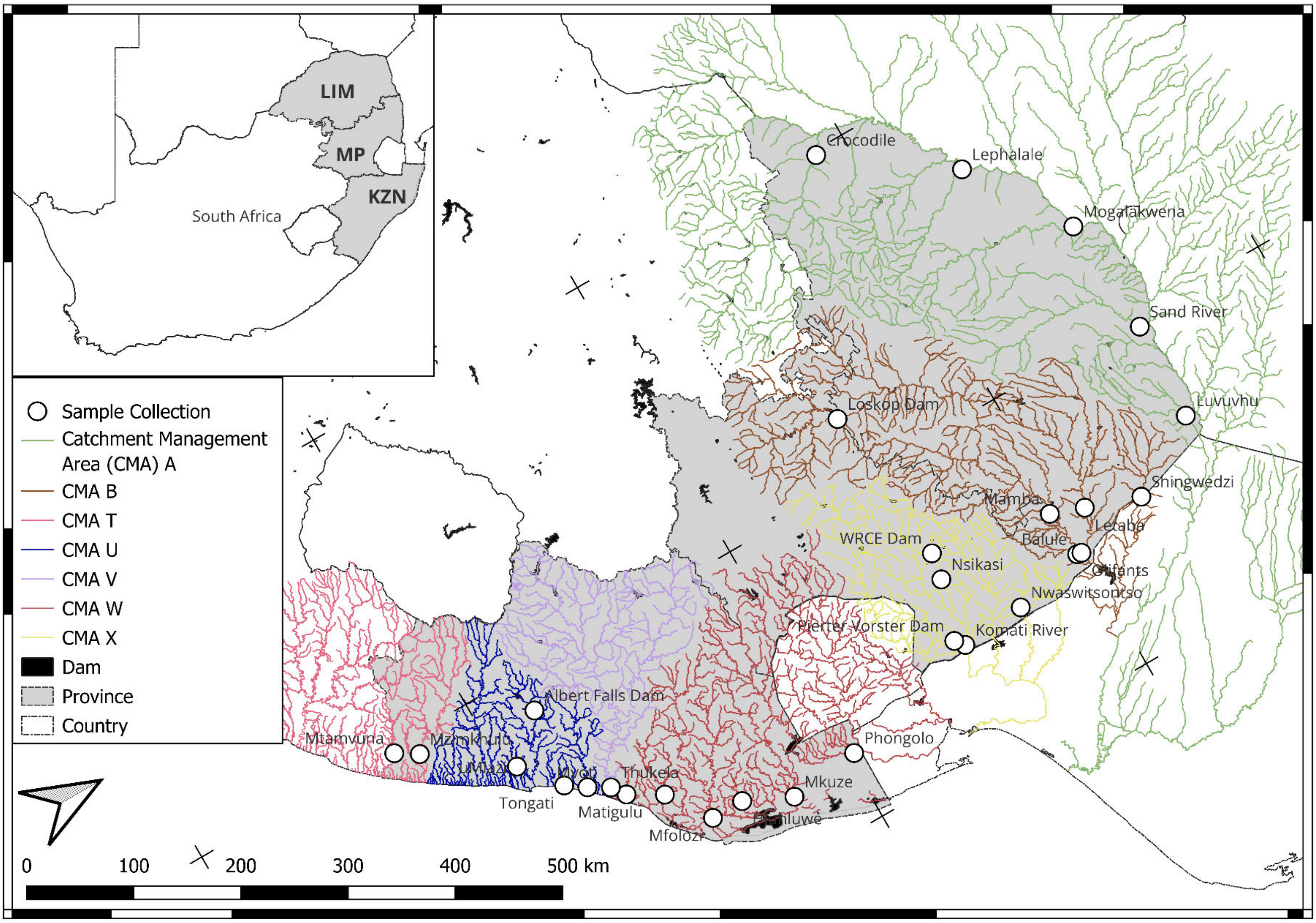

Figure 2.1: Map showing the geographic distribution of sampling localities within various catchment management areas (CMAs) in the Limpopo (LIM), Mpumalanga (MP), and KwaZulu-Natal (KZN) Provinces, South Africa. The 29 surveyed locations where *Oreochromis mossambicus* samples were collected are marked. The marked CMAs are relevant to illustrating river connectivity.

2.3.2 DNA extraction and amplification

We extracted DNA from fin clips using the Nucleospin®Tissue kit (Macherey-Nagel, Germany) following the standard protocol for animal tissue. Extracted DNA samples were stored at -20°C. For this study, 14 microsatellites loci originally isolated from *O. niloticus* (Hall 2001, Simbine et al. 2014) and *O. mossambicus* (Saju et al. 2010) were amplified (Table 3.1). In each primer pair, the forward primer was fluorescently labelled. Polymerase Chain Reaction (PCR) profiles were performed in a 10µl volume containing 0.1 µl of each primer, 5 µl of 2G Fast Multiplex Mix KAPA Taq (KAPA Biosystems, Cape Town, South Africa), 0.3 µl of BSA, 4.2 µl distilled water (dH2O) and 0.3 µl of DNA. The thermocycler conditions were 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 15 s, annealing temperature for 30 s followed by extension at 72 °C for 1 min, and final extension step at 72 °C for 10 min. We first conducted PCR profiles as single plexes for each of the 14 microsatellites. Primers were selected for multiplexes based on annealing temperature, fluorescence dye, and product size into multiplexes (A, B, C, and D). The annealing temperature was 60°C for multiplex A and D, and 61°C for multiplex B and C (Table 3.1). Fragment analyses were conducted at the Central Analytical Facilities (CAF), University of Stellenbosch, South Africa, using a 3500XL Genetic Analyser from Applied Biosystems (Thermo Fisher). To ensure genotype accuracy, 20% of all individuals were re-genotyped and checked for consistency. No inconsistent genotypes were found.

Table 2.1: Microsatellite loci used in the present study for genotyping of *Oreochromis mossambicus*. Fluorescent dye and annealing temperature used in the present study are provided.

Multiplex loci	Locus	Sequence	Fluorescent Dye	Annealing Temperature °C
A	OM01[c]	F: TTTAAAGTTACACAGCAGTACAAAG R: TTGTAGCATTTCAACACAGTCTC	Fam	60
D	OM02[c]	F: TGTGAATTTGACAACTTCCTTTC R: ATCCTTGCAATAAGGTTACAG	Fam	60
D	OM03[c]	F: CTTTTTAATGAGCAACTTTTAAGTC R: TGTGAATTTGACAACTTCCTTTC	Hex	60
A	OM04[c]	F: AGCTCAAAACCTCATACAAAGG R: GCAGAGATGTCAGATGTTGTTC	Fam	60
B	OM05[c]	F: GTAAAGTTTGGAACAGAAATGCT R: GATCACTTTTGGACAGACTGG	Hex	61
B	OM06[c]	F: TGAGCTACCGTAAGGATGTAC R: GTTATTTCAATTATATTTGCATG	Fam	61
C	OM07[c]	F: TTGGCTCAGAGTGGTCAGG R: CGCGTGGACTAAAAGCCAG	Hex	61
B	OM08[c]	F: TGTTGGTTGGATTACTGGG R: GCTGTAATGGTTTTGAGGC	Fam	61
B	OM09[c]	F: GGCTACAACACCTGGATGG R: TTGGGCTTACTGAAGCTGAC	Hex	61
C	UNH104[a]	F: GCAGTTATTTGTGGTCACTA R: GGTATATGTCTAACTGAAATC	Tet	61
C	UNH129[a]	F: AGAAGTCGTGCATCTCTC R: TGTACATCATCTGTGGG	Tet	61
B	UNH142[b]	F: CTTTACGTTGACGCAGT R: GTGACATGCAGCAGATA	Tet	61
B	UNH222[b]	F: CTCTAGCACACGTGCAT R: TAACAGGTGGGAACTCA	Tet	61
C	UNH231[b]	F: GCCTATTATAGTCAAAGCGT R: ATTTCTGCAAAAGTTTTCC	Tet	61

Primers taken from Hall (2001) [a] Simbine et al. (2014) [b] and Saju et al. (2010) [c]

2.3.3 Molecular data analyses

We used GeneMarker® v2.4 (Hulce et al. 2011) for scoring genotypes. To assess the presence of null alleles, we estimated null allele frequencies using FreeNA (Chapuis and Estoup 2007). To evaluate whether these null alleles had a significant impact on our population structure results, we conducted analyses both with and without null allele correction (ENA corrected and non-corrected) and computed global F_{ST}-values using FreeNA. We compared the results from these two sets of analyses using a t-test. The p-value obtained for the t-test was adjusted for multiple comparisons using a Bonferroni correction (Bland and Altman 1995). To evaluate the informativeness of each microsatellite locus, we calculated the Polymorphic Information Content (PIC) using Cervus version 3.0.7 (Marshall et al. 1998, Kalinowski et al. 2007). Loci with PIC values exceeding 0.5 were considered highly informative, while those with PIC values below 0.25 were regarded as less informative, following the criteria established by Botstein et al. (1980). We investigated Linkage Disequilibrium (LD) using Fisher's Exact test in Genepop version 4.7.0 (Raymond 1995, Rousset 2008). The Chi-square p-values were generated using the Markov Chain method, with the test parameters set at 10,000 replicates, 100 groups, and 5,000 iterations per group. Bonferroni corrections were applied to account for multiple comparisons and ensure the robustness of the statistical analyses.

2.3.4 Genetic diversity

To assess genetic diversity within sampling localities, we computed various indices using GenAlEx version 6.5 (Peakall and Smouse 2012). These included the total number of alleles (Na), the number of effective alleles (Ne), unbiased expected and observed heterozygosity (uH_E and H_O), and the inbreeding coefficient (F_{is}). Deviations from deviations from Hardy-Weinberg Equilibrium were also investigated using GenAlEx and applying Bonferroni corrections to account for multiple comparisons and ensure the robustness of the statistical analyses. To further assess genetic diversity, we calculated allelic richness (Ar) using FSTAT (version 2.9.3.2; Goudet, 2001). Unlike measures based on allele frequencies, Ar considers the raw number of alleles present at the 14 microsatellite loci, providing insights into the overall allelic variation within the populations.

2.3.5 Population genetic structure

In our study, we employed Bayesian clustering analysis using STRUCTURE version 2.3.4 (Pritchard et al. 2000) to explore potential genetic structure within *O. mossambicus* across 30 sampled localities distributed within and among river catchments across Limpopo, Mpumalanga, and KwaZulu-Natal provinces. This analysis aimed to detect patterns of genetic differentiation. We used the admixture model, which is suitable when dealing with shallow population structure, and incorporated correlated allele frequencies. To enhance the accuracy of our analysis, we used the sampling localities as prior information by applying the LOCPRIOR parameter. To facilitate geographical visualisation of the potential genetic structure, we organised individuals by their respective provinces and river catchments. The STRUCTURE analyses involved 500, 000 Markov-Chain Monte Carlo (MCMC) replicates, with a 50,000-step burn-in period. We conducted 25 iterations, with K values ranging from 1 to 29. To determine the optimal number of genetic clusters, we used the STRUCTURE selector version 2.3 (Li and Liu 2018) and applied the Puechmaille method (Puechmaille 2016) to calculate the K-value using the STRUCTURE harvester (Earl and Von Holdt 2012). The obtained membership coefficient values (Q-values) for the optimal K were used to create bar plots, and this process was facilitated by Clumpak software (Kopelman et al. 2015). Additionally, we conducted a Principal Coordinate Analysis (PCoA) based on Nei's genetic distances (Nei 1972) using GenAlEx. This analysis aimed to provide further insights into the genetic relationships among populations.

To assess the level of genetic differentiation among *O. mossambicus* populations across the 29 sampled localities, we employed an Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992). The AMOVA utilised 10,000 permutations to analyse variation both within and across the sampled loci. GenAlEx software facilitated this analysis, and we applied a Bonferroni correction to adjust the significance level for multiple comparisons. To quantify genetic differentiation, we computed pairwise F_{ST}-values between all pairs of *O. mossambicus* sampled localities, employing the ENA correcting method through FreeNA. To test for significant genetic differentiation between populations, we computed probability (p-values) using FSTAT software (version 2.9.3.2; Goudet 2001). A permutation test with 9,300 replicates was employed to account for multiple comparisons. The significance level was set at 0.000108 after the Bonferroni correction.

2.3.6 Population genetic structure based on the twelve formulated scenarios.

We formulated multiple scenarios representing different genetic structures to assess the impact of various river and water resource management strategies implemented in South Africa (Supplementary Table 2.1). These scenarios were designed to explore genetic differentiation among *O. mossambicus* from the 29 sampling localities based on a range of factors, including rivers, dams, catchment orders (primary, secondary, tertiary, and quaternary), the old 2004 Water Management Areas (WMAs), the new 2012 WMAs, ecoregions, local municipalities, districts, provinces, and the biomes to which these sampling localities belong (Supplementary Table 2.1). In total, we conducted 12 Analysis of Molecular Variance (AMOVA) tests and calculated the associated p-values for each comparison, each corresponding to one of the formulated scenarios for river and water resource management in

South Africa (Supplementary Table 2.1). To account for multiple tests, we applied the Bonferroni correction to adjust the p-values for all rounds of AMOVA performed. Furthermore, we performed multiple AMOVAs within each of the 12 scenarios to estimate the extent of genetic differentiation within those scenarios. This additional level of analysis was conducted when a given scenario contained more than one sampled locality that fell under a specific criterion. For example, if multiple localities surveyed were part of the Olifants WMA, we conducted an AMOVA to assess genetic differentiation within that WMA. All the AMOVA analyses were conducted with 10,000 permutations, both across and within the sampled loci, using GenAlEx. This approach allowed us to comprehensively evaluate the genetic differentiation patterns among *O. mossambicus* from the 29 sampled localities under various water management scenarios and criteria.

2.4 Results

Genotyping was successfully carried out on all 370 *O. mossambicus* individuals using the 14 microsatellite markers. It is important to note that while not all microsatellites could be amplified in every individual, the amount of missing data remained relatively low. The extent of missing data for the different loci ranged from 6% (for loci OM04, UNH142, UNH222, UNH129, OM07, and OM03) to 33% (for locus OM05), reflecting the challenges associated with some markers. To determine if loci with more than 20% missing data (OM08 and OM05) negatively biased analyses, assignment tests were performed both including and excluding the two markers, and found that the overall genetic structure and clustering patterns remained consistent. In the dataset, variations in null allele frequencies were observed across the different loci, ranging from 0.03 (for locus OM07) to 0.21 (for locus UNH222). A critical analysis comparing the F_{ST}-values between datasets that included null alleles (ENA corrected) and those that did not (non-corrected) revealed that the presence of null alleles did not introduce significant bias into the analysis of population structure (Supplementary Table 2.3). The t-test showed a non-significant p-value of 0.06 after Bonferroni correction. Consequently, all 14 microsatellite loci were retained for subsequent analyses. All microsatellite loci exhibited high levels of polymorphism, as indicated by Polymorphic Information Content (PIC) values. The PIC values ranged from 0.50 (for locus UNH231) to 0.90 (for loci OM07 and OM03), underlining the high genetic diversity captured by these markers (Supplementary Table 2.3). Additionally, no significant linkage disequilibrium was detected among any of the loci, further supporting the independence of these markers and their suitability for subsequent genetic analyses.

2.4.1 Genetic diversity

The analyses of genetic diversity at various loci revealed a range in the number of alleles, ranging from 2.20 (for locus UNH231) to 9.10 (for locus OM07). The allelic richness of loci also exhibited variation, with values ranging from 1.85 for locus OM06 to 4.47 for locus OM07 (Supplementary Table 2.3). The observed heterozygosity values ranged from 0.09 (UNH231 and OM06) to 0.87 (for locus OM07). Notably, the observed heterozygosity values were lower than the unbiased expected heterozygosity values. The unbiased expected heterozygosity ranged from 0.23 (for locus UNH231) to 0.87 (for locus OM07) (Supplementary Table 2.3).

The number of alleles recorded from each sampled locality varied from 2.86 in the Mtamvuna to 7.79 in the Pieter Vorster Dam (Table 2.2). The number of effective alleles within sampled localities varied from 2.46 in the Albert Falls Dam to 5.07 in the Sand River. When comparing observed heterozygosity (H_O) with unbiased expected heterozygosity (uH_E), it was evident that observed heterozygosity tended to be lower. The H_O values ranged from

0.27 in the Nsikasi River to 0.56 in both the Sand River and Shingwedzi River, whereas uH_E varied from 0.44 in the Mtamvuna River to 0.76 in the Luvuvhu River (Table 2.2).

A broad pattern emerged when considering genetic diversity across provinces. High observed heterozygosity was recorded from sampled localities located in the Limpopo Province (mean H_O = 0.5), followed by those in the Mpumalanga Province (mean H_O = 0.42), while sampled localities in the KwaZulu-Natal Province were less genetically diverse (mean H_O = 0.38). Our analysis revealed non-significant deviations from Hardy-Weinberg equilibrium (HWE) in most sampled localities, except for Albert Falls, WRCE, and Pieter Vorster dams (Table 2.2). The inbreeding index (Fis) recorded in all sampled localities varied from 0.09 in the Shingwedzi River to 0.58 in the Nwaswitsontso River, indicating excess homozygosity across all sampled localities (Table 2.2).

Table 2.2: Genetic diversity indices for 370 *Oreochromis mossambicus* from 29 sampled localities in Limpopo, Mpumalanga, and KwaZulu-Natal, South Africa estimated using 14 microsatellite loci. (Note: WRCE Dam = White River Country Estate Dam, Pieter Dam = Pieter Vorster Dam).

Sampled localities	**N**	**Na**	**Ne**	**Ar**	**H_O**	**uH_E**	**F_{is}**	**P_{HWE}**
Phongolo	14	5.93	3.82	2.78	0.38	0.70	0.45	ns
Mkuze	18	5.71	3.48	3.08	0.37	0.58	0.30	ns
Hluhluwe	11	5.43	3.40	2.25	0.36	0.55	0.29	ns
Mfolozi	8	3.86	3.05	1.82	0.31	0.51	0.37	ns
Mhlathuze	10	4.93	3.28	2.21	0.46	0.57	0.20	ns
Matigulu	10	3.43	2.61	2.02	0.36	0.46	0.32	ns
Thukela	19	6.07	3.53	0.62	0.37	0.58	0.37	ns
Mvoti	25	5.57	2.98	0.72	0.39	0.54	0.26	ns
Tongati	7	4.71	3.38	1.90	0.39	0.71	0.43	ns
Albert Falls Dam	20	4.36	2.46	2.74	0.30	0.52	0.50	*
uMlazi	7	3.21	2.56	1.62	0.45	0.53	0.11	ns
Mzimkhulu	10	3.64	2.61	2.13	0.45	0.54	0.18	ns
Mtamvuna	15	2.86	1.94	2.34	0.36	0.44	0.17	ns

Mamba	15	7.07	4.05	2.93	0.50	0.68	0.28	ns
Balule	9	6.07	4.50	2.30	0.48	0.74	0.34	ns
Olifants	10	6.07	4.27	6.76	0.55	0.68	0.13	ns
Loskop Dam	16	6.71	4.50	8.93	0.45	0.69	0.41	ns
Nwaswitsontso	9	4.79	3.33	5.66	0.29	0.64	0.58	ns
Nsikasi	9	3.64	2.54	4.99	0.27	0.54	0.54	ns
WRCE Dam	10	4.71	2.99	5.88	0.37	0.63	0.42	*
Komati River	10	6.14	4.43	6.81	0.45	0.73	0.38	ns
Pierter Dam	32	7.79	3.12	14.42	0.37	0.65	0.45	*
Crocodile	10	4.57	3.22	5.93	0.44	0.63	0.32	ns
Lephalale	10	6.43	4.10	6.79	0.52	0.70	0.26	ns
Mogalakwena	10	6.57	4.29	6.90	0.55	0.73	0.26	ns
Sand River	10	6.79	5.07	7.26	0.56	0.74	0.21	ns
Luvuvhu	10	7.07	5.03	7.34	0.47	0.76	0.39	ns
Shingwedzi	9	5.07	3.24	5.70	0.56	0.63	0.09	ns
Letaba	10	5.71	4.23	6.62	0.49	0.71	0.30	ns
Mean	**12**	**5.34**	**3.52**	**4.53**	**0.42**	**0.62**	**0.32**	**-**

N: number of individuals, Na: number of alleles, Ne: effective number of alleles, Ar: Allelic richness, uHe: unbiased expected heterozygosity, Ho: observed heterozygosity, F_{is}: inbreeding coefficient and P_{HWE}: deviation from Hardy-Weinberg equilibrium (* $P<0.05$), are provided for each population.

2.4.2 Population structure

The STRUCTURE analyses conducted on *O. mossambicus* genetic data identified K = 15 as the most likely number of genetic partitions within the dataset, determined by the Puechmaille method (Figure 2.2). Despite the overall high admixture observed, distinct clustering patterns emerged. Several sampled localities exhibited similar allelic frequencies, with mean Q-values ranging from 0.00 to 0.94 (Mean = 0.06; S.D. = 0.02). Specifically, the Crocodile, Lephalale, and Mogalakwena rivers in Catchment Management Area A (CMA-A) displayed a commonality in allelic frequencies (Figure 2.2). In contrast, the Sand River, Luvuvhu, Shingwedzi, and Letaba rivers in CMA-A exhibited substantially different allelic frequencies, with the Sand River and Shingwedzi River showing particularly high genetic differentiation compared to other rivers within the same CMA. The Nwaswitsontso, Nsikasi, and Komati rivers shared similar allelic frequencies (Figure 2.2). A prevalent trend was the observation of localities within the same river sharing high genetic similarity (Mamba, Balule, and Olifants). In contrast, the Pieter Vorster Dam in Mpumalanga appeared to be highly genetically distinct compared to other populations.

The Phongolo, Mkuze, Hluhluwe, Mfolozi, Mhlathuze, and Matigulu rivers in KwaZulu-Natal also demonstrated a higher degree of genetic similarity (Figure 2.2). Similarly, the Thukela, Mvoti, Tongati, uMlazi, Mzimkhulu rivers, and the Albert Falls Dam (uMngeni) exhibited shared allelic frequencies. However, the Mtamvuna River exhibited genetic distinctiveness from all other CMAs in the KwaZulu-Natal Province, sharing only limited allelic frequencies with the Albert Falls Dam, uMlazi, and Mzimkhulu rivers (Figure 2.2). While localities within the same CMAs generally displayed genetic similarity, exceptions were observed. For example, certain allelic frequencies present in the Olifants River in CMA-B were also shared with the Phongolo River in CMA-W. Additionally, genotypic frequencies from the Olifants River, Shingwedzi, and Letaba within CMA-B were observed in the Luvuvhu, Crocodile, Lephalale, and Mogalakwena rivers belonging to CMA-A (Figure 2.2).

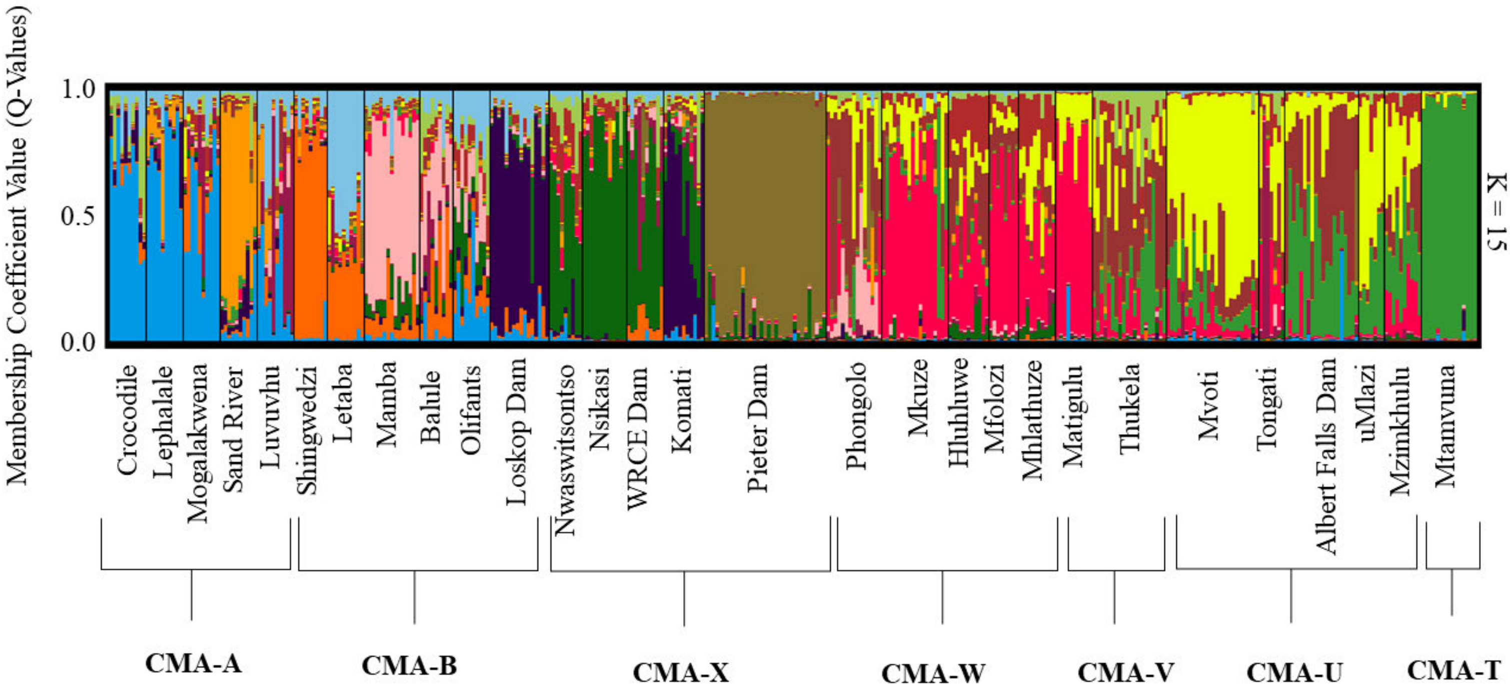

Figure 2.2: STRUCTURE bar plots showing assignment of the 370 *Oreochromis mossambicus* collected from 29 sampled localities in Limpopo, Mpumalanga, and KwaZulu-Natal provinces, South Africa. Each vertical line in the bar plot represents an individual and is coloured according to individual's estimated membership coefficient (Q) values. Individuals sampled from rivers and dams belonging to the same catchment management areas (CMAs) are also shown and localities are grouped based on the CMAs. K = 15 was estimated as the optimal genetic partitions present. (Note: WRCE Dam = White River Country Estate Dam, Pieter Dam = Pieter Vorster Dam).

The unbiased Nei's genetic distances within the various sampled localities ranged from 0.03 between the Mhlathuze River and Hluhluwe River in the CMA-W to 1.29 between WRCE Dam in CMA-X and uMlazi in CMA-U (Supplementary Table 2.4). Generally, unbiased genetic distances were higher when comparing the sampled localities belonging to the various CMAs (Supplementary Table 2.4). The PCoA plot (Figure 2.3), based on unbiased Nei's genetic distances among individuals from the 29 sampled localities across the CMAs in Limpopo, Mpumalanga, and KwaZulu-Natal provinces, provided a visual representation that complemented the STRUCTURE analysis by highlighting the genetic differentiation and clustering patterns among the localities. In this plot, the Y-axis (representing 16.68% of the variation) primarily captured the genetic variation observed within individuals from the sampled localities, while the X-axis (accounting for 34.84% of the variation) reflected the genetic differentiation among the 29 sampled localities (Figure 3.3a). This interpretation is supported by the clustering patterns observed in the PCoA plot (Figure 2.3), where individuals from the same locality tend to cluster together along the Y-axis, indicating within-locality variation. Conversely, the separation of localities along the X-axis corresponds to the genetic distances among them, as indicated by Nei's unbiased genetic distances (Supplementary Table 2.4), confirming the differentiation observed in the STRUCTURE analysis.

The PCoA indicated that individuals from the Lephalale, Crocodile, and Mogalakwena rivers in CMA-A cluster together genetically. Similarly, individuals from the Nwaswitsontso, Nsikasi, rivers, and the WRCE Dam populations in the CMB-X clustered closely (Figure 2.4). Interestingly, this trend was also observed between the Loskop Dam in CMA-B and the Komati River belonging within the CMA-X (Figure 2.4). Similar patterns of overlap were evident within the various CMAs in the KwaZulu-Natal Province, with individuals from the Matigulu, Mhlathuze, Hluhluwe, Mfolozi, Mkuze, and Phongolo rivers displaying close clustering patterns. Additionally, the Albert Falls Dam (uMngeni), uMlazi, Mvoti, Thukela, and Mzimkhulu rivers clustered closely, indicating genetic relatedness among their respective individuals. In contrast, certain *O. mossambicus* sampled localities, including the Luvuvhu, Sand River, and Mtamvuna appeared more genetically distinct from the others, displaying distant relatedness with neighbouring sampled localities within the same CMAs (Figure 2.3).

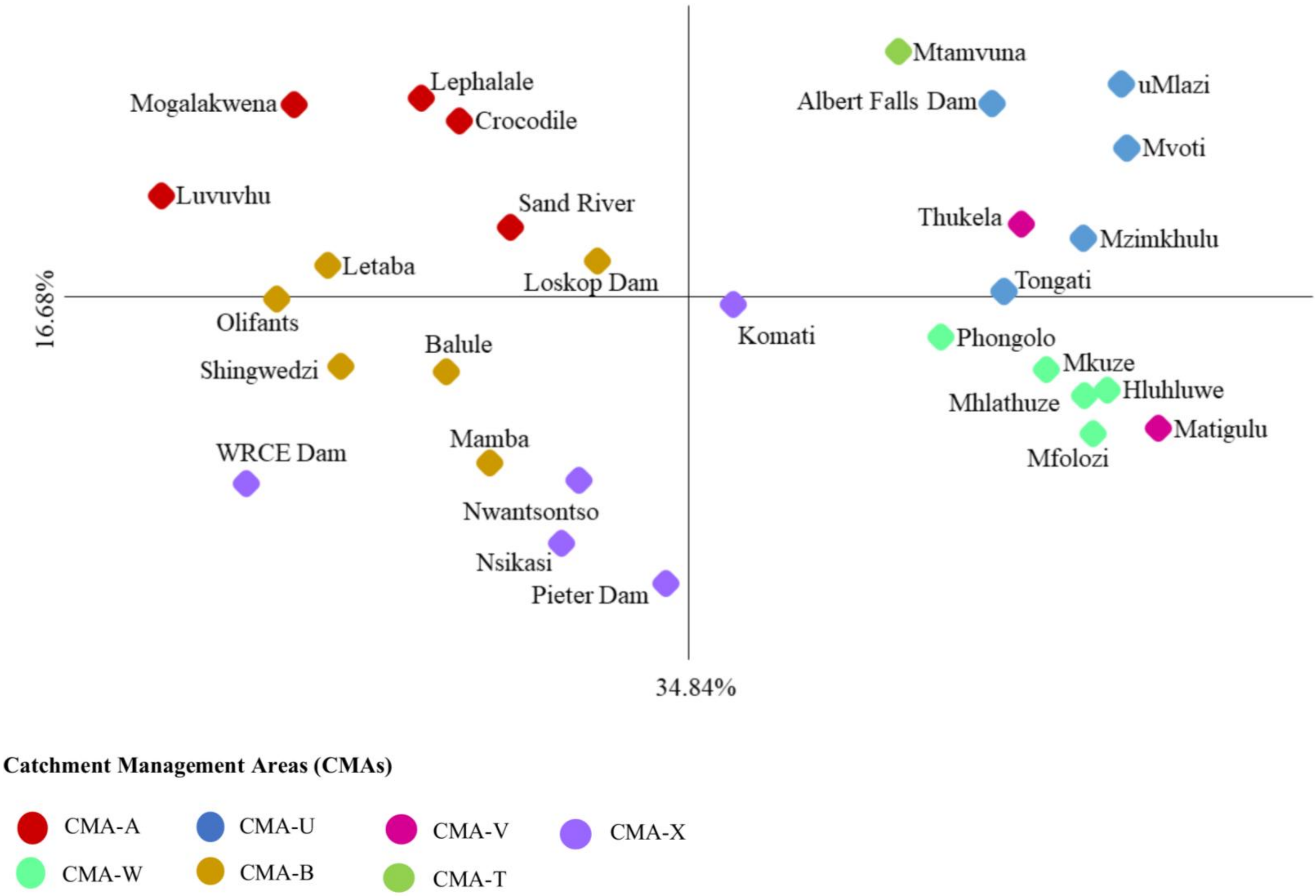

Figure 2.3: A Principal coordinate analysis (PCoA) of *Oreochromis mossambicus* grouped by the 29 sampled localities across various CMAs in the Limpopo, Mpumalanga, and KwaZulu-Natal Provinces, South Africa, plotted using Nei's (1972) unbiased genetic distance between sampling localities colour-coded by CMAs. (Note: WRCE Dam = White River Country Estate Dam, Pieter Dam = Pieter Vorster Dam).

The AMOVA analysis provided significant insights into the genetic structure of *O. mossambicus* across the 29 sampled localities. The results revealed a relative level of genetic differentiation among groups (19%) and a slightly higher differentiation among sites within groups (29%). The highest genetic variation was observed within sites (52%), indicating significantly high genetic differentiation between localities sampled (Table 2.3). Pairwise F_{ST}-values showed a range of genetic differences among localities within the various CMAs (Supplementary Table 2.5). These values ranged from 0.03 (between Tongati and Phongolo, and between Mhlathuze and Hluhluwe) to 0.38 (between Mtamvuna and Matigulu rivers). Corresponding pairwise P-values indicated a spectrum of significant and non-significant genetic differentiations, ranging from 0.0001 (between most sampled localities) to 0.0770 (between Phongolo and Tongati). Even within CMAs, significant genetic differences were evident. For instance, in CMA-W, Phongolo and Mkuze showed notable differentiation. In CMA-X, Pieter Vorster Dam displayed high genetic distinctiveness from other localities. Similarly, within CMA-A, the Sand River exhibited substantial genetic differentiation from other rivers. In CMA-B the Mamba River indicated significant genetic differences from all other sites except the Balule River. The Pieter Vorster Dam in CMA-X and Mtamvuna River in CMA-T also showed high genetic divergence from other localities within their respective CMAs.

Additional significant genetic differences were observed among other localities. The Crocodile and Sand rivers in CMA-A were genetically distinct from the Luvuvhu and Letaba rivers. Furthermore, the Luvuvhu and Letaba rivers were genetically distinct from the Shingwedzi River (Supplementary Table 2.5). In CMA-B, the Olifants River exhibited significant differentiation from the Nsikasi River, WRCE, and Pieter Vorster dams in CMA-X. The Matigulu River in CMA-W showed significant genetic differences with all rivers except Phongolo within the same CMA. Lastly, the Mhlathuze and Mfolozi rivers in CMA-W were genetically distinct from the Albert Falls Dam and uMlazi River in CMA-U, with Mfolozi and Hluhluwe rivers also showing significant differences from the Mvoti River (Supplementary Table 2.5).

The multiple rounds of AMOVA performed consistently revealed pronounced genetic structuring across all 12 scenarios, as evidenced by the significant genetic differentiation coefficients (Table 2.4). Notably, even within each scenario examined, substantial genetic differentiation was observed among the various groups. Nevertheless, it is important to note that there were specific instances where exceptions to this trend were observed. The rivers situated within the Waterberg district (specifically Crocodile and Lephalale) and those falling within Ecoregion 13 (Mkuze and Mfolozi) exhibited non-significant genetic differences (Supplementary Table 2.6). The associated p-values for the comparisons between Crocodile and Lephalale, and Mkuze and Mfolozi, were 0.001 and 0.008, respectively. After applying the Bonferroni correction for multiple tests, these p-values remained non-significant.

Table 2.3: Analysis of Molecular Variance (AMOVA) Groupings at 14 microsatellite loci for *O. mossambicus* from 29 sampled localities in Limpopo, Mpumalanga, and KwaZulu-Natal.

Source of variation	**Sum of squares**	**Variance components**	**Percentage variation**	**P value <0.001**
Among groups	959.78	1.07	19%	0.000
Among sites within groups	2118.62	1.61	29%	0.000
Within sites	1086.00	2.86	52%	0.000
Total	**4164.39**	**5.53**	**100%**	**0.000**

Table 2.4: Analysis of Molecular Variance (AMOVA) Groupings (12 scenarios tested) at 14 microsatellite loci for *O. mossambicus* from 29 sampled localities in Limpopo, Mpumalanga, and KwaZulu-Natal.

Grouping/Scenario	Source of variation	Sum of squares	Variance components	Percentage of variation	P-Value <0.001
Rivers and Dams	Among groups	600.26	0.78	14%	0.000
	Among sites within groups	2478.13	1.94	35%	0.000
	within sites	1086.00	2.86	51%	0.000
WMA19_2004-old	Among groups	471.94	0.66	12%	0.000
	Among sites within groups	2606.45	2.07	37%	0.000
	within sites	1086.00	2.86	51%	0.000
WMA9_2012-new	Among groups	354.02	0.63	11%	0.000
	Among sites within groups	2724.37	2.19	39%	0.000
	within sites	1086.00	2.86	50%	0.000
Primary catchment	Among groups	468.71	0.67	12%	0.000
	Among sites within groups	2609.68	2.07	37%	0.000
	within sites	1086.00	2.86	51%	0.000
Secondary catchment	Among groups	801.98	0.92	17%	0.000
	Among sites within groups	2276.41	1.76	32%	0.000
	within sites	1086.00	2.86	52%	0.000
Tertiary catchment	Among groups	837.48	0.95	17%	0.000
	Among sites within groups	2240.91	1.73	31%	0.000
	within sites	1086.00	2.86	52%	0.000
Quaternary catchment	Among groups	946.20	1.06	19%	0.000

	Among sites within groups	2132.19	1.62	29%	0.000
	within sites	1086.00	2.86	52%	0.000
Municipality	Among groups	737.28	0.86	15%	0.000
	Among sites within groups	2341.12	1.83	33%	0.000
	within sites	1086.00	2.86	52%	0.000
District	Among groups	538.72	0.71	13%	0.000
	Among sites within groups	2539.67	2.01	36%	0.000
	within sites	1086.00	2.86	51%	0.000
Province	Among groups	298.43	0.59	10%	0.000
	Among sites within groups	2779.96	2.26	40%	0.000
	within sites	1086.00	2.86	50%	0.000
Ecoregion	Among groups	399.76	0.57	10%	0.000
	Among sites within groups	2678.63	2.18	39%	0.000
	within sites	1086.00	2.86	51%	0.000
Biome	Among groups	148.88	0.45	8%	0.000
	Among sites within groups	2929.51	2.46	43%	0.000
	within sites	1086.00	2.86	50%	0.000

2.4.3 Best-fit management strategy for O. mossambicus

Based on comprehensive AMOVA analyses, the genetic differentiation of *O. mossambicus* populations from the 29 sampled localities reflects the hierarchical organisation of rivers within and between catchment management areas (CMAs). Specifically, populations within the same CMAs show higher genetic similarity, whereas fish from different CMAs exhibit genetic divergence. However, some populations showed high genetic divergence even within the same CMA. To effectively preserve and sustain the genetic diversity of *O. mossambicus* within the study area, we proposed integrating traditional, contemporary water management areas (WMAs), and catchment management areas (CMAs) practices. This strategic approach has led to the delineation of nine newly defined *O. mossambicus* management areas (OMAs). These OMAs optimise the conservation of genetic diversity by grouping localities with shared genetic affinities and segregating genetically distinct localities into separate OMAs (Figure 2.4). To determine the most suitable groupings, we considered several criteria including genetic differentiation, geographic proximity, and ecological factors. Our primary goal was to maximise the preservation of genetic diversity, while also ensuring practical management within existing administrative frameworks. Although AMOVA results indicated there was little difference in genetic variation partitioning among the tested scenarios (Table 2.4), the chosen integration of CMAs and WMAs offered a balance between genetic differentiation and management feasibility. For instance, the delineation of OMAs prioritised the grouping of populations with higher genetic similarity, thereby facilitating cohesive management strategies. Additionally, integrating the WMAs and CMAs was deemed beneficial as it aligned more closely with existing water management practices, enhancing the practicality and sustainability of conservation efforts. Specifically, the Sand River, Pieter Vorster Dam, and Mtamvuna River are particularly genetically distinct, warranting their management as independent OMAs. The Shingwedzi and Letaba rivers, though genetically distinct from other populations within CMA-B, are genetically similar to each other and could be managed as a single OMA. Conversely, the Loskop Dam shows genetic similarities with the Komati River, justifying their co-management within the same OMA. Additionally, the Thukela River in CMA-V shares genetic affinities with the uMvoti and Tongati rivers, and Albert Falls Dam (uMngeni) in CMA-U, recommending its inclusion within the same OMA as other localities managed within CMA-U, rather than as a standalone entity (Figure 2.4). The Mtamvuna River should be managed separately due to its high genetic differentiation from other localities within CMAs in KwaZulu-Natal Province. This aligns with CMA strategies rather than the new WMAs that lump all rivers within various CMAs into one WMA.

These newly proposed *O. mossambicus* management areas (OMAs) are outlined as follows: the Limpopo OMA encompasses the Crocodile, Lephalale, Mogalakwena, and Luvuvhu rivers, while the Sand River OMA is dedicated to the Sand River. The Letaba OMA is dedicated to the Shingwedzi and Letaba rivers. The Olifants OMA encompasses the Olifants river localities (Figure 2.4). The Komati OMA is dedicated to the Nwaswitsontso, Nsikasi, and Komati rivers, WRCE, and Loskop dams. The Pieter Dam OMA focuses on the Pieter Vorster Dam. The Phongolo-Matigulu OMA includes the Phongolo, Mkuze, Hluhluwe, Mfolozi, Mhlathuze, and Matigulu rivers. The Thukela-Mzimkhulu OMA covers the Thukela, Mvoti, Tongati, uMngeni (Albert Falls Dam), uMlazi, and Mzimkhulu localities. Lastly, the Mtamvuna OMA is dedicated to the Mtamvuna River (Figure 2.4).

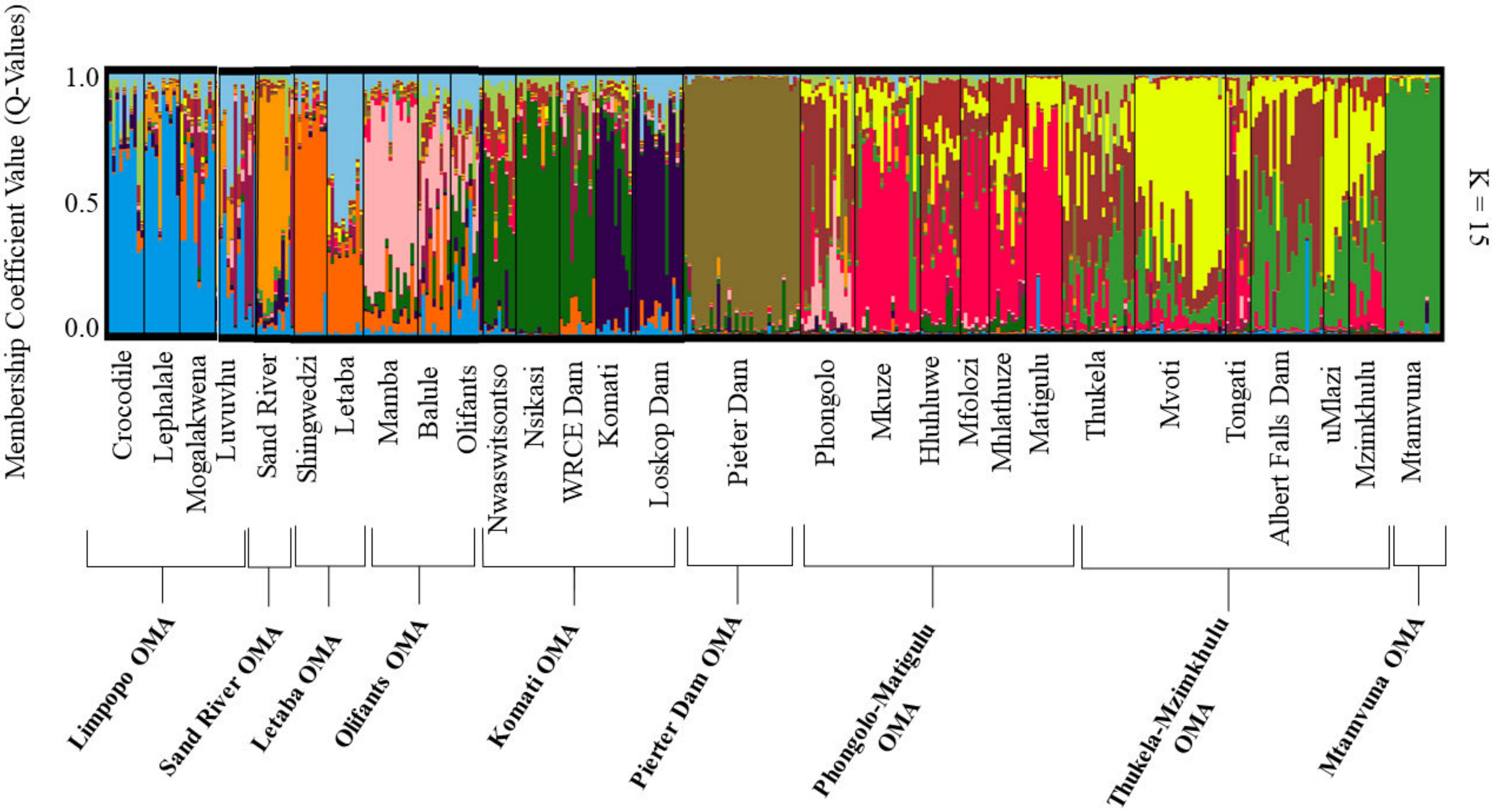

Figure 2.4: A STRUCTURE bar plot showing the assignment of the *Oreochromis mossambicus* from the 29 sampled localities into the proposed nine *O. mossambicus* Management Areas (OMAs) in Limpopo, Mpumalanga, and KwaZulu-Natal provinces, South Africa. Each vertical line in the bar plot represents an individual and is coloured according to individual's estimated membership coefficient (Q) values. (Note: WRCE Dam = White River Country Estate Dam, Pieter Dam = Pieter Vorster Dam).

2.5 Discussion

2.5.1 Genetic diversity in O. mossambicus

Genetic diversity plays a pivotal role in predicting the fitness of populations (Frankham, Bradshaw, and Brook 2014, Gu et al. 2014, Dieleman et al. 2019, Väli et al. 2019, Fitzpatrick and Wade 2022). Multiple factors, such as inbreeding, genetic drift, restricted gene flow, and small population size, can lead to decreased genetic diversity (Nikolic et al. 2009, Coleman et al. 2018, Poirier et al. 2019, Amoussou et al. 2019, Teixeira and Huber 2021). Microsatellite data from this study supports our predictions, revealing low observed heterozygosity (H_O) and elevated inbreeding coefficients across *O. mossambicus* populations in Catchment Management Areas (CMAs) throughout the three provinces. Interestingly, populations from three dams (Albert Falls, WRCE, and Pieter Vorster) deviated significantly from the Hardy-Weinberg Equilibrium despite exhibiting high allelic richness. The observed deviations from Hardy-Weinberg Equilibrium in *O. mossambicus* populations within the studied dams might be attributed to factors such as recent population bottlenecks, non-random mating within populations, high sample sizes, or sub structuring within the populations themselves, reduced gene flow or smaller effective population sizes compared to riverine populations (Crookes and Shaw 2016, Klütsch et al. 2019, Machado et al. 2022), Furthermore, another significant contributor might be the introduction of genetically distinct fish during dam stocking events, as reported by Anane-Taabeah Attu et al. (2022). Additionally, while sample size plays a role, particularly in the case of Pieter Vorster Dam with its high number of individuals, it's also important to consider the possibility of Type II error in some observed non-significant results. In contrast, some significant results at other sites, such as Nwaswitsonsto and Nsikasi, where observed heterozygosity is less than half of the expected, might be due to Type II errors, indicating insufficient power to detect differences between observed and expected values.

High genetic diversity within a population is often linked to greater adaptability and resilience in the face of environmental changes and emerging diseases (Buza et al., 2000; Basiita et al., 2018; Barmentlo et al., 2018; Leroy et al., 2018; Martinez et al., 2018; Kardos et al., 2021; Teixeira & Huber, 2021). Examining the genetic diversity of *O. mossambicus* across different CMAs revealed interesting patterns. Within Limpopo CMAs, the Lephalale, Luvuvhu, Sand River, and Mogalakwena rivers in CMA-A exhibited the highest genetic diversity. In Mpumalanga, the Balule and Olifants rivers (CMA-B) and the Komati River, Loskop Dam, and Pieter Vorster Dam (CMA-X) displayed the most genetic diversity. In KwaZulu-Natal, the highest diversity was observed in the Phongolo and Mkuze rivers (CMA-W) and the Thukela River (CMA-V). The high genetic diversity observed within these sampling localities may reflect *O. mossambicus* capacity for adaptation and persistence within their native environments. Some prior studies on tilapia species have also reported high genetic diversity within populations (Firmat et al. 2013, Mireku et al. 2017, Fatsi et al. 2020, Anane-Taabeah Attu et al., 2022).

Interestingly, *O. mossambicus* from Loskop Dam exhibited higher genetic diversity than some riverine populations within the same CMA-B. This finding might be explained by potential historical or ongoing gene flow with surrounding rivers, facilitated by factors like flooding events or upstream migration. In contrast, *O. mossambicus* from Albert Falls Dam in the KwaZulu-Natal, White River Country Estate Dam in Mpumalanga exhibited low genetic diversity and elevated inbreeding coefficients relative to *O. mossambicus* from rivers within the same catchments, likely attributable to anthropogenic barriers to gene flow and inbreeding (Crispo and Chapman 2010, Coleman et al. 2018, Poirier et al. 2019). However, given that the dams in question vary in age, with many being established several decades ago (particularly, Loskop Dam and Albert Falls Dam), for water storage, irrigation, and recreational fishing purposes, the low genetic diversity and high inbreeding observed in these dams could be due to the consequences of founder effects when populations were initially established, followed by subsequent genetic drift and inbreeding (Poirier et al. 2019, Amoussou et al. 2019, Teixeira and Huber 2021). Though additional sampling is needed to comprehensively investigate the genetic implications of anthropogenic river impoundments on native *O. mossambicus* populations, similar studies on other freshwater fish species such as the rainbow trout (*Oncorhynchus mykiss*, Deiner et al. 2007), brown trout (*Salmo trutta*, Klütsch et al. 2019), and migratory catfish (*Pseudoplatystoma corruscans*, Machado et al. 2022) have indicated a net loss of genetic diversity as a result of impoundments.

Factors such as overfishing, stocking, and pollution are also known to influence the genetic diversity of freshwater fish populations (Hauser et al. 2002, Hassanien and Gilbey 2005, Soliman et al. 2017, Faria et al. 2018). Only about 50% of South Africa's primary rivers are located within protected areas (Nel et al. 2007), and as such anthropogenic factors including overfishing may also be important factors affecting genetic diversity levels in the present study. Soliman et al. (2017) reported low genetic diversity for red belly tilapia (*Captodon zillii*) from regions impacted by overfishing in Egypt. Overfishing and habitat modifications were also reported to have led to a genetic diversity reduction in the population of wild *O. niloticus* from the drainage basins of Shana (Anane-Taabeah Attu 2019). Nevertheless, there is a need for continued monitoring of *O. mossambicus* to evaluate the influence of overfishing on the genetic diversity in the region.

2.5.2 Population structure and connectivity

The genetic structure of populations typically conforms to a hierarchical framework that mirrors the dendritic character of river systems and their unidirectional flow patterns (Hurwood and Hughes 1998). This hierarchical structure is expected to align with the drainage hierarchy, resulting in gene flow primarily occurring within, rather than between, catchments.

In accordance with this expectation, a consistent trend emerged across most sampled localities within each CMA. This suggests a relatively shallow population structure and a high degree of gene flow among *O. mossambicus* within these CMAs. However, some notable exceptions were observed. The Sand River population in CMA-A displayed distinct genetic patterns compared to other rivers in the broader Limpopo River basin. Similarly, the Shingwedzi and Letaba rivers within CMA-B, the Pieter Voster Dam in CMA-X, and the Mtamvuna River in CMA-T, all exhibited unique genetic signatures. This may suggest an influence of various factors, including anthropogenic activities, physical barriers, river structure, flow dynamics, and fluctuations in environmental conditions, which are known to exert considerable influence on the genetic population structure of aquatic species (Hudson et al. 2013, Ishiyama et al. 2015, Jaisuk and Senanan 2018, Amoussou et al. 2019). These findings align with the notion that the genetic population structure in tilapia species is inherently shaped by both biological and geographical processes (Romana-Eguia et al. 2004, Bezault et al. 2011, Yoboue et al. 2014, Amoussou et al. 2019). Tibihika et al. (2020) reported similar genetic trends in *O. niloticus* populations in East Africa, corroborating our findings. Comparable results were reported by Anane-Taabeah Attu (2019) in an evaluation of genetic variation in *O. niloticus* within and among drainage basins in Ghana. Moreover, prior studies on cichlid species have also documented high genetic differentiation among populations (D'Amato et al. 2007, Mireku et al. 2017, Richmond 2018, Fatsi et al. 2021, Hashem et al. 2020; 2022,).

Despite the ecological and economic significance of genetic variation in fish populations, these populations are increasingly susceptible to multiple stressors (Casey et al. 2016, FAO 2019, Friedman et al. 2022). Alterations in drainage networks, basin size, and habitat quality can significantly impact the distribution and diversity of freshwater organisms (Burridge et al. 2006, Albert et al. 2017. Val et al. 2022). South Africa has experienced substantial habitat transformations, with over 50% of land cover changes observed in many provinces, resulting in the classification of most of its main river systems as severely modified (Driver et al. 2012, Lemley et al. 2015). Moreover, approximately 60% of South Africa's rivers remain free-flowing, while the rest have been dammed or substantially altered (Nel et al. 2011, Maree et al. 2016). These modifications may have led to the emergence of highly genetically distinct *O. mossambicus* from the 29 sampled localities, such as the Sand River, Shingwedzi, Letaba, and Mtamvuna rivers, including the Pieter Vorster Dam, which could potentially serve as management units (MUs). Management units represent populations that have achieved demographic independence and warrant separate management (Moritz 1994, 2002). Identifying such units informs conservation strategies by acknowledging historical lineage isolation and the functional and demographic autonomy of distinct populations (Biun et al. 2021). Accurate quantification of these units for the present study may contribute positively to the conservation and management of *O. mossambicus*, and this may suggest that the Sand River, Shingwedzi, Mtamvuna, and Letaba rivers, and the Pieter Vorster Dam localities should be managed separately. However, understanding how these localities are connected in broad is important and needs verification with more intensive surveys.

Additionally, water resource management strategies may have inadvertently contributed to the observed genetic patterns. South Africa's water scarcity necessitates careful water management, leading to the consolidation of Water Management Areas (WMAs) from nineteen to nine. These changes, however, could potentially introduce genetic mismatches by mixing previously isolated populations from unconnected river systems. Such mismatches can disrupt co-evolutionary adaptations to local environments, potentially reducing overall fitness and compromising the long-term sustainability of these fish populations through outbreeding depression (Brauer et al. 2013, Ralls et al. 2018, Fitzpatrick et al. 2020). The new nine WMAs fail to account for fine-scale river connectivity, potentially jeopardising the genetic integrity of *O. mossambicus* populations through the integration of genetically distinct populations. Our microsatellite data supports this observation, as genetically distinct localities are presently managed together with those belonging to the same WMAs and CMAs. For instance, the Sand River locality exhibits high genetic distinctiveness and is managed alongside other localities within the Limpopo WMA.

Furthermore, genetic mismatches are evident within river catchments, where *O. mossambicus* from some dams appear genetically distinct from rivers. The Komati Catchment is exemplary, with the Pieter Vorster dam displaying high genetic distinctiveness yet being managed together with rivers within the same WMA. Fine-scale research is imperative to elucidate the connections between this distinct dam and river *O. mossambicus*. Our study highlights the necessity for refined management of *O. mossambicus* in South Africa, particularly in the major river catchments under investigation. It is suggested that *O. mossambicus* from sampling localities exhibiting high genetic distinctiveness should be managed separately to preserve the existing genetic integrity. The mixing of highly distinct populations with others poses the risk of diminished overall fitness because of the loss of local adaptations and potential outbreeding depression (Brauer et al. 201, Ralls et al. 2018, Fitzpatrick et al. 2020). Furthermore, such highly distinct populations may also be representing sub-species or introduced species, given the known instances of invasive tilapia species introductions in South Africa, particularly in the provinces under scrutiny (Firmat et al. 2013, Ellender and Wely 2014, Bills 2019, Evans et al. 2022). However, a comprehensive assessment of hybridisation and the presence of introduced species in surveyed localities, employing markers such as mitochondrial DNA in conjunction with microsatellite markers, will be essential in constructing phylogenetic analyses and confirming this hypothesis.

2.5.3 Proposed management of O. mossambicus in South Africa

Considering the ongoing natural processes and extensive anthropogenic activities impacting South African river systems, it is imperative to recognise that O. *mossambicus* within the same river catchment or interconnected river catchments have become geographically isolated. This isolation has rendered the conventional management approaches, aligned with South Africa's water resource management, inadequate for safeguarding *O. mossambicus*. The multitude of anthropogenic stressors, including overfishing, the introduction of non-native species, alterations in water flow dynamics, and landscape modifications within and across river catchments encompassing various Water Management Areas (WMAs), poses a substantial threat to *O. mossambicus*.

The present state of WMAs necessitates immediate attention, as unidentified *O. mossambicus* exhibiting unique genetic profiles may be at risk of extinction, representing an irreplaceable loss of vital genetic diversity. Given the observed genetic mismatches both within and among *O. mossambicus* sampling localities across our study area, a critical consideration revolves around the formulation of effective management strategies that can ensure the long-term viability of this vulnerable species. After a comprehensive evaluation of the diverse water resource and catchment management strategies employed in South Africa, our findings propose maybe a more effective approach. This approach entails the refinement and integration of the existing nine WMAs with the old 19 WMAs and the existing Catchment Management Areas (CMAs). Under this new Ideal, we advocate for the establishment of nine distinct *O. mossambicus* management areas (OMAs). This proposed management approach clusters *O. mossambicus* sharing genetic similarities within a single OMA while segregating those genetically distinct into new OMAs. These distinct *O. mossambicus* would be managed as Independent OMAs, thereby preventing any mixing that might result in the irreversible loss of this species. This strategy holds considerable promise for preserving the existing genetic integrity of *O. mossambicus* in Limpopo, Mpumalanga, and KwaZulu-Natal provinces. Significantly, the implementation of a dedicated national strategy for managing *O. mossambicus* becomes paramount, given the increasing threat posed by hybridisation with *O. niloticus*, particularly because of pressures stemming from the aquaculture sector. This forward-looking approach contributes substantially to the long-term sustainability of this Vulnerable species, offering a viable pathway for its continued existence.

2.6 Conclusions

Our study highlighted the intricate interplay of multiple factors in shaping the genetic diversity and population structure of freshwater fish. Our examination of the O. *mossambicus* from the 29 sampled localities in the Limpopo, Mpumalanga, and KwaZulu-Natal provinces, South Africa, has revealed a landscape where genetic diversity is notably relatively low, likely attributable to the escalating anthropogenic pressures exerted on these rivers. Moreover, the prevalence of impoundments and restricted free-flowing sections in these river systems (Rivers-Moore 2007, Nel and Drivers 2015, O'Brien et al. 2019, Evans et al. 2022) may have undoubtedly contributed to this observed decline in genetic diversity. The clustering revealed a shallow genetic structure, with certain geographically proximate localities expected to exhibit genetic similarities, appearing as highly distinct entities. This compelling outcome highlights the pivotal roles played by anthropogenic activities, the historical context of catchments, connectivity dynamics, and water management strategies in shaping the genetic population structure of *O. mossambicus*. Considering the prevailing pronounced genetic patterns within wild *O. mossambicus* across these three provinces, it is imperative that any management strategy devised for conserving these fish populations steadfastly prioritises the preservation of their existing genetic diversity, with a commitment to preventing any further deterioration. This approach holds the key to securing the long-term sustainability of this Vulnerable fish species. The findings from this study carry significant implications for decision-making in river management and the development of inland water resources in South Africa. They advocate for a careful consideration of the conservation status of fish populations inhabiting these systems. Furthermore, this study stands to make a positive contribution towards the monitoring and conservation of critical genetic units within the vulnerable *O. mossambicus*, ensuring their sustainable utilisation and fostering the responsible growth of aquaculture in South Africa, particularly within these three provinces. Additionally, the methodology and insights from this research present an opportunity for replication in other river systems within South Africa and beyond. Such practices can aid in the considerate and informed management of freshwater systems while safeguarding the conservation status of resident fish populations.

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2.9 Supplementary information

Supplementary Table 2.1: Groupings of the 29 sampled localities from Limpopo, Mpumalanga and KwaZulu-Natal based on various levels of water resource management strategies fall within. (Note: WRCE Dam = White River Country Estate Dam, Pieter Dam = Pieter Vorster Dam).

Localities sampled	Scenario 1	Scenario 2	Scenario 3	Scenario 4	Scenario 5	Scenario 6	Scenario 7	Scenario 8	Scenario 9	Scenario 10	Scenario 11	Scenario 12
Sites	**Dam/River**	**WMA19_2004**	**WMA9_2012**	**Primary catchment**	**Secondary catchment**	**Tertiary catchment**	**Quaternary catchment**	**Province**	**Municipality**	**District**	**Ecoregions**	**Biomes**
Phongolo	River	uSuthu-Mhlathuze	Pongola-Mtamvuna	W	W4	W45	W45B	KwaZulu-Natal	uMhlabuyalingana	uMkhanyakude	3	Grassland
Mkuze	River	uSuthu-Mhlathuze	Pongola-Mtamvuna	W	W3	W32	W32A	KwaZulu-Natal	Jozini	uMkhanyakude	13	Grassland
Hluhluwe	River	uSuthu-Mhlathuze	Pongola-Mtamvuna	W	W3	W32	W32F	KwaZulu-Natal	Big Five Hlabisa	uMkhanyakude	3	Forest
Mfolozi	River	uSuthu-Mhlathuze	Pongola-Mtamvuna	W	W2	W23	W23D	KwaZulu-Natal	Mfolozi	uMkhanyakude	13	Forest
Mhlathuze	River	uSuthu-Mhlathuze	Pongola-Mtamvuna	W	W1	W12	W12E	KwaZulu-Natal	Mhlathuze	uThungulu	14	Forest
Matigulu	River	uSuthu-Mhlathuze	Pongola-Mtamvuna	W	W1	W11	W11C	KwaZulu-Natal	Mandeni	iLembe	17	Forest
Thukela	River	Thukela	Pongola-Mtamvuna	V	V5	V50	V50D	KwaZulu-Natal	Mandeni	iLembe	17	Grassland
Mvoti	River	uMvoti-Mzimkhulu	Pongola-Mtamvuna	U	U4	U40	U40J	KwaZulu-Natal	Kwadukuza	iLembe	17	Grassland
Tongati	River	uMvoti-Mzimkhulu	Pongola-Mtamvuna	U	U3	U30	U30D	KwaZulu-Natal	Kwadukuza	iLembe	17	Savanna
Albert Falls Dam	Dam	uMvoti-Mzimkhulu	Pongola-Mtamvuna	U	U2	U20	U20E	KwaZulu-Natal	uMngeni	uMgungundlovu	16	Savanna
uMlazi	River	uMvoti-Mzimkhulu	Pongola-Mtamvuna	U	U6	U60	U60D	KwaZulu-Natal	eThekwini	eThekwini	17	Savanna
Mzimkhulu	River	uMvoti-Mzimkhulu	Pongola-Mtamvuna	T	T5	T52	T52M	KwaZulu-Natal	Ray Nkonyeni	Ugu	16	Savanna
Mtamvuna	River	uMvoti-Mzimkhulu	Pongola-Mtamvuna	T	T4	T40	T40E	KwaZulu-Natal	Mbizana	Ugu	17	Savanna
Mamba	River	Olifants (Limpopo South-MP North)	Olifants	B	B7	B73	B73C	Mpumalanga	Ba-Phalaborwa	Mopani	3	Grassland
Balule	River	Olifants (Limpopo	Olifants	B	B7	B73	B73H	Mpumalanga	Ba-Phalaborwa	Mopani	3	Grassland

		South-MP North)										
Olifants	River	Olifants (Limpopo South-MP North)	Olifants	B	B7	B73	B73H	Mpumalanga	Ba-Phalaborwa	Mopani	3	Grassland
Loskop Dam	Dam	Olifants (Limpopo South-MP North)	Olifants	B	B3	B32	B32A	Mpumalanga	Steve Tshwete	Nkangala	9	Grassland
Nwaswitsontso	River	Komati	Komati-uSuthu	X	X4	X40	X40D	Mpumalanga	Bushbuckridge	Ehlanzeni	12	Grassland
Nsikasi	River	Komati	Komati-uSuthu	X	X2	X24	X24B	Mpumalanga	Mbombela	Ehlanzeni	3	Grassland
WRCE Dam	Dam	Komati	Komati-uSuthu	X	X2	X22	X22H	Mpumalanga	Mbombela	Ehlanzeni	3	Grassland
Komati	River	Komati	Komati-uSuthu	X	X1	X13	X13L	Mpumalanga	Nkomazi	Ehlanzeni	12	Grassland
Pieter Dam	Dam	Komati	Komati-uSuthu	X	X1	X13	X13K	Mpumalanga	Nkomazi	Ehlanzeni	3	Grassland
Crocodile	River	Crocodile-West	Limpopo	A	A2	A24	A24J	Limpopo	Thabazimbi	Waterberg	1	Grassland
Lephalale	River	Limpopo	Limpopo	A	A5	A50	A50H	Limpopo	Lephalale	Waterberg	1	Grassland
Mogalakwena	River	Limpopo	Limpopo	A	A6	A63	A63D	Limpopo	Blouberg	Vhembe	1	Grassland
Sand River	River	Limpopo	Limpopo	A	A7	A71	A71K	Limpopo	Musina	Vhembe	1	Grassland
Luvuvhu	River	Luvuvhu-Letaba	Limpopo	A	A9	A92	A92D	Limpopo	Musina	Vhembe	2	Grassland
Shingwedzi	River	Luvuvhu-Letaba	Olifants	B	B9	B90	B90H	Limpopo	Great Giyani	Mopani	3	Grassland
Letaba	River	Luvuvhu-Letaba	Olifants	B	B8	B83	B83A	Limpopo	Ba-Phalaborwa	Mopani	3	Grassland

Supplementary Table 2.2: Details of sampled localities where *Oreochromis mossambicus* were collected in Limpopo, Mpumalanga, and KwaZulu-Natal provinces, South Africa. A nested sampling approach was used. Multiple rivers per catchment and localities per river were sampled, where possible. (Note: WRCE Dam= White River Country Estate Dam, Pieter Dam = Pieter Vorster Dam).

Catchment	River/Dam/locality	Region	Catchment Management Area (CMA)	Sample size	Population ID	Longitude	Latitude
Phongolo	Phongolo	KwaZulu-Natal	CMA-W	15	Pop1	-26.929964	32.324218
Mkuze	Mkuze	KwaZulu-Natal	CMA-W	18	Pop2	-27.653055	32.403056
Hluhluwe	Hluhluwe	KwaZulu-Natal	CMA-W	11	Pop3	-28.119305	32.183157
Mfolozi	Mfolozi	KwaZulu-Natal	CMA-W	8	Pop4	-28.451579	32.182396
Mhlathuze	Mhlathuze	KwaZulu-Natal	CMA-W	10	Pop5	-28.746950	31.747450
Matigulu	Matigulu	KwaZulu-Natal	CMA-V	10	Pop6	-29.072961	31.557560
Thukela	Thukela	KwaZulu-Natal	CMA-V	20	Pop7	-29.170560	31.421092
Mvoti	Mvoti	KwaZulu-Natal	CMA-U	25	Pop8	-29.370141	31.304451
Tongati	Tongati	KwaZulu-Natal	CMA-U	7	Pop9	-29.559913	31.174085
uMngeni	Albert Falls Dam	KwaZulu-Natal	CMA-U	20	Pop10	-29.444805	30.389196
uMlazi	uMlazi	KwaZulu-Natal	CMA-U	7	Pop11	-29.869000	30.781200
Mzimkhulu	Mzimkhulu	KwaZulu-Natal	CMA-U	10	Pop12	-30.635981	30.196877
Mtamvuna	Mtamvuna	KwaZulu-Natal	CMA-T	15	Pop13	-30.849236	30.064003
Olifants	Mamba	Mpumalanga	CMA-B	15	Pop14	-24.086417	31.250944
Olifants	Balule	Mpumalanga	CMA-B	9	Pop15	-24.052139	31.728778

Olifants	Olifants	Mpumalanga	CMA-B	10	Pop16	-24.008221	31.737847
Olifants	Loskop Dam	Mpumalanga	CMA-B	16	Pop17	-25.429315	29.398656
Crocodile	Nwaswitsontso	Mpumalanga	CMA-X	9	Pop18	-24.794159	31.904441
Crocodile	Nsikasi	Mpumalanga	CMA-X	12	Pop19	-25.333133	31.275137
Crocodile	WRCE Dam	Mpumalanga	CMA-X	10	Pop20	-25.285210	31.006546
Komati	Komati River	Mpumalanga	CMA-X	11	Pop21	-25.450801	31.951443
Komati	Pieter Vorster Dam	Mpumalanga	CMA-X	33	Pop22	-25.525341	31.862491
Limpopo	Crocodile	Limpopo	CMA-A	10	Pop23	-24.314167	27.046139
Limpopo	Lephalale	Limpopo	CMA-A	10	Pop24	-23.141278	27.885028
Limpopo	Mogalakwena	Limpopo	CMA-A	10	Pop25	-22.473444	28.919500
Limpopo	Sand River	Limpopo	CMA-A	10	Pop27	-22.399278	30.099417
Limpopo	Luvuvhu	Limpopo	CMA-A	10	Pop28	-22.444444	31.083444
Olifants	Shingwedzi	Limpopo	CMA-B	9	Pop29	-23.221944	31.554917
Olifants	Letaba	Limpopo	CMA-B	10	Pop30	-23.758333	31.369972

Supplementary Table 2.3: Genetic diversity indices for each of the 14 microsatellite loci used in this *Oreochromis mossambicus* study.

Locus	Na	Null allele frequencies	Ar	F_{ST}^{A}	F_{ST}^{B}	H_O	uH_E	P_{HWE}	F_{is}	PIC
OM04	7.2	0.04	3.92	0.14	0.14	0.79	0.79	*	-0.05	0.88
OM01	8.0	0.12	4.00	0.14	0.13	0.54	0.80	ns	0.28	0.86
UNH142	3.5	0.16	2.49	0.21	0.17	0.32	0.55	ns	0.38	0.77
OM08	3.6	0.09	2.68	0.21	0.20	0.52	0.61	ns	0.09	0.77
OM09	6.0	0.11	3.45	0.22	0.21	0.47	0.69	ns	0.28	0.81
OM05	5.6	0.13	3.48	0.17	0.15	0.52	0.74	ns	0.25	0.89
OM06	2.5	0.17	1.85	0.55	0.52	0.09	0.32	ns	0.69	0.78
UNH222	4.3	0.21	2.97	0.18	0.16	0.29	0.65	ns	0.53	0.79
UNH104	5.3	0.16	3.25	0.18	0.16	0.41	0.70	ns	0.38	0.82
UNH231	2.2	0.10	1.63	0.19	0.25	0.09	0.23	ns	0.57	0.50
UNH129	4.7	0.10	2.75	0.17	0.15	0.39	0.54	ns	0.23	0.70
OM07	9.1	0.03	4.47	0.07	0.08	0.87	0.87	ns	-0.05	0.90
OM03	5.7	0.17	3.19	0.21	0.19	0.35	0.64	ns	0.42	0.90
OM02	5.6	0.20	3.17	0.20	0.18	0.29	0.64	ns	0.52	0.88
Mean	**5.24**	**0.13**	**3.09**	**0.20**	**0.19**	**0.43**	**0.62**	**-**	**0.32**	**0.80**

Na: number of alleles, Ar: Allelic richness, F_{ST}^{A}: non-corrected F_{ST}, F_{ST}^{B}: ENA corrected F_{ST}, H_O: observed heterozygosity, uH_E: unbiased expected heterozygosity. PIC: polymorphic information content.

Supplementary Table 2. 4: Unbiased Nei's genetic distance matrix for the 370 *O. mossambicus* individuals across the 29 sampling localities in Limpopo, Mpumalanga, and KwaZulu-Natal, South Africa estimated using 14 microsatellite loci. (Note: WRCE Dam = White River Country Estate Dam, Pieter Dam = Pieter Vorster Dam).

Locality	Phongolo	Mkuze	Hluhluwe	Mfolozi	Mhlathuze	Matigulu	Thukela	Mvoti	Tongati	uMngeni	uMlazi	Mzimkhulu	Mtamvuna	Mamba	Balule	Olifants	Nwantsontso	Nsikasi	WRCE Dam	Komati	Pieter Dam	Loskop Dam	Crocodile	Lephalale	Mogalakwena	Sand River	Luvuvhu	Shingwedzi	Letaba
Phongolo	0.00																												
Mkuze	0.22	0.00																											
Hluhluwe	0.18	0.14	0.00																										
Mfolozi	0.21	0.07	0.09	0.00																									
Mhlathuze	0.18	0.12	0.03	0.10	0.00																								
Matigulu	0.38	0.07	0.29	0.19	0.26	0.00																							
Thukela	0.18	0.17	0.14	0.18	0.13	0.34	0.00																						
Mvoti	0.26	0.23	0.25	0.37	0.21	0.30	0.10	0.00																					
Tongati	0.19	0.20	0.16	0.14	0.14	0.37	0.10	0.20	0.00																				
uMngeni	0.27	0.22	0.26	0.33	0.30	0.41	0.11	0.12	0.25	0.00																			
uMlazi	0.33	0.33	0.33	0.41	0.30	0.46	0.20	0.13	0.20	0.22	0.00																		
Mzimkhulu	0.17	0.18	0.18	0.22	0.15	0.28	0.12	0.15	0.18	0.19	0.13	0.00																	
Mtamvuna	0.52	0.48	0.76	0.72	0.63	0.69	0.38	0.35	0.60	0.30	0.33	0.32	0.00																
Mamba	0.35	0.41	0.52	0.39	0.46	0.61	0.56	0.81	0.51	0.72	0.87	0.60	0.81	0.00															
Balule	0.37	0.43	0.51	0.46	0.51	0.59	0.46	0.62	0.46	0.53	0.71	0.56	0.92	0.12	0.00														
Olifants	0.55	0.68	0.86	0.74	0.75	0.86	0.68	0.80	0.75	0.72	0.96	0.74	0.72	0.27	0.18	0.00													
Nwantsontso	0.39	0.30	0.31	0.25	0.29	0.47	0.46	0.69	0.40	0.63	0.76	0.47	0.92	0.29	0.27	0.33	0.00												
Nsikasi	0.47	0.43	0.44	0.38	0.38	0.52	0.60	0.81	0.57	0.83	0.99	0.59	0.96	0.37	0.39	0.31	0.09	0.00											
WRCE Dam	0.70	0.68	0.74	0.71	0.70	0.83	0.77	**1.06**	0.79	**1.05**	**1.29**	0.85	**1.06**	0.32	0.19	0.20	0.28	0.20	0.00										
Komati	0.42	0.31	0.52	0.49	0.48	0.47	0.40	0.47	0.46	0.48	0.56	0.57	0.53	0.49	0.46	0.54	0.59	0.63	0.55	0.00									
Pieter Dam	0.46	0.53	0.57	0.48	0.59	0.72	0.73	**1.03**	0.67	0.94	0.92	0.78	**1.14**	0.39	0.48	0.67	0.35	0.34	0.60	0.63	0.00								
Loskop Dam	0.61	0.38	0.72	0.60	0.56	0.45	0.48	0.48	0.63	0.51	0.68	0.64	0.47	0.47	0.37	0.33	0.52	0.61	0.51	0.14	0.86	0.00							
Crocodile	0.67	0.64	0.78	0.71	0.82	0.87	0.55	0.67	0.68	0.48	0.50	0.68	0.68	0.61	0.34	0.26	0.55	0.68	0.60	0.69	0.81	0.52	0.00						
Lephalale	0.58	0.69	0.78	0.73	0.82	0.98	0.53	0.61	0.67	0.40	0.55	0.65	0.63	0.58	0.34	0.17	0.52	0.63	0.57	0.71	0.85	0.57	0.07	0.00					
Mogalakwena	0.75	0.79	0.76	0.81	0.83	**1.23**	0.59	0.74	0.60	0.55	0.65	0.79	0.80	0.58	0.31	0.23	0.49	0.72	0.48	0.72	**1.04**	0.67	0.19	0.06	0.00				
Sand River	0.64	0.66	0.69	0.69	0.68	0.89	0.50	0.67	0.61	0.52	0.65	0.62	0.79	0.35	0.23	0.43	0.57	0.79	0.62	0.66	0.74	0.49	0.42	0.37	0.36	0.00			
Luvuvhu	0.68	0.81	0.98	0.96	0.92	**1.09**	0.73	0.88	0.88	0.71	**1.01**	0.78	0.77	0.47	0.34	0.15	0.42	0.52	0.31	0.61	0.95	0.49	0.32	0.20	0.19	0.58	0.00		
Shingwedzi	0.99	0.96	**1.20**	**1.14**	**1.00**	**1.01**	0.97	0.94	**1.02**	**1.02**	**1.27**	**1.11**	**1.17**	0.44	0.41	0.62	0.83	0.94	0.70	0.87	0.84	0.59	0.95	0.90	0.79	0.37	0.78	0.00	
Letaba	0.90	0.86	**1.01**	0.84	0.89	0.97	0.84	0.87	0.79	0.87	0.90	0.89	**1.11**	0.66	0.46	0.47	0.57	0.69	0.57	0.82	**1.01**	0.50	0.53	0.61	0.50	0.60	0.50	0.61	0.00

Unbiased Nei's unbiased genetic distance (Nei 1972) values ranging from 0- 0.05 indicate low genetic distance (high genetic similarity), 0.1-0.30 = moderate genetic distance (indicating moderate differentiation), 0.30-1 = high genetic distance (high genetic differentiation), and ≥ 1= very great genetic distance (given in bold).

Supplementary Table 2. 5: ENA corrected pairwise F_{ST}-values and the probability (P-values) of genetic differences for the 370 *Oreochromis mossambicus* from the 29 sampled localities in the present study. (Note: WRCE Dam = White River Country Estate Dam, Pieter Dam = Pieter Vorster Dam).

Sampling locality	Phongolo	Mkuze	Hluhluwe	Mfolozi	Mhlathuze	Matigulu	Thukela	Mvoti	Tongati	uMngeni	uMlazi	Mzimkhulu	Mtamvuna	Mamba	Balule	Olifants	Loskop Dam	Nwantsontso	Nsikasi	WRCE Dam	Komati River	Pieter Dam	Crocodile	Lephalale	Mogalakwena	Sand River	Luvuvhu	Shingwedzi	Letaba
Phongolo		0.0001*	0.0002^{NS}	0.0027^{NS}	0.0007^{NS}	0.0003^{NS}	0.0001*	0.0001*	0.0770^{NS}	0.0001*	0.0008^{NS}	0.0001*	0.0001*	0.0001*	0.0002^{NS}	0.0001*	0.0001*	0.0002^{NS}	0.0001*	0.0001*	0.0003^{NS}	0.0001*	0.0001*	0.0001*	0.0002^{NS}	0.0001*	0.0001*	0.0002^{NS}	0.0001*
Mkuze	0.09		0.0001*	0.0244^{NS}	0.0003^{NS}	0.0001*	0.0001*	0.0001*	0.0152^{NS}	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0002^{NS}	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*
Hluhluwe	0.10	0.09		0.0045^{NS}	0.0277^{NS}	0.0001*	0.0001*	0.0001*	0.0045^{NS}	0.0001*	0.0003^{NS}	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0002^{NS}	0.0004^{NS}	0.0001*	0.0003^{NS}	0.0001*	0.0001*	0.0002^{NS}	0.0001*	0.0001*	0.0001*	0.0002^{NS}	0.0001*
Mfolozi	0.11	0.06	0.05		0.0003^{NS}	0.0001*	0.0001*	0.0001*	0.0318^{NS}	0.0001*	0.0003^{NS}	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0005^{NS}	0.0010^{NS}	0.0002^{NS}	0.0008^{NS}	0.0001*	0.0001*	0.0001*	0.0003^{NS}	0.0001*	0.0002^{NS}	0.0009^{NS}	0.0001*
Mhlathuze	0.09	0.06	0.03	0.08		0.0001*	0.0001*	0.0001*	0.0542^{NS}	0.0001*	0.0004^{NS}	0.0002^{NS}	0.0001*	0.0001*	0.0003^{NS}	0.0001*	0.0001*	0.0003^{NS}	0.0005^{NS}	0.0001*	0.0004^{NS}	0.0001*	0.0001*	0.0001*	0.0002^{NS}	0.0001*	0.0001*	0.0003^{NS}	0.0002^{NS}
Matigulu	0.20	0.09	0.20	0.18	0.15		0.0001*	0.0001*	0.0017^{NS}	0.0001*	0.0002^{NS}	0.0001*	0.0001*	0.0001*	0.0001*	0.0002^{NS}	0.0001*	0.0002^{NS}	0.0002^{NS}	0.0001*	0.0005^{NS}	0.0001*	0.0001*	0.0001*	0.0002^{NS}	0.0001*	0.0001*	0.0002^{NS}	0.0001*
Thukela	0.07	0.08	0.12	0.13	0.11	0.24		0.0001*	0.0174^{NS}	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0002^{NS}	0.0001*	0.0001*	0.0001*	0.0001*
Mvoti	0.15	0.11	0.16	0.20	0.11	0.18	0.11		0.0005^{NS}	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*
Tongati	0.03	0.07	0.09	0.08	0.06	0.18	0.06	0.12		0.0005^{NS}	0.0019^{NS}	0.0017^{NS}	0.0004^{NS}	0.0004^{NS}	0.0016^{NS}	0.0020^{NS}	0.0010^{NS}	0.0015^{NS}	0.0048^{NS}	0.0010^{NS}	0.0051^{NS}	0.0001*	0.0009^{NS}	0.0023^{NS}	0.0026^{NS}	0.0013^{NS}	0.0012^{NS}	0.0048^{NS}	0.0016^{NS}
uMngeni	0.09	0.10	0.16	0.19	0.17	0.26	0.06	0.12	0.11		0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*
uMlazi	0.14	0.14	0.16	0.19	0.15	0.26	0.11	0.10	0.10	0.14		0.0002^{NS}	0.0001*	0.0001*	0.0003^{NS}	0.0001*	0.0001*	0.0005^{NS}	0.0008^{NS}	0.0001*	0.0010^{NS}	0.0001*	0.0001*	0.0003^{NS}	0.0003^{NS}	0.0002^{NS}	0.0001*	0.0009^{NS}	0.0001*
Mzimkhulu	0.10	0.09	0.10	0.14	0.08	0.18	0.08	0.08	0.09	0.11	0.07		0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0004^{NS}	0.0001*	0.0001*	0.0004^{NS}	0.0001*	0.0001*	0.0001*	0.0002^{NS}	0.0001*	0.0001*	0.0003^{NS}	0.0001*
Mtamvuna	0.23	0.24	0.33	0.34	0.30	0.38	0.20	0.24	0.26	0.19	0.23	0.21		0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*
Mamba	0.13	0.16	0.20	0.16	0.16	0.25	0.19	0.25	0.12	0.23	0.23	0.20	0.28		0.0156^{NS}	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0002^{NS}	0.0001*
Balule	0.12	0.16	0.18	0.19	0.16	0.23	0.16	0.21	0.10	0.19	0.20	0.18	0.30	0.03		0.0001*	0.0002^{NS}	0.0005^{NS}	0.0005^{NS}	0.0020^{NS}	0.0007^{NS}	0.0001*	0.0001*	0.0003^{NS}	0.0003^{NS}	0.0001*	0.0001*	0.0005^{NS}	0.0001*
Olifants	0.14	0.20	0.25	0.23	0.21	0.29	0.19	0.25	0.14	0.22	0.25	0.22	0.28	0.10	0.08		0.0001*	0.0010^{NS}	0.0001*	0.0001*	0.0004^{NS}	0.0001*	0.0002^{NS}	0.0004^{NS}	0.0008^{NS}	0.0001*	0.0001*	0.0001*	0.0001*
Loskop Dam	0.14	0.14	0.22	0.20	0.18	0.21	0.16	0.19	0.12	0.18	0.20	0.19	0.23	0.09	0.06	0.10		0.0002^{NS}	0.0002^{NS}	0.0001*	0.0311^{NS}	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*
Nwantsontso	0.11	0.13	0.15	0.13	0.14	0.25	0.15	0.23	0.08	0.20	0.21	0.17	0.30	0.08	0.06	0.07	0.11		0.0266^{NS}	0.0002^{NS}	0.0012^{NS}	0.0001*	0.0003^{NS}	0.0004^{NS}	0.0005^{NS}	0.0001*	0.0002^{NS}	0.0013^{NS}	0.0002^{NS}
Nsikasi	0.15	0.18	0.20	0.17	0.17	0.28	0.19	0.27	0.14	0.26	0.27	0.21	0.33	0.14	0.15	0.12	0.16	0.03		0.0001*	0.0005^{NS}	0.0001*	0.0001*	0.0007^{NS}	0.0001*	0.0002^{NS}	0.0004^{NS}	0.0008^{NS}	0.0002^{NS}
WRCE Dam	0.18	0.23	0.25	0.26	0.22	0.31	0.23	0.30	0.16	0.29	0.30	0.26	0.34	0.10	0.08	0.09	0.13	0.09	0.10		0.0003^{NS}	0.0001*	0.0001*	0.0001*	0.0002^{NS}	0.0001*	0.0002^{NS}	0.0003^{NS}	0.0001*
Komati River	0.09	0.10	0.18	0.18	0.14	0.19	0.13	0.16	0.07	0.15	0.17	0.16	0.22	0.12	0.09	0.13	0.04	0.12	0.17	0.14		0.0001*	0.0001*	0.0003^{NS}	0.0003^{NS}	0.0003^{NS}	0.0001*	0.0009^{NS}	0.0003^{NS}
Pieter Dam	0.13	0.18	0.19	0.17	0.18	0.25	0.22	0.28	0.13	0.25	0.25	0.23	0.33	0.14	0.15	0.20	0.19	0.12	0.15	0.17	0.16		0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*
Crocodile	0.16	0.21	0.25	0.24	0.24	0.30	0.19	0.25	0.16	0.19	0.21	0.22	0.30	0.14	0.09	0.11	0.12	0.13	0.21	0.18	0.15	0.22		0.0059^{NS}	0.0009^{NS}	0.0002^{NS}	0.0001*	0.0002^{NS}	0.0001*
Lephalale	0.13	0.20	0.23	0.22	0.22	0.29	0.17	0.23	0.13	0.16	0.19	0.20	0.26	0.14	0.10	0.05	0.13	0.10	0.17	0.15	0.13	0.20	0.05		0.0109^{NS}	0.0002^{NS}	0.0003^{NS}	0.0002^{NS}	0.0002^{NS}
Mogalakwena	0.13	0.20	0.22	0.22	0.20	0.30	0.17	0.24	0.10	0.18	0.19	0.20	0.27	0.12	0.07	0.08	0.12	0.10	0.18	0.12	0.11	0.20	0.06	0.03		0.0001*	0.0195^{NS}	0.0005^{NS}	0.0001*
Sand River	0.11	0.16	0.19	0.20	0.17	0.27	0.13	0.21	0.10	0.15	0.18	0.17	0.25	0.09	0.05	0.11	0.09	0.09	0.17	0.14	0.11	0.17	0.10	0.09	0.06		0.0001*	0.0003^{NS}	0.0001*
Luvuvhu	0.14	0.21	0.24	0.24	0.21	0.28	0.20	0.26	0.13	0.22	0.24	0.21	0.27	0.09	0.06	0.08	0.09	0.09	0.16	0.10	0.10	0.19	0.08	0.07	0.04	0.10		0.0004^{NS}	0.0001*
Shingwedzi	0.20	0.25	0.30	0.29	0.25	0.31	0.25	0.28	0.18	0.27	0.30	0.27	0.36	0.14	0.13	0.18	0.16	0.18	0.24	0.20	0.18	0.21	0.23	0.20	0.17	0.11	0.17		0.0004^{NS}
Letaba	0.18	0.23	0.26	0.25	0.23	0.28	0.23	0.26	0.14	0.26	0.25	0.24	0.34	0.12	0.08	0.14	0.14	0.19	0.15	0.15	0.20	0.11	0.15	0.16	0.12	0.13	0.10	0.17	

F_{ST}-values < 0.05 indicate little genetic differentiation, 0.05-0.15 = moderate differentiation, 0.15-0.25 = significant differentiation, and > 0.25 = very great differentiation (given in bold) (Wright 1965). * Significant P-values and not significant (NS) at P< 0.0001

Supplementary Table 2.6: Analysis of Molecular Variance (AMOVA) Groupings at 14 microsatellite loci for *O. mossambicus* from the 29 sampling localities in Limpopo, Mpumalanga, and KwaZulu-Natal. Various groupings within each level of water resource management strategy are shown.

Grouping/ scenario	Source of variation	Sum of squares	Variance components	Percentage of variation	P value <0.001
Within old WMA_2004					
uSuthu-Mhlathuze	Among groups	78.85	0.42	9%	0.000
	Among sites within groups	379.81	1.59	35%	0.000
	within sites	185.50	2.58	56%	0.000
uMvoti-Mzimkhulu	Among groups	113.87	0.67	15%	0.000
	Among sites within groups	389.50	1.20	27%	0.000
	within sites	217.50	2.59	58%	0.000
Olifants	Among groups	56.61	0.51	9%	0.000
	Among sites within groups	298.90	1.54	28%	0.000
	within sites	171.00	3.42	63%	0.000
Komati	Among groups	122.09	0.79	14%	0.000
	Among sites within groups	474.21	2.18	40%	0.000
	within sites	188.50	2.51	46%	0.000
Limpopo	Among groups	68.63	0.813	14%	0.000
	Among sites within groups	237.95	1.530	26%	0.000
	within sites	142.00	3.550	60%	0.000

Luvuvhu-Letaba	Among groups	47.80	0.90	15%	0.000
	Among sites within groups	172.08	1.57	26%	0.000
	within sites	101.00	3.48	59%	0.000
Within new WMA_2012					
Pongola-Mtamvuna	Among groups	274.70	0.66	14%	0.000
	Among sites within groups	881.63	1.43	31%	0.000
	within sites	453.00	2.57	55%	0.000
Olifants	Among groups	109.90	0.69	12%	0.000
	Among sites within groups	402.23	1.48	26%	0.000
	within sites	239.50	3.47	62%	0.000
Komati-uSuthu	Among groups	122.90	0.80	14%	0.000
	Among sites within groups	474.21	2.18	40%	0.000
	within sites	188.50	2.51	46%	0.000
Limpopo	Among groups	99.08	0.66	12%	0.000
	Among sites within groups	360.55	1.63	29%	0.000
	within sites	205.00	3.42	60%	0.000
Within primary catchment					
W	Among groups	78.85	0.42	9%	0.000
	Among sites within groups	379.81	1.59	35%	0.000

	within sites	185.50	2.58	56%	0.000
U	Among groups	53.64	0.47	11%	0.000
	Among sites within groups	296.40	1.44	32%	0.000
	within sites	148.50	2.52	57%	0.000
T	Among groups	27.52	0.98	22%	0.000
	Among sites within groups	93.10	0.64	15%	0.000
	within sites	69.00	2.76	63%	0.000
B	Among groups	109.90	0.69	12%	0.000
	Among sites within groups	402.23	1.46	26%	0.000
	within sites	239.50	3.47	62%	0.000
X	Among groups	122.90	0.79	14%	0.000
	Among sites within groups	474.21	2.18	40%	0.000
	within sites	188.50	2.51	46%	0.000
A	Among groups	99.08	0.66	12%	0.000
	Among sites within groups	360.55	1.63	29%	0.000
	within sites	205.00	3.42	60%	0.000
Within secondary catchment					
W3	Among groups	13.62	0.29	7%	0.000
	Among sites within groups	152.03	1.54	35%	0.000

	within sites	74.00	2.55	58%	0.000
W1	Among groups	18.70	0.70	16%	0.000
	Among sites within groups	83.40	0.92	21%	0.000
	within sites	56.00	2.80	63%	0.000
B7	Among groups	28.341	0.35	7%	0.000
	Among sites within groups	196.89	1.39	26%	0.000
	within sites	121.50	3.57	67%	0.000
X2	Among groups	25.83	0.51	11%	0.000
	Among sites within groups	115.87	1.85	39%	0.000
	within sites	53.00	2.41	51%	0.000
X1	Among groups	36.83	0.90	16%	0.000
	Among sites within groups	298.56	2.22	38%	0.000
	within sites	117.50	2.67	46%	0.000
A7	Among groups	34.48	1.39	22%	0.000
	Among sites within groups	119.45	1.61	25%	0.000
	within sites	68.50	3.42	53%	0.000
Within tertiary catchment					
W32	Among groups	13.62	0.29	7%	0.000
	Among sites within groups	152.03	1.54	35%	0.000
	within sites	74.00	2.55	58%	0.000

B73	Among groups	28.34	0.35	7%	0.000
	Among sites within groups	196.89	1.39	26%	0.000
	within sites	121.50	3.57	67%	0.000
X13	Among groups	36.83	0.90	16%	0.000
	Among sites within groups	298.56	2.22	38%	0.000
	within sites	117.50	2.67	46%	0.000
A71	Among groups	34.48	1.39	22%	0.000
	Among sites within groups	119.45	1.61	25%	0.000
	within sites	68.50	3.43	53%	0.000
Within quaternary catchment					
B73H	Among groups	13.57	0.37	7%	0.000
	Among sites within groups	111.06	1.46	27%	0.000
	within sites	68.50	3.61	66%	0.000
Within municipalities					
Mandeni	Among groups	37.84	1.22	24%	0.000
	Among sites within groups	149.73	1.43	28%	0.000
	within sites	74.50	2.48	48%	0.000
KwaDukuza	Among groups	16.18	0.49	11%	0.000
	Among sites within groups	167.03	1.46	32%	0.000

	within sites	85.00	2.66	58%	0.000
Ba-Phalaborwa	Among groups	54.08	0.534	10%	0.000
	Among sites within groups	258.39	1.463	26%	0.000
	within sites	155.50	3.534	64%	0.000
Nkomazi	Among groups	50.71	0.87	15%	0.000
	Among sites within groups	307.06	2.13	37%	0.000
	within sites	128.00	2.72	48%	0.000
Mbombela	Among groups	15.26	0.47	10%	0.000
	Among sites within groups	107.37	2.04	43%	0.000
	within sites	42.50	2.24	47%	0.000
Musina	Among groups	48.43	0.86	14%	0.000
	Among sites within groups	188.20	1.80	30%	0.000
	within sites	101.00	3.37	56%	0.000
Within districts					
uMkhanyakude	Among groups	44.14	0.35	7%	0.000
	Among sites within groups	291.10	1.86	40%	0.000
	within sites	126.50	2.48	53%	0.000
iLembe	Among groups	72.70	0.65	14%	0.000
	Among sites within groups	316.75	1.44	31%	0.000
	within sites	159.50	2.57	55%	0.000

Ugu	Among groups	27.52	0.98	22%	0.000
	Among sites within groups	93.10	0.64	15%	0.000
	within sites	69.00	2.76	63%	0.000
Mopani	Among groups	81.43	0.67	12%	0.000
	Among sites within groups	300.23	1.34	24%	0.000
	within sites	190.00	3.59	64%	0.000
Ehlanzeni	Among groups	122.09	0.79	14%	0.000
	Among sites within groups	474.21	2.18	40%	0.000
	within sites	188.50	2.51	46%	0.000
Waterberg	Among groups	10.28	0.20	4%	0.001
	Among sites within groups	112.25	1.46	29%	0.000
	within sites	66.50	3.33	67%	0.000
Vhembe	Among groups	63.00	0.71	12%	0.000
	Among sites within groups	248.30	1.72	29%	0.000
	within sites	138.50	3.46	59%	0.000
Within provinces					
Limpopo	Among groups	159.80	0.83	14%	0.000
	Among sites within groups	463.88	1.54	26%	0.000
	within sites	273.50	3.46	59%	0.000

Mpumalanga	Among groups	226.85	0.77	14%	0.000
	Among sites within groups	773.11	1.92	35%	0.000
	within sites	359.50	2.88	52%	0.000
KwaZulu-Natal	Among groups	274.70	0.66	14%	0.000
	Among individuals within groups	881.63	1.417	31%	0.000
	within sites	453.00	2.57	55%	0.000
Within ecoregions					
Eco3	Among groups	284.73	0.94	17%	0.000
	Among sites within groups	793.07	1.75	31%	0.000
	within sites	394.00	2.94	52%	0.000
Eco13	Among groups	8.53	0.13	3%	0.008
	Among sites within groups	135.18	1.59	38%	0.000
	within sites	64.00	2.46	59%	0.000
Eco17	Among groups	139.06	0.85	18%	0.000
	Among sites within groups	395.52	1.23	26%	0.000
	within sites	219.00	2.61	56%	0.000
Eco16	Among groups	19.15	0.53	12%	0.000
	Among sites within groups	143.70	1.35	31%	0.000
	within sites	73.00	2.43	56%	0.000

Eco12	Among groups	21.50	0.69	12%	0.000
	Among sites within groups	142.60	2.71	46%	0.000
	within sites	50.00	2.50	42%	0.000
Eco1	Among groups	87.04	0.76	13%	0.000
	Among sites within groups	291.80	1.52	26%	0.000
	within sites	172.50	3.45	60%	0.000
Within biomes					
Grassland	Among groups	692.96	1.01	18%	0.000
	Among sites within groups	1711.23	1.74	31%	0.000
	within sites	851.50	2.94	52%	0.000
Forest	Among groups	31.81	0.53	12%	0.000
	Among sites within groups	137.13	1.10	25%	0.000
	within sites	83.50	2.69	62%	0.000
Savanna	Among groups	86.12	0.73	16%	0.000
	Among sites within groups	270.26	1.22	27%	0.000
	within sites	151.00	2.56	57%	0.000

CHAPTER 3

Genetic assessment of farmed *Oreochromis mossambicus* populations in KwaZulu-Natal and Mpumalanga Provinces, South Africa

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Running header: Genetic diversity of farmed *O. mossambicus* in South Africa

3.1 Abstract

The global utilisation of *Oreochromis* spp. in freshwater aquaculture extends to South Africa, where the native Mozambique tilapia (*Oreochromis mossambicus*) has been proposed as a priority species for regional aquaculture projects, although it is still not preferred over *O. niloticus*. Despite its significance, there is limited understanding of the genetic diversity, integrity, and genetic differentiation of four farmed (University of Zulu-Land aquaculture ponds, Zini Fish Farm, Fresca Fisheries Farm, and uMphafa ponds ponds) O. *mossambicus* populations in KwaZulu-Natal and Mpumalanga provinces, South Africa. Using a suite of 14 microsatellite markers, the present study aimed to determine the origin and genetic diversity of the farmed *O. mossambicus* populations. Wild *O. mossambicus* from rivers surrounding the farms were included to trace the origin of farmed individuals. The results indicated higher genetic diversity in the farmed sampled localities compared with individuals from surrounding wild sampled localities, except for the University of Zululand sample locality. While the farmed sample localities generally showed genetic similarity to wild sample localities, the uMphafa ponds exhibited distinctive genetic characteristics. Notably, some individuals from the uMphafa ponds shared genetic affinities with individuals in the Thukela River, suggesting either that the Thukela River could be the source of this farmed sample locality or that some of the farmed individuals have escaped or were introduced into the Thukela River. This finding underscores the need for careful monitoring and potential supplementation from other sources. The study indicated the potential use of farmed fish from uMphafa and Fresca Fisheries for selective breeding and broodstock supplementation, emphasising the importance of maintaining genetic diversity in aquaculture practices. Recommendations include supplementing farmed broodstock from the University of Zululand with individuals from other farms or wild sample localities, with close monitoring of genetic diversity levels across all farmed sample localities.

Keywords: *Oreochromis mossambicus*, aquaculture, broodstock, conservation genetic diversity, microsatellite markers.

3.2 Introduction

Anthropogenic activities have a major impact on both wild and farmed fish stocks, and freshwater aquaculture practices have led to changes in gene flow patterns of wild fish populations and the release of invasive species into native ecosystems (Svåsand et al. 2007, Crispo et al. 2011, Firmat et al. 2013, Garg et al. 2014, Bolstad et al. 2021). Baseline data on the genetic diversity and population structure of both farmed and wild populations of species used in aquaculture are essential for the sustainable management of farmed stocks and ensuring the long-term persistence of wild populations (Bert 2007, Cossu et al. 2021, Sonesson et al. 2023). Wild populations are an important genetic Dam for aquaculture (Brummett 2008, Brummett and Ponzoni 2009, Lind et al. 2012, Firmat et al. 2013, Garg et al. 2014, Aguiar et al. 2018, Liu et al. 2018, Nyinondi et al. 2020, Fagbѐmi et al. 2021, Sonesson et al. 2023). Monitoring the change in genetic variation in farmed populations is very important because inbreeding and genetic drift in small, farmed populations can reduce reproductive output and survivorship, reducing productivity (Glover et al. 2010, Khadher et al. 2016, Aguiar et al. 2018, Lal et al. 2021).

Members of the *Oreochromis* genus are used extensively in aquaculture around the world (Gupta and Acosta 2004, D'Amato et al. 2007, Eknath and Huluta 2009, Briñez et al. 2011, Wu and Yang 2012, Simbine et al. 2014, Shoemaker et al. 2017, Diedericks et al. 2021). Among the approximately 70 tilapia species, the Nile tilapia (*Oreochromis niloticus*), Mozambique tilapia (*O. mossambicus*), and blue tilapia (*O. aureus*) are the main focus of aquaculture, driven by their hardiness, ease of cultivation, and rapid growth (FAO 2002, FAO 2019). Over the past decades, tilapia genetic resources, both in the wild and in culture, have been inadequately managed (Kocher et al. 1998). Management of tilapia farming in Africa, including South Africa, needs to be prioritised. This can be achieved by enhancing current management and legislative practices, which involve protecting wild tilapia stocks and developing stricter regulations for farmed stock. Current measures include various national policies and guidelines aimed at sustainable aquaculture (Weyl et al. 2021, Naylor and Fanzo 2023), but there is a need for more rigorous enforcement and updated strategies to address emerging challenges in tilapia farming.

Oreochromis mossambicus is one of the crucial species utilised in aquaculture (D'Amato et al. 2007, Firmat et al. 2013, FAO 2019, Fagbėmi et al. 2021), but natural populations of this species are at risk because of genetic admixture with *O. niloticus* (Firmat et al. 2013) and other *Oreochromis* species (Romana-Eguia et al. 2004). According to the IUCN Red List, *O. mossambicus* is now classified as Vulnerable due to the potential for hybridisation with the introduced Nile tilapia (*O. niloticus*), as well as competition with other tilapia species (Bills 2019, Mojekwu et al. 2021). Anthropogenic translocation of *O. niloticus* within South Africa has led to reported cases of interspecific hybridisation with native *O. mossambicus* (D'Amato et al. 2007, Firmat et al. 2013, Bills 2019). Additionally, Nile tilapia often competes with Mozambique tilapia for growth, food, shelter, territory, and breeding sites within their shared habitat (Esterhuyse 2002). Important genetically linked features of *O. mossambicus*, such as adaptation to a wide range of environmental conditions (resistance to drought, tolerance of high salinity and low temperature), are threatened by both hybridisation and competition (Moralee et al. 2000, D'Amato et al. 2007).

Based on estimates by the Tilapia Association of South Africa, there may be as many as 74 producers of tilapia in South Africa (DAFF 2016). None of these facilities implement routine genetic monitoring of their farmed stock. This study evaluated the genetic diversity, and differentiation within four farmed *O. mossambicus* localities: three aquaculture facilities from KwaZulu-Natal (Zini Fish Farm, University of Zululand Fish Farm and uMphafa ponds) and one (Fresca Fisheries Farm) from Mpumalanga, South Africa were included in the study. In the KwaZulu-Natal and Mpumalanga provinces of South Africa, *O. mossambicus* occurs in all the major river catchments and is also farmed. Aquaculture practices suggest that the founder stock for farmed stocks comes from rivers surrounding the fish farms (various pers comm.). There is potential, however, for translocations to have occurred, with farms stocked using fish from many different river catchments. If these fish are released into natural systems, this could disrupt existing patterns of genetic variation, leading to genetic homogenisation, reduced resilience, and adaptability of natural populations. To determine the genetic admixture of the farmed individuals, wild *O. mossambicus* from five rivers in KwaZulu-Natal (Mfolozi, Mhlathuze, Matigulu, Thukela, and Mvoti) and three potential sources of fish in Mpumalanga (Komati River, Loskop Dam and Pieter Vorster Dam) surrounding the aquaculture farms were included. Importantly, this study provides baseline information about the genetic diversity of farmed *O. mossambicus* individuals used as broodstock in KwaZulu-Natal and Mpumalanga. This information is crucial for informing conservation strategies and improving management practices in aquaculture to ensure the long-term sustainability and genetic health of these populations.

3.3 Methods

3.3.1 Study area

The study was conducted in the KwaZulu-Natal and Mpumalanga provinces of South Africa (Figure 3.1). KwaZulu-Natal, being a coastal province, presents opportunities for both marine and freshwater aquaculture because of its warm climate (DAFF 2016, Oyeleke 2017). Similarly, Mpumalanga, with its favourable climate, is conducive to freshwater aquaculture (Oyeleke 2017). In the KwaZulu-Natal Province, three localities engage in the cultivation of Mozambique tilapia. These farms include Zini Fish Farm, which is connected to the Mlalazi estuary, the University of Zululand Aquaculture Research Unit, and the uMphafa Private Nature Reserve, which utilises natural ponds for Mozambique tilapia breeding. The *O. mossambicus* at Zini Fish Farm is thought (Gordon O'Brien, pers comm) to have originated from the Mlalazi estuary and surrounding northern KwaZulu-Natal rivers (Mfolozi, Matigulu, Mhlathuze, Thukela, and Mvoti). The establishment of *O. mossambicus* at Zini Fish Farm resulted from their thriving in earthen ponds initially designated for dusky kob (*Argyrosomus japonicus*) breeding. The University of Zululand likely (Gordon O'Brien, pers comm) originated from fish collected in northern KwaZulu-Natal, although the exact source remains unclear. The broodstock source for the uMphafa ponds is also uncertain. However, considering the proximity of these ponds to the Thukela River in northern KwaZulu-Natal, individuals from the Thukela River were included in the study to investigate if it could be the source of the uMphafa ponds locality. In Mpumalanga, one farm (Fresca Fisheries Farm), presently cultivates *O. mossambicus* believed (Holt Lance, pers comm) to have originated from the Komati River and the Loskop Dam, which are linked to the Olifants catchment.

3.3.2 Collection of DNA samples

The study adhered to all necessary ethical and legal protocols. Permits for sampling were diligently obtained from Ezemvelo KwaZulu-Natal Wildlife (OP1583/2017, OP1432/2018, and OP871/2021) and Mpumalanga Tourism and Parks Agency (MPB. 56932). Moreover, the study received ethical clearance from the University of KwaZulu-Natal Animal Ethics Subcommittee (REF: AREC/023/020). DNA sampling involved the collection of ten samples from each of the four aquaculture farms: Zini Fish Farm, University of Zululand Aquaculture Facility, uMphafa ponds, and Fresca Fisheries (Figure 3.1, Table 3.3). Wild samples were obtained from the surrounding river catchments of these farms during 2017-2018. In KwaZulu-Natal, wild individuals were sourced from the Mfolozi, Mhlathuze, Matigulu, Thukela, and Mvoti rivers. In Mpumalanga, wild individuals were collected from the Komati River, Pieter Vorster Dam, and Loskop Dam (Figure 3.1, Table 3.3).

Collection methods varied based on the habitat. Fish from farms were obtained from ponds and tanks using a scooping net and seine net. Wild specimens, on the other hand, were collected using established passive techniques, including fyke nets, electro-fishing, seine nets, and gill nets. Upon capture, *Oreochromis mossambicus* individuals were identified to species level following Skelton (2001) and anesthetised using clove oil (0.5 ml/L) as per Bennett et al. (2016). Each fish underwent measurements for weight (g) and length (standard and total length in mm) and was photographed for reference. A non-lethal fin clip, approximately $5mm^2$ to $10mm^2$, was taken from each anesthetised fish, promptly preserved in 99% ethanol, stored in a refrigerator, and transported to the laboratories of the University of KwaZulu-Natal for subsequent genetic analyses. The captured fish were then placed in a recovery bucket and released back into their respective capture locations.

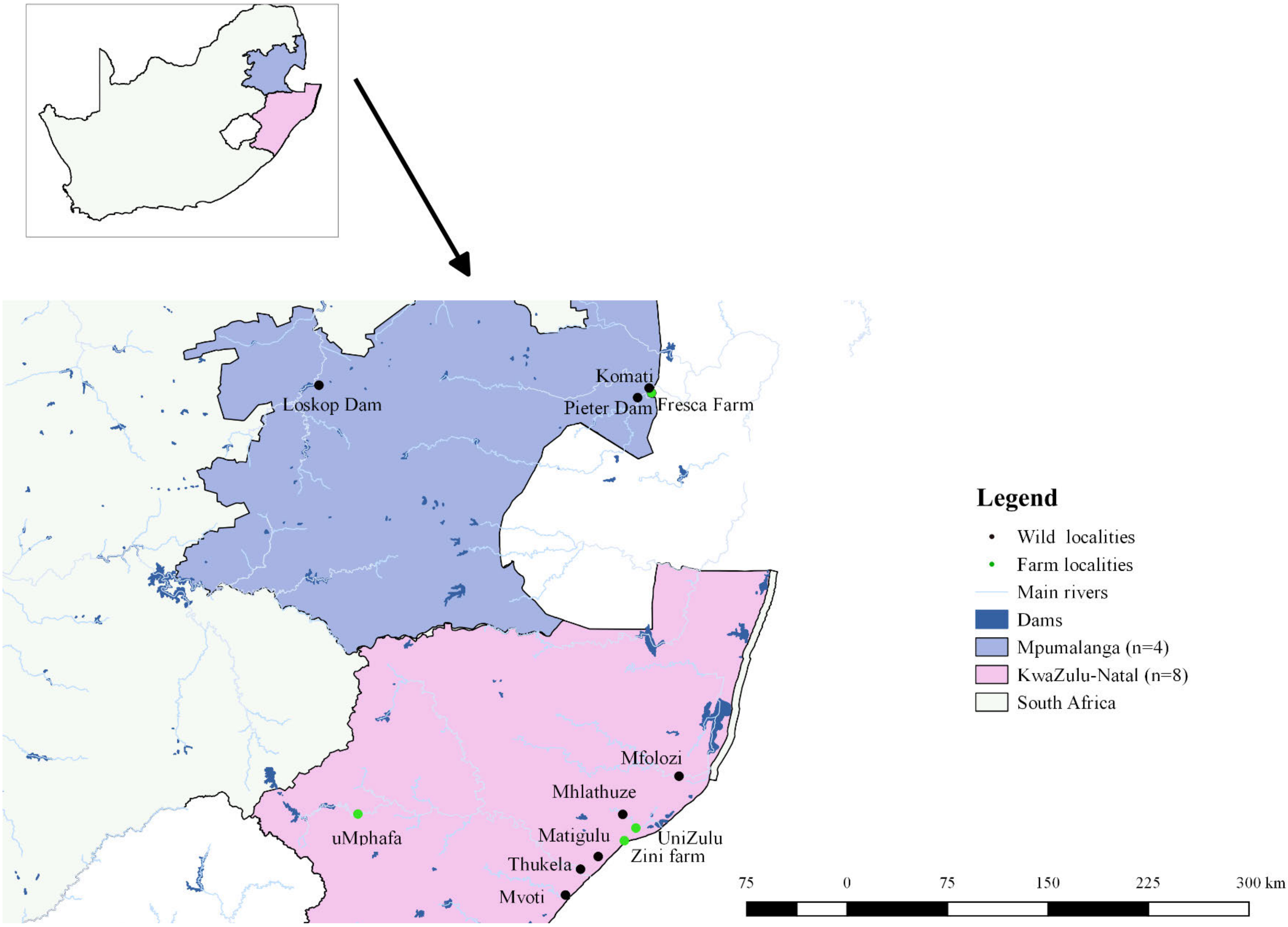


Figure 3.1: Sampling localities of farmed and wild-caught *O. mossambicus* in KwaZulu-Natal and Mpumalanga provinces, South Africa. The figure illustrates four sampling localities of farmed *O. mossambicus* from KwaZulu-Natal (Zini Fish Farm, University of Zululand Fish Farm, and uMphafa ponds) and Mpumalanga (Fresca Fisheries Farm). Additionally, it shows wild fish sampling locations from river catchments in KwaZulu-Natal (Mfolozi, Mhlathuze, Matigulu, Thukela, and Mvoti) and three sources in Mpumalanga (Komati River, Loskop Dam, and Pieter Vorster Dam), which could be potential sources of the farmed individuals.

3.3.3 DNA extraction and amplification

The DNA extraction from fin clips was performed using the Nucleospin®Tissue kit (Macherey-Nagel, Germany) following the standard animal tissue protocol. Extracted DNA samples were then stored at -20°C. This study utilised 14 microsatellite loci, originally isolated from *O. niloticus* (Hall 2001, Simbine et al. 2014) and *O. mossambicus* (Saju et al. 2010) (Table 3.,1). In each primer pair, the forward primer was fluorescently labelled. The PCR profiles were initially conducted as singleplexes for each of the 14 microsatellites. Subsequently, working primers were selected and grouped into multiplexes (A, B, C, and D) based on annealing temperature, fluorescence dye, and product size. Multiplex A and D had an annealing temperature of 60°C, while multiplex B and C had an annealing temperature of 61°C. Each PCR reaction was prepared to a final volume of 10 µl, comprising 0.1 µl of each primer, 5 µl of 2G Fast Multiplex Mix (KAPA Biosystems, Cape Town, South Africa), 0.3 µl of BSA, 4.2 µl of distilled water, and 0.3 µl of DNA. Thermocycler conditions included an initial 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 15 s, annealing for 30 s, extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. The annealing temperature was set at 60°C for multiplex A and D, and 61°C for multiplex B and C. Fragment analyses were conducted at the Central Analytical Facilities (CAF), University of Stellenbosch, South Africa, using a 3500XL Genetic Analyzer from Applied Biosystems (Thermo Fisher). To ensure genotype accuracy, 20% of all individuals underwent re-amplification.

Table 3.1: Microsatellite loci used in the present study for genotyping of *O. mossambicus* farmed and wild individuals. Fluorescent dye and annealing temperature used in the present study are provided. In some cases, the annealing temperature differs from that presented in the literature; the temperatures provided here were optimised for each multiplex.

Multiplex loci	Locus	Sequence	Fluorescent Dye	Annealing Temperature °C
A	OM01[c]	F: TTTAAAGTTACACAGCAGTACAAAG R: TTGTAGCATTTCAACACAGTCTC	Fam	60
D	OM02[c]	F: TGTGAATTTGACAACTTCCTTTC R: ATCCTTGCAATAAGGTTACAG	Fam	60
D	OM03[c]	F: CTTTTTAATGAGCAACTTTTAAGTC R: TGTGAATTTGACAACTTCCTTTC	Hex	60
A	OM04[c]	F: AGCTCAAAACCTCATACAAAGG R: GCAGAGATGTCAGATGTTGTTC	Fam	60
B	OM05[c]	F: GTAAAGTTTGGAACAGAAATGCT R: GATCACTTTTGGACAGACTGG	Hex	61
B	OM06[c]	F: TGAGCTACCGTAAGGATGTAC R: GTTATTTCAATTATATTTGCATG	Fam	61
C	OM07[c]	F: TTGGCTCAGAGTGGTCAGG R: CGCGTGGACTAAAAGCCAG	Hex	61
B	OM08[c]	F: TGTTGGTTGGATTACTGGG R: GCTGTAATGGTTTTGAGGC	Fam	61
B	OM09[c]	F: GGCTACAACACCTGGATGG R: TTGGGCTTACTGAAGCTGAC	Hex	61
C	UNH104[a]	F: GCAGTTATTTGTGGTCACTA R: GGTATATGTCTAACTGAAATC	Tet	61
C	UNH129[a]	F: AGAAGTCGTGCATCTCTC R: TGTACATCATCTGTGGG	Tet	61
B	UNH142[b]	F: CTTTACGTTGACGCAGT R: GTGACATGCAGCAGATA	Tet	61
B	UNH222[b]	F: CTCTAGCACACGTGCAT R: TAACAGGTGGGAACTCA	Tet	61
C	UNH231[b]	F: GCCTATTATAGTCAAAGCGT R: ATTTCTGCAAAAGTTTTCC	Tet	61

Primers taken from Hall (2001)[a] Simbine et al. (2014) [b] and Saju et al. (2010) [c]

3.3.4 Molecular data analyses

Genotypes were scored using GeneMarker® version 2.4 (Hulce et al. 2011). Null allele frequencies were computed for both farmed and wild *O. mossambicus* individuals using FreeNA version 20091116 (Chapuis and Estoup 2007). To assess whether null alleles significantly influenced population structure estimates, both null allele-excluded (ENA) corrected and non-corrected global F_{ST} values were calculated in FreeNA. Subsequently, these values were compared using a student t-test in Microsoft Excel 2016.

3.3.5 Genetic diversity

Polymorphic Information Content (PIC) for each microsatellite locus was computed using Cervus version 3.0.7 (Marshall et al. 1998, Kalinowski et al. 2007). PIC serves as a metric indicating the suitability of each microsatellite locus for assessing genetic differentiation between farmed and wild sampling localities (Botstein et al. 1980). Loci with PIC > 0.5 are highly informative, while those with PIC < 0.25 are considered less informative (Botstein et al. 1980). The total number of alleles (Na), effective number of alleles (Ne), unbiased expected and observed heterozygosity (uHe and Ho) for the 14 microsatellites amplified from both farmed and wild *O. mossambicus* were calculated using GenAlEx version 6.5 (Peakall and Smouse 2012). Allelic richness values were obtained using FSTAT version 2.9.3.2 (Goudet 2001). Tests for deviation from Hardy-Weinberg equilibrium were performed using GenAlEx. Linkage disequilibrium (LD) was estimated using Genepop version 4.7.0 (Raymond 1995, Rousset 2008). In the latter analyses, chi-square p-values were obtained using the Markov Chain method with 10,000 replicates, 100 groups, and 5000 iterations per group. Bonferroni corrections were applied to both the HWE and LD tests to account for multiple comparisons and ensure the robustness of the statistical analyses. The inbreeding coefficient (Fis) was calculated using GenAlEx. Positive high Fis-values indicate high levels of inbreeding, characterised by an excess of homozygotes, while negative Fis-values indicate outbreeding and an excess of heterozygosity in the population (Simbine et al. 2014).

3.3.6 Population genetic structure

The Bayesian clustering method, implemented in STRUCTURE version 2.3.4 (Pritchard et al. 2000), was employed to identify potential genetic divisions within the farmed and wild *O. mossambicus* sampling localities from KwaZulu-Natal and Mpumalanga. Assignment tests were conducted using the admixture model with correlated allele frequencies. The STRUCTURE analyses comprised 500,000 Markov-Chain Monte Carlo (MCMC) replicates with a 50,000 generation burn-in period. Ten iterations were performed for K values ranging from 1 to 10. The STRUCTURE selector software version 2.3 (Li and Liu 2018) was utilised to determine the optimal number of genetic clusters (K-value) using the Puechmaille method (Puechmaille 2016). STRUCTURE harvester version 0.6.94 (Earl and VonHoldt, 2012) was employed to obtain membership coefficient values (Q-values) for the optimal K. Bar plots for optimal K-values were generated using Clumpak version 1.1 (Kopelman et al. 2015). Population structure was also visualised by plotting Nei's genetic distances (Nei 1972) through Principal Coordinate Analysis (PCoA) in GenAlEx.

Analysis of Molecular Variance (AMOVA) in GenAlEx was employed to assess the significance of genetic differentiation among all sites (Excoffier et al. 1992). The test, based on 9999 permutations, first evaluated the significance of genetic differentiation between farmed and wild sampling localities from KwaZulu-Natal. Subsequently, another AMOVA tested the significance of genetic differentiation between the farmed and wild *O. mossambicus* in

Mpumalanga. Pairwise F_{ST}-values, quantifying genetic differentiation between farmed and wild *O. mossambicus* using the ENA correction method in FreeNA, were calculated. The significance of genetic differentiation between farmed and wild *O. mossambicus* was assessed using FSTAT software (version 2.9.3.2; Goudet 2001). The probability values (p-values) were calculated based on 1,320 permutations to account for multiple comparisons. A significance level of 0.000758 was adopted after the Bonferroni correction to control for error rate.

3.4 Results

A total of 173 individuals from four farmed and eight wild *O. mossambicus* from KwaZulu-Natal and Mpumalanga were successfully genotyped using 14 microsatellite loci. Not all microsatellites were amplifiable in all individuals, and some missing data were included in the data matrix, but no microsatellite locus had more than 27% missing data. Fourteen loci were highly polymorphic for the farmed and wild *O. mossambicus,* with PIC values ranging from 0.50 (UNH231) to 0.90 (OM05 and OM07) (Table 2.2). Null allele frequencies of the 14 loci varied from 0.02 (OM07) to 0.25 (UNH222) (Table 2.2). Both corrected (excluding null alleles, ENA) and non-corrected F_{ST}-values were computed and there were no discernible differences between the F_{ST}-values, indicating that the inclusion of null alleles did not influence the estimation of population structure. All data, including those with null alleles, were retained for subsequent analyses.

3.4.1 Genetic diversity

The microsatellite loci employed in this study exhibited a mean of 4.98 alleles per locus. The allelic diversity ranged from 2.17 (OM06) to 9.25 (OM07), demonstrating substantial variability across the markers (Table 3.2). Allelic richness, a measure accounting for sample size differences, varied from 1.94 (OM06) to 7.04 (OM07). However, this variation in Ar across all loci could have been influenced by sample sizes across loci as some loci had missing data because of genotyping failures. The observed heterozygosity showed considerable variation, ranging from 0.10 (UNH231) to 0.92 (OM07). Interestingly, the unbiased expected heterozygosity was consistently higher than the observed heterozygosity across all loci, accounting for some of the notable deviations from Hardy-Weinberg Equilibrium. Particularly, locus OM07 exhibited pronounced observed heterozygosity in comparison to unbiased expected heterozygosity. The unbiased expected heterozygosity ranged from 0.22 (UNH231) to 0.84 (OM07) (Table 3.2). Inbreeding coefficients (F_{is}) for most loci were relatively low, indicative of limited inbreeding, but notable exceptions included UNH142, UNH222, UNH231, and OM02, where F_{is} -values approached 1, reflecting a deficiency of heterozygotes. The spectrum of F_{is}-values varied from -0.04 (OM04) to 0.74 (UNH222) (Table 3.2). These results provide insights into the genetic characteristics of *O. mossambicus*, revealing both diversity and specific loci with potential deviations from expected genetic patterns.

Table 3.2: Genetic diversity indices of the 14 microsatellite loci of the four farmed and eight wild *O. mossambicus* in the present study.

Locus	**Na**	**Null allele frequencies**	**F_{ST}^{A}**	**F_{ST}^{B}**	**Ar**	**H_O**	**uH_E**	**F_{is}**	**PIC**
OM01	7.75	0.11	0.15	0.13	6.06	0.57	0.80	0.26	0.87
OM02	5.25	0.14	0.21	0.21	4.17	0.29	0.54	0.45	0.82
OM03	5.42	0.13	0.22	0.22	4.22	0.33	0.55	0.37	0.85
OM04	8.25	0.04	0.09	0.09	6.43	0.82	0.83	-0.04	0.88
OM05	5.67	0.15	0.20	0.16	4.96	0.51	0.75	0.28	0.90
OM06	2.17	0.13	0.52	0.49	1.94	0.16	0.30	0.43	0.68
OM07	9.25	0.02	0.09	0.09	7.04	0.92	0.84	-0.14	0.90
OM08	2.83	0.08	0.27	0.27	2.65	0.43	0.48	0.07	0.73
OM09	4.83	0.08	0.30	0.30	3.83	0.45	0.56	0.15	0.77
UNH104	4.67	0.17	0.21	0.18	3.90	0.38	0.65	0.38	0.78
UNH142	2.92	0.18	0.30	0.25	2.61	0.20	0.45	0.53	0.75
UNH129	4.83	0.07	0.13	0.13	3.56	0.42	0.48	0.09	0.69
UNH222	3.42	0.25	0.25	0.22	3.08	0.13	0.53	0.74	0.75
UNH231	2.42	0.09	0.10	0.18	2.08	0.10	0.22	0.55	0.50
Mean	**4.98**	**0.12**	**0.22**	**0.21**	**4.04**	**0.41**	**0.57**	**0.30**	**0.78**

Na: number of alleles, Ar: Allelic richness, F_{ST}^{A}: non-corrected F_{ST}, F_{ST}^{B}: ENA corrected F_{ST}, H_O: observed heterozygosity, uH_E: unbiased expected heterozygosity. F_{is}: Inbreeding coefficient, P PIC: polymorphic information content.

The farmed O. *mossambicus* sampling localities exhibited a mean recovery of 3.81 alleles, whereas the wild sampling localities displayed a higher mean of 5.56 alleles. The number of alleles (Na) observed in both farmed and wild sampling localities varied across locations, ranging from 2.86 (uMphafa ponds) to 7.79 (Pieter Vorster Dam) (Table 3.3). The effective number of alleles (Ne) for farmed sampling localities ranged from 2.17 (uMphafa ponds) to 3.42 (Zini Fish Farm), while wild sampling localities showed variability from 2.61 (Matigulu) to 4.50 (Loskop Dam). The mean observed heterozygosity (H_O) in farmed sampling localities was 0.43, while wild sampling localities were 0.40 (Table 3.3). Ho across both farmed and wild sampling localities were generally lower compared with unbiased expected heterozygosity (uH_E), with the uMphafa ponds *O. mossambicus* being an exception as it exhibited higher H_O than uHe (Table 3.3). The mean uH_E in farmed sampling localities was 0.53, while wild sampling localities had a mean of 0.59. The observed heterozygosity ranged from 0.31 to 0.51, and unbiased expected heterozygosity varied from 0.46 to 0.73 across all sampling localities (Table 3.3). The mean Inbreeding coefficient (F_{is}) was relatively lower in farmed sampling localities (0.17) than in wild sampling localities (0.35), with values ranging from -0.10 in the uMphafa ponds to 0.45 in the Pieter Vorster Dam (Table 3.3). No significant deviations from Hardy-Weinberg Equilibrium (HWE) were observed in any farmed or wild sampling localities except for the Pieter Vorster Dam**.** This finding is consistent with the high F_{is} value reported for this sampling locality.

Based on the genetic diversity analyses, the Zini Fish Farm exhibited higher genetic diversity followed by the Fresca Fisheries Farm compared with the University of Zululand and uMphafa ponds sampling localities. Among the eight wild sampling localities, the Mhlathuze, Komati, and Loskop Dam sampling localities displayed higher genetic diversity than the other wild sampling localities. The overall genetic diversity was lower in farmed sampling localities compared to wild sampling localities.

Table 3.3: Genetic diversity indices of the eight wild and four farmed *O. mossambicus* in the present study. (Note: Zini farm = Zini Fish Farm; UniZulu ponds = University of Zululand, Fresca farm= Fresca Fisheries Farm, Pieter Dam = Pieter Vorster Dam).

Population	N	Na	Ne	Ar	H_O	uH_E	F_{is}	P_{HWE}
Farmed populations								
Zini farm	10	4.93	3.42	2.74	0.38	0.59	0.31	ns
uniZulu ponds	10	3.57	2.54	2.50	0.36	0.48	0.19	ns
uMphafa ponds	10	2.86	2.17	2.01	0.51	0.46	-0.10	ns
Fresca Farm	10	3.86	2.65	2.28	0.47	0.58	0.28	ns
Mean	**10**	**3.81**	**2.70**	**2.38**	**0.43**	**0.53**	**0.17**	**-**
Wild populations								
Mfolozi	8	3.86	3.05	1.97	0.31	0.51	0.37	ns
Mhlathuze	10	4.93	3.28	2.44	0.46	0.57	0.20	ns
Matigulu	10	3.43	2.61	2.37	0.36	0.46	0.32	ns
Thukela	19	6.07	3.53	3.35	0.37	0.58	0.37	ns
Mvoti	25	5.57	2.98	3.90	0.39	0.54	0.26	ns
Komati	10	6.14	4.43	2.42	0.45	0.73	0.38	ns
Pieter Dam	32	7.79	3.12	4.88	0.37	0.65	0.45	*
Loskop Dam	16	6.71	4.50	3.65	0.45	0.69	0.41	ns
Mean	**16.25**	**5.56**	**3.44**	**3.12**	**0.40**	**0.59**	**0.35**	**-**

N: number of individuals, Na: number of alleles, Ne: effective number of alleles, Ar: Allelic richness, uH_E: unbiased expected heterozygosity, H_O: observed heterozygosity, F_{is}: inbreeding coefficient and P_{HWE}: deviation from Hardy-Weinberg equilibrium (* $P<0.05$), are provided for each population.

3.4.2 Population structure and genetic differentiation

The STRUCTURE analyses conducted on both farmed and wild sampling localities, utilising the admixture model, identified K = 9 as the optimal genetic partitioning strategy, as determined by the Puechmaille method. Additionally, K = 8 and K = 10 were presented for comparative purposes (Figure 3.2). The overall STRUCTURE clustering pattern revealed that the farmed *O. mossambicus* had genetic connections with their wild counterparts from surrounding rivers. Specifically, the Zini Fish Farm and University of Zululand *O. mossambicus* showed genetic similarities with the wild *O. mossambicus* from the Mfolozi, Mhlathuze, and Matigulu rivers (Figure 3.2). Additionally, some genetic connection was observed between the Zini Fish Farm and wild *O. mossambicus* from the Mvoti and Thukela rivers. The farmed uMphafa ponds displayed distinctive genetic patterns, with evidence suggesting that it was founded by individuals from the Thukela River. Interestingly, the uMphafa ponds also shared genetic components with individuals from the Loskop Dam and the Komati River. Additionally, the Fresca Fisheries Farm exhibited distinct genetic characteristics, sharing admixture components mostly observed in farmed sampling localities in KwaZulu-Natal, suggesting possible movement of broodstock. The wild *O. mossambicus* from Pieter Vorster Dam appeared to be highly genetically distinct, forming its exclusive genetic cluster (Figure 3.2).

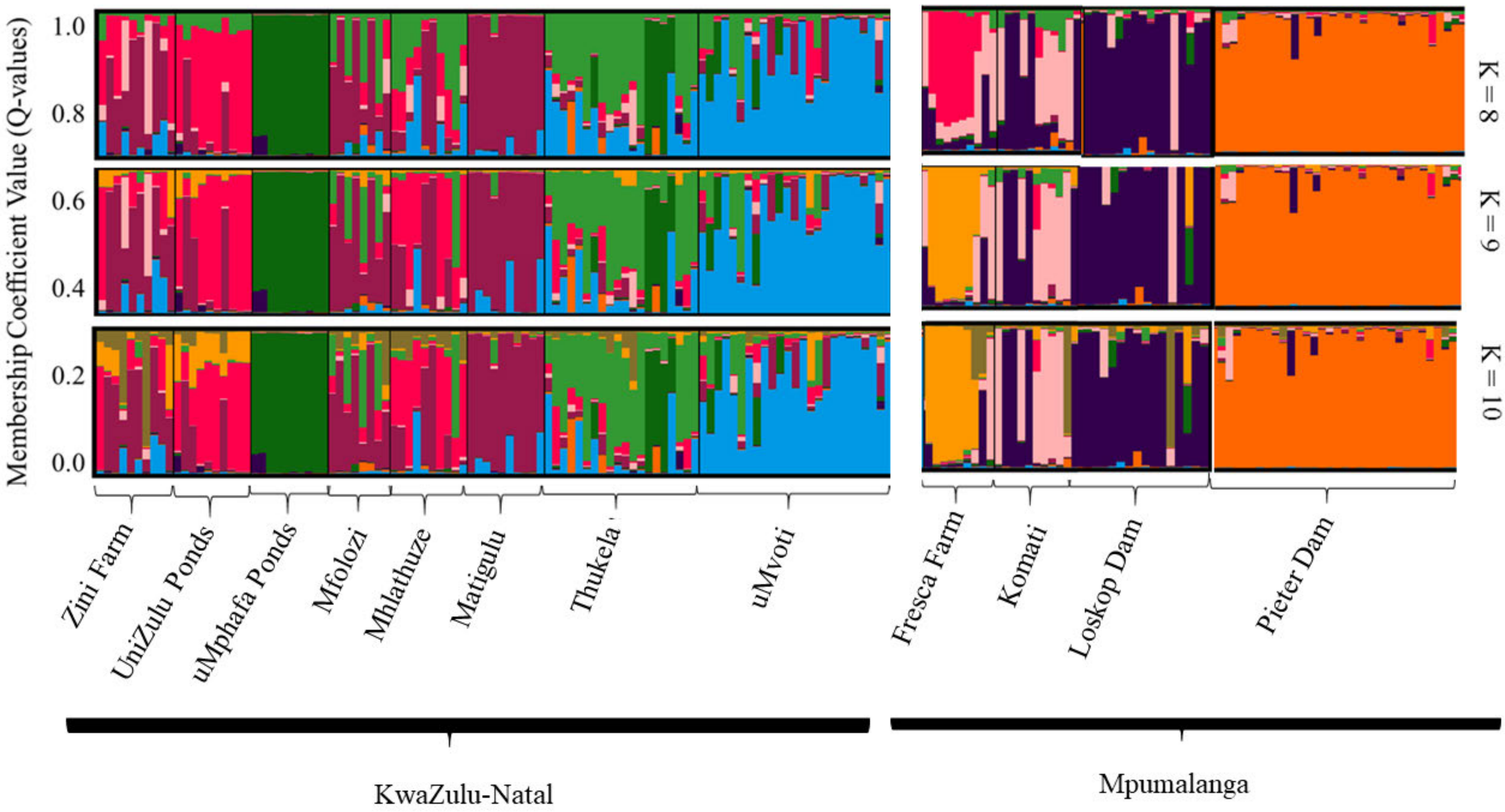
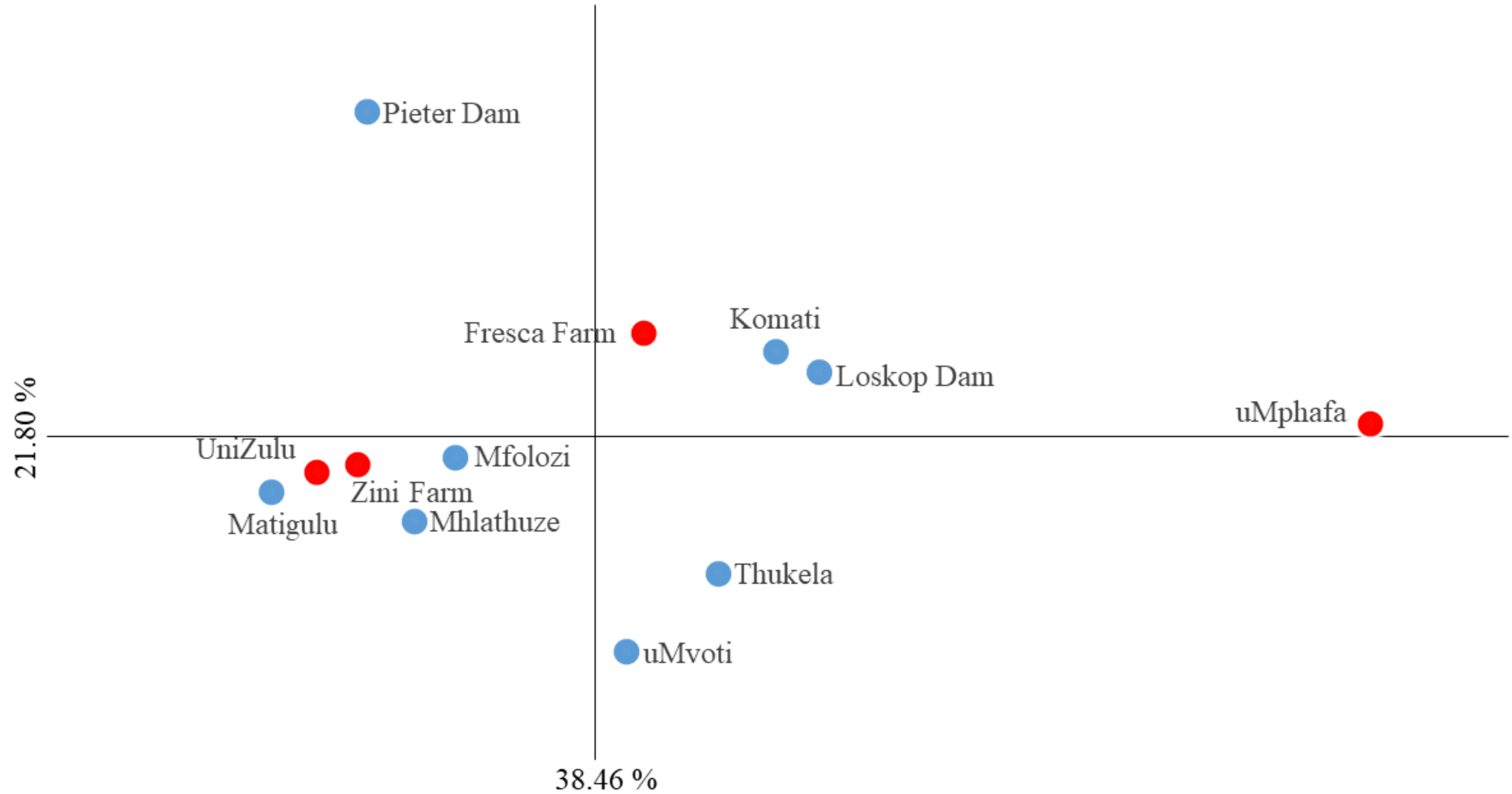


Figure 3.2: STRUCTURE bar plots showing the assignment of individuals from the four farmed and eight wild *O. mossambicus* from KwaZulu-Natal and Mpumalanga based on membership coefficient values (Q-values). Three K-values are provided, K = 8, K = 9, and K = 10. The most likely recovered optimal value of genetic clusters was K = 9. (Note: Zini farm = Zini Fish Farm; UniZulu ponds = University of Zululand, Fresca farm= Fresca Fisheries Farm, Pieter Dam = Pieter Vorster Dam).

The Principal Coordinate Analysis (PCoA) further underscored the genetic relationships among the farmed and wild *O. mossambicus*. In particular, the farmed *O. mossambicus* from the Zini Fish Farm and the University of Zululand exhibited a close clustering, indicating potential shared source populations (Figure 3.3a). The genetic proximity of these two farmed *O. mossambicus* to the wild *O. mossambicus* from Matigulu, Mfolozi, and Mhlathuze in suggested a degree of genetic similarity (Figure 3.3a). Consistent with the STRUCTURE results, the uMphafa ponds appeared isolated and did not cluster clearly with any of the wild *O.*

mossambicus populations (Figure 3.3a). However, some individuals from the Thukela River shared genetic affinities with those from the uMphafa ponds. This finding suggests that the Thukela River could be the source of the uMphafa ponds' broodstock, or that some individuals from the uMphafa ponds may have escaped into the Thukela River. The Fresca Fisheries Farm clustered with the wild *O. mossambicus* from the Komati River and Loskop Dam but did not show a genetic relationship with the *O. mossambicus* from Pieter Vorster Dam (Figure 3.3a).

The Nei's unbiased genetic distances supported the observed PCoA clustering patterns, with distances ranging from 0.06 between the University of Zululand and Mhlathuze River to 0.98 between uMphafa and Mhlathuze River (Supplementary Table 3.1). Generally, the farmed sampling localities displayed genetic similarity to the surrounding wild *O. mossambicus*, except for the uMphafa ponds, which exhibited genetic distinctiveness from the nearby wild populations in KwaZulu-Natal. However, these particular farmed individuals showed limited genetic similarities with wild *O. mossambicus* from the Thukela River, corroborating the STRUCTURE results. The shared alleles between the farmed *O. mossambicus* (Zini Fish Farm and University of Zululand) suggested a common source for their establishment. The Fresca Fisheries Farm demonstrated genetic similarity to the wild *O. mossambicus* from the Komati River and Loskop Dam, supporting these as potential sources for this farm (Supplementary Table 3.1). Based on Nei's unbiased genetic distances, the *O. mossambicus* from the uMphafa ponds also clustered more closely with farmed *O. mossambicus* from Fresca Fisheries and wild *O. mossambicus* from the Komati River and Loskop Dam (Supplementary Table 3.1). This clustering suggests potential movement or exchange of broodstock between the two provinces.


Pieter Dam
Fresca Farm
Komati
Loskop Dam
uMphafa
21.80 %
UniZulu
Mfolozi
Zini Farm
Matigulu
Mhlathuze
Thukela
uMvoti
38.46 %

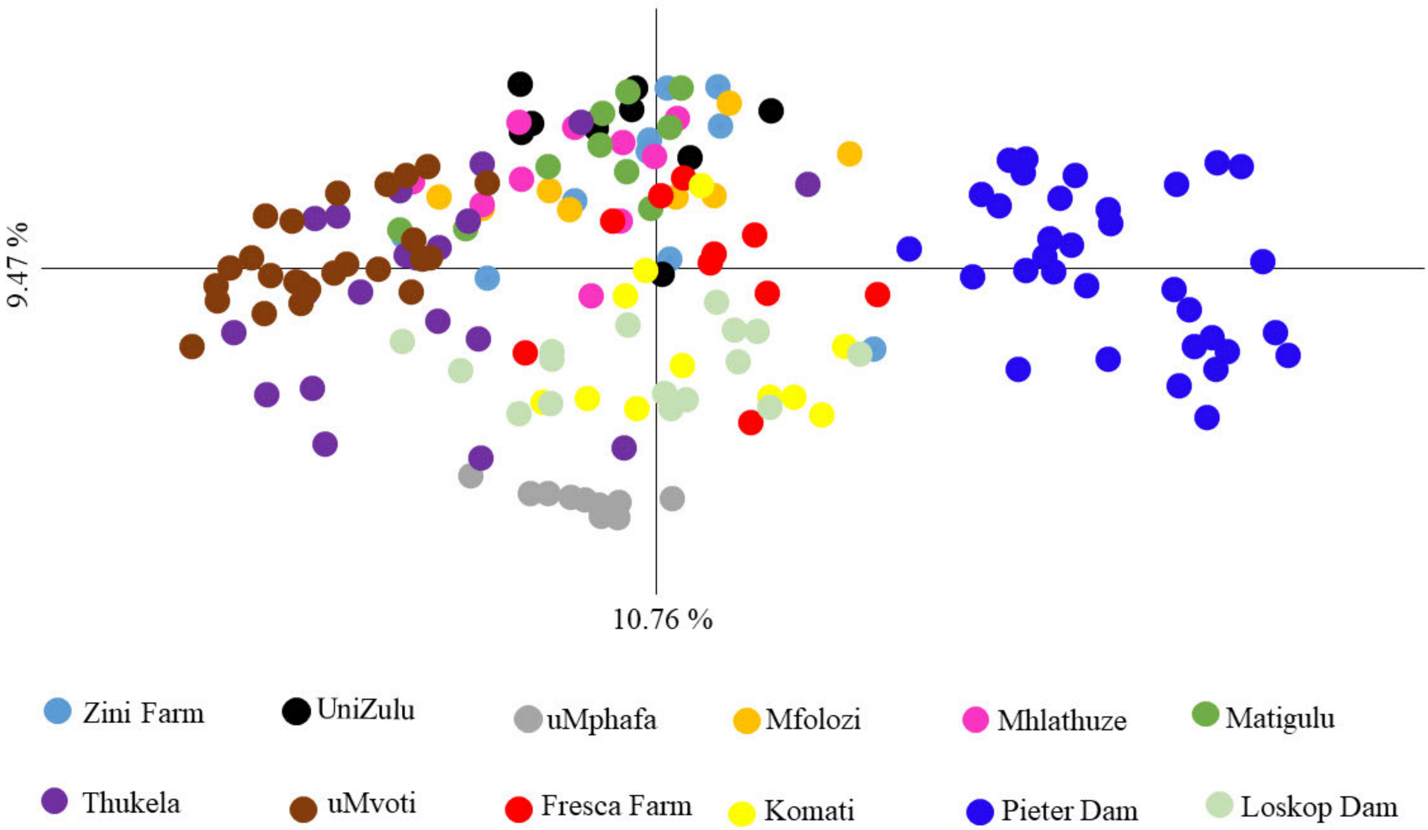

9.47 %
10.76 %
Zini Farm
UniZulu
uMphafa
Mfolozi
Mhlathuze
Matigulu
Thukela
uMvoti
Fresca Farm
Komati
Pieter Dam
Loskop Dam

Figure 3.3: a) Principal coordinate analysis (PCoA) of *Oreochromis mossambicus* grouped by the four farmed and eight wild sampling localities in Mpumalanga and KwaZulu-Natal provinces, South Africa. b) PCoA of *Oreochromis mossambicus* individuals plotted using a genetic distance between individuals color-coded by sampling locality. The PCoAs were plotted using Nei's (1972) genetic distance. (Note: Zini farm = Zini Fish Farm; UniZulu = University of Zululand, uMphafa = uMphafa ponds, Fresca farm= Fresca Fisheries Farm, Pieter Dam = Pieter Vorster Dam).

The AMOVA results revealed significant genetic differentiation between the farmed and wild *O. mossambicus*. The genetic variations observed among the farmed and wild *O. mossambicus* accounted for 21% among groups (farmed vs. wild), 27% among sites within groups, and 52% within sites (Table 3.4). Pairwise F_{ST} comparisons between various sampling localities highlighted genetic distinctions. The genetic differentiation between farmed and wild *O. mossambicus* ranged from minimal (0.04 between the University of Zululand and Mhlathuze) to substantial (0.45 between uMphafa ponds and Matigulu) (Table 3.6). Importantly, pairwise F_{ST} values indicated that the uMphafa ponds exhibited greater genetic similarity to the Fresca Fisheries Farm and Komati River sampling localities than to all sampling localities in KwaZulu-Natal. The probability estimates (P-values) for comparisons between various farmed and wild sampling localities were predominantly significant, suggesting the presence of genetic distinctions (Table 3.5). Exceptions included comparisons between the Zini Fish Farm and two wild sampling localities (Mfolozi and Mhlathuze rivers), as well as between Fresca Fisheries Farm and the wild sampling localities from Komati and Mfolozi rivers, where no significant genetic differentiation was observed (Table 3.5). The P-values for genetic differentiation between farmed and wild sampling localities ranged from 0.001 for most farmed and wild sampling localities comparisons to 0.026 between Komati River and Loskop Dam sampling localities (Table 3.5).

Table 3.4: Analysis of Molecular Variance (AMOVA) Groupings at 14 microsatellite loci for *O. mossambicus* from four farmed and eight wild sampling localities in Mpumalanga and KwaZulu-Natal. AMOVAs are also shown separately for sampling localities from KwaZulu-Natal and Mpumalanga.

Source of variation	**Sum of squares**	**Variance components**	**Percentage variation**	**P value <0.001**
Among groups (river or farm)	400.09	1.09	21%	0.000
Among sites within groups	915.25	1.46	27%	0.000
Within sites	478.00	2.76	52%	0.000
Total	**1793.34**	**5.31**	**100%**	**0.000**

Table 3.5: Estimates of ENA-corrected pairwise F_{ST}-values and probability (P-values) of the genetic differences for the four farmed and eight wild *O. mossambicus* from Mpumalanga and KwaZulu-Natal. (Note: Zini farm = Zini Fish Farm; UniZulu ponds = University of Zululand, Fresca farm= Fresca Fisheries Farm, Pieter Dam = Pieter Vorster Dam).

Population	Zini farm	uniZulu ponds	uMphafa ponds	Mfolozi	Mhlathuze	Matigulu	Thukela	Mvoti	Fresca Farm	Komati	Pieter Dam	Loskop Dam
Zini farm		0.0008^{*}	0.0008^{*}	0.0015^{NS}	0.0053^{NS}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}
uniZulu ponds	0.09		0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}
uMphafa ponds	**0.36**	**0.42**		0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}
Mfolozi	0.12	0.09	**0.36**		0.0008^{*}	0.0015^{NS}	0.0008^{*}	0.0008^{*}	0.0015^{NS}	0.0030^{NS}	0.0008^{*}	0.0008^{*}
Mhlathuze	0.04	0.04	**0.34**	0.08		0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}
Matigulu	0.11	0.19	**0.45**	0.18	0.15		0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}
Thukela	0.15	0.18	**0.27**	0.13	0.11	0.24		0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}
Mvoti	0.12	0.18	**0.33**	0.20	0.11	0.18	0.11		0.0008^{*}	0.0008^{*}	0.0008^{*}	0.0008^{*}
Fresca Farm	0.15	0.14	**0.30**	0.13	0.15	0.24	0.16	0.23		0.0023^{NS}	0.0008^{*}	0.0008^{*}
Komati	0.13	0.20	**0.26**	0.18	0.14	0.19	0.13	0.16	0.11		0.0008^{*}	0.0258^{NS}
Pierter Dam	0.16	0.20	**0.33**	0.17	0.18	0.25	0.22	**0.28**	0.17	0.16		0.0008^{*}
Loskop Dam	0.13	0.21	**0.27**	0.20	0.18	0.21	0.16	0.19	0.09	0.04	0.19	

F_{ST}-values < 0.05 indicate little genetic differentiation, 0.05-0.15 = moderate differentiation, 0.15-0.25 = significant differentiation, and > 0.25 = very great differentiation (given in bold) (Wright 1965). * P-values and not significant (NS) at P<0.0008

3.5 Discussion

In this study, I investigated the genetic diversity and differentiation of farmed and wild *O. mossambicus* in KwaZulu-Natal and Mpumalanga provinces, South Africa, using 14 microsatellite loci. Samples from surrounding rivers were included to examine the origin of the farmed *O. mossambicus*. While the amplification of microsatellite loci was generally successful, resulting in some missing data in the matrix, the 14 microsatellite loci proved highly polymorphic, providing valuable insights into genetic diversity and population structure in both farmed and wild *O. mossambicus*. Specifically, the analysis revealed that farmed fish showed genetic similarity to nearby river populations, suggesting potential admixture. Genetic diversity was lower in farmed *O. mossambicus*, indicating limited admixture, while isolated natural populations exhibited distinct genetic characteristics.

3.5.1 Genetic Diversity

Genetic diversity indices revealed lower genetic diversity in farmed *O. mossambicus* from the uMphafa ponds compared to both other farmed sampling localities and the potential wild source counterparts. The diminished genetic diversity in the uMphafa ponds may be attributed to genetic drift, inbreeding, selection for captive conditions, imbalanced sex ratios of breeders through selection, and the effects of several generations of closed breeding (Tave 1993, Tave 1999, Wang 2002, Duong and Scribner 2018, Lal et al. 2021). Hardy-Weinberg equilibrium deviations, potentially because of founder effects, non-random mating, and sampling method, were observed, aligning with previous studies (Mitton 1989, Selkoe and Toonen 2006, Simbine et al. 2014, Abramovs et al. 2020). This reduction in genetic diversity raises concerns as it may limit the use of the uMphafa ponds in breeding or stock enhancement programs, hindering its potential contribution to wild population supplementation (Yusuf et al. 2017).

Genetic monitoring of farmed stock is imperative, and baseline data are crucial for guiding future population-specific conservation programs and research efforts (Liu et al. 2018). For the uMphafa ponds farmed *O. mossambicus*, efforts should focus on maximising genetic variation and minimising inbreeding to maintain the heritability of valuable culture strains, especially if there is potential for these fish to be released or if they are already significant for breeding programs (Lal et al. 2021). Similarly to our findings, reduced genetic diversity in farmed populations, compared with wild ones, has been reported in various aquaculture studies (Norris et al. 1999, Pampoulie et al. 2006, Li et al. 2009, Briñez et al. 2011, Aguiar et al. 2018, Ukenye & Megbowon 2023). The observed high genetic diversity in Zini Fish Farm and Fresca Fisheries farmed *O. mossambicus* may suggest effective genetic resource management utilised in the farms. However, it is crucial to consider that these farmed *O. mossambicus* are cultured under different conditions, obtained from various generations, and raised using distinct aquaculture systems. For instance, the observed diversity at Fresca Fisheries Farm in Mpumalanga is consistent with the conjecture that its broodstock may have been sourced from different localities within the Komati River catchment. The Zini Fish Farm *O. mossambicus*, introduced to dusky kob grow-out pans connected to the Mlalazi River, may have retained high genetic diversity by having access to their natural environment for breeding. This suggests that different aquaculture practices and broodstock sourcing strategies can significantly influence the genetic makeup of cultured populations (Boyd et al. 2005, Ying et al. 2018, Sonesson et al. 2023).

Genetic diversity is crucial in aquaculture and conservation, contributing significantly to broodstock adaptability (Xu et al. 2001, Hassanien and Gilbey 2005, Yusuf et al. 2017, Aguiar et al. 2018, de Oliveira et al. 2018, Dehmord et al. 2023). In this study, positive inbreeding values were recorded for both farmed and natural *O. mossambicus*, except for the uMphafa ponds, which showed a negative inbreeding value. Elevated inbreeding levels might result from a smaller number of founders, leading to inbreeding depression in farmed populations (Briñez et al. 2011, Aguiar et al. 2018). Previous aquaculture studies reported negative inbreeding coefficient values, indicative of excess heterozygosity (Li et al. 2009, Khadher et al. 2016, Aguiar et al. 2018, Fagbḕmi et al. 2021). However, this was not the case for the uMphafa ponds *O. mossambicus* in the present study. Despite the negative inbreeding coefficient, the genetic diversity indices for this sampling locality indicated low genetic diversity. This could be due to sampling bias (Nei, 1987) or the Wahlund effect, where the population might represent a mixture of subpopulations with distinct allele frequencies (DeGiorgio and Rosenberg 2009). To prevent inbreeding depression, maintaining inbreeding coefficients below 5% is crucial (Tave 1999). Inbreeding depression can increase susceptibility to diseases, reduce fitness, and hinder environmental adaptation. These factors might contribute to the genetic differences observed between farmed and wild *O. mossambicus* populations.

Conserving genetic variability in farmed fish populations involves strategies such as restocking from the wild or exchanging stock between farms to counteract inbreeding depression (Duong et al. 2017, Yusuf et al. 2017, Liu et al. 2018). Annual broodstock exchange, as suggested by Duong and Scribner (2018), could maintain heterozygosity levels in farmed fish populations, with the caveat that such exchanges should consider geographic proximity to prevent potential genetic disruptions. Implementing good farming practices is essential for promoting adaptability, growth, low disease susceptibility, and increased fish production, benefiting the aquaculture industry (Yusuf et al. 2017). Considering a stock exchange between the University of Zululand, Zini Fish Farm, and uMphafa ponds *O. mossambicus* may enhance genetic diversity in all three sampling localities by introducing distinct genetic stocks from each farm. The genetic integrity and diversity observed in the initial generation of the Fresca

Fisheries in Mpumalanga should be preserved to ensure the production of high-quality *O. mossambicus* seed for future aquaculture in the region.

3.5.2 Genetic population structure and genetic differentiation

The Bayesian STRUCTURE analysis revealed nine distinct genetic clusters encompassing four farmed and eight wild *O. mossambicus* in the present study. Notably, the genetic composition indicated a close genetic affinity between the farmed *O. mossambicus* from the University of Zululand and Zini Fish Farm and the wild *O. mossambicus* from Mfolozi, Matigulu, and Mhlathuze Rivers in KwaZulu-Natal. The clustering of these two farmed *O. mossambicus* implies a shared genetic origin, possibly sourced from the same rivers or interconnected water systems. This aligns with our understanding that the Zini Fish Farm is linked to the Mlalazi estuary, connecting it to various river systems, including Mfolozi, Matigulu, Mhlathuze, Mvoti, and the Thukela River, emphasising the likelihood of genetic exchange between sampling localities.

While the specific origin of the University of Zululand *O. mossambicus* remains unknown, the present study suggests a potential connection to the wild *O. mossambicus* from Mfolozi, Matigulu, and Mhlathuze Rivers, supported by the clustering analyses. Particularly, the high-level sharing of genotypic frequencies with the Mhlathuze River suggests a possible genetic link. These findings are consistent with findings in other aquaculture studies that reported genetic similarities between farmed and wild populations of *O. niloticus* (Fagbẻmi et al. 2021), *Cyprinus carpio* (Napora-Rutkowski et al. 2017), *Colossoma macropomum* (Aguiar et al. 2018), and *Brycon amazonicus* (de Oliveira et al. 2018). The uMphafa ponds *O. mossambicus* stands out as the most genetically distinct group compared to all other farmed and wild *O. mossambicus* analysed in KwaZulu-Natal. This distinctiveness raises the possibility that it may not be directly representative of the native *O. mossambicus* in the region. Interestingly, a small number of individuals sampled from the Thukela River share this distinct genetic profile with the uMphafa ponds. This shared ancestry suggests a potential historical connection between these two groups. Further investigation, including a larger sample size from the Thukela River and analyses using more genetic markers, could shed light on the origins of the uMphafa ponds and its potential relationship with the Thukela River *O. mossambicus*.

Similarly, the *O. mossambicus* at Fresca Fisheries Farm exhibited genetic distinctiveness from the surrounding wild *O. mossambicus*. However, some individuals from this farm clustered genetically with the wild *O. mossambicus* from the Komati River and Loskop Dam, suggesting that these wild sampling localities may serve as sources for Fresca Fisheries Farm. In contrast, the Pieter Vorster Dam *O. mossambicus* appeared highly genetically distinct, despite the belief that some farmed individuals at Fresca Fisheries Farm were sourced from this sampling locality. This indicates that the farmed *O. mossambicus* at Fresca Fisheries Farm does not accurately represent the genetic diversity of the Pieter Vorster Dam. The non-significant genetic differentiation further supports the notion that this farmed *O. mossambicus* originated from an admixture of *O. mossambicus* in the Komati River and Loskop Dam. However, this differentiation could also result from a founder event when establishing the farmed *O. mossambicus* and from broodstock input from other sources, which may be contributing to the observed high genetic diversity in this farm. D'Amato et al. (2007) previously reported similar observations on genetic differences between farmed and wild *O. mossambicus* in southern Africa using microsatellite loci, aligning with our results. The Nei's genetic distance further corroborates our findings by indicating genetic similarities and differences between wild and farmed *O. mossambicus*. The genetic distance observed between the University of Zululand and Zini Fish Farm *O. mossambicus* suggests a shared source. These results align with previous studies that have reported similar genetic distances (Macaranas et al., 1995; de Silva, 1997; Hall, 2001; Hassanien and Gilbey, 2005; Yusuf et al., 2017).

In the context of aquaculture, selective breeding emerges as a powerful tool for enhancing production and fostering a sustainable industry by cultivating strains with desirable traits (Hulata 2001, Gjedrem et al. 2012, Lind et al. 2012, Janssen et al. 2017, de Assis Lago et al. 2017, Robledo et al. 2018, Causey et al. 2019). However, caution is imperative to prevent potential threats posed by selective breeding, such as the loss of genetic variation because of inbreeding. While this study did not focus directly on aquaculture practices or selective breeding and their impact on diversity, the recommendations by Lind et al. (2012) for maintaining long-term genetic variation through selective breeding practices align with the importance of preserving high genetic diversity for sustainable aquaculture (Lal et al., 2021). The unique genetic characteristics identified in the Zini Fish Farm and Fresca Fisheries Farmed *O. mossambicus* in KwaZulu-Natal and Mpumalanga offer potential for future selective breeding efforts aimed at developing a local *O. mossambicus* strain. These sampling localities can potentially serve as valuable resources for supplementing broodstock in other *O. mossambicus* farms, contributing to increased genetic diversity in farmed stocks. However, it is important to note that uncontrolled mixing of genetically divergent individuals carries the risk of outbreeding depression, potentially leading to offspring with reduced fitness and adaptability (Ward 2006, Huff et al. 2011, Tsaparis et al. 2022, Vitt et al. 2023). This underscores the necessity of careful management when considering gene flow between populations to maintain genetic health and long-term viability, particularly in the context of potential farm leakages that could contaminate wild populations (Tsaparis et al. 2022, Vitt et al. 2023).

Additionally, using cultured fish for restocking or augmenting wild populations must be approached with caution. While aquaculture can significantly contribute to the sustainability of the industry by enhancing the genetic diversity of wild stocks, it also carries potential pitfalls. Genetic swamping, where the genetic integrity of wild populations is overwhelmed by the introduction of cultured fish, can lead to homogenisation and loss of local adaptations (Ward 2006, Vitt et al. 2023). This process might reduce the overall fitness of the wild population and its ability to adapt to local environmental conditions. Moreover, the introduction of farmed individuals into the wild can result in the spread of diseases and parasites, further threatening the health of native populations (Ward 2006, Huff et al. 2011, Tsaparis et al. 2022, Vitt et al. 2023). It is crucial to implement rigorous health screening protocols for farmed fish before their release into the wild to mitigate these risks. Overall, the enhancement of genetic diversity in farmed and wild *O. mossambicus* stocks through the use of cultured fish requires a balanced and carefully managed approach. Strategies should include the establishment of genetic management plans, regular monitoring of genetic health, and adherence to best practices in aquaculture to ensure the long-term sustainability and resilience of both farmed and wild populations.

3.6 Conclusions

Studies focused on assessing the genetic diversity and variation of both farmed and wild *O. mossambicus* in South Africa have been relatively scarce (Hall 2001, Esterhuyse 2002, D'Amato et al. 2007, Simbine et al. 2014). This investigation specifically aimed to fill this knowledge gap by thoroughly examining the genetic diversity and differentiation of farmed *O. mossambicus* in KwaZulu-Natal and Mpumalanga. The results from population structure and genetic differentiation analyses revealed a genetic similarity between the farmed *O.*

mossambicus and their neighbouring wild counterparts. In contrast, the uMphafa ponds *O. mossambicus* in KwaZulu-Natal stand out as genetically distinct. Interestingly, some individuals sampled from the Thukela River share this distinct genetic profile with the uMphafa ponds, suggesting a potential historical connection between these two groups. This may indicate that the uMphafa ponds *O. mossambicus* was sourced from the Thukela River or has already escaped and introgressed into that River. Importantly, the farmed *O. mossambicus* from the University of Zululand, Zini Fish Farm, and Fresca Fisheries Farm demonstrated a close resemblance to wild *O. mossambicus* from surrounding rivers. This may indicate their potential utility in the future for restocking or supplementing wild *O. mossambicus* populations. However, caution must be exercised to avoid farming *O. mossambicus* that exhibit significant genetic differences from their wild counterparts. Such disparities could pose challenges in the event of fish escapes, potentially leading to adverse impacts on wild populations. uMphafa ponds

On a positive note, both the Zini Fish Farm and Fresca Fisheries *O. mossambicus* displayed high genetic diversity. This suggests their suitability for supplementing farms characterised by low genetic diversity. Moreover, these *O. mossambicus* individuals hold promise for developing good genetic local strains conducive to sustainable seed production and tilapia farming in KwaZulu-Natal and Mpumalanga, contributing to the broader aquaculture landscape in South Africa. Considering these findings, it is imperative to emphasise the importance of quantifying the genetic diversity and population structure of farmed *O. mossambicus* not only in KwaZulu-Natal and Mpumalanga provinces but across South Africa. This not only enhances our understanding of local populations but also contributes significantly to the sustainable management and conservation of wild populations. Recommendations for improved management practices are warranted to safeguard the genetic integrity of farmed *O.*

mossambicus. This involves implementing enhanced biosecurity measures to prevent the entry of wild fish into farms and ensuring improved confinement of cultured fish. Such measures will not only maintain the genetic integrity of farmed populations but also contribute positively to the conservation efforts aimed at maintaining the genetic diversity of wild populations.

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3.9 Supplementary information

Supplementary Table 3.1: Pairwise Nei's genetic distance estimates for the four farmed and eight wild *O. mossambicus* populations in Mpumalanga and KwaZulu-Natal. (Note: Zini farm = Zini Fish Farm; UniZulu ponds = University of Zululand, Fresca farm= Fresca Fisheries Farm, Pieter Dam = Pieter Vorster Dam).

Population	Zini farm	uniZulu ponds	uMphafa ponds	Mfolozi	Mhlathuze	Matigulu	Thukela	Mvoti	Fresca Farm	Komati	Pieter Dam	Loskop Dam
Zini farm	0.00											
uniZulu ponds	0.12	0.00										
uMphafa ponds	1.15	1.23	0.00									
Mfolozi	0.22	0.16	0.91	0.00								
Mhlathuze	0.09	0.06	0.98	0.12	0.00							
Matigulu	0.16	0.27	1.43	0.21	0.24	0.00						
Thukela	0.33	0.31	0.62	0.20	0.20	0.47	0.00					
Mvoti	0.24	0.30	0.80	0.34	0.20	0.30	0.17	0.00				
Fresca Farm	0.34	0.24	0.78	0.24	0.33	0.44	0.38	0.52	0.00			
Komati	0.41	0.48	0.74	0.44	0.45	0.45	0.37	0.44	0.30	0.00		
Pieter Dam	0.49	0.53	1.32	0.49	0.56	0.66	0.82	0.98	0.54	0.65	0.00	
Loskop Dam	0.41	0.59	0.81	0.61	0.60	0.51	0.55	0.52	0.31	0.13	0.93	0.00

Unbiased Nei's unbiased genetic distance (Nei 1972) values ranging from 0- 0.05 indicate low genetic distance (high genetic similarity), 0.1-0.30 = moderate genetic distance (indicate moderate differentiation), 0.30-1 = high genetic distance (high genetic differentiation), and > 1= very great genetic distance (given in bold).

CHAPTER 4

Genetic introgression of farmed and wild *Oreochromis mossambicus* with introduced *Oreochromis* species in Limpopo, Mpumalanga, and KwaZulu-Natal provinces, South Africa

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Running header: Hybridisation between introduced *Oreochromis* species with *Oreochromis mossambicus*

4.1 Abstract

Non-indigenous *Oreochromis* species, including *O. niloticus* and *O. aureus*, were introduced in South Africa to boost the economy, primarily through aquaculture and fisheries. However, *Oreochromis* species hybridise and now pose a threat to wild populations of native *O. mossambicus*. *Oreochromis mossambicus* naturally inhabits several major river catchments in South Africa, particularly in warmer regions, and wild populations are used in aquaculture across various provinces. This study employed 14 microsatellite loci to assess the presence of introduced genetic material from *O. niloticus* and *O. aureus* within *O. mossambicus* populations from major river catchments across KwaZulu-Natal, Mpumalanga, and Limpopo provinces. Farmed *O. mossambicus* stocks from three KwaZulu-Natal farms and one Mpumalanga farm were also evaluated, considering their potential wild origins. Analyses revealed evidence suggestive of hybridisation with *O. niloticus* in seven wild populations: four in Limpopo (Mogalakwena, Mapungubwe, Luvuvhu, and Letaba), two in Mpumalanga (Olifants and White River Country Estate Dam), and one in KwaZulu-Natal (Tongati). Additionally, analyses indicated potential genetic overlap between farmed *O. mossambicus* from uMphafa ponds (KwaZulu-Natal) and Fresca Fisheries Farm (Mpumalanga) with the introduced *O. aureus*. These findings highlight the importance of implementing strict measures to conserve the genetic integrity of remaining unadmixed *O. mossambicus* populations, particularly those showing no signs of introduced genetic material. Such conservation efforts are crucial for the sustainable management of this potentially vulnerable species.

Keywords: conservation, hybridisation, microsatellite markers, *Oreochromis mossambicus, O. niloticus*, South Africa.

4.2 Introduction

In the realm of freshwater fish conservation, the introduction of invasive species poses a substantial and well-documented threat, contributing significantly to the decline in biodiversity (Ricciardi and Simberloff 2009, Crispo et al. 2011, Deines et al. 2014, Shechonge et al. 2018, Dieleman et al. 2019, Yongo et al. 2023). This threat is particularly noteworthy due to its pervasive impact on native fish populations worldwide (Dieleman et al. 2019, Yongo et al. 2023). Introduction of invasive freshwater fish can lead to a variety of ecological consequences, which include increased predation, competitive pressures, disease propagation, and, on occasion, hybridisation events among closely related species, leading to the genetic contamination of indigenous populations (Garroway et al. 2010, Crispo et al. 2011, Firmat et al. 2013, Shechonge et al. 2018, Richmond 2018, Duenas et al. 2021, Green and Grosholz 2021). In particular, the hybridisation of invasive and native species not only threatens unique genetic resources but, in extreme cases, may lead to the complete loss of native species (D'Amato et al. 2007, Bradbeer et al. 2019, Anane-Taabeah 2019, Rougemont et al. 2022, Geletu and Zhao 2023). The impact of invasive species, driven by inadvertent or deliberate introductions, is predicted to increase with urbanisation and intensified food production demands, despite natural barriers (Hulme et al. 2008, Westphal et al. 2008, Bradbeer et al. 2019, Lopez et al. 2022, Artaev 2023). Freshwater habitats, in particular, are highly susceptible to invasion (Baron et al. 2002, Falkenmark 2003, Deeksha and Shukla 2022, Lynch et al. 2023). Despite their crucial value to human societies, many of these ecosystems face severe degradation owing to escalating human activities (O'Brien et al. 2019, Bănăduc et al. 2022, Masese et al. 2023), thus threatening the genetic diversity and population structure across various aquatic taxa (Templeton et al. 2001, Cantonati et al. 2020, Petit-Marty et al. 2022).

The introduction of non-native fish species on a global scale has occurred through various means, including recreational angling, the ornamental fish trade, biocontrol initiatives, fisheries and aquaculture establishment, and inter-basin water transfer schemes (Bruton and Van As 1986, Gozlan et al. 2010, Ellender and Weyl 2014, Zengeya et al. 2015, Yongo et al. 2023). While deliberate introductions may offer economic and social advantages, they perpetuate and are a persistent threat to native freshwater taxa (Genner et al. 2013, FAO 2019, FAO 2020, Sax et al. 2022, Banha et al. 2023, Muñoz-Mas et al. 2023). The consequences of invasive species introductions often remain unclear during the early stages of invasion, with negative impacts on native fish populations only becoming apparent years later (Barel et al. 1985, Genner et al. 2013, Mayfield et al. 2021, Muñoz-Mas et al. 2023). Moreover, conservation strategies aimed at safeguarding the natural populations of fish species essential to the aquaculture industry are often characterised by delayed implementation and inadequacies, leading to a rapid decline in many freshwater fish species (Canonico et al. 2005, Angienda et al. 2011, Reid et al. 2013, Woodford et al. 2017, Xie et al. 2019, Troell et al. 2023).

Cichlids, particularly the *Oreochromis* genus, occupy a central role in global fisheries and aquaculture because of their marketable size, rapid growth rates, adaptability to captive breeding, and the availability of wild source stocks (D'Amato et al. 2007; Lind et al. 2012, Firmat et al. 2013, Amoussou et al. 2019, Shechonge et al. 2019, Geletu et al. 2023, Koblmüller et al. 2023). The demand for cichlids has surged in developing countries, fuelling increased farming and wild harvesting activities (FAO 2014, FAO 2022, Geletu et al. 2023). In Africa, species such as *Oreochromis mossambicus* (Peters 1852), *O. niloticus* (Linnaeus 1758), and *O. aureus* (Steindachner 1864) are very popular for aquaculture (Li et al. 2006, Toguyeni et al. 2009, FAO 2010, Wu et al. 2012, Ansah et al. 2014, El-Sayed and Fitzsimmons 2023). However, poorly regulated aquaculture practices have unintentionally introduced some cichlid species beyond their native ranges, threatening native *Oreochromis* populations (D'Amato et al. 2007; Firmat et al. 2013; Zengeya et al. 2015; Amoussou et al. 2019). These introduced fish can hybridise with native species, leading to a loss of genetic integrity (D'Amato et al. 2007, Lind et al. 2012, Firmat et al. 2013, Zengeya et al. 2015, Marr et al. 2018, Weyl et al. 2020).

South Africa is categorised as one of the six major global hotspots for fish invasions (Leprieur et al., 2009), and non-native fish species have become prevalent in many major river systems in the region (van Rensburg et al., 2011; Ellender & Weyl, 2014; Weyl et al., 2020). Poorly regulated aquaculture practices have facilitated the escape and establishment of some cichlid species beyond their native ranges (D'Amato et al. 2007, Firmat et al. 2013, Zengeya et al. 2015, Amoussou et al. 2019). Currently, there is evidence supporting the establishment of *O. aureus* and *O. niloticus* in South Africa, primarily driven by the fisheries and aquaculture industries (Ellender & Weyl, 2014; Marr et al., 2018; Weyl et al., 2020). These introductions pose a substantial threat to native *Oreochromis* populations, as these introduced species are known to hybridise with other *Oreochromis* species, leading to loss of genetic integrity (D'Amato et al. 2007, Lind et al. 2012, Firmat et al. 2013, Zengeya et al. 2015, Marr et al. 2018, Weyl et al. 2020, Stauffer et al. 2022, Ciezarek et al. 2023).

Oreochromis niloticus was introduced to South Africa for aquaculture in 1955. Initially, it was brought to the Cape Flats area (Cape Town, Western Cape Province) and KwaZulu-Natal during the 1950s and has since expanded its range and is now primarily confined to the Limpopo River system and small coastal river systems in KwaZulu-Natal, though its current status in KwaZulu-Natal remains uncertain (D'Amato et al. 2007, Zengeya et al. 2013, Ellender & Weyl 2014). While no concrete evidence exists of established populations in Mpumalanga, the proximity of water systems, particularly those connected to the Limpopo basin, raises the possibility of its presence. *Oreochromis aureus* was imported from Israel to the Jonkershoek

Hatchery near Stellenbosch in 1959 and released into farm dams in the Western Cape's Lourens and Eerste River catchments in the early 1960s and has since established (Marr et al. 2018, Weyl et al. 2020). Both *O. niloticus* and *O. aureus* were introduced into KwaZulu-Natal at the Amatikulu hatchery in 1978. Since then, they have escaped into the wild, posing a threat to native species, particularly *O. mossambicus*, through competition and hybridisation (Ellender & Weyl 2014, Marr et al. 2018). Unlike *O. niloticus*, there are no reports of *O. aureus* in Limpopo or Mpumalanga, although evaluating all populations in these provinces for the presence of *O. aureus* is crucial due to water management practices that might facilitate its spread. Given the potential for these species to spread and establish in new areas, ongoing monitoring and management strategies are essential. This includes evaluating all populations in the affected provinces to understand the full extent of their distribution and impacts.

Endemic to southern Africa, *Oreochromis mossambicus* inhabits the region's southeastern flowing rivers, spanning from the Ligonha River in northern Mozambique to the Boesmans River in South Africa (Scott et al. 2006). This species naturally occurs in numerous major river catchments in South Africa, predominantly in the warmer regions of the country. However, native populations of *O. mossambicus* face genetic admixture threats from the introduced Nile tilapia (*O. niloticus*) and other *Oreochromis* species, resulting in its classification as Vulnerable on the IUCN Red List (Ellender and Weyl 2014, Zengeya et al. 2015, Bills, 2019). Nile tilapia often competes with Mozambique tilapia for resources such as food, shelter, and territory (Ellender and Weyl et al. 2014, Zengeya et al. 2015, Stauffer et al. 2022, Robin et al. 2023). The genetic characteristics that have enabled *O. mossambicus* to adapt to various environmental conditions, including resistance to drought, tolerance of high salinity, and low-temperature resilience, are also jeopardised by hybridisation events. While there has been substantial research on the introduction of invasive *Oreochromis* species in South Africa, particularly O. *niloticus* (D'Amato et al. 2007, Lind et al. 2012, Firmat et al. 2013, Zengeya et al. 2015, Marr et al. 2018, Mboweni et al. 2020, Weyl et al. 2020), a comprehensive understanding of the extent of these introductions and their impact on the genetic diversity and population structure of native *Oreochromis* populations in South Africa remains elusive. Consequently, there is a pressing need for a comprehensive assessment of the present status of wild tilapia genetic resources to inform effective management strategies and enhance sustainable aquaculture practices (Falk et al. 2004, Hallerman and Hilsdorf 2014, Abwao et al. 2023, Sonesson et al. 2023).

In the provinces of Limpopo, Mpumalanga, and KwaZulu-Natal in South Africa, *O. mossambicus* naturally populates all major river catchments and wild populations of this species are presently used as broodstock in various tilapia farms. Given the favourable climate conditions prevailing in these regions, they are recognised as some of the suitable sites for expanding the freshwater aquaculture industry in South Africa by leveraging the wild populations of *O. mossambicus* (DAFF 2016, Dti 2020). However, embarking on such endeavours without a solid foundation of genetic data concerning the purity and integrity of these populations is not encouraged. This study employed microsatellite analysis to assess the presence of introduced *Oreochromis* species, specifically *O. niloticus* and *O. aureus*, within populations initially identified as *O. mossambicus* based on morphological characteristics across Limpopo, Mpumalanga, and KwaZulu-Natal Provinces in South Africa. I focused on detecting the presence of introduced genetic material, which could potentially indicate hybridisation between these species. Additionally, I evaluated the genetic purity of farmed *O. mossambicus* stock, considering the possibility that the broodstock originated from wild populations. Ultimately, this study aimed to inform future conservation and management strategies for *O. mossambicus* by identifying river systems with potentially unadmixed populations. Furthermore, this information can be valuable in establishing sustainable tilapia farming ventures in South Africa by pinpointing suitable populations.

4.3 Methods

4.3.1 Sampling

I collected samples for this study in the Limpopo, Mpumalanga, and KwaZulu-Natal provinces of South Africa from 2017 to 2021. All necessary permits for sampling were obtained from Ezemvelo KwaZulu-Natal Wildlife (OP1583/2017, OP1432/2018, and OP871/2021). The study received ethical clearance from the University of KwaZulu-Natal Animal Ethics Subcommittee (REF: AREC/023/020). I conducted surveys based on historical distribution records for *O. mossambicus* in the three provinces (Cambray and Swartz 2007, GBIF 2017, 2024). A total of 380 wild *O. mossambicus* samples were collected from 29 distinct localities, with eight in Limpopo, eight in Mpumalanga, and 13 in KwaZulu-Natal. Where possible, we surveyed multiple rivers and dams within various catchments to determine the spatial scale of population structuring and assess the extent of contamination with the introduced species within river catchments (Figure 4.1, Supplementary information Table 4.1).

Additionally, DNA samples were collected from a total of 40 farmed fish: ten samples each from three locations in KwaZulu-Natal (Zini Fish Farm, University of Zululand Aquaculture Facility, and uMphafa ponds), and one location in Mpumalanga (Fresca Fisheries Farm). I employed standard passive techniques when sampling for the wild stock, including using fyke nets, electro-fishing, seine nets, and gill nets. The farmed stock was collected from ponds and tanks using a scooping net and seine nets. Upon capture, *O. mossambicus* was identified at the species level using established morphological characteristics (Skelton 2001). However, these methods may not be sufficient to definitively identify hybrids, particularly later generation hybrids (F3 and F4) which can closely resemble parental species (Esterhuyse 2002). While some hybrids can be distinguished based on morphological features, in many cases, genetic analysis is necessary for accurate identification. A non-lethal fin clip, approximately 5 mm^2 to 10 mm^2, was taken from each anesthetised fish, immediately preserved in 99% ethanol, stored in a refrigerator, and transported to the laboratories of the University of KwaZulu-Natal, Pietermaritzburg, for subsequent genetic analyses. The captured fish were placed in a recovery bucket and released back at their original capture location.

Reference samples of genetically pure *O. niloticus* (n = 10) and *O. aureus* (n = 10) were collected from specific sources to investigate potential genetic introgression into *O. mossambicus* in this study. The *O. niloticus* samples were sourced from farmed strains within Mega-Fish Tanks, renowned for maintaining genetically pure populations. Similarly, *O. aureus* samples were collected from a farm dam located at Faure, between Somerset-West and Kuils River in the Western Cape, where genetically pure stocks are maintained. Including reference samples from the introduced *Oreochromis* species was critical for detecting genetic material from these introduced species within the surveyed farmed and wild *O. mossambicus* populations across the three provinces. By comparing the genetic makeup of the reference samples with the *O. mossambicus* populations, we were able to assess the extent of introgression from these introduced species.

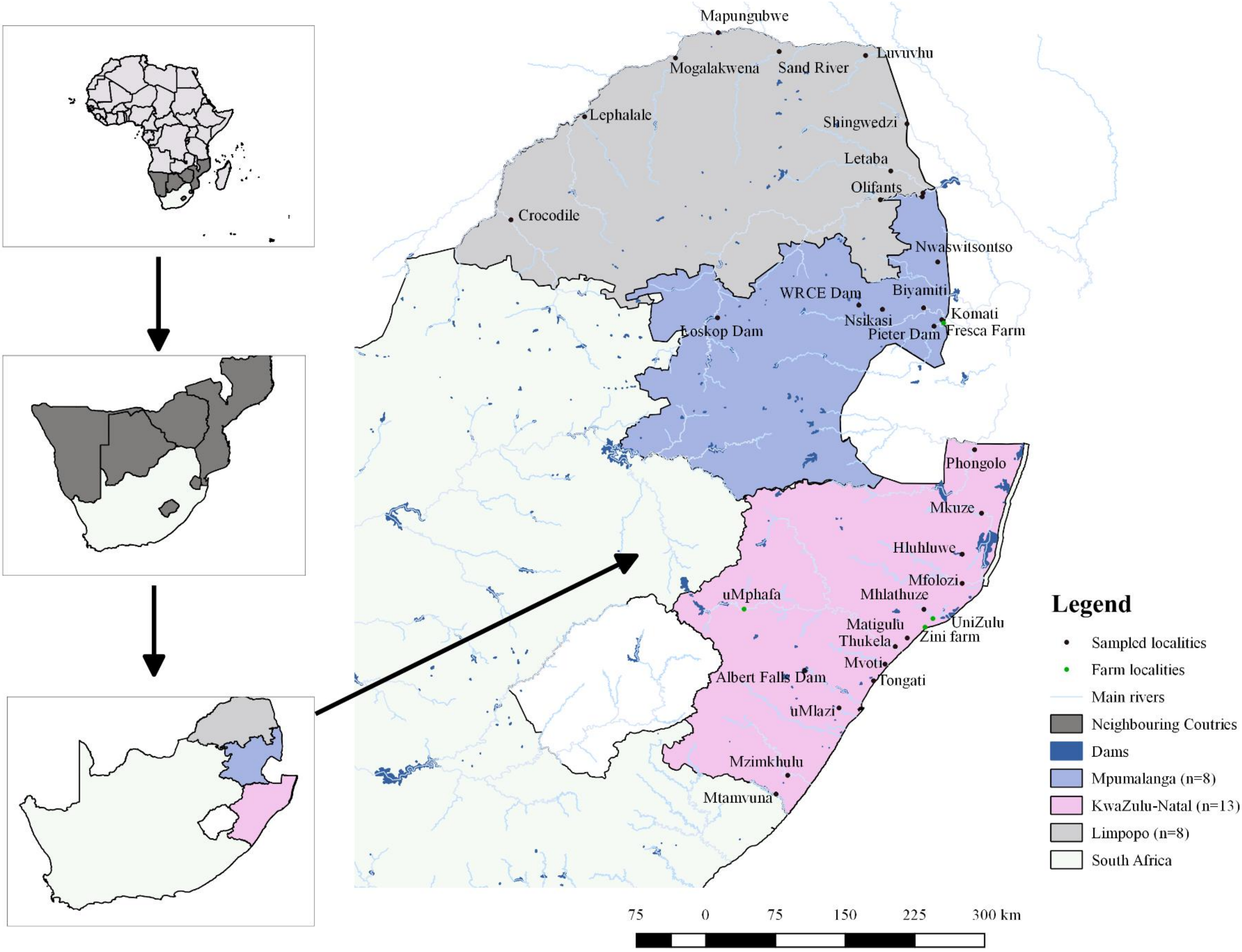


Figure 4.1: Sampling localities of farmed and wild-caught *O. mossambicus* in Limpopo, Mpumalanga, and KwaZulu-Natal provinces, South Africa.

4.3.2 DNA extraction and amplification

Following the standard animal tissue protocol, I extracted DNA from fin clips using the Nucleospin® Tissue kit (Macherey-Nagel, Germany). Extracted DNA samples were stored at -20°C. In this study, 14 microsatellite loci, originally isolated from *O. niloticus* (Hall 2001, Simbine et al. 2014) and *O. mossambicus* (Saju et al. 2010), were amplified in all three species

(Table 4.1). Each primer pair had a fluorescently labelled forward primer. Polymerase Chain Reaction (PCR) profiles were executed in a final volume of 10 µl, comprising 0.1 µl of each primer, 5 µl of 2G Fast Multiplex Mix KAPA Taq (KAPA Biosystems, Cape Town, South Africa), 0.3 µl of BSA, 4.2 µl distilled water (dH_2O), and 0.3 µl of DNA. The thermocycler conditions included an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 15s, annealing at the specified temperature for 30s, extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR profiles were initially conducted as singleplexes for each of the 14 microsatellites. Subsequently, working primers were selected and grouped into multiplexes (A, B, C, and D) based on annealing temperature, fluorescence dye, and product size. Multiplex A and D had an annealing temperature of 60°C, while multiplex B and C had an annealing temperature of 61°C (Table 4.1). Fragment analyses were performed at the Central Analytical Facilities (CAF), University of Stellenbosch, South Africa, using a 3500XL Genetic Analyser from Applied Biosystems (Thermo Fisher). To ensure genotype accuracy, 20% of all individuals were re-genotyped and checked for consistency, with no inconsistent genotypes detected.

Table 4.1: Microsatellite loci used in the present study for genotyping of farmed and wild *Oreochromis mossambicus* individuals and the two introduced species (*O. niloticus* and *O. aureus*). Fluorescent dye and annealing temperature used in the present study are provided.

Multiplex loci	**Locus**	**Sequence**	**Fluorescent Dye**	**Annealing Temperature °C**
A	OM01[c]	F: TTTAAAGTTACACAGCAGTACAAAG R: TTGTAGCATTTCAACACAGTCTC	Fam	60
D	OM02[c]	F: TGTGAATTTGACAACTTCCTTTC R: ATCCTTGCAATAAGGTTACAG	Fam	60
D	OM03[c]	F: CTTTTTAATGAGCAACTTTTAAGTC	Hex	60

		R: TGTGAATTTGACAACTTCCTTTC		
A	OM04[c]	F: AGCTCAAAACCTCATACAAAGG	Fam	60
		R: GCAGAGATGTCAGATGTTGTTC		
B	OM05[c]	F: GTAAAGTTTGGAACAGAAATGCT	Hex	61
		R: GATCACTTTTGGACAGACTGG		
B	OM06[c]	F: TGAGCTACCGTAAGGATGTAC	Fam	61
		R: GTTATTTCAATTATATTTGCATG		
C	OM07[c]	F: TTGGCTCAGAGTGGTCAGG	Hex	61
		R: CGCGTGGACTAAAAGCCAG		
B	OM08[c]	F: TGTTGGTTGGATTACTGGG	Fam	61
		R: GCTGTAATGGTTTTGAGGC		
B	OM09[c]	F: GGCTACAACACCTGGATGG	Hex	61
		R: TTGGGCTTACTGAAGCTGAC		
C	UNH104[a]	F: GCAGTTATTTGTGGTCACTA	Tet	61
		R: GGTATATGTCTAACTGAAATC		
C	UNH129[a]	F: AGAAGTCGTGCATCTCTC	Tet	61
		R: TGTACATCATCTGTGGG		
B	UNH142[b]	F: CTTTACGTTGACGCAGT	Tet	61
		R: GTGACATGCAGCAGATA		
B	UNH222[b]	F: CTCTAGCACACGTGCAT	Tet	61
		R: TAACAGGTGGGAACTCA		
C	UNH231[b]	F: GCCTATTATAGTCAAAGCGT	Tet	61
		R: ATTTCTGCAAAAGTTTTCC		

Primers taken from Hall (2001)[a] Simbine et al. (2014) [b] and Saju et al. (2010) [c]

4.3.3 Molecular data analyses

Genotypes were scored using GeneMarker® (version 2.4 software; Hulce et al. 2011). Given the reliance of various population genetic analyses on populations being in Hardy-Weinberg equilibrium and the potential bias introduced by high null allele frequencies (Chapuis and Estoup 2006, Carlsson 2008), null allele frequencies for the three tilapia species (*O. mossambicus*, *O. niloticus*, and *O. aureus*) were computed using FreeNA software Excluding Null Alleles (ENA) correction (Chapuis and Estoup 2006). I assessed the impact of null alleles on population structure results by comparing ENA-corrected and non-corrected global F_{ST}-values across all fourteen microsatellites for the three tilapia species. I used Student t-tests in Microsoft Excel 2016 for these comparisons, and p-values were adjusted for multiple comparisons using Bonferroni correction (Bland and Atman 1995).

4.3.4 Genetic diversity

I computed various indices to evaluate genetic diversity within populations. The total number of alleles (Na), unbiased expected and observed heterozygosity (uH_E and H_O) for the 14 microsatellites amplified from both farmed and wild *O. mossambicus* and the two introduced species (*O. niloticus* and *O. aureus*) were calculated using GenAlEx software version 6.5 (Peakall and Smouse 2012). I assessed Hardy-Weinberg equilibrium deviations using GenAlEx software. Linkage disequilibrium (LD) across populations of the three tilapia species was estimated using Genepop version 4.7.0 (Raymond 1995, Rousset 2008). Chi-square p-values were determined using the Markov Chain method with 10,000 replicates, 100 groups, and 5000 iterations per group. Bonferroni corrections were applied to account for multiple comparisons and ensure the robustness of the statistical analyses. I obtained the inbreeding coefficient (Fis) using GenAlEx software, where positive high Fis-values indicated high inbreeding, characterised by excess homozygosity, and negative Fis-values indicated low inbreeding with excess heterozygosity in the population (Simbine et al. 2014). I calculated each microsatellite locus' polymorphic information content (PIC) using Cervus software version 3.0.7 (Marshall et al. 1998, Kalinowski et al. 2007). The PIC serves as a measure of the suitability of each microsatellite locus for evaluating genetic differentiation between populations (Botstein et al. 1980), with loci having PIC > 0.5 considered highly informative and those with PIC < 0.25 deemed less informative (Botstein et al. 1980). I obtained allelic richness values using FSTAT Software, version 2.9.3.2 (Goudet 2001).

4.3.5 Genetic pollution: Potential hybridisation of O. mossambicus with introduced species

To evaluate the extent to which introduced genetic material from *O. niloticus,* and *O. aureus* might be present within wild and farmed *O. mossambicus* populations, I employed a Bayesian clustering method implemented in STRUCTURE software (version 2.3.4; Pritchard et al., 2000). I specifically focused on the potential for hybridisation between these species. The initial STRUCTURE analysis examined the likelihood of hybridisation and the potential presence of introduced genetic material within 29 wild *O. mossambicus* populations across Limpopo, Mpumalanga, and KwaZulu-Natal provinces in South Africa. I utilised the admixture model with correlated allele frequencies for the assignment tests. Furthermore, to facilitate the visualisation of any geographically associated patterns, I organised the individuals by both province and river catchment. The STRUCTURE analyses comprised 500,000 Markov-Chain Monte Carlo (MCMC) replicates, with a 50,000-step burn-in period and 20 iterations, exploring K values ranging from 10 to 29. The optimal number of genetic clusters (K-value) was determined using the STRUCTURE selector, version 2.3 (Li & Liu, 2018) and the Puechmaille method (Puechmaille 2016). STRUCTURE harvester (Earl and Holdt 2012) was employed to obtain membership coefficient values (Q-values) for optimal K, and bar plots were generated for optimal K-values using Clumpak, version 1.1 (Kopelman et al. 2015).

I conducted a subsequent STRUCTURE analysis to assess potential introgression in farmed stocks by the two introduced species, maintaining the same settings as for the wild populations, except for the number of iterations set to 20, with K ranging from 1 to 10.

To further investigate the population genetic structure of *O. mossambicus* and assess the extent to which genetic material from the introduced *O. niloticus* and *O. aureus* might be present, we employed Nei's genetic distances (Nei 1972) and principal coordinate analyses (PCoA) implemented in GenAlEx software. To investigate the genetic structure of *O. mossambicus* populations and assess the potential for introgression from introduced *Oreochromis* species, I employed an Analysis of Molecular Variance (AMOVA). Here, I compared farmed and wild *O. mossambicus* populations combined across provinces to the two introduced species (*O. niloticus* and *O. aureus*). This allowed for evaluation of the overall genetic differentiation between farmed and wild populations and identified potential genetic differentiation between native *O. mossambicus* and the introduced *Oreochromis* species. GenAlEx software facilitated this analysis, and we applied a Bonferroni correction to adjust the significance level for multiple comparisons. Pairwise F_{ST}-values were also calculated, implementing the ENA correcting method in FreeNA, and to test for significant genetic differentiation between the three species, we computed probability (p-values) using FSTAT software (version 2.9.3.2; Goudet 2001). This test included 11,900 permutations, with an indicative adjusted nominal level (5%) for multiple comparisons set at 0.000084 after Bonferroni correction.

4.4 Results

A total of 440 individuals were successfully genotyped using 14 microsatellite loci. These samples included 29 wild and 4 farmed *O. mossambicus* populations, along with 10 individuals each for the introduced species (*O. niloticus* and *O. aureus*). Some individuals across all three species presented challenges in amplifying all microsatellite markers, resulting in a small amount of missing data. The extent of missing data for individual loci in *O. mossambicus* (both farmed and wild) ranged from 6% (for loci OM04, UNH142, and UNH222) to 33% (for locus OM05). Cross-amplification of these markers was even less successful for *O. aureus*, with several loci (OM08, OM09, OM05, UHN104, and UNH231) exhibiting 20% missing data and locus OM05 reaching 40% missing data. Interestingly, amplification using these 14 microsatellite loci was successful for all *O. niloticus* individuals, with no missing data observed. The presence of missing data highlighted potential limitations associated with certain markers for amplifying individuals across the latter two species (*O. mossambicus* and *O. aureus*). To assess the potential impact of missing data, we conducted assignment tests with and without loci exceeding 20% missing data. These tests revealed that the overall genetic structure and observed clustering patterns remained consistent, suggesting minimal bias from the missing data.

The 14 loci displayed high polymorphism for the three *Oreochromis* species, except for locus OM06, which was monomorphic for both *O. aureus* and *O. niloticus*, while locus OM08 was also monomorphic for *O. aureus*. Polymorphic information content (PIC) values ranged from 0.50 (UNH231) to 0.90 (OM07) for *O. mossambicus*, 0.38 (OM06) to 0.83 (OM05) for *O. niloticus*, and 0.38 (OM06 and OM08) to 0.79 (OM04) for *O. aureus* (Supplementary Table 4.2). Null allele frequencies varied from 0.03 (OM07) to 0.21 (UNH222) for *O. mossambicus*, 0.00 (OM009) to 0.40 (OM08) for *O. niloticus*, and 0.00 (OM04, OM08, OM06, UNH104, and

UNH142) to 0.36 (UNH222) for *O. aureus* (Supplementary Table 4.2). Comparison of ENA-corrected and non-corrected F_{ST} values revealed no substantial impact on population structure analysis for the three *Oreochromis* species. Both uncorrected and ENA-corrected F_{ST} values yielded non-significant p-values (0.55 for *O. mossambicus* and 0.39 for both *O. niloticus* and *O. aureus*) after Bonferroni correction. This suggests that accounting for the potential presence of null alleles did not meaningfully alter the interpretation of genetic differentiation among these species. The majority of the 14 microsatellite loci analysed for *O. mossambicus* populations did not exhibit significant deviations from Hardy-Weinberg equilibrium. Only three loci, OM04, OM01, and OM07, were in equilibrium, showing no significant deviations (Supplementary Table 4.2). Eight microsatellite loci significantly deviated from Hardy-Weinberg equilibrium in *O. niloticus*, with only loci OM05, UNH231, UNH129, OM03, OM02, and OM06 being at equilibrium. In *O. aureus*, nine microsatellite loci deviated from equilibrium, with only loci UNH231, UNH129, OM07, OM08, and OM06 being at equilibrium (Supplementary Table 4.2). Linkage disequilibrium was not significant for any of the loci in all three species.

4.4.1 Genetic diversity

A mean of 5.21, 4.64, and 3.43 alleles were observed for *O. mossambicus*, *O. niloticus*, and *O. aureus*, respectively. The number of alleles varied across loci, ranging from 2.30 (UNH231) to 8.67 (OM04) for *O. mossambicus*, 1.00 (OM01) to 7.00 (OM05) for *O. niloticus*, and from 1.00 (OM08 and OM06) to 6.00 (OM04) for *O. aureus* (Supplementary Table 4.2). The effective number of alleles ranged from 1.41 (UNH231) to 6.27 (OM07) for *O. mossambicus*, 1.00 (OM06) to 4.26 for *O. niloticus*, and 1.00 (OM08 and OM06) for *O. aureus*. Allelic richness displayed variation, with values ranging from 1.65 (UNH231) to 4.34 (OM07) for *O.*

mossambicus, 1.00 (OM01) to 7.00 (OM05) for *O. niloticus* (Supplementary Table 4.2), and from 1.00 (OM08 and OM06) to 5.30 (OM04) for *O. aureus*. Observed heterozygosity (H_O) varied from 0.10 (UNH231) to 0.88 (OM04) for *O. mossambicus*, 0.00 (OM06 and OM08) to 0.80 (OM05) for *O. niloticus*, and 0.00 (OM05, OM06, OM08, UNH222) to 0.80 (OM04) for *O. aureus*. Unbiased expected heterozygosity (uH_E) was generally higher than observed heterozygosity in all three species. Loci OM04 and OM05 in *O. mossambicus*, locus OM02 in *O. niloticus*, and locus OM04 in *O. aureus* exhibited higher observed heterozygosity than unbiased expected heterozygosity. Unbiased expected heterozygosity ranged from 0.23 (UNH231) to 0.85 (OM07) for *O. mossambicus*, 0.00 (OM06) to 0.81 (UNH222) for *O. niloticus*, and 0.00 (OM06 and OM08) to 0.78 (OM04) for *O. aureus* (Supplementary Table 4.2). Inbreeding coefficients (Fis) for most loci in all three species were relatively high and close to 1, particularly for *O. aureus*. The Fis values for *O. mossambicus* ranged from -0.03 (OM04) to 0.61 (OM06), from -0.18 (OM05) to 1.00 (OM08) for *O. niloticus*, and from -0.10 (OM04) to 1.00 (OM05, UNH222, and UNH231) for *O. aureus* (Supplementary Table 4.2).

Genetic diversity observed in most wild and farmed *O. mossambicus* populations was much higher than that observed in O. *aureus*, while the genetic diversity estimates for *O. niloticus* fell within the range of those for *O. mossambicus*. The number of alleles observed across the three species varied from 2.86 (uMphafa and Mtamvuna) to 7.79 (Pieter Dam), while the effective number of alleles varied from 1.93 (*O. aureus*) to 5.07 (Sand River). Allelic richness varied from 0.62 in the Thukela population to 14.42 in the Pieter Dam population (Table 4.2). Observed heterozygosity varied from 0.18 (*O. aureus*) to 0.56 (Sand River and Shingwedzi), and uH_E varied from 0.44 in *O. aureus* and Mtamvuna populations to 0.76 in the Luvuvhu population (Table 4.2). Overall, the uH_E in all populations was relatively lower than that expected under Hardy-Weinberg equilibrium (HWE) expectations, possibly because of high homozygosity across the populations of the three species (Table 4.2). Among the *O. mossambicus* populations, only the individuals from uMphafa ponds exhibited a higher H_O compared to uH_E. Overall, most populations across the three species conformed to Hardy-Weinberg equilibrium with few exceptions. Significantly deviant populations included all *O. aureus* individuals and three *O. mossambicus* populations (Pieter Dam, White River Country Estate Dam, and Albert Falls Dam). Across all sampling localities and species, inbreeding coefficient (F_{is}) values ranged from moderate to high, with the lowest observed values of -0.10 and -0.12 found in uMphafa ponds and Biyamiti populations of *O. mossambicus* respectively. Conversely, the highest value of 0.60 was observed in the *O. aureus* population (Table 4.2).

Table 4.2: Genetic diversity indices of the 29 wild populations of *O. mossambicus,* four farmed populations of *O. mossambicus,* and the farmed populations of the two introduced species (*O. niloticus and O. aureus*). (Note: WRCE Dam = White River Country Estate Dam, Pieter Dam = Pieter Vorster Dam, Zini farm = Zini Fish Farm, UniZulu ponds = University of Zululand tilapia ponds, Fresca farm = Fresca Fisheries Farm).

Sampled localities	N	Na	Ne	Ar	H_O	uH_E	F_{is}	P_{HWE}
O. mossambicus								
Phongolo	14.20	5.93	3.82	2.78	0.38	0.70	0.45	ns
Mkuze	17.80	5.71	3.48	3.08	0.37	0.58	0.30	ns
Hluhluwe	10.90	5.43	3.40	2.25	0.36	0.55	0.29	ns
Mfolozi	7.90	3.86	3.05	1.82	0.31	0.51	0.37	ns
Mhlathuze	9.90	4.93	3.28	2.21	0.46	0.57	0.20	ns
Matigulu	9.90	3.43	2.61	2.02	0.36	0.46	0.32	ns
Thukela	19.40	6.07	3.53	0.62	0.37	0.58	0.37	ns
Mvoti	24.70	5.57	2.98	0.72	0.39	0.54	0.26	ns
Tongati	6.80	4.71	3.38	1.90	0.39	0.71	0.43	ns
Albert Falls Dam	19.80	4.36	2.46	2.74	0.30	0.52	0.50	*
uMlazi	7.00	3.21	2.56	1.62	0.45	0.53	0.11	ns
Mzimkhulu	9.90	3.64	2.61	2.13	0.45	0.54	0.18	ns
Mtamvuna	14.90	2.86	1.94	2.34	0.36	0.44	0.17	ns
Olifants	34.00	6.40	4.27	4.00	0.51	0.70	0.25	ns
Loskop Dam	15.60	6.71	4.50	8.93	0.45	0.69	0.41	ns
Nwaswitsontso	8.90	4.79	3.33	5.66	0.29	0.64	0.58	ns

Biyamiti	3.00	2.93	2.61	2.85	0.50	0.54	-0.12	ns
Nsikasi	8.80	3.64	2.54	4.99	0.27	0.54	0.54	ns
WRCE Dam	9.90	4.71	2.99	5.88	0.37	0.63	0.42	*
Komati River	9.90	6.14	4.43	6.81	0.45	0.73	0.38	ns
Pieter Dam	32.40	7.79	3.12	14.42	0.37	0.65	0.45	*
Crocodile	10.00	4.57	3.22	5.93	0.44	0.63	0.32	ns
Lephalale	9.90	6.43	4.10	6.79	0.52	0.70	0.26	ns
Mogalakwena	9.90	6.57	4.29	6.90	0.55	0.73	0.26	ns
Mapungubwe	9.90	4.50	3.34	5.92	0.42	0.67	0.39	ns
Sand River	9.90	6.79	5.07	7.26	0.56	0.74	0.21	ns
Luvuvhu	9.90	7.07	5.03	7.34	0.47	0.76	0.39	ns
Shingwedzi	8.80	5.07	3.24	5.70	0.56	0.63	0.09	ns
Letaba	9.90	5.71	4.23	6.62	0.49	0.71	0.30	ns
Zini farm[a]	10.00	4.93	3.42	2.74	0.38	0.59	0.31	ns
uniZulu ponds[a]	10.00	3.57	2.54	2.50	0.36	0.48	0.19	ns
uMphafa ponds[a]	10.00	2.86	2.17	2.01	0.51	0.46	-0.10	ns
Fresca Farma	10.00	3.86	2.65	2.28	0.47	0.58	0.28	ns
Mean	**12.54**	**4.99**	**3.34**	**4.30**	**0.42**	**0.61**	**0.30**	**-**
Introduced species								
O. niloticus[a]	10.00	4.64	2.92	4.64	0.39	0.64	0.37	ns
O. aureus[a]	9.43	3.43	1.93	3.09	0.18	0.44	0.60	*
Mean	**12.38**	**4.94**	**3.29**	**4.27**	**0.41**	**0.60**	**0.31**	**-**

Na: number of alleles, Ar: Allelic richness, F_{ST}^{A}: non-corrected F_{ST}, F_{ST}^{B}: ENA corrected F_{ST}, H_O: observed heterozygosity, uH_E: unbiased expected heterozygosity. P_{HWE}: deviation from Hardy-Weinberg equilibrium (*P<0.05), F_{is}: Inbreeding coefficient, PIC: polymorphic information content, [a] represents populations collected from farms.

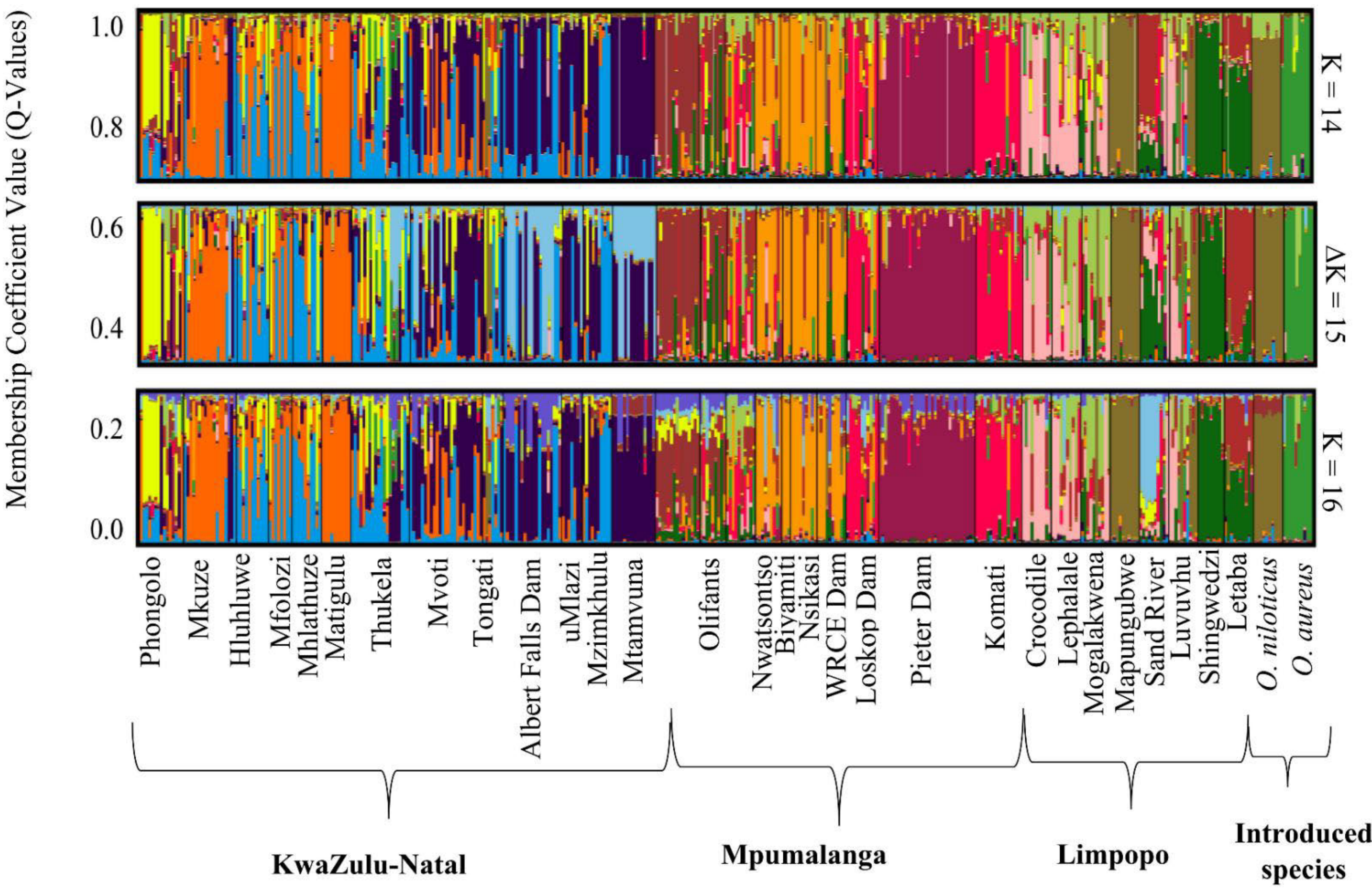
4.4.2 Genetic signals of potential hybridisation in wild O. mossambicus

The STRUCTURE analyses, using the admixture model with K = 15 identified as the optimal partitioning strategy according to the Puechmaille method (Figure 4.2). Comparative bar plots for K = 14 and K = 16 are included, supporting a consistent clustering pattern. Overall, the STRUCTURE results revealed genetic differentiation among populations of the three *Oreochromis* species. Notably, certain wild *O. mossambicus* populations shared genotypes with *O. niloticus*, including those from Mapungubwe, Luvuvhu, Mogalakwena, Letaba (Limpopo Province), White River Country Estate Dam, and Olifants (Mpumalanga Province), and Tongati

(KwaZulu-Natal Province) (Figure 4.2). Similarly, *O. mossambicus* in the Thukela River (KwaZulu-Natal) exhibited shared genotypes with *O. aureus* (Figure 4.2). The analysis suggested a greater degree of genetic overlap between *O. mossambicus* and *O. niloticus* compared to *O. aureus*. Specifically, the Mapungubwe population in the Limpopo Province indicated a more extensive presence of introduced genetic material from *O. niloticus*.

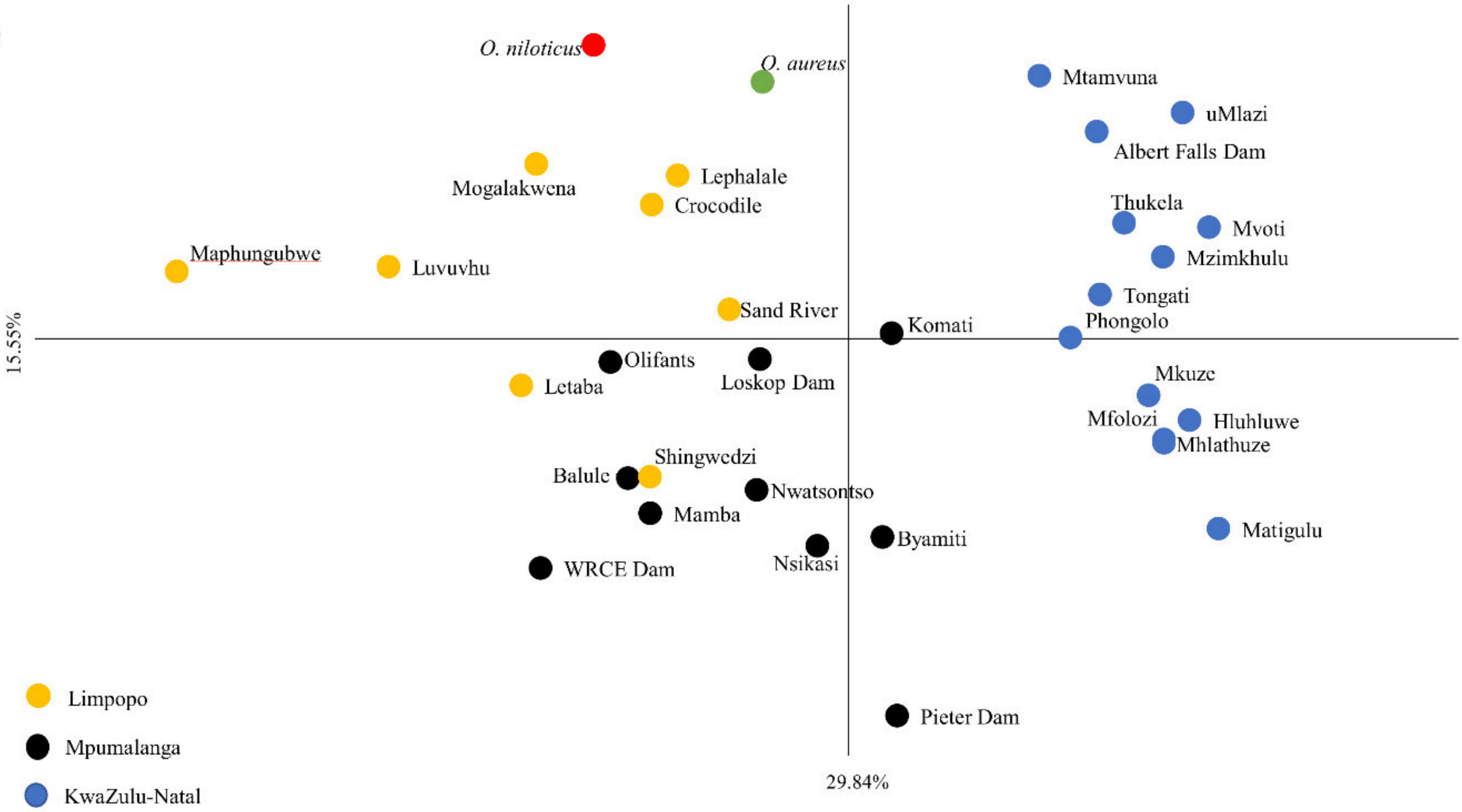
The PCoA reinforced genetic distinctions between the introduced species (*O. niloticus* and *O. aureus*) and the wild *O. mossambicus* populations. However, some wild *O. mossambicus* populations shared genetic overlap with the introduced species (Figure 4.3b). Notably, individuals from *O. mossambicus* populations in Mapungubwe, Mogalakwena, Tongati, Luvuvhu, Sand River, Letaba, Olifants, and the White River Country Estate Dam clustered with some of the *O. niloticus* individuals, while *O. aureus* individuals clustered with some of the *O. mossambicus* individuals from the Komati, Thukela, and Crocodile populations (Figure 4.3b). The Nei's genetic distances corroborated the PCoA clustering pattern, ranging from 1.05 between Tongati and *O. niloticus* to 2.31 between Pieter Dam and *O. niloticus* (Supplementary Table 4.3). Genetic distances across the three species varied from 0.56 between the Crocodile River population and *O. aureus* to 1.63 between *O. niloticus* and *O. aureus* (Supplementary Table 4.3).

Figure 4.2: STRUCTURE bar plots showing the assignment of the 380 individuals from the



29 wild *O. mossambicus* populations in Limpopo, Mpumalanga, and KwaZulu-Natal Provinces, South Africa. The two introduced species populations (*O. niloticus* and *O. aureus*) are also indicated. The assignment is based on membership coefficient values (Q-values). Three K-values are provided, K = 14, K = 15, and K = 16. The most likely recovered optimal value of genetic clusters was K = 15. (Note: WRCE Dam = White River Country Estate Dam, Pieter Dam = Pieter Vorster Dam).

a)


O. niloticus
O. aureus
Mtamvuna
uMlazi
Albert Falls Dam
Mogalakwena
Lephalale
Crocodile
Thukela
Mvoti
Mzimkhulu
Maphungubwe
Luvuvhu
Tongati
Sand River
Komati
Phongolo
15.55%
Olifants
Loskop Dam
Letaba
Mkuze
Mfolozi
Hluhluwe
Mhlathuze
Shingwedzi
Balule
Nwatsontso
Mamba
Byamiti
Matigulu
Nsikasi
WRCE Dam
Limpopo
Mpumalanga
KwaZulu-Natal
Pieter Dam
29.84%

b)

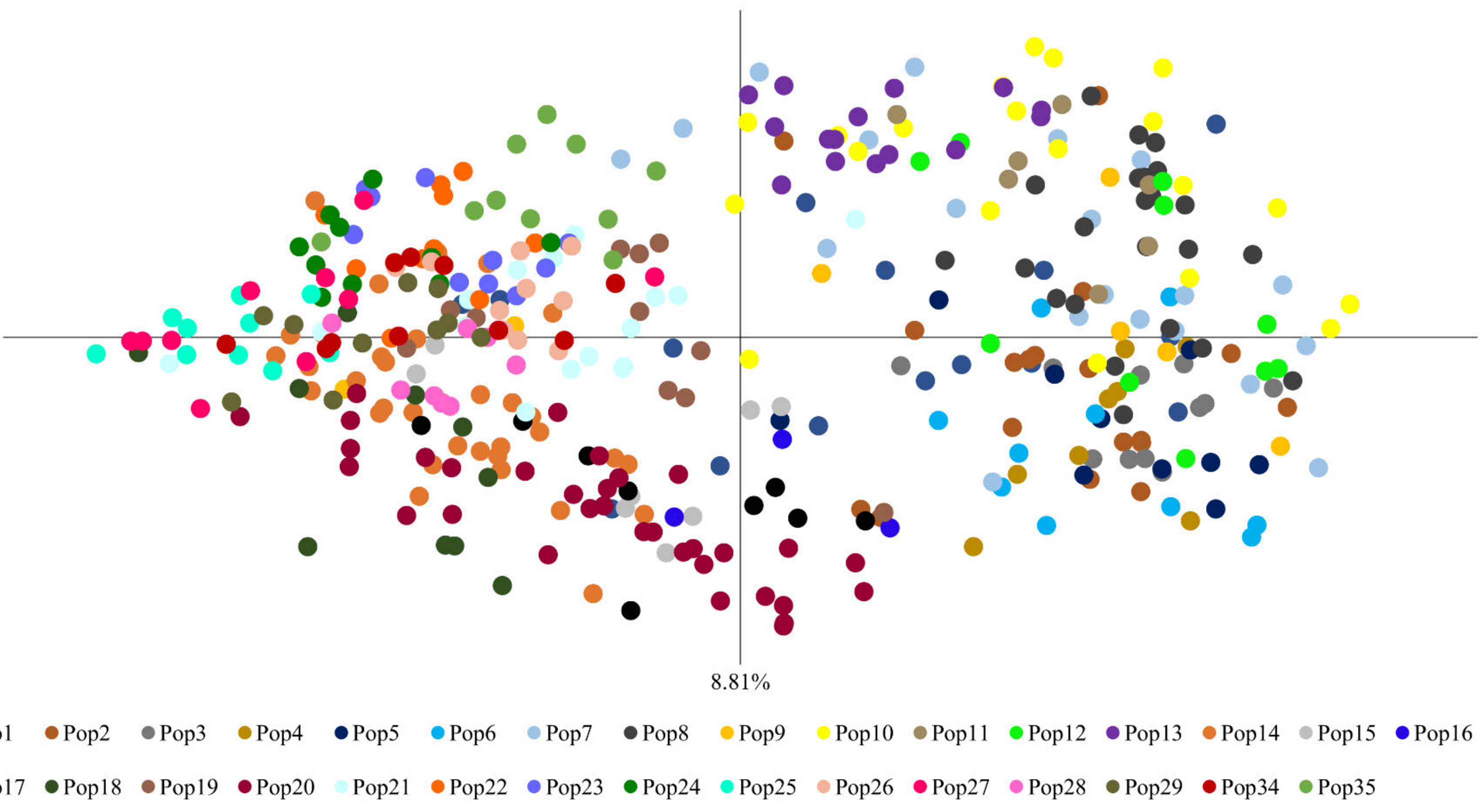

16.08%
8.81%
Pop1 Pop2 Pop3 Pop4 Pop5 Pop6 Pop7 Pop8 Pop9 Pop10 Pop11 Pop12 Pop13 Pop14 Pop15 Pop16
Pop17 Pop18 Pop19 Pop20 Pop21 Pop22 Pop23 Pop24 Pop25 Pop26 Pop27 Pop28 Pop29 Pop34 Pop35

Figure 4.3: A Principal coordinate analysis (PCoA) of wild *Oreochromis mossambicus* and the two introduced species (*O. niloticus and O. aureus*). a) grouped by the sampled localities in the Limpopo, Mpumalanga, and KwaZulu-Natal provinces, South Africa, plotted using a genetic distance between individuals colour-coded by Province and species, and b) individuals collected from sampled localities, plotted using Nei's unbiased genetic distances (1972), colour-coded by population. (Note: Pop = Population, and the number relates to the name of sampled localities (Supplementary information Table 4.1). (Note: WRCE Dam = White River Country Estate Dam, Pieter Dam = Pieter Vorster Dam).

4.4.3 Genetic signals of potential hybridisation in farmed O. mossambicus

When the four farmed *O. mossambicus* populations and the two introduced species (*O. niloticus* and *O. aureus*) were included in STRUCTURE analyses using the admixture model, K = 5 emerged as the optimal genetic partitioning strategy according to the Puechmaille method (Figure 4.4). Comparative bar plots for K = 4 and K = 6 are included, supporting a consistent clustering pattern. The STRUCTURE results indicated that most farmed *O. mossambicus* populations from KwaZulu-Natal and Mpumalanga showed minimal evidence of introduced genetic material from only the introduced *O. aureus*. However, the uMphafa ponds population displayed shared genotypic frequencies with *O. aureus*, albeit at relatively low levels. Additionally, there was also a minor component of *O. aureus* genotypes in the Zini Fish Farm population.

The principal coordinate analysis (PCoA), plotted using Nei's unbiased genetic distances (Supplementary Information Table 4.3), provided additional support for the STRUCTURE results. It revealed a lack of overlap between the three species, except for fish from uMphafa ponds and Fresca Fisheries Farm, which clustered with *O. aureus* (Figure 4.4). This congruence between STRUCTURE and PCoA outcomes strengthens the evidence for distinct genetic profiles among the farmed *O. mossambicus* populations and the introduced species but also indicates limited introgression, especially with *O. aureus*.

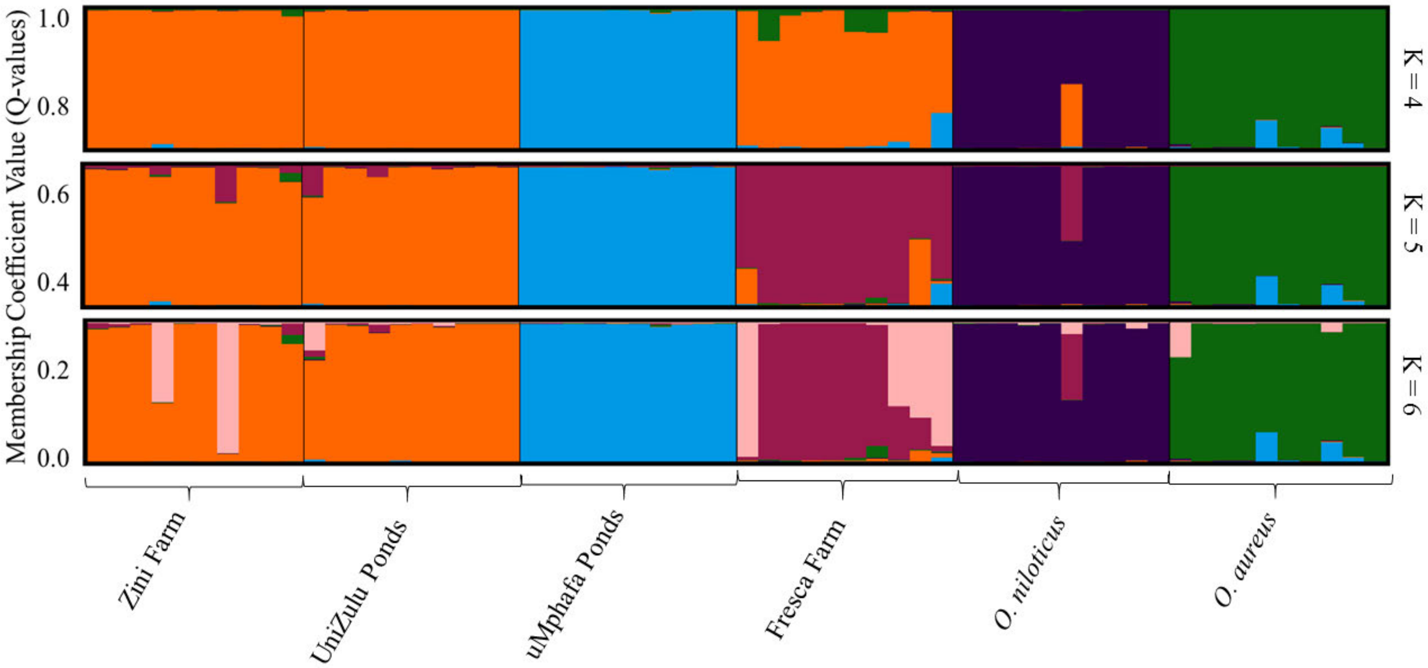
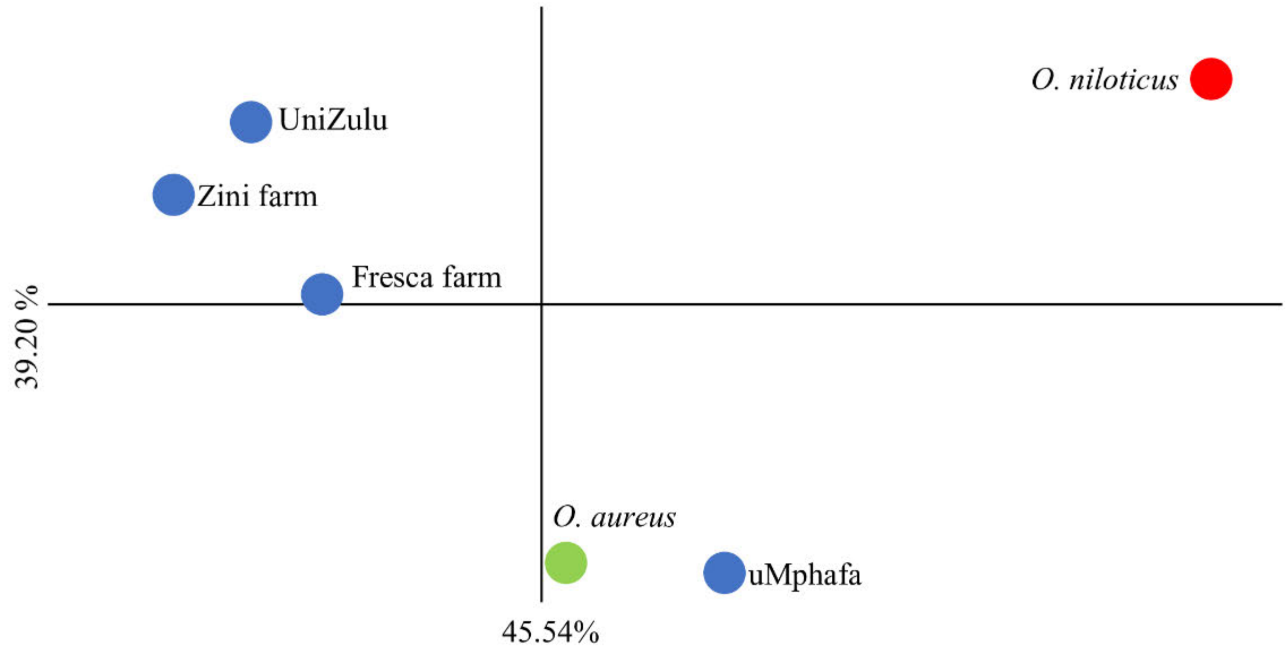


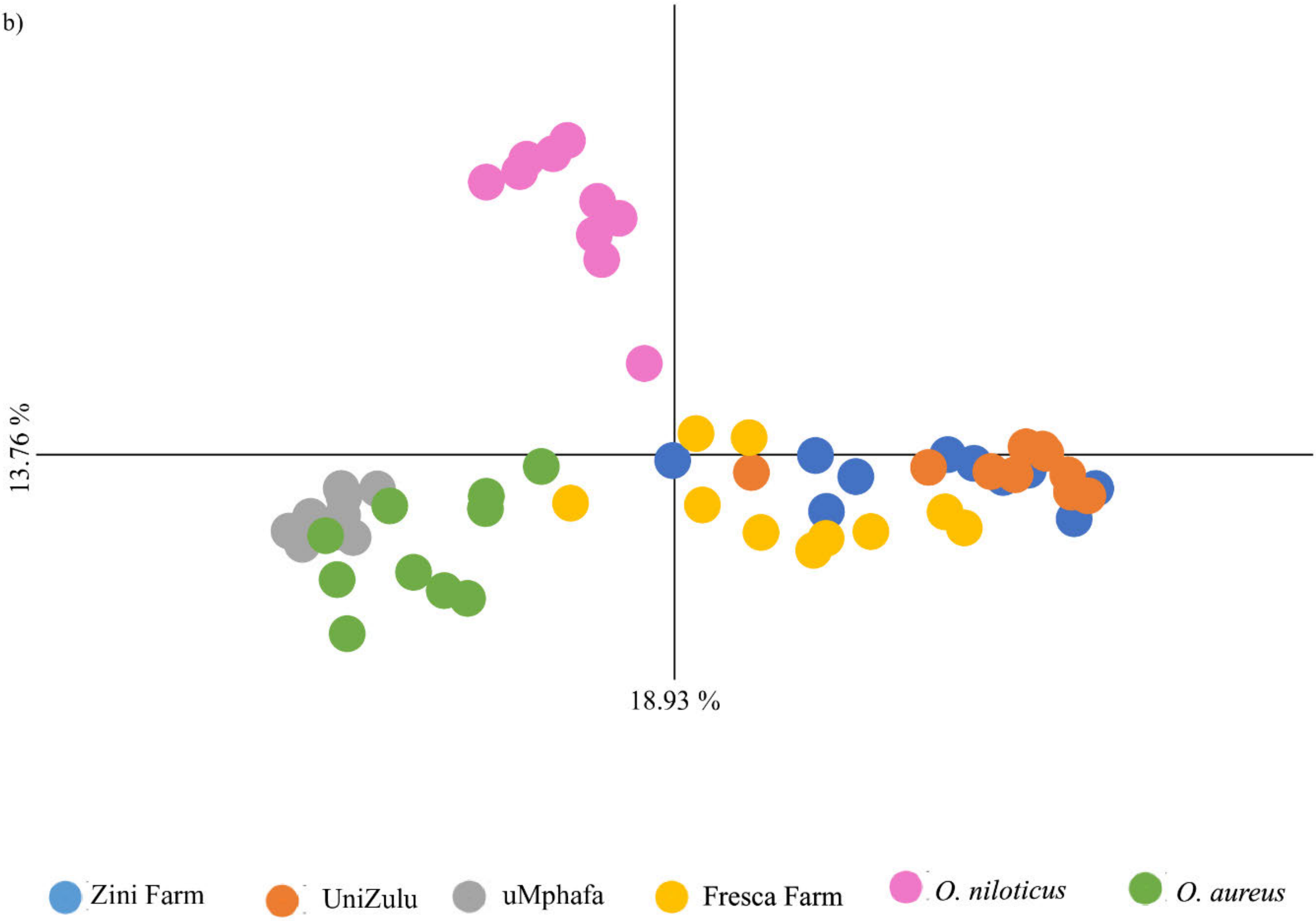
Figure 4.4: STRUCTURE bar plots showing the assignment of individuals from the four farmed *O. mossambicus* populations in Mpumalanga and KwaZulu-Natal, with the two introduced species populations (*O. niloticus* and *O. aureus*) based on membership coefficient values (Q-values). Three K-values are provided, K = 4, K = 5, and K = 6. The most likely recovered optimal value of genetic clusters was K = 5. (Note: Zini farm = Zini Fish Farm; UniZulu ponds = University of Zululand, = Fresca farm= Fresca Fisheries Farm).

a)



O. niloticus
UniZulu
Zini farm
Fresca farm
39.20 %
O. aureus
uMphafa
45.54%

b)



13.76 %
18.93 %
Zini Farm
UniZulu
uMphafa
Fresca Farm
O. niloticus
O. aureus

Figure 4.5: a) Principal Coordinates Analysis (PCoA) a) of the four farmed *O. mossambicus* populations and the two introduced species *(O. niloticus* and *O. aureus)* based on Nei's (1972) unbiased genetic distance, with individuals grouped by population or species, and b) of the farmed *O. mossambicus* and the two introduced species *(O. niloticus and O. aureus)* individuals plotted using a genetic distance between individuals and colour-coded by population. (Note: Zini farm = Zini Fish Farm; UniZulu = University of Zululand, uMphafa = uMphafa ponds, Fresca farm= Fresca Fisheries Farm, Pieter Dam = Pieter Vorster Dam).

The results from the Analysis of Molecular Variance (AMOVA) highlighted significant genetic differentiation between both farmed and wild populations of *O. mossambicus* and the introduced species (*O. niloticus* and *O. aureus*). However, genetic similarities were observed among individuals of the three tilapia species. Grouping individuals into populations of the three species revealed that 21% of the observed genetic variation was attributed to differences among species, indicating distinct genetic profiles. Meanwhile, a slightly higher level of differentiation (28%) was observed among samples/individuals within species, suggesting some intraspecific variation. Additionally, a substantial proportion (51%) of the variation was found among individuals within samples, underscoring genetic diversity within populations. These findings collectively demonstrate significant genetic structuring among the studied *Oreochromis* species (Table 4.3).

The pairwise F_{ST}-values provided a spectrum of genetic differentiation among the studied populations. For wild *O. mossambicus* populations, F_{ST}-values ranged from 0.21 between Luvuvhu and *O. niloticus* populations to 0.42 between Mtamvuna and *O. niloticus* populations. F_{ST}-values between *O. mossambicus* and *O. aureus* varied from 0.25 (Olifants and Pieter Dam) to 0.46 for Matigulu (Supplementary Table 4.4). For the farmed *O. mossambicus* populations and *O. niloticus*, F_{ST}-values varied from 0.32 in Fresca Farm to 0.38 for uMphafa ponds. Between *O. aureus* and farmed *O. mossambicus*, the F_{ST}-values ranged from 0.28 for

Fresca Farm to 0.43 in UniZulu (Supplementary Table 4.4). The significance values associated with these F_{ST}-values reflected the confidence in the observed genetic differentiation.

Table 4.3: Analysis of molecular variance (AMOVA) groupings at 14 microsatellite loci for farmed and wild *O. mossambicus* and the two introduced species (*O. niloticus* and *O. aureus*) in Limpopo, Mpumalanga, and KwaZulu-Natal provinces, South Africa.

Source of variation	**Sum of squares**	**Variance components**	**Percentage variation (%)**	**P-value <0.001**
Among species	1169.46	1.14	21	0.001
Among samples within species	2421.36	1.57	28	0.001
Among individuals within samples	1246.00	2.83	51	0.001
Total	**4836.82**	5.54	**100**	**-**

4.5 Discussion

The present study evaluated the presence of introduced genetic material from the two introduced *Oreochromis* species (*O. niloticus* and *O. aureus*) within wild and farmed populations of the native *O. mossambicus* in South Africa, specifically in the Limpopo, Mpumalanga, and KwaZulu-Natal provinces. I aimed to assess whether this genetic material might be indicative of potential hybridisation between the species in the studied sampling localities. The study findings showed distinctive patterns in genetic diversity, with *O. aureus* displaying relatively lower diversity than *O. mossambicus* and *O. niloticus*. The observed lower genetic diversity in *O. aureus* might be partially attributed to technical limitations associated with the efficiency of the primers used in this study. These primers were designed specifically for *O. mossambicus* and *O. niloticus*, and their amplification efficiency in *O. aureus* may not be optimal, as reported in studies evaluating primer performance across cichlid species (Saju et al. 2010; Simbine et al. 2014). However, it's important to acknowledge that these limitations likely reflect issues with primer design, cross-amplification success, or PCR conditions, rather than underlying ecological differences in diversity. The polymorphic nature of the 14 microsatellite loci, despite the challenges in *O. aureus*, underscores their utility, providing evidence for the genetic complexities within these populations. Given that the reference samples for *O. niloticus* and *O. aureus* were sourced from farmed stocks, this could have potentially contributed to the observed low genetic diversity, particularly in *O. aureus*. Inbreeding depression, stemming from genetic drift, inbreeding, selection for captive conditions, and imbalanced sex ratios of breeders, are well-documented factors influencing genetic diversity in farmed populations (Tave 1993, Wang 2002, Duong and Scribner 2018).

While almost all *O. mossambicus* and *O. niloticus* populations-maintained equilibrium with Hardy-Weinberg expectations, *O. aureus* displayed significant deviations. These deviations might be attributed to factors such as potential founder effects, non-random mating, and Wahlund effects (Wahlund 1928, Mitton 1989, Selkoe & Toonen 2006, Briñez et al. 2011, Simbine et al. 2014, Parreira et al. 2015, Aguiar et al. 2018, Sainz-Escudero et al. 2023). However, it's important to consider that the observed deviations could also be partially due to the use of primers with reduced amplification efficiency in *O. aureus*, as reported in previous studies (Saju et al. 2010, Simbine et al., 2014). This reduced efficiency could lead to an overestimation of homozygotes and a consequent deviation from HWE expectations. Inbreeding values varied across species, with *O. aureus* exhibiting higher levels than *O. mossambicus* and *O. niloticus*. The low reported negative inbreeding coefficient values in *O. mossambicus* and *O. niloticus* populations are consistent with findings in related studies (Li et al. 2009, Khadher et al. 2016, Aguiar et al. 2018, da Silva et al. 2020, Ahmed et al., 2023), which might suggest hight levels of heterozygosity or that these populations are likely outbred.

4.5.1 Genetic admixture and conservation implications

The introduction of non-native species has profound implications for the viability of natural populations (Shechonge et al. 2018, Bradbeer et al. 2019, Gu et al. 2022). In the case of tilapia species, hybridisation is a well-documented phenomenon (Rognon and Guyomard 2003, D'Amato et al. 2007, Nyingi and Agnèse 2007, Angienda et al. 2011, Wu et al. 2012, Deines et al. 2014, Richmond 2018, Shechonge et al. 2018, Kwikiriza et al. 2023) and represents a significant threat to the genetic integrity of vulnerable species like *O. mossambicus* (Moralee and Van Der Waal 2000, D'Amato et al. 2007, Firmat et al. 2013, Stauffer et al. 2022, Ciezarek et al. 2023). Hybridisation poses challenges to the adaptation potential of *O. mossambicus* in the wild, emphasising the need for continuous monitoring both in natural habitats and aquaculture settings (Moralee and Van Der Waal 2000, Stauffer et al. 2022, Ciezarek et al.

2023). This study investigated the potential for hybridisation between the native *O. mossambicus* and two introduced species, the *O. niloticus*, and the O. aureus, in South African freshwater systems. Previous research documented evidence of hybridisation in the Changane River, Limpopo Province (Firmat et al. 2013). Both *O. niloticus* and *O. aureus* have established populations in South Africa after introductions (Ellender & Weyl 2014, Marr et al. 2018, Weyl et al. 2020). Our analyses revealed shared genetic material between wild *O. mossambicus* and introduced *O. niloticus*. This was particularly evident in Limpopo Province River systems (Mapungubwe, Mogalakwena, Letaba, Luvuvhu). Notably, all individuals from the Mapungubwe population displayed high assignment to the *O. niloticus* cluster. While this suggests potential genetic introgression, it's important to acknowledge the possibility of misidentification in field sampling. Further investigation is needed to definitively determine the origin of these individuals. The presence of introduced genetic material in these populations underscores the ongoing threat of genetic contamination, especially considering the initial introduction points in Limpopo Province (Firmat et al. 2013, Weyl et al. 2020). Shared alleles between *O. niloticus* and *O. mossambicus* from the Olifants River suggest a potential connection with the Limpopo River Basin. This interconnectedness raises concerns about the persistence of genetic contamination in other connected Mpumalanga Province River systems. The presence of *O. niloticus* genetic material in the White River Country Estate Dam (WRCE Dam) suggests potential human-mediated introductions (Barnett et al. 2021), posing a further threat to native species' genetic resources. Similarly, the presence of *O. niloticus* genetic material in the Tongati River, KwaZulu-Natal, potentially linked to historical introductions at the Matigulu hatchery, emphasise the need for continued monitoring, especially in previously unaffected areas (Ellender & Weyl 2014, Marr et al. 2018). The interconnectedness of South African river systems managed by Water Management Areas (WMAs) presents an additional challenge (Driver et al. 2012, O'Brien et al. 2019). Increased connectivity, coupled with existing threats, elevates the risk of genetic integrity loss for uncontaminated *O. mossambicus* populations through hybridisation, either from the invasive species themselves or from conspecifics with contaminated genotypes. Therefore, a reassessment of river system management is crucial to mitigate these threats and protect native *O. mossambicus* (Mashaphu et al. 2024).

While the aquaculture industry often utilises hybrids (Esterhuyse 2002, Lind 2012, Deines et al. 2014, Amoussou et al. 2019, Kang et al. 2023), this study did not detect shared genotypic frequencies between *O. aureus* and wild *O. mossambicus*. This suggests that the absence of shared genotypic frequencies could be due to differences in hybridisation dynamics, or it might simply reflect differences in the introduction, establishment, demographics, and distributions of these species (*O, niloticus* and *O. aureus*). However, common genotypes were observed between farmed *O. mossambicus* from uMphafa ponds (KwaZulu-Natal) and Fresca Fisheries Farm (Mpumalanga) and *O. aureus*. These shared genotypes suggest potential genetic interaction, which could imply either minimal hybridisation or the retention of ancestral alleles. This observation aligns with the possibility of ancestral polymorphism, which is known to occur in closely related species like tilapiines (Wohlfarth & Huluta 1981, Trewavas 1983, D'Amato et al. 2007, Firmat et al. 2013, Astudillo-Clavijo et al. 2023). Importantly, these findings raise caveats for the interpretation of uMphafa ponds as a unique population for conservation genetics, as previously indicated in Chapter 3. If contamination by *O. aureus* is confirmed, it would suggest a significant genetic influence from introduced species, challenging the assumption of genetic purity and uniqueness in this population. This revaluation underscores the complexity of genetic interactions in aquaculture settings and highlights the need for further research to elucidate the extent and implications of these findings. Regardless, close monitoring of these farmed stocks is essential to prevent escapes into the wild, particularly considering the established *O. aureus* presence in the Western Cape (Marr et al. 2018, Weyl et al. 2020).

The evaluation of genetic purity before cultivation is paramount for South African tilapia farms. Utilising genetically pure *O. mossambicus* not only mitigates contamination risks but also presents an opportunity for supplementing wild populations facing extinction threats (Fraser et al. 2008, Lorenzen et al. 2012, Bouchard et al. 2022, Baerwald et al. 2023). Our findings emphasise the need for rigorous monitoring and conservation efforts for *O. mossambicus* in South Africa. I recommend stringent measures to assess and preserve the genetic integrity of farmed stocks, advocating for the prohibition of farming *O. mossambicus* without prior genetic purity evaluation. Utilising genetically pure *O. mossambicus* in tilapia farming mitigates contamination risks and contributes significantly to the conservation and management of this vulnerable species in South Africa. While this study focused on microsatellite markers, further investigation using mitochondrial DNA (mtDNA) could provide valuable insights into potential historical hybridisation events and maternal lineages within these populations. This additional information would complement the current findings and enhance understanding of the extent and dynamics of genetic interactions between introduced and native tilapia species.

The information generated in this study serves as baseline genetic data for farmed *O. mossambicus* in KwaZulu-Natal and Mpumalanga, emphasising the need for improved management practices to maintain the genetic integrity of farmed and wild *O. mossambicus* populations. The recommendation is for the continued monitoring and evaluation of wild and farmed *O. mossambicus* populations used for supplementation, ensuring they remain free from contamination with introduced species. This proactive approach is essential, especially considering the historical introductions of *O. niloticus* and *O. aureus* in South Africa and the known impact on wild populations. The data from this study can serve as a valuable baseline for future comparisons, aiding in the sustainable use and development of aquaculture in South Africa.

4.6 Conclusions

This study investigated the presence of genetic material from introduced *O. niloticus* and *O. aureus* within wild and farmed populations of the native *O. mossambicus* across South Africa. Using microsatellite data, I found evidence of this introduced genetic material, particularly from *O. niloticus*, in wild *O. mossambicus* populations across Limpopo, Mpumalanga, and KwaZulu-Natal provinces. These findings suggest potential hybridisation events and highlight the ongoing threat of genetic contamination, especially in Limpopo Province. The study also assessed the genetic purity of farmed *O. mossambicus* stocks, detecting common shared alleles with introduced *O. aureus* in specific locations. This underscores the importance of evaluating genetic purity across all farmed populations and implementing regulations to prevent escapes impacting wild populations. Furthermore, this study highlights the critical need to preserve genetic integrity in farmed *O. mossambicus* populations to reduce the risks of hybridisation in interconnected river systems. Enhanced management practices can achieve a dual objective by mitigating threats to native fish while promoting sustainable growth in South Africa's aquaculture industry. By exploring the invasion dynamics of introduced *Oreochromis* species, this research equips stakeholders with essential tools for monitoring and conserving vulnerable *O. mossambicus* populations, thereby supporting the long-term success of conservation efforts and aquaculture development in the region.

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4.9 Supplementary information

Supplementary Table 4.1: Details of sampled localities where wild and farmed *Oreochromis mossambicus* were collected in Limpopo, Mpumalanga, and KwaZulu-Natal provinces, South Africa. Details for the two introduced Species (*O. niloticus* and *O. aureus*) are also indicated. (Note: WRCE Dam = White River Country Estate Dam, Pieter Dam = Pieter Vorster Dam, Zini farm = Zini Fish Farm, UniZulu ponds = University of Zululand tilapia ponds, Fresca farm = Fresca Fisheries Farm**).**

Catchment	River/Dam/locality	Region	Sample size	Population ID	Longitude	Latitude
Phongolo	Phongolo	KwaZulu-Natal	15	Pop1	-26.929964	32.324218
Mkuze	Mkuze	KwaZulu-Natal	18	Pop2	-27.653055	32.403056
Hluhluwe	Hluhluwe	KwaZulu-Natal	11	Pop3	-28.119305	32.183157
Mfolozi	Mfolozi	KwaZulu-Natal	8	Pop4	-28.451579	32.182396
Mhlathuze	Mhlathuze	KwaZulu-Natal	10	Pop5	-28.746950	31.747450
Matigulu	Matigulu	KwaZulu-Natal	10	Pop6	-29.072961	31.557560
Thukela	Thukela	KwaZulu-Natal	20	Pop7	-29.170560	31.421092
Mvoti	Mvoti	KwaZulu-Natal	25	Pop8	-29.370141	31.304451
Tongati	Tongati	KwaZulu-Natal	7	Pop9	-29.559913	31.174085
uMngeni	Albert Falls Dam	KwaZulu-Natal	20	Pop10	-29.444805	30.389196
uMlazi	uMlazi	KwaZulu-Natal	7	Pop11	-29.869000	30.781200
Mzimkhulu	Mzimkhulu	KwaZulu-Natal	10	Pop12	-30.635981	30.196877
Mtamvuna	Mtamvuna	KwaZulu-Natal	15	Pop13	-30.849236	30.064003
Olifants	Olifants	Mpumalanga	10	Pop14	-24.008221	31.737847
Olifants	Loskop Dam	Mpumalanga	16	Pop15	-25.429315	29.398656
Crocodile	Nwaswitsontso	Mpumalanga	9	Pop16	-24.794159	31.904441
Crocodile	Biyamiti	Mpumalanga	3	Pop17	-25.316351	31.744484
Crocodile	Nsikasi	Mpumalanga	9	Pop18	-25.333133	31.275137
Crocodile	WRCE Dam	Mpumalanga	10	Pop19	-25.285210	31.006546
Komati	Komati River	Mpumalanga	11	Pop20	-25.450801	31.951443
Komati	Pieter Vorster Dam	Mpumalanga	33	Pop21	-25.525341	31.862491

Limpopo	Crocodile	Limpopo	10	Pop22	-24.314167	27.046139
Limpopo	Lephalale	Limpopo	10	Pop23	-23.141278	27.885028
Limpopo	Mogalakwena	Limpopo	10	Pop24	-22.473444	28.919500
Limpopo	Mapungubwe	Limpopo	10	Pop25	-22.183833	29.405194
Limpopo	Sand River	Limpopo	10	Pop26	-22.399278	30.099417
Limpopo	Luvuvhu	Limpopo	10	Pop27	-22.444444	31.083444
Olifants	Shingwedzi	Limpopo	9	Pop28	-23.221944	31.554917
Olifants	Letaba	Limpopo	10	Pop29	-23.758333	31.369972
Farmed	Zini Farm	KwaZulu-Natal	10	Pop30	-28.950300	31.760000
Farmed						
	UniZulu Ponds	KwaZulu-Natal	10	Pop31	-28.852400	31.849200
Farmed	uMphafa Ponds	KwaZulu-Natal	10	Pop32	-28.744400	29.699800
Farmed	Fresca Farm	Mpumalanga	10	Pop33	-25.489700	31.970800
Introduced	*O. niloticus*	Western Cape	10	Pop34	-18.865825	33.942777
Introduced	*O. aureus*	Western Cape	10	Pop35	-18.865825	33.942777

Supplementary Table 4.2: Genetic diversity indices of the 14 microsatellite loci amplified for *O. mossambicus*. *O. niloticus* and *O. aureus* in the present study.

Oreochromis mossambicus

Locus	Na	Ne	Ar	Null allele frequencies	F_{ST}^{A}	F_{ST}^{B}	H_O	uH_E	P_{HWE}	F_{is}	PIC
OM01	7.97	5.17	3.95	0.13	0.14	0.12	0.52	0.79	*	0.30	0.87
OM02	5.52	3.46	3.06	0.18	0.21	0.19	0.30	0.60	ns	0.49	0.87
OM03	5.64	3.57	3.07	0.16	0.21	0.20	0.34	0.61	ns	0.41	0.89
OM04	7.21	4.91	3.90	0.04	0.14	0.14	0.77	0.79	*	-0.03	0.88
OM05	5.67	3.78	3.51	0.13	0.16	0.14	0.53	0.74	ns	0.26	0.89
OM06	2.48	1.61	1.83	0.17	0.53	0.49	0.12	0.32	ns	0.61	0.77
OM07	8.67	6.27	4.34	0.03	0.08	0.09	0.88	0.85	*	-0.08	0.90
OM08	3.42	2.51	2.59	0.08	0.21	0.21	0.53	0.58	ns	0.04	0.76
OM09	5.67	3.58	3.27	0.11	0.22	0.21	0.46	0.66	ns	0.26	0.80
UNH104	5.15	3.13	3.13	0.16	0.19	0.17	0.40	0.67	ns	0.37	0.81
UNH129	4.45	2.68	2.61	0.09	0.18	0.16	0.39	0.51	ns	0.20	0.70
UNH142	3.42	2.19	2.41	0.15	0.21	0.17	0.30	0.52	ns	0.39	0.77
UNH222	4.09	2.73	2.87	0.21	0.19	0.17	0.26	0.63	ns	0.56	0.78
UNH231	2.30	1.41	1.65	0.10	0.18	0.24	0.10	0.23	ns	0.55	0.50
Mean	**5.12**	**3.36**	**3.01**	**0.13**	**0.20**	**0.19**	**0.42**	**0.61**	**-**	**0.31**	**0.80**

Oreochromis niloticus

Locus	Na	Ne	Ar	Null allele frequencies	F_{ST}^{A}	F_{ST}^{B}	H_O	uH_E	P_{HWE}	F_{is}	PIC
OM01	7.00	3.85	7.00	0.12	-0.10	-0.11	0.50	0.78	*	0.31	0.74
OM02	4.00	2.60	4.00	0.18	-0.09	-0.08	0.30	0.65	ns	0.49	0.75
OM03	4.00	2.60	4.00	0.18	-0.09	-0.08	0.30	0.65	ns	0.49	0.74
OM04	3.00	2.30	3.00	0.04	-0.03	-0.03	0.70	0.59	*	-0.27	0.44
OM05	5.00	3.23	5.00	0.00	-0.07	-0.07	0.80	0.73	ns	-0.18	0.83
OM06	1.00	1.00	1.00	0.00	N/A	-0.13	0.00	0.00	ns	N/A	0.38
OM07	6.00	3.64	6.00	0.10	0.12	0.16	0.50	0.76	*	0.21	0.69
OM08	4.00	2.94	4.00	0.40	-0.04	-0.02	0.00	0.69	*	1.00	0.65
OM09	7.00	2.53	7.00	0.00	-0.05	-0.05	0.50	0.64	*	0.14	0.77
UNH104	3.00	1.65	3.00	0.23	-0.19	-0.13	0.10	0.42	*	0.74	0.60
UNH129	6.00	4.00	6.00	0.03	-0.01	-0.01	0.70	0.79	ns	0.01	0.78
UNH142	5.00	2.70	5.00	0.26	-0.03	-0.05	0.20	0.66	*	0.66	0.68
UNH222	5.00	4.26	5.00	0.32	0.00	0.01	0.20	0.81	*	0.71	0.82
UNH231	5.00	3.57	5.00	0.09	-0.08	-0.04	0.60	0.76	ns	0.14	0.74
Mean	**4.64**	**2.92**	**4.64**	**0.14**	**-0.05**	**-0.05**	**0.39**	**0.64**	**-**	**0.34**	**0.68**

Oreochromis aureus

Locus	Na	Ne	Ar	Null allele frequencies	F_{ST}^{A}	F_{ST}^{B}	H_O	uH_E	P_{HWE}	F_{is}	PIC
OM01	5.00	2.47	4.53	0.24	0.03	0.03	0.20	0.63	*	0.62	0.68
OM02	4.00	1.53	3.32	0.19	0.13	0.30	0.10	0.36	*	0.66	0.56
OM03	5.00	1.72	4.02	0.16	0.02	0.16	0.20	0.44	*	0.47	0.66
OM04	6.00	3.85	5.30	0.00	-0.08	-0.08	0.80	0.78	*	-0.10	0.79
OM05	2.00	1.80	2.00	0.32	-0.23	-0.14	0.00	0.47	*	1.00	0.67
OM06	1.00	1.00	1.00	0.00	N/A	-0.13	0.00	0.00	ns	N/A	0.38

OM07	3.00	1.81	3.00	0.15	0.05	0.11	0.29	0.48	ns	0.30	0.66
OM08	1.00	1.00	1.00	0.00	N/A	-0.15	0.00	0.00	ns	N/A	0.38
OM09	4.00	2.75	3.74	0.32	0.22	0.13	0.11	0.67	*	0.79	0.72
UNH104	3.00	1.59	2.92	0.00	-0.20	-0.20	0.22	0.39	*	0.40	0.50
UNH129	4.00	1.87	3.40	0.20	-0.12	-0.10	0.20	0.49	ns	0.56	0.73
UNH142	5.00	1.92	4.08	0.00	-0.10	-0.10	0.40	0.51	*	0.15	0.61
UNH222	3.00	2.17	2.92	0.36	0.00	0.04	0.00	0.57	*	1.00	0.58
UNH231	2.00	1.53	2.00	0.28	-0.28	-0.17	0.00	0.37	*	1.00	0.50
Mean	**3.43**	**1.93**	**3.09**	**0.16**	**-0.05**	**-0.02**	**0.18**	**0.44**	**-**	**0.63**	**0.60**

Na: number of alleles, Ar: Allelic richness, F_{ST}^{A}: non-corrected F_{ST}, F_{ST}^{B}: ENA corrected F_{ST}, H_O: observed heterozygosity, uH_E: unbiased expected heterozygosity. P_{HWE}: deviation from Hardy-Weinberg equilibrium (*P<0.05), PIC: polymorphic information content.

Supplementary Table 4.3: Pairwise Nei's genetic distance estimates for the four farmed *O. mossambicus* populations, 25 *O. mossambicus* populations and two introduced species populations (*O. niloticus* and *O. aureus*). (Note: WRCE Dam = White River Country Estate Dam, Pieter Dam = Pieter Vorster Dam, Zini farm = Zini Fish Farm, UniZulu ponds = University of Zululand tilapia ponds, Fresca farm = Fresca Fisheries Farm).

Sampled localities	Phongolo	Mkuze	Hluhluwe	Mfolozi	Mhlathuze	Matigulu	Thukela	Mvoti	Tongati	Albert Falls Dam	uMlazi	Mzimkhulu	Mtamvuna	Olifants	Loskop Dam	Nwaswitsontso	Biyamiti	Nsikasi	WRCE Dam	Komati River	Pieter Dam	Crocodile	Lephalale	Mogalakwena	Mapungubwe	Sand River	Luvuvhu	Shingwedzi	Letaba	Zini farm	uniZulu ponds	uMphafa ponds	Fresca Farm	*O. niloticus*
Mkuze	0.19																																	
Hluhluwe	0.18	0.13																																
Mfolozi	0.22	0.07	0.10																															
Mhlathuze	0.21	0.12	0.04	0.12																														
Matigulu	0.44	0.10	0.30	0.21	0.24																													
Thukela	0.15	0.17	0.16	0.20	0.20	0.47																												
Mvoti	0.29	0.22	0.25	0.34	0.20	0.30	0.17																											
Tongati	0.16	0.16	0.14	0.12	0.12	0.36	0.11	0.18																										
Albert Falls Dam	0.22	0.20	0.25	0.31	0.33	0.48	0.09	0.16	0.22																									
uMlazi	0.32	0.30	0.30	0.37	0.30	0.48	0.20	0.13	0.18	0.21																								
Mzimkhulu	0.17	0.16	0.16	0.21	0.14	0.30	0.15	0.14	0.15	0.20	0.12																							
Mtamvuna	0.49	0.46	0.70	0.69	0.66	0.78	0.36	0.39	0.57	0.29	0.30	0.33																						
Olifants	0.41	0.45	0.56	0.46	0.51	0.62	0.57	0.70	0.51	0.65	0.79	0.58	0.79																					
Loskop Dam	0.42	0.33	0.35	0.30	0.37	0.54	0.49	0.68	0.40	0.62	0.73	0.51	0.86	0.24																				
Nwaswitsontso	0.44	0.28	0.27	0.19	0.25	0.40	0.53	0.66	0.41	0.66	0.68	0.43	0.93	0.38	0.14																			
Biyamiti	0.45	0.39	0.42	0.34	0.39	0.55	0.55	0.71	0.49	0.73	0.83	0.54	0.86	0.34	0.08	0.20																		
Nsikasi	0.70	0.66	0.71	0.70	0.65	0.78	0.79	1.01	0.75	1.02	1.20	0.81	1.04	0.19	0.26	0.41	0.23																	
WRCE Dam	0.37	0.27	0.47	0.44	0.45	0.45	0.37	0.44	0.38	0.43	0.51	0.51	0.52	0.40	0.52	0.56	0.54	0.52																
Komati River	0.50	0.54	0.58	0.49	0.56	0.66	0.82	0.98	0.63	0.96	0.94	0.76	1.21	0.50	0.44	0.47	0.42	0.61	0.65															
Pieter Dam	0.65	0.42	0.73	0.61	0.60	0.51	0.55	0.52	0.64	0.55	0.69	0.66	0.53	0.31	0.50	0.53	0.59	0.50	0.13	0.93														
Crocodile	0.68	0.67	0.80	0.72	0.87	0.91	0.58	0.71	0.69	0.51	0.54	0.71	0.72	0.33	0.48	0.72	0.67	0.58	0.58	0.92	0.44													
Lephalale	0.57	0.66	0.76	0.70	0.81	0.98	0.53	0.61	0.63	0.40	0.54	0.63	0.64	0.35	0.44	0.89	0.61	0.57	0.65	0.90	0.58	0.12												
Mogalakwena	0.72	0.77	0.77	0.78	0.84	1.25	0.59	0.74	0.60	0.56	0.64	0.76	0.79	0.32	0.42	0.75	0.69	0.45	0.64	1.08	0.62	0.17	0.10											
Mapungubwe	1.37	1.67	1.75	1.92	1.66	2.20	1.58	1.95	1.39	1.47	1.80	1.64	1.49	0.87	0.91	1.10	1.14	0.71	1.16	1.41	1.12	1.22	1.17	0.74										
Sand River	0.55	0.59	0.63	0.64	0.64	0.86	0.45	0.67	0.54	0.46	0.61	0.58	0.73	0.28	0.51	0.66	0.73	0.56	0.55	0.74	0.46	0.38	0.36	0.32	1.26									
Luvuvhu	0.80	0.90	1.04	1.01	0.98	1.12	0.86	0.96	0.96	0.82	1.06	0.86	0.87	0.24	0.42	0.64	0.61	0.30	0.60	1.06	0.45	0.28	0.28	0.17	0.41	0.54								
Shingwedzi	0.85	0.85	1.08	0.98	0.92	0.91	0.90	0.88	0.86	0.90	1.16	1.02	1.12	0.45	0.77	0.97	0.89	0.68	0.77	0.72	0.60	0.89	0.85	0.73	1.32	0.35	0.80							
Letaba	1.02	0.94	1.05	0.91	0.94	0.96	1.01	0.95	0.88	1.00	1.00	0.96	1.25	0.42	0.70	0.56	0.82	0.58	0.82	1.05	0.50	0.53	0.73	0.54	0.99	0.63	0.43	0.67						
Zini farm	0.32	0.15	0.17	0.22	0.09	0.16	0.33	0.24	0.22	0.40	0.30	0.23	0.57	0.52	0.31	0.24	0.38	0.67	0.41	0.49	0.41	0.81	0.88	0.96	1.57	0.72	0.94	0.86	0.82					
uniZulu ponds	0.25	0.17	0.11	0.16	0.06	0.27	0.31	0.30	0.17	0.45	0.43	0.21	0.82	0.49	0.36	0.25	0.34	0.60	0.48	0.53	0.59	0.93	0.92	1.00	1.61	0.82	0.97	0.88	0.95	0.12				
uMphafa ponds	0.72	0.79	0.90	0.91	0.98	1.43	0.62	0.80	0.76	0.44	0.56	0.75	0.59	0.72	0.93	0.67	1.36	1.20	0.74	1.32	0.81	0.42	0.41	0.42	1.13	0.58	0.63	1.25	0.73	1.15	1.23			
Fresca Farm	0.35	0.27	0.37	0.24	0.33	0.44	0.38	0.52	0.44	0.54	0.70	0.46	0.66	0.33	0.30	0.16	0.31	0.41	0.30	0.54	0.31	0.70	0.71	0.77	0.99	0.68	0.59	0.74	0.60	0.34	0.24	0.78		
O. niloticus	1.41	1.44	1.49	1.37	1.41	1.83	1.42	1.70	1.05	1.43	1.41	1.29	1.82	1.24	1.26	1.42	1.44	1.44	1.33	2.31	1.77	1.52	1.47	1.10	1.27	1.40	1.13	1.81	1.49	1.69	1.39	1.47	1.44	
O. aureus	0.75	0.79	1.08	0.87	1.06	1.31	0.68	0.90	0.85	0.65	0.71	0.81	0.59	0.79	0.77	0.63	0.86	1.21	0.72	1.34	0.68	0.56	0.72	0.82	1.25	0.88	0.75	1.14	0.85	0.93	1.09	0.44	0.59	1.63

Unbiased Nei's unbiased genetic distance (Nei 1972) values ranging from 0- 0.05 indicate low genetic distance (high genetic similarity), 0.1-0.30 = moderate genetic distance (indicate moderate differentiation), 0.30-1 = high genetic distance (high genetic differentiation), and ≥ 1= very great genetic distance

Supplementary Table 4.4: Estimates of ENA-corrected pairwise F_{ST}-values and probability (P-values) of the genetic differences for the four farmed *O. mossambicus* populations, 25 *O. mossambicus* populations and two introduced species populations (*O. niloticus* and *O. aureus*). (Note: WRCE Dam = White River Country Estate Dam, Pieter Dam = Pieter Vorster Dam, Zini farm = Zini Fish Farm, UniZulu ponds = University of Zululand tilapia ponds, Fresca farm = Fresca Fisheries Farm).

Sampled localities	Phongolo	Mkuze	Hluhluwe	Mfolozi	Mhlathuze	Matigulu	Thukela	Mvoti	Tongati	Albert Falls Dam	uMlazi	Mzimkhulu	Mtamvuna	Olifants	Nwaswitsontso	Biyamiti	Nsikasi	WRCE Dam	Komati River	Pieter Dam	Loskop Dam	Crocodile	Lephalale	Mogalakwena	Mapungubwe	Sand River	Luvuvhu	Shingwedzi	Letaba	Zini farm	uniZulu ponds	uMphafa ponds	Fresca Farm	*O. niloticus*	*O. aureus*
Phongolo		0.00025^{NS}	0.00017^{NS}	0.00134^{NS}	0.00025^{NS}	0.00008^{*}	0.00059^{NS}	0.00008^{*}	0.07706^{NS}	0.00008^{*}	0.00042^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00370^{NS}	0.00008^{*}	0.00008^{*}	0.00025^{NS}	0.00008^{*}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00034^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00042^{NS}
Mkuze	0.08		0.00008^{*}	0.02345^{NS}	0.00017^{NS}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.01471^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00185^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}
Hluhluwe	0.08	0.07		0.00395^{NS}	0.02689^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00370^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00034^{NS}	0.00479^{NS}	0.00017^{NS}	0.00008^{*}	0.00025^{NS}	0.00008^{*}	0.00008^{*}	0.00017^{NS}	0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00067^{NS}	0.00008^{*}	0.00008^{*}	0.00025^{NS}	0.00008^{*}	0.00067^{NS}
Mfolozi	0.10	0.03	0.05		0.00101^{NS}	0.00042^{NS}	0.00008^{*}	0.00008^{*}	0.02992^{NS}	0.00008^{*}	0.00034^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00042^{NS}	0.00874^{NS}	0.00034^{NS}	0.00008^{*}	0.00067^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00025^{NS}	0.00034^{NS}	0.00008^{*}	0.00025^{NS}	0.00034^{NS}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00109^{NS}
Mhlathuze	0.09	0.06	0.02	0.07		0.00025^{NS}	0.00008^{*}	0.00008^{*}	0.05378^{NS}	0.00008^{*}	0.00050^{NS}	0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00017^{NS}	0.00630^{NS}	0.00042^{NS}	0.00008^{*}	0.00042^{NS}	0.00008^{*}	0.00008^{*}	0.00025^{NS}	0.00008^{*}	0.00008^{*}	0.00025^{NS}	0.00008^{*}	0.00067^{NS}	0.00017^{NS}	0.00773^{NS}	0.00034^{NS}	0.00017^{NS}	0.00017^{NS}	0.00008^{*}	0.00084^{NS}
Matigulu	0.20	0.07	0.19	0.15	0.16		0.00008^{*}	0.00008^{*}	0.00202^{NS}	0.00008^{*}	0.00017^{NS}	0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00042^{NS}	0.00504^{NS}	0.00059^{NS}	0.00008^{*}	0.00025^{NS}	0.00008^{*}	0.00008^{*}	0.00017^{NS}	0.00017^{NS}	0.00017^{NS}	0.00025^{NS}	0.00008^{*}	0.00025^{NS}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00017^{NS}	0.00025^{NS}	0.00008^{*}	0.00050^{NS}
Thukela	0.07	0.09	0.09	0.11	0.11	0.24		0.00008^{*}	0.01756^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00059^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00017^{NS}
Mvoti	0.14	0.13	0.15	0.19	0.12	0.19	0.11		0.00034^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00059^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}
Tongati	0.04	0.07	0.07	0.06	0.05	0.18	0.05	0.11		0.00092^{NS}	0.00218^{NS}	0.00151^{NS}	0.00042^{NS}	0.00008^{*}	0.00168^{NS}	0.00193^{NS}	0.02857^{NS}	0.00437^{NS}	0.00168^{NS}	0.00605^{NS}	0.00017^{NS}	0.00126^{NS}	0.00185^{NS}	0.00202^{NS}	0.00176^{NS}	0.00143^{NS}	0.00084^{NS}	0.00462^{NS}	0.00101^{NS}	0.00840^{NS}	0.00109^{NS}	0.00126^{NS}	0.00210^{NS}	0.00151^{NS}	0.00756^{NS}
Albert Falls Dam	0.11	0.12	0.15	0.18	0.18	0.27	0.06	0.11	0.12		0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00076^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}
uMlazi	0.12	0.15	0.17	0.20	0.16	0.28	0.11	0.09	0.08	0.13		0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00025^{NS}	0.00731^{NS}	0.00076^{NS}	0.00008^{*}	0.00084^{NS}	0.00008^{*}	0.00008^{*}	0.00017^{NS}	0.00017^{NS}	0.00017^{NS}	0.00034^{NS}	0.00034^{NS}	0.00059^{NS}	0.00017^{NS}	0.00050^{NS}	0.00025^{NS}	0.00017^{NS}	0.00034^{NS}	0.00008^{*}	0.00126^{NS}
Mzimkhulu	0.08	0.09	0.10	0.13	0.09	0.20	0.09	0.09	0.08	0.13	0.08		0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00025^{NS}	0.00429^{NS}	0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00042	0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00076^{NS}
Mtamvuna	0.23	0.26	0.34	0.35	0.33	0.40	0.22	0.25	0.27	0.20	0.22	0.23		0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00126^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}
Olifants	0.11	0.15	0.18	0.16	0.16	0.22	0.18	0.22	0.12	0.21	0.21	0.18	0.26		0.00008^{*}	0.00008^{*}	0.00218^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}
Nwaswitsontso	0.17	0.16	0.23	0.21	0.20	0.22	0.19	0.21	0.15	0.22	0.22	0.22	0.25	0.09		0.00050^{NS}	0.00378^{NS}	0.00017^{NS}	0.00008^{*}	0.02748^{NS}	0.00008^{*}	0.00008^{*}	0.00017^{NS}	0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00025^{NS}	0.00017^{NS}	0.00050^{NS}
Biyamiti	0.13	0.14	0.15	0.14	0.15	0.24	0.19	0.26	0.11	0.25	0.24	0.20	0.34	0.07	0.15	0.54882^{NS}	0.02723^{NS}	0.00017^{NS}	0.00160^{NS}	0.00008^{NS}	0.00008^{*}	0.00017^{NS}	0.00042^{NS}	0.00034^{NS}	0.00017^{NS}	0.00017^{NS}	0.00050^{NS}	0.00017^{NS}	0.00034^{NS}	0.00017^{NS}	0.00008^{*}	0.00025^{NS}	0.00008^{*}	0.00143^{NS}	0.54882^{NS}
Nsikasi	0.13	0.13	0.14	0.11	0.13	0.25	0.21	0.28	0.11	0.28	0.29	0.22	0.40	0.11	0.16	0.02		0.02210^{NS}	0.02244^{NS}	0.02412^{NS}	0.00034^{NS}	0.00445^{NS}	0.00756^{NS}	0.00647^{NS}	0.00504^{NS}	0.00487^{NS}	0.01294^{NS}	0.01185^{NS}	0.00445^{NS}	0.01882^{NS}	0.00454^{NS}	0.00412^{NS}	0.02950^{NS}	0.00319^{NS}	0.01782^{NS}
WRCE Dam	0.17	0.19	0.21	0.19	0.19	0.29	0.24	0.30	0.18	0.31	0.31	0.25	0.38	0.13	0.20	0.03	0.09		0.00025^{NS}	0.00134^{NS}	0.00008^{*}	0.00008^{*}	0.00034^{NS}	0.00067^{NS}	0.00059^{NS}	0.00017^{NS}	0.00017^{NS}	0.00101^{NS}	0.00034^{NS}	0.00025^{NS}	0.00017^{NS}	0.00008^{*}	0.00025^{NS}	0.00017^{NS}	0.00244^{NS}
Komati River	0.19	0.23	0.25	0.26	0.23	0.30	0.26	0.32	0.19	0.32	0.32	0.27	0.37	0.06	0.15	0.09	0.15	0.11		0.00042^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00076^{NS}
Loskop Dam	0.09	0.11	0.17	0.16	0.15	0.20	0.14	0.18	0.08	0.18	0.17	0.18	0.24	0.09	0.03	0.13	0.14	0.18	0.14		0.00008^{*}	0.00008^{*}	0.00076^{NS}	0.00050^{NS}	0.00025^{NS}	0.00017^{NS}	0.00034^{NS}	0.00134^{NS}	0.00025^{NS}	0.00034^{NS}	0.00034^{NS}	0.00017^{NS}	0.00193^{NS}	0.00025^{NS}	0.00261^{NS}
Pieter Dam	0.16	0.20	0.21	0.19	0.20	0.25	0.25	0.29	0.17	0.29	0.27	0.25	0.35	0.14	0.23	0.15	0.16	0.17	0.19	0.17		0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}
Crocodile	0.18	0.23	0.27	0.26	0.26	0.32	0.22	0.27	0.18	0.22	0.21	0.25	0.31	0.10	0.14	0.16	0.23	0.24	0.19	0.15	0.24		0.00588^{NS}	0.00160^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00017^{NS}
Lephalale	0.14	0.21	0.24	0.23	0.23	0.31	0.19	0.23	0.14	0.18	0.19	0.22	0.28	0.09	0.15	0.13	0.22	0.20	0.16	0.14	0.22	0.04		0.01017^{NS}	0.00017^{NS}	0.00017^{NS}	0.00008^{*}	0.00042^{NS}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00151^{NS}
Mogalakwena	0.16	0.22	0.23	0.24	0.23	0.32	0.19	0.25	0.13	0.21	0.20	0.23	0.30	0.08	0.15	0.12	0.19	0.21	0.13	0.13	0.23	0.06	0.02		0.00008^{*}	0.00017^{NS}	0.02050^{NS}	0.00025^{NS}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00025^{NS}	0.00008^{*}	0.00076^{NS}

Mapungubwe	0.24	0.32	0.34	0.35	0.32	0.40	0.32	0.37	0.23	0.35	0.34	0.34	0.39	0.18	0.23	0.22	0.26	0.29	0.20	0.21	0.27	0.26	0.23	0.17		0.00008^{*}	0.00017^{NS}	0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00059^{NS}
Sand River	0.13	0.19	0.20	0.21	0.19	0.28	0.16	0.23	0.12	0.19	0.20	0.20	0.29	0.07	0.12	0.13	0.17	0.22	0.15	0.11	0.19	0.12	0.09	0.08	0.22		0.00008^{*}	0.00025^{NS}	0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00017^{NS}	0.00017^{NS}	0.00008^{*}	0.00084^{NS}
Luvuvhu	0.16	0.23	0.25	0.25	0.23	0.30	0.23	0.27	0.16	0.25	0.25	0.23	0.30	0.06	0.11	0.11	0.15	0.18	0.09	0.11	0.22	0.09	0.07	0.03	0.10	0.11		0.00034^{NS}	0.00017^{NS}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00017^{NS}	0.00008^{*}	0.00050^{NS}
Shingwedzi	0.21	0.27	0.31	0.31	0.28	0.33	0.28	0.30	0.21	0.31	0.32	0.31	0.38	0.13	0.17	0.22	0.28	0.28	0.21	0.18	0.22	0.24	0.21	0.19	0.27	0.11	0.18		0.00017^{NS}	0.00025^{NS}	0.00017^{NS}	0.00025^{NS}	0.00025^{NS}	0.00025^{NS}	0.00227^{NS}
Letaba	0.20	0.25	0.27	0.26	0.24	0.30	0.26	0.28	0.17	0.29	0.26	0.26	0.36	0.10	0.13	0.17	0.16	0.24	0.16	0.16	0.23	0.15	0.16	0.13	0.21	0.14	0.10	0.18		0.00008^{*}	0.00008^{*}	0.00008^{*}	0.00025^{NS}	0.00008^{*}	0.00042^{NS}
Zini farm	0.12	0.07	0.09	0.12	0.04	0.11	0.16	0.14	0.08	0.20	0.15	0.12	0.30	0.16	0.15	0.12	0.10	0.18	0.22	0.13	0.18	0.25	0.23	0.23	0.31	0.20	0.22	0.26	0.22		0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00017^{NS}	0.00067^{NS}
uniZulu ponds	0.13	0.11	0.08	0.11	0.04	0.20	0.18	0.19	0.10	0.25	0.25	0.15	0.40	0.18	0.23	0.18	0.17	0.21	0.26	0.20	0.22	0.32	0.29	0.29	0.36	0.26	0.27	0.32	0.29	0.08		0.00017^{NS}	0.00008^{*}	0.00008^{*}	0.00025^{NS}
uMphafa ponds	0.26	0.32	0.36	0.38	0.37	0.47	0.29	0.35	0.29	0.26	0.31	0.35	0.35	0.23	0.28	0.33	0.35	0.43	0.37	0.26	0.34	0.22	0.21	0.20	0.34	0.24	0.24	0.38	0.27	0.38	0.44		0.00017^{NS}	0.00008^{*}	0.00017^{NS}
Fresca Farm	0.13	0.13	0.18	0.14	0.16	0.24	0.18	0.24	0.16	0.25	0.27	0.22	0.32	0.12	0.12	0.13	0.08	0.16	0.17	0.11	0.19	0.23	0.21	0.21	0.26	0.20	0.17	0.24	0.19	0.16	0.16	0.33		0.00008^{*}	0.00126^{NS}
O. niloticus	0.26	0.32	0.34	0.34	0.32	0.40	0.32	0.37	0.22	0.36	0.33	0.33	0.42	0.23	0.28	0.27	0.30	0.33	0.29	0.23	0.32	0.30	0.26	0.22	0.26	0.24	0.21	0.31	0.26	0.33	0.36	0.38	0.32		0.00034^{NS}
O. aureus	0.27	0.32	0.39	0.37	0.37	0.46	0.30	0.37	0.29	0.32	0.34	0.35	0.35	0.25	0.26	0.30	0.31	0.36	0.36	0.25	0.35	0.26	0.27	0.28	0.35	0.28	0.26	0.36	0.29	0.35	0.43	0.30	0.28	0.39	

P-Values: NS=not significant, * P<0.00008). F_{ST}-values < 0.05 indicate little genetic differentiation, 0.05-0.15 = moderate differentiation, 0.15-0.25 = great differentiation, and > 0.25 = very great differentiation (Wright 1965).

CHAPTER 5

The status of COI and 12S rRNA DNA barcode reference libraries for freshwater fish in South Africa: Implications for future eDNA projects

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Running header: DNA barcode reference libraries of freshwater fish in South Africa

5.1 Abstract

Environmental DNA metabarcoding (eDNA) is a rapidly emerging field in which high-throughput sequencing is used to catalogue the biodiversity of ecosystems through the amplification of DNA extracted from environmental samples (water, air, faeces, and soil). Although eDNA has strong links to DNA barcoding, the molecular marker most often used to detect vertebrates in eDNA studies is a portion of the mitochondrial 12S ribosomal RNA (12S rRNA) and not the standard cytochrome oxidase I (COI) marker used in traditional DNA barcoding. eDNA methods rely on a comprehensive reference library to link sequence data to species, which are often lacking in hyper-diverse countries such as South Africa. In this study, we review the present state of DNA barcode reference databases for both 12S rRNA and COI for freshwater fish (native and introduced) found in South African aquatic systems. Analysis of DNA records available on GenBank and the Barcode of Life Database (BOLD) revealed incomplete records of the examined taxa for both markers. Our findings showed that 34 species, six genera, and zero families of native South African freshwater fish lack COI barcode records, while 86 species, 22 genera, and eight families lack 12S rRNA records. Unlike the native freshwater fish, the non-native fish all had barcode records available for both COI and 12S rRNA. Producing comprehensive reference libraries for both markers is an important first step in developing an eDNA protocol for the non-invasive monitoring of native and non-native freshwater fish in South Africa.

Keywords: biomonitoring, COI gene, DNA barcoding, metabarcoding, 12S rRNA gene

5.2 Introduction

DNA barcoding has accelerated species identification and has been used to monitor changes in species composition in ecosystems (Hebert et al. 2003, da Silva and Willows-Munro 2016, Elsaied et al. 2021, Singh et al. 2021). Metabarcoding extends DNA barcoding by using high-throughput sequencing technology to allow for the rapid production of species inventories from complex bulk samples (Singh et al. 2021). Environmental DNA (eDNA) uses DNA extracted from environmental samples such as soil, air, or water (Taberlet et al. 2012, Belle et al. 2019) and provides the opportunity for non-invasive sampling and monitoring (Miya et al. 2015, Thomsen and Willersleve 2015, Valentini et al. 2016, Belle et al. 2019, Alam et al. 2020, Keck et al. 2022). In particular, many studies have demonstrated the utility of eDNA in monitoring species linked to aquatic systems (Hänfling et al. 2016, Vasselon et al. 2017, Fernández et al. 2018, Mächler et al. 2019, Keck et al. 2022). Despite the promise of providing important global baseline data on species distribution and abundance essential for conservation and management (Heywood 2011, Belle et al. 2019), eDNA research in African systems is still limited (Belle et al. 2019).

Globally, freshwater ecosystems are particularly vulnerable to multiple stressors derived from anthropogenic activities (Revenga et al. 2005, Dudgeon 2010, Dudgeon 2019, Belle et al. 2019, Fierro et al. 2019, Reid et al. 2019, Alam et al. 2020). This is particularly true in South Africa, which is a water-scarce country (Dallas and Rivers-Moore 2014, Govender et al. 2022). Freshwater ecosystems in South Africa are species-rich (Dudgeon 2019, O'Brien et al. 2019, Dallas et al. 2022), with high levels of endemism (Ellender et al. 2017, Dallas et al. 2022) and are affected by factors including pollution, water extraction, the introduction of invasive species and the overexploitation of aquatic resources (Dudgeon et al. 2006, Dallas and Rivers-Moore 2014, Riddell et al. 2019, Adams et al. 2020, Desai et al. 2021, Dallas et al. 2022, Evans et al. 2022). These activities pose a major threat to the freshwater biodiversity in the region (Dallas and Rivers-Moore 2014, O'Brien et al. 2019, Desai et al. 2021, Dallas et al. 2022). There is an ever-increasing need to effectively monitor changes in biodiversity, identify the most affected areas and establish priority conservation areas for vulnerable taxa. Species identification, discovery and monitoring have become an essential research theme for the conservation and management of biodiversity (Tsoupas et al. 2022). Sustainable conservation of freshwater biodiversity requires baseline knowledge of the community structure of natural ecosystems (Fierro et al. 2019). This will aid in understanding the impact of anthropogenic and natural activities on biodiversity loss (Fierro et al. 2019, Desai et al. 2021).

Environmental DNA could provide an important tool for monitoring biodiversity in aquatic systems in South Africa (and other countries). Future eDNA research may be held back by the lack of comprehensive DNA reference libraries linking DNA barcodes to taxonomically verified reference (voucher) specimens (Elbrecht et al. 2017, Leese et al. 2018, Weigand et al. 2019, Garcia de Amézaga Quintanilla 2021, Singh et al. 2021, Li et al. 2022). The molecular marker most widely used in the DNA barcode community for identifying animal taxa is the standardised 658-base pair (bp) portion of the cytochrome *c* oxidase subunit I gene (COI, Hebert et al. 2003). These data are curated mainly in two popular databases, the Barcode of Life Database (BOLD, Ratnasingham, and Hebert 2007) and GenBank. In contrast, recent eDNA metabarcoding studies have relied on the 12S ribosomal mitochondrial gene (12S rRNA) for the detection of vertebrate taxa (Riaz et al. 2011, Kelly et al. 2014, Miya et al. 2015, Hänfling et al. 2016, Yamamoto et al. 2017, Polanco et al. 2021), including the characterisation of fish communities in freshwater habitats (Thomsen et al. 2012, Evans et al. 2016, Valentini et al. 2016, Bylemans et al. 2018, Cilleros et al. 2019, Fujii et al. 2019, Lecaudey et al. 2019, Berger et al. 2020, Antognazza et al. 2021, Hallam et al. 2021, Sales et al. 2021, García-Machado et al. 2022). As eDNA research for biodiversity monitoring is still being established in South Africa, this review aims to summarise the available DNA barcode reference libraries for freshwater fish (both native and introduced). Specifically, we compared the COI and 12S rRNA data available for fish found in South African freshwaters and made some suggestions for the standardisation of techniques used in future aquatic eDNA research.

5.3 Methods

Our review of available 12S rRNA and COI records focused on all current native freshwater fish; we only considered primary freshwater fish that are restricted to and complete their life cycle in freshwater (Myers 1938). We collated a list of native freshwater fish using Skelton (2001), Chakona et al. (2022), and FishBase (Froese and Pauly 2023). Our review also included introduced freshwater fish that have naturalised in South African freshwaters (Ellender and Weyl 2014, Weyl et al. 2020). We compiled a list of introduced fish species from Weyl et al. (2020) and FishBase (Froese and Pauly 2023). We checked the availability of COI and 12S rRNA sequence data for each species by searching the BOLD (Ratnasingham and Hebert 2007) and National Centre for Biotechnology Information (NCBI, GenBank) databases. Our study included all data available up to and including October 2023. Given that eDNA often makes use of short-read high-throughput sequencing technologies such as Illumina, we included COI and 12S rRNA sequences longer than 300 bp to ensure comprehensive taxonomic resolution and accuracy. This approach allows for more informative genetic data, even when using shorter Illumina reads, and considers the availability of genes from both whole genomes and mitogenomes to enhance taxonomic assignment for eDNA studies. Where possible, we also noted if the reference individual was collected in South Africa or another country. This was only noted if the country of collection was reported for the sequences in both BOLD and

GenBank. If the barcode specimen was collected outside of South Africa, we still considered the record available for that species.

5.4 Results

The species list that we compiled included 106 native South African freshwater fish species (37 genera and 17 families; Supplementary Table 5.1) and 20 non-native species (15 genera and seven families; Supplementary Table 5.2). The alien species were introduced to South African aquatic systems through release from the pet trade, for recreational fishing, or aquaculture (Ellender and Weyl 2014, Weyl et al. 2020). For native fish, only 72 COI records were available, representing only 65% of the native species found in South Africa. Of these, only 47 records (65%) were collected from localities in South Africa, and 42 (58%) were full-length (>600 bp) COI barcodes (Table 5.1). At higher taxonomic levels, 84% of native fish genera had at least one COI record, while all (100%) families were represented by at least one record. For 12S rRNA, only 20 (19%) native species, 15 genera (41%), and nine families (53%) were represented by at least one record (Table 5.1). All the 12S rRNA data (100%) were sequenced from individuals collected outside of South Africa. Of the 20 non-native freshwater species found in South Africa, all had COI and 12S rRNA barcode records. Of these records, only 12 (60%) COI records were from specimens collected in South Africa (Table 5.1). Consequently, the non-native species were also fully represented at higher taxonomic levels, with 100% of genera and families covered for both COI and 12S rRNA (Table 5.1).

Table 5.1: Number of COI and 12S rRNA records available for native and introduced freshwater fish in South Africa (SA). The numbers of records for each species, genus, and family are presented. We distinguished between records from specimens collected in South Africa and those only available from specimens collected outside the borders of South Africa

Taxonomy	**Total in SA**	**Total COI records**	**COI records <600 bp**	**SA COI records**	**COI records from other countries**	**Total 12S rRNA records**	**12S rRNA records <600 bp**	**SA 12S rRNA records**	**12S rRNA records from other countries**
Native taxa									
Species	106	72	31	47	25	20	4	0	20
Genus	37	31	7	21	10	15	5	0	15
Family	17	17	2	12	5	9	2	0	9
Introduced taxa									
Species	20	20	2	12	8	20	10	0	20
Genus	15	15	0	9	11	15	8	0	15
Family	7	7	0	6	1	7	1	0	7

Not all the examined genera of South African freshwater fish had available COI or 12S rRNA barcode records. The genera *Ctenopoma, Microctenopoma, Serranochromis, Amatolacypris, Namaquacypris,* and *Silhouettea* lacked barcode records for both markers (Figure 5.1a). Among the native freshwater fish families found in South Africa, both COI and 12S rRNA records were available for nine families, while the remaining eight families had records for only COI (Figure 5.1b). For non-native freshwater fish in South Africa, all the genera and families had barcode records for both markers (Figure 5.2).

Given that not all the South African freshwater fish genera and families we examined contained the same number of species, we also present the data as a proportion of coverage (% of species with records in each genus and family). Among the native South African freshwater fish genera, *Pseudobarbus* followed by *Labeo* had the lowest proportion coverage for COI, while *Enteromius* followed by *Labeo* and *Amphilius* had the lowest proportion coverage for 12S rRNA (Supplementary Figure 5.3a). Among the various families of native South African freshwater fish, the lowest proportion of coverage for COI was recorded in the Anabantidae family, followed by the Gobiidae family (Supplementary Figure 5.3b). The lowest proportion coverage for 12S rRNA was found in the Cyprinidae family, followed by the Amphiliidae family (Supplementary Figure 5.3b). For non-native freshwater fish in South Africa, there was complete proportion coverage (100%) for COI and 12S rRNA barcode records for all genera and families (Supplementary Figure 5.4).

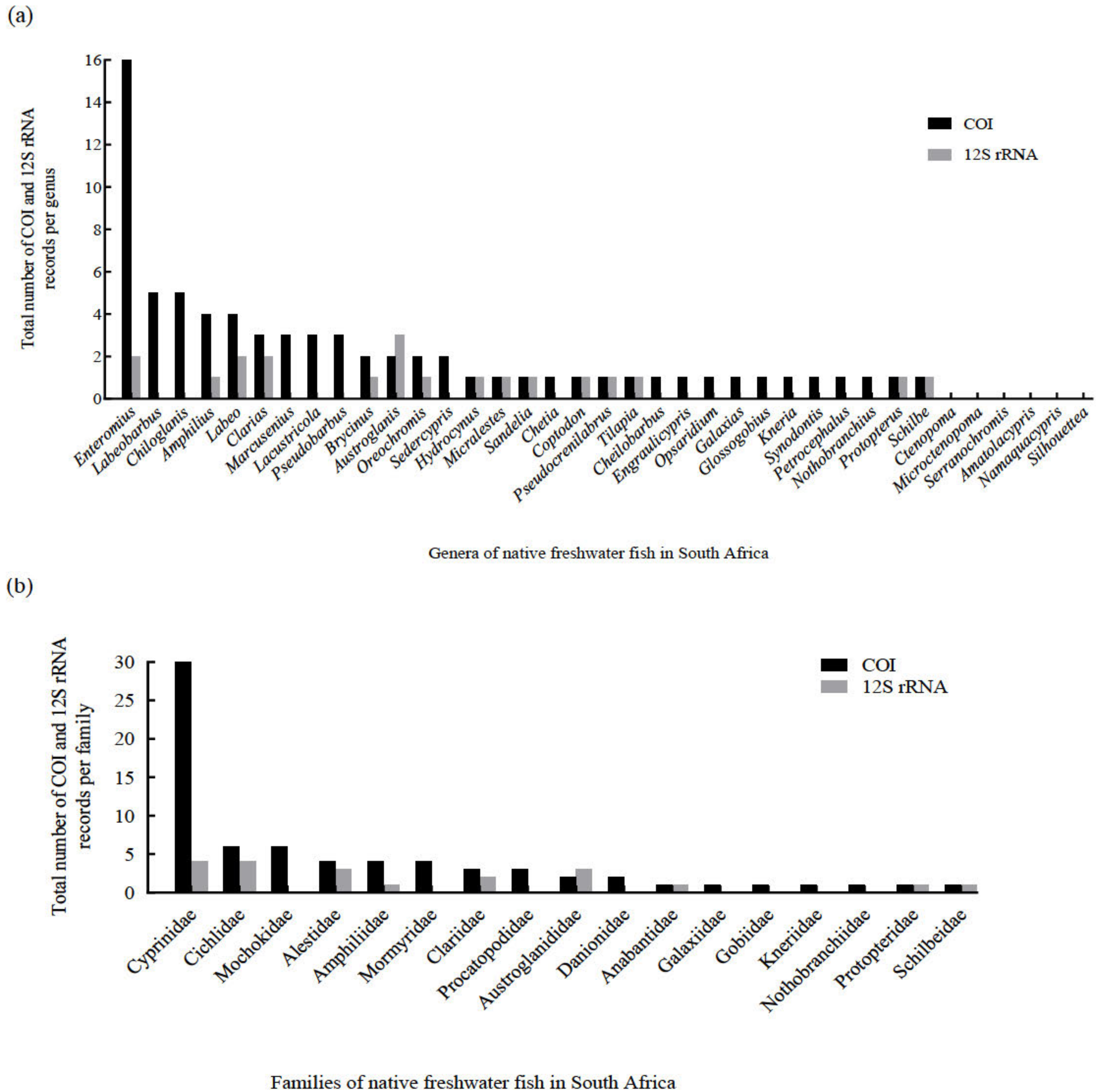
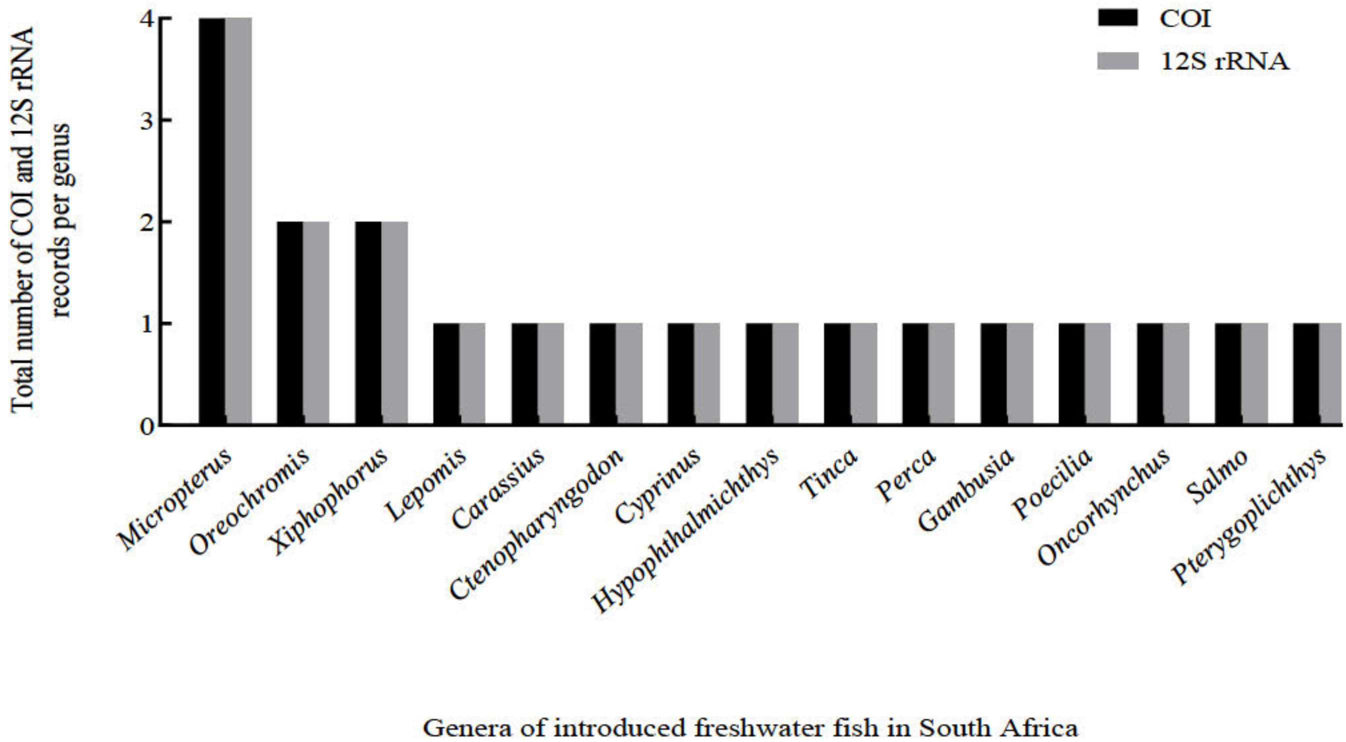


Figure 5.1: Total number of native freshwater fish species with (a) COI and 12S rRNA records per genus (genera are ranked according to the number of COI data records) and (b) COI and 12S rRNA records per family (families are ranked according to the number of COI data records).

(a)


Total number of COI and 12S rRNA records per genus
4
3
2
1
0
COI
12S rRNA
Micropterus
Oreochromis
Xiphophorus
Lepomis
Carassius
Ctenopharyngodon
Cyprinus
Hypophthalmichthys
Tinca
Perca
Gambusia
Poecilia
Oncorhynchus
Salmo
Pterygoplichthys
Genera of introduced freshwater fish in South Africa

(b)

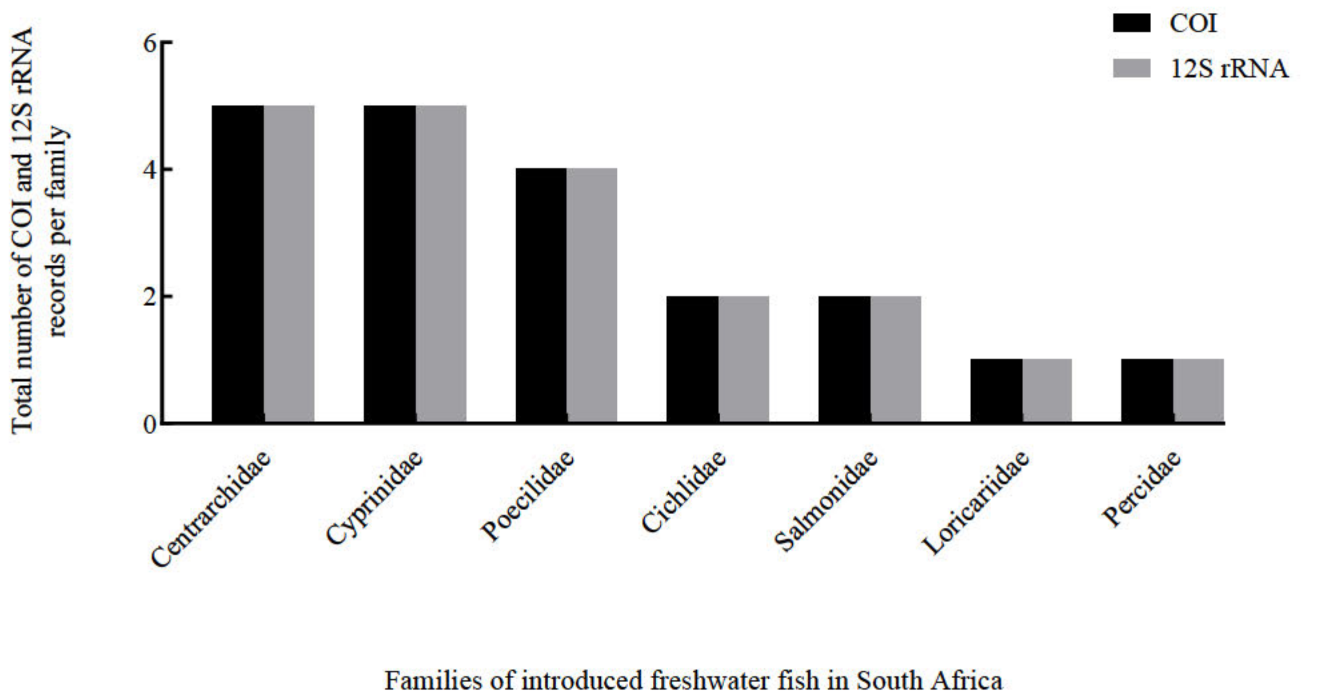

Total number of COI and 12S rRNA records per family
6
4
2
0
COI
12S rRNA
Centrarchidae
Cyprinidae
Poecilidae
Cichlidae
Salmonidae
Loricariidae
Percidae
Families of introduced freshwater fish in South Africa

Figure 5.2: Total number of introduced freshwater fish species with (a) COI and 12S rRNA records per genus (genera are ranked according to the number of COI data records) and (b) COI and 12S rRNA records per family (families are ranked according to the number of COI data records).

5.5 Discussion

This study reviewed the present state of COI and 12S rRNA DNA barcode reference libraries for native and introduced freshwater fish in South Africa. Although COI is the marker traditionally used in the DNA barcoding of animal species (Hebert et al. 2003, Bucklin et al. 2011, Leray et al. 2019, Li et al. 2022), 12S rRNA has recently been used in eDNA studies in other countries (Thomsen et al. 2012, Kelly et al. 2014, Miya et al. 2015, Noble et al. 2015, Evans et al. 2016, Valentini et al. 2016, Bylemans et al. 2018, Milan et al. 2020, Shu et al. 2021, Polanco et al. 2021, Xiong et al. 2022). This review highlights the incompleteness of the DNA reference libraries for South African native fish species for both COI and 12S rRNA sequences; in particular, the 12S rRNA DNA barcode reference library is poorly populated. The South African node of the International Barcode of Life (http:// www.ibolproject.org) was established in 2010 and has been coordinating research efforts leading to significant growth of the COI DNA sequence reference library for South African taxa (da Silva and Willows-Munro 2016). This has likely led to a higher number of COI records than 12S rRNA records, despite the 12S rRNA marker becoming an increasingly important gene for the identification and monitoring of fish using eDNA methods (Deagle et al. 2014, Collins et al. 2019, Zhang et al. 2020, Polanco et al. 2021). For future eDNA studies in South Africa to be comparable to those conducted in other parts of the globe, this review suggests that a multi-marker approach (using both COI and 12S rRNA) be used. Moreover, using other genes, such as 12S rRNA and 16S rRNA, in addition to COI will allow for more accurate species assignment and the elucidation of phylogenetic relationships at higher taxonomic levels (Duarte et al. 2020, Ahmed et al. 2022).

To this end, it is also essential that the present 12S rRNA reference library for freshwater fish in South Africa be improved, particularly for native species. The DNA barcode reference libraries for the Cyprinidae family, which represents an important component of the freshwater fish in South Africa, are still lacking. The Cyprinidae family contains 56 species belonging to eight genera, of which 18 species are threatened (six Vulnerable, nine Endangered, and three Critically Endangered). In particular, the *Pseudobarbus* genus is the most threatened in South Africa, with most species in this genus listed as Endangered or Critically Endangered on the IUCN Red List (Chakona et al. 2022). The findings of this review indicate that most of these threatened species are not present in the current reference libraries for either COI or 12S rRNA DNA sequences. Of the 18 threatened species belonging to the Cyprinidae family, only three Vulnerable species (*Enteromius anoplus s.s.*, *Pseudobarbus swartzi,* and *Pseudobarbus burgi*), and two Critically Endangered species (*Enteromius treurensis* and *Sedercypris erubescens*) have COI barcode records, and there are no 12S rRNA records available. Nevertheless, genes such as cytochrome *b* (Cyt*b*), which have also been used in fish species identification (Tobe et al. 2009, Ficetola et al. 2010), are becoming increasingly popular in eDNA research (Rees et al. 2015, Shu et al. 2020, 2021). One advantage of including Cyt*b* in a multi-marker panel for eDNA is that the substitution rate of this marker could provide support for higher taxonomic associations (Gillet et al. 2018). Although this review highlights the incompleteness of the 12S rRNA sequence reference library, we also reviewed the Cyt*b* sequence reference library (results not presented in this review), and it is more complete, with records available for 65 species, representing 61% compared with the 19% representation reported for 12S rRNA in this review. Furthermore, although the *Pseudobarbus* genus lacks COI and 12S rRNA barcodes, almost all the species belonging to this genus have Cyt*b* records available in GenBank. This further highlights the importance of using multi-marker approaches, which include the use of genes such as Cyt*b* that have more complete reference libraries, for eDNA studies in South Africa.

Environmental DNA methods have also been used successfully for the detection and monitoring of invasive fish (Takahara et al. 2013, Keskin 2014, Bylemans et al. 2016, Keskin et al. 2016, Hinlo et al. 2017, Clusa and García-Vázquez 2018, Jo et al. 2021, Minett et al. 2021, Dubreuil et al. 2022, Jeunen et al. 2022). Considerable efforts have been made to barcode introduced freshwater fish species in South Africa (van der Walt et al. 2017). As a result, introduced freshwater fish species in South Africa are fully represented (100%) in the reference libraries for both COI and 12S rRNA DNA sequences, and this may promote the use of eDNA metabarcoding for the early warning, detection, monitoring, and management of these introduced species. The identification of taxa using DNA-based approaches also depends on the geographical coverage of local species in barcode reference databases (Li et al. 2022). This has been shown to improve species assignment by increasing taxonomic resolution during sequencing (Singh et al. 2021). According to Jones et al. (2021), complete DNA barcoding databases for regions or countries are still scarce. An important finding from this review is that most COI data (65%) for native freshwater fish were from specimens collected in South Africa. In contrast, all the 12S rRNA barcodes were from specimens collected outside the borders of

South Africa (100%). This further highlights the need to build the 12S rRNA barcode reference library for South Africa to improve taxonomic resolution during eDNA analyses.

Our review suggests that gaps in the reference libraries for COI and 12S rRNA sequences will negatively affect the use of eDNA metabarcoding to monitor freshwater fish in South Africa. Therefore, priority should be given to filling these gaps, especially at the species level, as this could increase the efficiency and accuracy of species assignment (Duarte et al. 2020). However, we suggest that best practices for building reference libraries be employed for both genes. This will guarantee the best possible quality and traceability of the supporting information linked to the identification reference barcode. Although BOLD (Ratnasingham and Hebert 2007) and GenBank (Benson et al. 2012) are the main repositories of DNA barcodes, they have been associated with species misidentification attributed to a lack of expert taxonomic verification and supporting information linked to the barcodes (Meiklejohn et al. 2019, Weigand et al. 2019, Rimet et al. 2021), with particular emphasis on the limitations of GenBank (Meiklejohn et al. 2019). According to Remit et al. (2021), a barcode sequence can only be considered reliable if its metadata are available, including the primary data and all supporting information for that DNA barcode. This includes the accurately identified voucher specimen, photographs, taxonomic name, collection location, storage facility information, and barcode authors (Rimet et al. 2021). These practices should be observed for building high quality and reliable COI and 12S rRNA DNA barcode reference libraries for South African freshwater fish, which will enable efficiency when employing eDNA methods.

5.6 Conclusions

Species discovery, identification, biodiversity monitoring, and management are important measures for assessing the impacts of ecosystem management, climate change, habitat degradation, and other anthropogenic stressors and impacts on freshwater biodiversity in South Africa. Environmental DNA metabarcoding provides an opportunity for non-invasive monitoring and the identification of both native and introduced fish in freshwater systems. Despite this, the technique has not been established in South African inland waters and our study provides the initial step in the development of an eDNA metabarcoding protocol for monitoring freshwater fish in South Africa.

This review assessed the status of the DNA barcode reference libraries of the two main eDNA metabarcoding markers (COI and 12S rRNA) for native and introduced freshwater fish in South Africa. Our results highlighted the incomplete representation and coverage of Indigenous species in the barcode reference libraries for COI sequences particularly for 12S rRNA. These gaps limit the use of eDNA metabarcoding technologies for discovering and managing these species in the region. Therefore, there is an urgent need to build reliable DNA barcode reference libraries for both markers for South African freshwater fish. The present state of the South African DNA barcode libraries provides the impetus to coordinate ongoing efforts and stimulate new initiatives aimed at filling the gaps in the barcode libraries for freshwater fish in South Africa. eDNA methods are innovative, robust, and effective, and are contributing to sustainable water resource management and conservation globally. We have the same opportunities for this approach to contribute to South African freshwater research. This review identifies the foundational data needed to achieve this.

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5.9 Supplementary information

Supplementary Table 5.1: List of the 106 native South African freshwater fish species used in this study (Source: Skelton et al. (2001), Chakona et al. (2022), and Fishbase (Froese and Pauly 2023))

Family	Genus	Species	Common name
Alestidae	*Brycinus*	*imberi* [ab]	Imberi
	Brycinus	*lateralis* [a]	Striped robber
	Hydrocynus	*vittatus* [ab]	Tigerfish
	Micralestes	*acutidens* [ab]	Silver robber
Amphiliidae	*Amphilius*	*engelbrechti* [a]	Inkomati mountain catfish
	Amphilius	*natalensis s.s.* [ab]	Natal mountain catfish
	Amphilius	*zuluorum* [a]	Mkhomazi mountain catfish
	Amphilius	*uranoscopus* [a]	Stargazer mountain catfish
Anabantidae	*Ctenopoma*	*multispine**	Manyspined climbing perch
	Microctenopoma	*intermedium**	Swartkol-
	Sandelia	*bainsii**	Eastern Cape rocky
	Sandelia	*capensis* [ab]	Cape kurper
Austroglanididae	*Austroglanis*	*barnardi* [ab]	Barnard's rock catfish
	Austroglanis	*gilli* [ab]	Clanwilliam rock-catfish
	Austroglanis	*sclateri* [b]	Rock-catfish
Cichlidae	*Chetia*	*brevis**	Orange-fringed largemouth
	Chetia	*flaviventris* [a]	Canary kurper
	Coptodon	*rendalli* [ab]	Redbreast tilapia
	Oreochromis	*mossambicus* [ab]	Mozambique tilapia
	Oreochromis	*placidus* [a]	Black tilapia
	Pseudocrenilabrus	*philander* [ab]	Southern mouthbrooder
	Serranochromis	*meridianus**	Lowveld largemouth
	Tilapia	*sparrmanii* [ab]	Banded tilapia
Clariidae	*Clarias*	*gariepinus* [ab]	Sharptooth catfish
	Clarias	*ngamensis* [a]	Blunttooth catfish
	Clarias	*theodorae* [ab]	Snake catfish
Cyprinidae	*Amatolacypris*	*trevelyani**	Border barb
	Cheilobarbus	*capensis**	Cape whitefish
	Cheilobarbus	*serra* [a]	Sawfin
	Enteromius	*afrohamiltoni* [a]	Hamilton's barb
	Enteromius	*amatolicus**	Amatola barb
	Enteromius	*annectens**	Broadstriped barb
	Enteromius	*anoplus s.s.* [a]	Chubbyhead barb
	Enteromius	*argenteus* [a]	Rosefin barb
	Enteromius	*bifrenatus* [a]	Hyphen barb
	Enteromius	*brevipinnis* [a]	Shortfin barb

Enteromius	*cernuus**	
Enteromius	*eutaenia* [ab]	Orangefin barb
Enteromius	*gurneyi**	Redtail barb
Enteromius	*lineomaculatus* [a]	Line-spotted barb
Enteromius	*mandelai**	Eastern chubbyhead barb
Enteromius	*mattozi* [a]	Papermouth
Enteromius	*motebensis* [a]	Marico barb
Enteromius	*oraniensis**	Gariep chubbyhead barb
Enteromius	*pallidus ss* [a]	Goldie barb
Enteromius	*paludinosus* [a]	Straightfin barb
Enteromius	*radiatus* [a]	Beira barb
Enteromius	*toppini**	East coast barb
Enteromius	*treurensis* [a]	Treur river barb
Enteromius	*trimaculatus* [ab]	Threespot barb
Enteromius	*unitaeniatus* [a]	Longbeard barb
Enteromius	*viviparus* [a]	Bowstripe barb
Labeo	*capensis* [a]	Orange river labeo
Labeo	*congoro* [b]	Purple labeo
Labeo	*cylindricus* [ab]	Redeye labeo
Labeo	*molybdinus* [a]	Leaden labeo
Labeo	*rosae**	Rednose labeo
Labeo	*rubromaculatus**	Tugela labeo
Labeo	*ruddi* [a]	Silver labeo
Labeo	*seeberi**	Clanwilliam sandfish
Labeo	*umbratus**	Moggel
Labeobarbus	*aeneus**	Smallmouth yellowfish
Labeobarbus	*kimberleyensis**	Largemouth yellowfish
Labeobarbus	*marequensis* [a]	Largescale yellowfish
Labeobarbus	*natalensis* [a]	Scaly
Labeobarbus	*nelspruitensis* [a]	Incomati chiselmouth
Labeobarbus	*polylepis* [a]	Smallscale yellowfish
Labeobarbus	*seeberi* [a]	Clanwilliam yellowfish
Namaquacypris	*hospes**	Namaqua barb
Pseudobarbus	*afer**	Eastern cape redfin
Pseudobarbus	*asper**	Smallscale redfin
Pseudobarbus	*burchelli* [a]	Burchell's redfin
Pseudobarbus	*burgi* [a]	Berg river redfin
Pseudobarbus	*phlegethon**	Fiery redfin
Pseudobarbus	*quathlambae**	Drakensberg minnow
Pseudobarbus	*senticeps**	
Pseudobarbus	*skeltoni**	Giant redfin
Pseudobarbus	*swartzi* [a]	Gamtoos redfin
Pseudobarbus	*tenuis**	Slender redfin
Pseudobarbus	*verloreni**	Verlorenvlei redfin

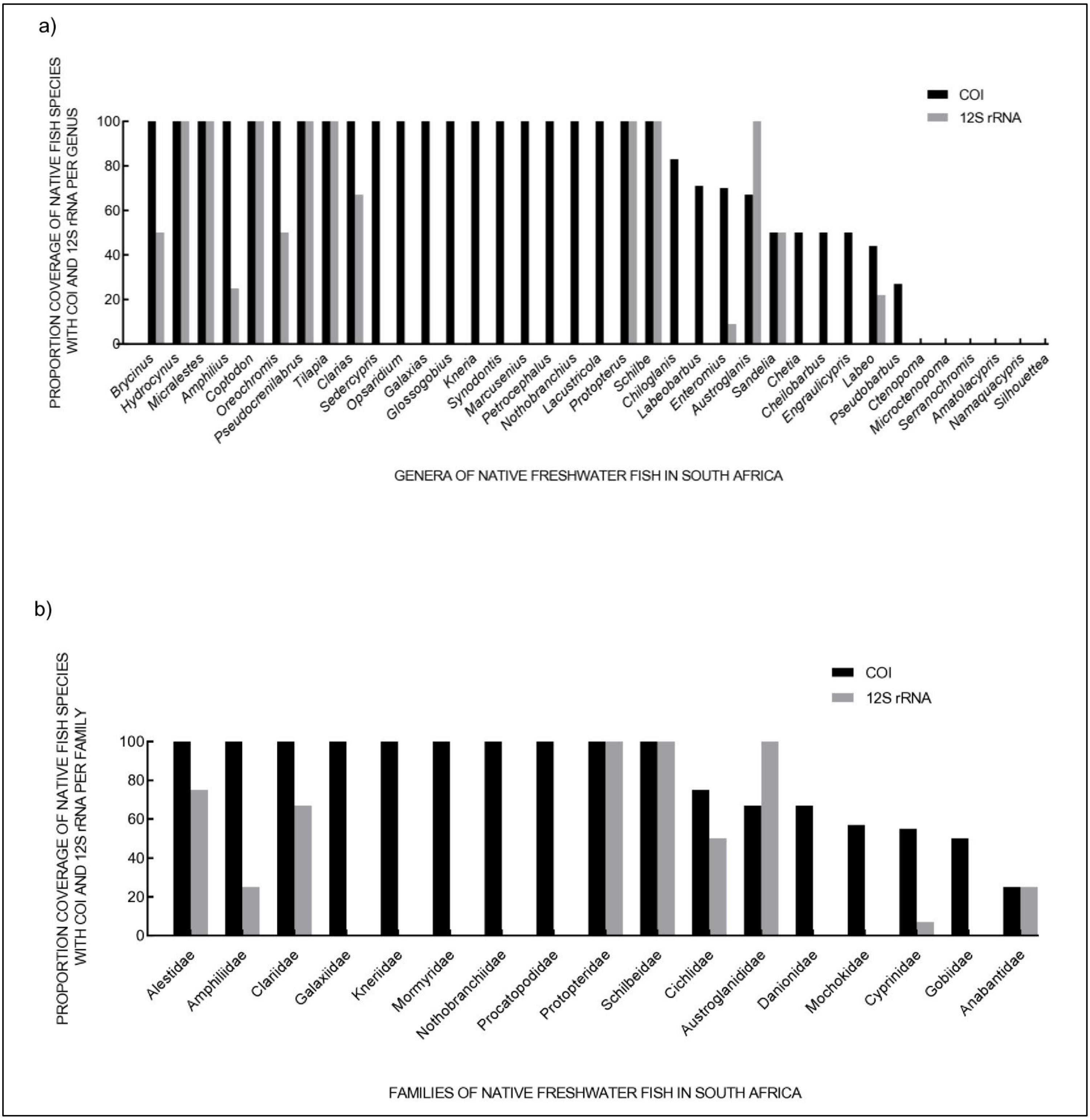
	Sedercypris	*calidus* [a]	Clanwilliam redfin
	Sedercypris	*erubescens* [a]	Twee river redfin
Danionidae	*Engraulicypris*	*brevianalis* [a]	River sardine
	Engraulicypris	*gariepinus* *	
	Opsaridium	*peringueyi* [a]	Southern barred minnow
Galaxiidae	*Galaxias*	*zebratus* [a]	Cape galaxias
Gobiidae	*Glossogobius*	*callidus* [a]	River goby
	Silhouettea	*sibayi* *	Sibayi goby
Kneriidae	*Kneria*	*auriculata* [a]	Southern kneria
Mochokidae	*Chiloglanis*	*anoterus* [a]	Pennant tail suckermouth
	Chiloglanis	*bifurcus* [a]	Incomati suckermouth
	Chiloglanis	*emarginatus* [a]	Pongolo suckermouth
	Chiloglanis	*paratus* *	Sawfin suckermouth
	Chiloglanis	*pretoriae* [a]	Shortspine suckermouth
	Chiloglanis	*swierstrai* [a]	Lowveld suckermouth
	Synodontis	*zambezensis* [a]	Brown squeaker
Mormyridae	*Marcusenius*	*caudisquamatus* [a]	Mhlatuze mormyrid
	Marcusenius	*krameri* [a]	Kramer's mormyrid
	Marcusenius	*pongolensis* [a]	Southern bulldog
	Petrocephalus	*wesselsi* [a]	Southern churchill
Nothobranchiidae	*Nothobranchius*	*orthonotus* [a]	Spotted killifish
Procatopodidae	*Lacustricola*	*johnstoni* [a]	Johnston's topminnow
	Lacustricola	*katangae* [a]	Striped topminnow
	Lacustricola	*myaposae* [a]	Natal topminnow
Protopteridae	*Protopterus*	*annectens brieni* [ab]	Lungfish
Schilbeidae	*Schilbe*	*depressirostris* [ab]	Silver catfish

[ab]Native freshwater fish with COI and 12S rRNA records in South Africa
[a]Native freshwater fish with only COI records in South Africa
[b]Native freshwater fish with only 12S rRNA records in South Africa
*Native freshwater fish without COI and 12S rRNA in South Africa

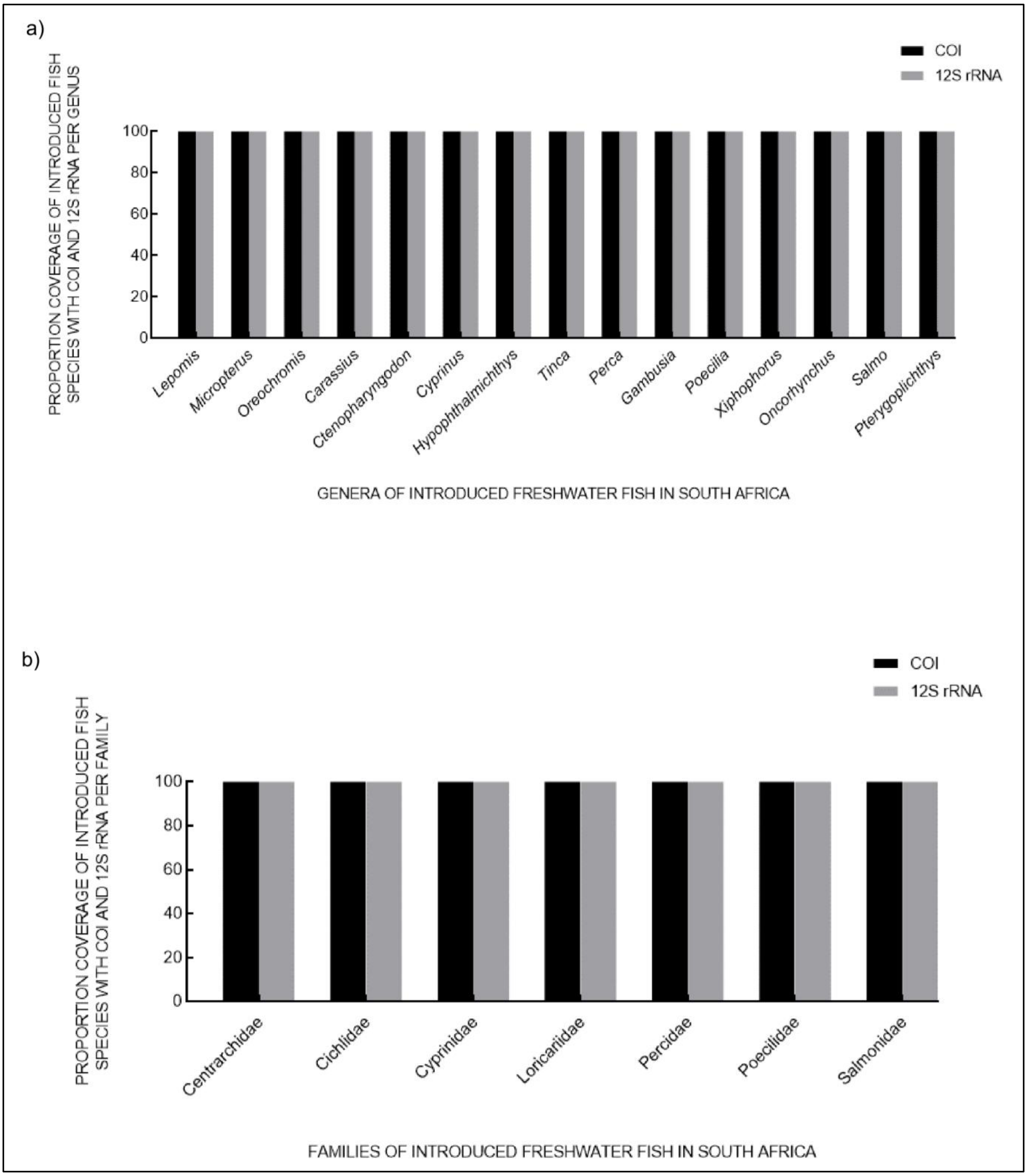
Supplementary Table 5.2: List of the 20 introduced freshwater fish species found in South Africa (Source: Weyl et al. 2020 and Fishbase (Froese and Pauly 2023))

Family	Genus	Species	Common name
Centrarchidae	*Lepomis*	*macrochirus* [ab]	Bluegill
	Micropterus	*dolomieu* [ab]	Smallmouth bass
	Micropterus	*floridanus* [ab]	Florida bass
	Micropterus	*punctulatus* [ab]	Spotted bass
	Micropterus	*salmoides* [ab]	Largemouth bass
Cichlidae	*Oreochromis*	*aureus* [ab]	Blue tilapia
	Oreochromis	*niloticus* [ab]	Nile tilapia
Cyprinidae	*Carassius*	*auratus* [ab]	Goldfish
	Ctenopharyngodon	*idella* [ab]	Grasscarp
	Cyprinus	*carpio* [ab]	Common carp
	Hypophthalmichthys	*molitrix* [ab]	Silver carp
	Tinca	*tinca* [ab]	Tench
Loricariidae	Pterygoplichthys	disjunctivus [ab]	Vermiculated sailfin catfish
Percidae	*Perca*	*fluviatilis* [ab]	European perch
Poecilidae	*Gambusia*	*affinis* [ab]	Western mosquitofish
	Poecilia	*reticulata* [ab]	Guppy
	Xiphophorus	*hellerii* [ab]	Green swordtail
	Xiphophorus	*maculatus* [ab]	Southern platyfish
Salmonidae	*Oncorhynchus*	*mykiss* [ab]	Rainbow trout
	Salmo	*trutta* [ab]	Brown trout

[ab]Introduced freshwater fish with COI and 12S rRNA records in South Africa



Supplementary Figure 5.1: Proportion coverage of South African native fish with a) COI and 12S rRNA records per genus and b) with COI and 12S rRNA records per family. Percentage coverage was calculated as the percentage of species per genus and family with records. This considers the size of each genus and family.



Supplementary Figure 5.2: Proportion coverage of introduced fish with a) COI and 12S rRNA records per genus and b) with COI and 12S rRNA records per family. Percentage coverage was calculated as the percentage of species per genus and family with records. This considers the size of each genus and family.

CHAPTER 6

Evaluating the efficacy of eDNA metabarcoding for detecting native *Oreochromis mossambicus* populations in KwaZulu-Natal, South Africa

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Running header: eDNA Metabarcoding of Native *Oreochromis mossambicus* in KwaZulu-Natal, South Africa

6.1 Abstract

Environmental DNA (eDNA) metabarcoding techniques have emerged as a rapid, non-invasive, and cost-effective approach for monitoring aquatic species. These methods play a crucial role in ecology and conservation by providing insights into the presence or absence of key freshwater taxa, biodiversity patterns, species presence-absence, and distribution. Few eDNA studies have been conducted in African rivers. This study aimed to develop and test the efficacy of eDNA metabarcoding for detecting and monitoring native and introduced *Oreochromis* species, with a primary emphasis on the vulnerable *O. mossambicus* populations in KwaZulu-Natal. Considering the rapid evolutionary radiation within the tilapiine group, a multi-marker system was employed, incorporating fragments of the cytochrome oxidase I (COI), 12S rRNA, and 16S rRNA gene regions. The eDNA metabarcoding results identified 211 fish-related sequences from 481,913 raw reads. COI primers proved effective, in detecting diverse fish species, including the vulnerable *O. mossambicus* and the introduced *O. niloticus*. Yet, achieving species-level identification remains challenging (18% confidence). The dominance of the Danionidae and Clariidae families highlights the efficacy of our method. However, the incomplete GenBank and BOLD databases emphasize the need for complete and accurate databases to assist with species-level assignments. Our results contribute valuable insights for freshwater fish monitoring, but ongoing refinement is crucial for maximizing eDNA metabarcoding potential.

Keywords: Biodiversity conservation; environmental DNA (eDNA); freshwater monitoring; metabarcoding; multi-marker system; *Oreochromis mossambicus*

6.2 Introduction

Freshwater ecosystems globally face substantial challenges because of anthropogenic activities, necessitating systematic tracking of alterations in biodiversity, identification of impacted areas, and designation of conservation zones (Revenga et al. 2005, Abell et al. 2007, Strayer and Dudgeon 2010, Geist 2011, Reid et al. 2019, Acreman et al. 2020, Kumar et al. 2024). Monitoring species has become a fundamental aspect of biodiversity conservation and management (Tsoupas et al. 2022, Kumar et al. 2024). Aquatic fish monitoring traditionally relies on field-based approaches such as gill netting, seine netting, fyke netting, and electrofishing (Birk et al. 2012, Geist 2015, Belle et al. 2019, Zhang et al. 2023). However, these methods involve physical capture, are labour-intensive, and may pose direct threats to surveyed species, causing stress, and indirect threats such as habitat disturbances (Mueller et al. 2017, Hering et al. 2018, Belle et al. 2019, Zhang et al. 2023). Moreover, these methods may fail to detect species not only due to rarity but also because of inherent biases related to sampling methodology, which may not align with the species' habitat, behaviour, and ecology (Belle et al. 2019).

In contrast, environmental DNA (eDNA) metabarcoding provides a fast and non-invasive alternative for monitoring aquatic species (Shaw et al. 2016, Belle et al. 2019, Alam et al. 2020, Sahu et al. 2023). These techniques are becoming increasingly valuable in ecology and conservation for assessing freshwater taxa, biodiversity patterns, species presence-absence, and distribution (Jerde et al. 2011, Thomsen et al. 2012, Hänfling et al. 2016, Belle et al. 2019, Alam et al. 2020, Erős et al. 2024). Additionally, eDNA approaches enable the identification of rare species, invasive species, and migratory species, providing crucial knowledge for conservation management (Dejean et al. 2012, Pilliod et al. 2013, Yamamoto et al. 2016, Belle et al. 2019, Sahu et al. 2023, Erős et al. 2024). An advantage over traditional survey methods is that eDNA can detect low abundances, even single individuals, making them critical for managing populations of threatened species and invasions (Thomsen 2012, Biggs et al. 2015, Stoeckle et al. 2016, Itakura et al. 2019, Belle et al. 2019, Crookes et al. 2020, Jeunen et al. 2022, Kalogianni et al. 2023).

Oreochromis mossambicus is a freshwater fish native to rivers in South Africa. This species plays a crucial role in the aquaculture industry within the region. However, the introduction of *Oreochromis niloticus* into South African rivers poses a significant threat to *O. mossambicus* through hybridisation although not seen in this study to any great extent (Chapter 4). As a consequence, *O. mossambicus* is now listed as Vulnerable on the International Union for Conservation of Nature (IUCN) Red List (Bills 2019). Previous surveys using active sampling techniques in major river catchments across KwaZulu-Natal Province indicated limited detection of *O. mossambicus*-*niloticus* hybrids, which aligns with our findings in Chapter 4 that most natural populations in this region remain genetically intact. While the limited presence of hybrids is relatively positive for *O. mossambicus*, considerable work is still required to improve the monitoring of *O. mossambicus* populations and detect other invasive *Oreochromis* species. This study tests the utility of eDNA to enhance the results of previous surveys.

A key consideration in eDNA accuracy is the choice of molecular markers used. The primers used in eDNA studies should be situated in conserved regions of the genome allowing for amplification across a wide taxonomic range of species. The amplicon size should not exceed 250 bp to ensure that degraded DNA is still detected and to make use of high through-put sequencing technologies such as Illumina to detect all DNA from mixed samples. Recent studies using eDNA to detect fish often use universal primers such as the MiFish primer set which targets a hypervariable region of the mitochondrial 12S rRNA gene (163–185 bp), that contains sufficient information to identify fishes to taxonomic family, genus, and species except for some closely related congeners (Miya et al. 2015, Shaw et al. 2016, Yamamoto et al. 2017, Alam et al. 2020). However, it is important to acknowledge the limitations of MiFish primers, especially in identifying closely related species, including cichlids (Yamamoto et al. 2017, Alam et al. 2020). To address this, using a multi-marker approach and incorporating additional genes such as the 16S rRNA, COI, 18S rRNA, and cytochrome *b* (cyt *b*) have been encouraged to enhance species assignment accuracy (Duarte et al. 2020, Ahmed et al. 2022).

The primary objective of this study was to develop and test an eDNA method for monitoring *Oreochromis* species in South African freshwater systems, focusing specifically on vulnerable *O. mossambicus* populations. Given the complex evolutionary history within the tilapiine group, encompassing species such as *O. mossambicus* and *O. niloticus*, characterised by rapid evolutionary radiation, hybridisation, shallow differentiation, and ancestral polymorphisms, this study aimed to evaluate the effectiveness of a multi-marker eDNA detection system. This system incorporates primers targeting the mitochondrial gene regions of cytochrome oxidase I (COI), 16S ribosomal RNA (16S rRNA), and 12S ribosomal RNA (12S rRNA).

6.3 Methods

6.3.1 Study sites

A total of seven sites were targeted for eDNA sampling within the KwaZulu-Natal Province South Africa, with five situated in the Thukela catchment and two in the uMngeni catchment (Figure 6.1). The selection of sampling sites was based on our prior active sampling and genetic assessments of O. *mossambicus* in KwaZulu-Natal using microsatellites (Chapter 3). The data acquired during previous surveys conducted during the 2017-2021 period were used as a reference in our study.

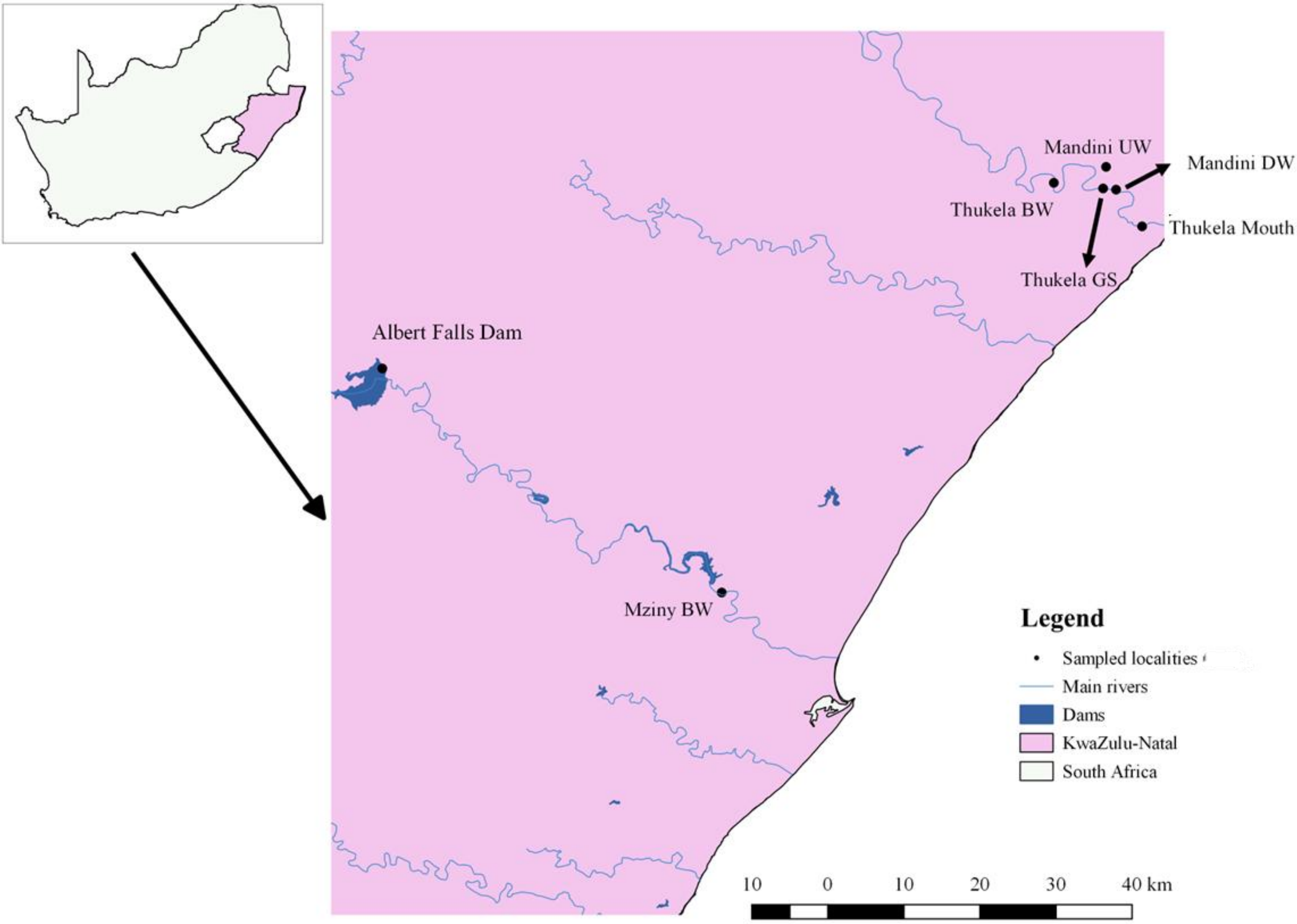

Figure 6.1: eDNA Sampling localities in KwaZulu-Natal Province, South Africa. Five sites are located within the Thukela catchment, and the remaining two are in the uMngeni catchment.

6.3.2 eDNA sampling and filtration

Sampling of water at the seven sites was conducted over 14 days in October 2023. Prior to the collection of eDNA samples, all water collection equipment underwent thorough sterilisation by immersion in a 10% bleach solution for 30 min., followed by a triple rinse with molecular-grade water. At each sampling site, sterile gloves were worn, and water samples were collected using sterile 1L Schott bottles. Each site yielded three replicates, totalling 3L of water, which were immediately placed in a sterile cooler box with ice for transportation to the University of KwaZulu-Natal's Conservation Genetics Laboratory, Pietermaritzburg campus, for filtration. Upon arrival at the laboratory, all tools and surfaces were sterilised with a 10% bleach solution, followed by wiping with molecular-grade water and disinfection using 70% ethanol. For the filtration process, a sterile 50 mL luer lock syringe was employed to withdraw water from each Schott bottle. The syringe was then connected to a Sterivex™ 0.22 µm filter (Figure 6.2). The water was systematically pushed through the filter unit in 50 mL subsamples. Each site's triplicates were individually filtered through their dedicated Sterivex™ 0.22 µm filter, utilising a fresh syringe for each triplicate.

Following the complete filtration of all triplicates, excess water was expelled through the filter outlet by introducing air into the syringe. Subsequently, each filter unit was filled with 2 mL of a tissue lysis buffer (ATL, Qiagen) using a sterile 2 mL syringe. The filter unit was then sealed at both ends with sterile parafilm and stored at -20°C until eDNA extraction. As a quality control measure, a 1L sterile DNA-free water sample served as a negative control during field collection and transport. This control underwent identical processing, including Sterivex filtration, alongside the river samples, allowing us to monitor for contamination throughout the process.

6.3.3 eDNA extraction

The extraction of environmental DNA (eDNA) was carried out in a sterile laboratory environment. Each Sterivex filter unit underwent eDNA extraction following the protocol outlined in the NucleoSpin eDNA Water kit (Macherey-Nagel, PA, USA) designed for extracting eDNA from water samples. Negative controls containing only the extraction reagents were included with each set of extractions to monitor potential contamination.

Quantification of eDNA was conducted using a Qubit 3.0 fluorometer using the Broad-Range Assay. All eDNA extracts were subsequently stored at -20°C.

6.3.4 In silico PCR primer check

Before selecting primers for use in eDNA amplification we assessed the utility of previously published primers to delimit different species of *Oreochromis*. I downloaded from GenBank all available *Oreochromis* sequence data for three mitochondrial genes:12S rRNA, 16S rRNA, and COI. Our data set included representative sequences from *Oreochromis* species including *O. mossambicus*, *O. niloticus*, and *O. aureus*. Gene sequences and primers were aligned using ClustalW 2.0 (Larkin et al., 2007). The primer selection process was guided by specific criteria, including (a) an amplicon length within the range of 15–250 bp (excluding primers), (b) a maximum of three mismatches between each primer and the target sequence, with no mismatches in the last two nucleotides at the 3′ end of the primer, and (c) enough variable characters to identify *O. mossambicus*, *O. niloticus,* and *O. aureus*. Subsequently, six primer sets were chosen for implementation in the present study (Table 6.1). Specifically, three sets of COI primers included coi.175f, coi.226r & coi.345r (Collins et al. 2019), OnilF & OnilR (Keskin 2014), and mlCOIintF, HCO2198 & FishR2 (Folmer 1994, Ward et al. 2005, Leray et al. 2013). For the 12S rRNA primer region, two primer sets were utilised, including MiFish-UF & MiFish-UR (Miya et al. 2015) and AcMDB07F & 12S OreoR (Noble et al. 2015, Bylemans et al. 2018). Only one primer set was employed for the 16S rRNA primer region (16S-FishF & 16S-FishR, McInnes et al. 2017).

6.3.5 Polymerase Chain Reaction (PCR) amplification and library preparation

PCR was conducted in triplicate for each primer set and sample (Figure 6.2) to enhance detection sensitivity while minimizing bias and amplification errors. Each PCR reaction (20 μL) contained 7.5 μL of Q5 High-Fidelity DNA Polymerase (0.02 U μL^{-1}, New England BioLabs Inc, Ipswich, Massachusetts, USA), 0.6 μL of forward primer (5 μmol/L), 0.6 μL of reverse primer (5 μmol/L) (0.3 μL each for primer pairs using two reverse primers), 4 μL of template DNA (10 ng/μL), 0.6 μL of additional MgCl2 (25 μmol/L), 1.2 μL of bovine serum albumin (BSA) (1 mg/mL, BioLabs Inc, Ipswich, Massachusetts, USA), and molecular-grade water. The thermocycler was preheated to 98°C. Thermal cycling comprised initial denaturation at 98°C for 30 s, followed by 30 cycles of denaturation at 98°C for 10 s, annealing (according to primer set specifications, Table 6.1) for 30s, extension at 72°C for 30 s, and a final extension at 72°C for 4 min.

A no-template negative control was included in each PCR series to monitor potential contamination. PCR products were visualized on 2% (w/v) agarose gel with TBE buffer containing CondaSafe (Condalab, Madrid). A 100-bp molecular weight marker (New England BioLabs Inc, Ipswich, Massachusetts, USA) was used to determine amplicon sizes. PCR products from each site were pooled (Figure 6.2). Subsequent pooling involved combining the three replicates of PCR for the same primer regions per site, followed by submission of the pooled PCR products for high-throughput sequencing (Figure 6.2).

3.5

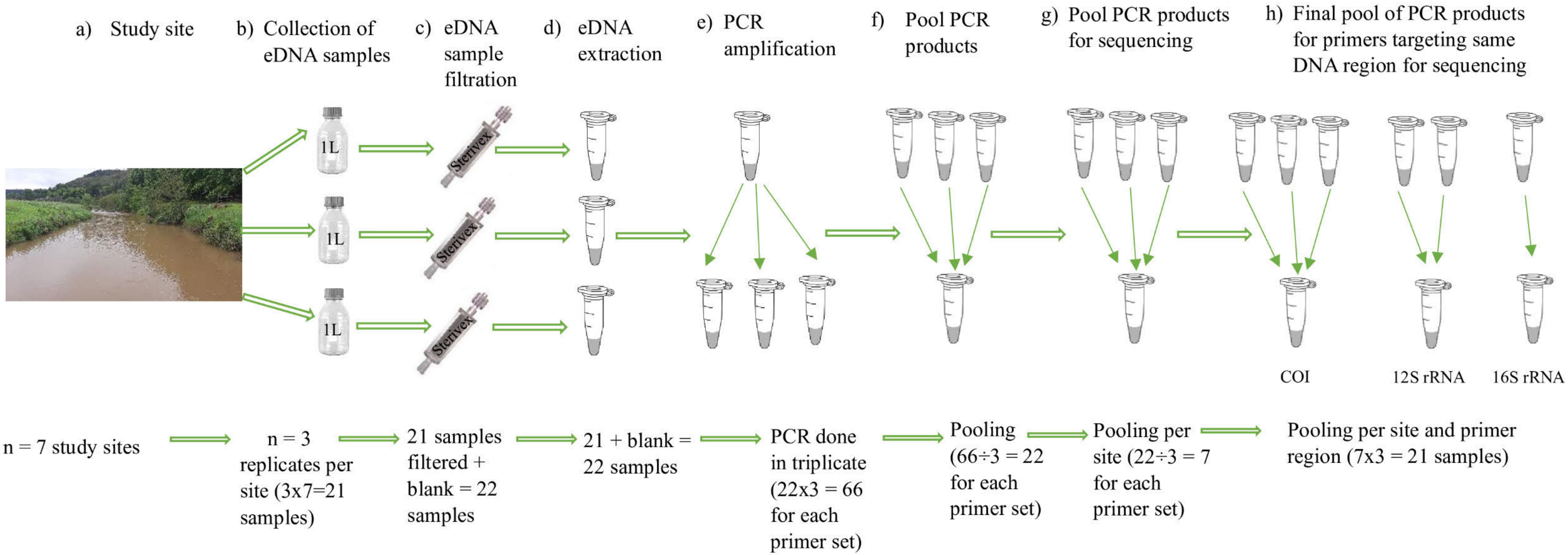

Figure 6.2: Comprehensive workflow for eDNA sample collection, filtration, extraction, and PCR amplification across seven sites in KwaZulu-Natal Province.

Table 6.1: Primers for amplification of eDNA. In some cases, the annealing temperature differs from that presented in the literature; the temperatures provided here were optimised for each primer set.

Fragment	Primer name	Primer sequence (5' - 3')	Direction	Amplicon length (bp)	Annealing Temperature °C	Reference
12S	AcMDB07F	GCCTATATACCGCCGTCG	F	85	54	Bylemans et al. 2018
	12S OreoR	CAGGGAATGTAGCCCATTTC	R		54	Noble et al. 2015
12S	MiFish-UF	GTCGGTAAAACTCGTGCCAGC	F	219	49	Miya et al. 2015
	MiFish-UR	CATAGTGGGGTATCTAATCCCAGTTTG	R			
16S	16S-FishF	AGCGYAATCACTTGTCTYTTAA	F	195	55	McInnes et al. 2017
	16S-FishR	CRBGGTCGCCCCAACCRAA	R			
COI	coi.175f	GGAGGCTTTGGMAAYTGRYT	F	55	49	Collins et al. 2019
	coi.226r	GGGGGAAGAARYCARAARCT	R	55	49	Collins et al. 2019
	coi.345r	TAGAGGRGGGTARACWGTYCA	R	130	49	Collins et al. 2019
COI	OnilF	GGCCGGGGTGTCATCTATTT	F	112	60	Keskin 2014
	OnilR	GGCAAGAACGGGTAGGGATAG	R			
COI	mlCOIintF	GGWACWGGWTGAACWGTWTAYCCYCC	F	319	46	Leray et al. 2013
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	R	319	46	Folmer et al. 1994
	FishR2	ACTTCAGGGTGACCGAAGAATCAGAA	R	319	46	Ward et al. 2005

6.3.6 eDNA sequencing

Illumina sequencing of eDNA amplicons was conducted at the KwaZulu-Natal Research and Innovation Platform (KRISP). Subsequent to library preparation, each library underwent purification using 1.89 Ampure XP purification beads (Beckman Coulter, High Wycombe, UK). The index PCR was performed using the Nextera XT Index Kit (Illumina, San Diego, USA). Following the index PCR, libraries were subjected to further purification using 0.69 Ampure XP purification beads, and their concentrations were quantified using a Qubit 4.0 device with the Qubit dsDNA High Sensitivity assay kit. Fragment sizes were determined using a LabChip GX Touch (Perkin Elmer, Hamburg, Germany) with an expected fragment size of 550 bp. Individual sample libraries were pooled, denatured with 0.2 N sodium acetate, and normalized to a concentration of 4 nmol/L. The sequencing process utilized an Illumina MiSeq platform (Illumina, San Diego, California, USA) with a MiSeq Nano Reagent Kit v2 (500 cycles), and a 5% PhiX Control (PhiX Control v3) was introduced into the library at a concentration of 12 pmol/L.

6.3.7 Bioinformatic analyses

For data processing, I employed the dada2 algorithm (Callahan et al. 2016) integrated into Qiime2 v.2022.10 (Bolyen et al. 2019). This algorithm was utilised for comprehensive quality control assessments, including filtering based on quality scores, trimming of primers, and truncation of reads to 300 base pairs for both forward and reverse sequences before merging into Amplicon Sequence Variants (ASVs). It also facilitated chimera detection and removal, denoising, deduplicating, and error modelling to ensure accurate sequence variant identification. Bioinformatics analysis was conducted using a high-performance computing platform (KRISP Server) to manage the computational demands effectively. Taxonomic assignments of ASVs were performed by cross-referencing against barcoding reference databases such as BOLD and GenBank.

6.4 Results

In the analysis of eDNA metabarcoding data, a total of 481,913 read counts were processed, resulting in 65,578 merged reads (Table 6.2). Subsequently, 2,275 Amplicon Sequence Variants (ASVs) were identified and subjected to comprehensive analysis across all sampling localities, ultimately revealing 211 ASVs taxonomically identified as fish-related through BLAST searches on GenBank and BOLD. Successful amplification was confirmed for the three targeted genes (12S rRNA, COI, and 16S rRNA) at varying levels across the sampling sites (Table 6.2). Specifically, COI amplified eDNA from four of the seven sites, while 12S rRNA amplified from six sites, and 16S rRNA successfully amplified from all seven sites. The total number of fish related ASVs recorded for each primer set were 119 for COI, 62 for 12S rRNA, and 30 for 16S rRNA (Table 6.2)

Despite these successes, species-level identification presented challenges, with only 38 out of the assigned ASVs associated with fish (18%) confidently identified to species level. Of these, COI identified 17 ASVs, 12S rRNA identified 16 ASVs and 16S rRNA identified 5 ASVs at the species level (Table 6.2). The majority of ASVs were classified at the genus or family level based on sequence similarity. Notably, two ASVs from the Albert Falls Dam and Thukela GS localities were assigned to *Oreochromis*, showing 100% sequence similarity to *O. mossambicus* and *O. niloticus*, respectively. The COI primers demonstrated superior performance in species identification across all sampling localities (Table 6.2). Among the 211 identified fish related ASVs, the Danionidae family was the most prevalent, followed closely by the Clariidae family (Table 6.3). However, species-level identification within the genus

Oreochromis proved challenging, with these species detected at only two of the seven sites investigated. Nevertheless, the COI primers showed promise in distinguishing between *O. mossambicus* and *O. niloticus*, as evidenced by their detection at these sites. In contrast, 12S showed moderate performance, while 16S exhibited poor ASV recovery.

Table 6.2: High-throughput summary statistics across all samples collected at seven localities in KwaZulu-Natal, South Africa. The results and duplication of site names are also represented based on the primer used.

Sampling locality & primer region	Read count	Merged reads	Total amplicon sequence variants	Merged amplicon sequence variants assigned to species level (97%)
COI				
Thukela mouth	18129	9924	7	3
Thukela GS	24363	8886	12	4
Albert Falls Dam	45483	1225	29	6
Mandini UW	42947	2065	71	4
	130922	**22100**	**119**	**17**
12S rRNA				
Thukela mouth	30151	4144	7	2
Thukela BW	23128	3068	6	2
Thukela GS	30203	7682	10	2
Albert Falls Dam	16887	4193	12	2
Mziny BW	22035	3117	11	2
Mandini UW	16505	8359	9	3
Mandini DW	13106	5156	7	3
	152015	**35719**	**62**	**16**
16S rRNA				
Thukela mouth	30770	943	6	1
Thukela BW	21889	151	2	0
Thukela GS	34176	869	3	0
Albert Falls Dam	31404	2533	7	1
Mziny BW	33411	1877	7	1
Mandini UW	47326	1386	5	2
	198976	**7759**	**30**	**5**
Total	**481913**	**65578**	**211**	**38**

Table 6.3: presents freshwater fish species identified across seven study sites in KwaZulu-Natal using eDNA metabarcoding. It shows species presence or absence detected by COI, 12S rRNA, and 16S rRNA markers at each site. Where species were detected by multiple markers, both are listed to reflect their occurrence accurately.

Family	**Genus**	**Species**	Thukela mouth	Thukela BW	Thukela GS	Albert Falls Dam	Mziny BW	Mandini UW	Mandini DW
Acanthoclininae	*Belonepterygion*	*fasciolatum*						COI	
Adrianichthyidae	*Oryzias*	*latipes*						COI	
Agonidae	*Podothecus*	*sachi*						COI	
Ailiidae	*Ailia*	*coila*						COI	
Alestidae	*Alestes*	*baremoze*						COI	
Allosidae	*Alosa*	*alosa*					12S		16S
Ambassidae	*Parambassis*	*ranga*							
Ammodytidae	*Hyperoplus*	*lanceolatus*				COI			
Anabantidae	*Anabas*	*testudineus*				16S			
Anthiinae	*Anthias*	spp						COI	
Aplocheilidae	*Aplocheilus*	*panchax*			12S				
Apogonidae	*Vincentia*	*novaehollandiae*				COI			
Aspredinidae	*Pseudobunocephalus*	*rugosus*						COI	
Atherinopsidae	*Chirostoma*	*humboldtianum*				COI		COI	
Auchenipteridae	*Auchenipterus*	*dentatus*						COI	
Badidae	*Badis*	*assamensis*						COI	
Bagridae	*Mystus*	spp						COI	
Balitoridae	*Bhavania*	*australis*						COI	
Blenniidae	*Aspidontus*	*teaenitus*						COI	
Blenniidae	*Blennius*	*ocellaris*			16S	16S			
Blenniidae	*Glyptoparus*	*delicatulus*				COI			
Blenniidae	*Lipophrys*	*pholi*				16S			
Bothidae	*Trichopsetta*	*ventralis*				COI			

Botiidae	*Sinibotia*	*superciliaris*						COI	
Carapidae	*Onuxodon*	*margaritiferae*						COI	
Catostomidae	*Myxocyprinus*	*asiaticus*						12S & 16S	
Catostomidae	*Xyrauchen*	*texanus*				16S			
Centrarchidae	*Micropterus*	*salmoides*						12S	
Chaetodontidae	*Chaetodon*	*declivis*				COI			
Characidae	*Astyanax*	*mexicanus*	COI						
Characidae	*Knodus*	spp						COI	
Characidae	*Paracheirodon*	*simulans*	COI						
Characide	*Creagrutus*	*barrigai*				COI			
Cichlidae	*Oreochromis*	*niloticus*			COI				
Cichlidae	*Oreochromis*	*mossambicus*				COI			
Clariidae	*Clarias*	spp	COI & 12S	12S	COI & 12S	12S	12S	12S	12S
Clariidae	*Clarias*	*batrachus*						COI	
Clariidae		spp	COI & 12S	12S	COI & 12S	COI & 12S	12S	12S	12S
Clariidae	*Clarias*	*gariepinus*			12S			12S & 16S	12S
Clupeidae	*spratelloides*	*gracillis*						COI	
Congridae	*Conger*	*conger*		16S					
Cyclopteridae	*Cyclopterus*	*lumpus*					12S		
Cynoglossidae	*Cynoglossus*	*itinus*						COI	
Cyprinidae	*Elopichthys*	*bambusa*						12S	
Cyprinidae	*Boraras*	spp						COI	
Cyprinidae	*Carassius*	*carassius*			16S				
Cyprinidae	*Cyprinus*	*carpio*			COI				12S
Cyprinidae	*Danio*	*rerio*	COI, 12S & 16S	COI & 12S	COI & 12S	12S	12S	12S	12S
Cyprinidae	*Danio*	*kyathit*					16S		
Cyprinidae	*Elopichthys*	*bambusa*	COI, 12S & 16S	COI & 12S	12S	12S	12S	12S	12S

Cyprinidae	*Megalobrama*	*amblycephala*							16S
Cyprinidae	*Osteobrama*	*cotio*						COI	
Cyprinidae	*Pethia*	*pollux*						COI	
Danionidae	*Esomus*	*metallicus*						COI	
Echeneidae	*Echeneis*	*naucrates*	COI			12S	12S		
Galaxiidae	*Galaxiiella*	*toourtkoourt*				COI			
Galaxiidae	*Galaxiiella*	*munda*				COI			
Gasterosteidae	*Pingutius*	*pungitius*			12S				
Gobiidae	*Gobiosoma*	*longipala*						COI	
Gobiidae	*Myersina*	*filifer*						COI	
Gobiidae	*Trimma*	*pentherum*						COI	
Gobionidae	*Coreius*	*heterodon*						COI	
Gobionidae	*Pseudorasbora*	*pumila*						COI	
Gymnotidae	*Electrophorus*	*electricus*	COI	16S	16S		16S		
Holocentridae	*Myripristis*	*murdjan*			COI				
Ictaluridae	*Noturus*	*exilis*						COI	
Labridae	*Psuedocoris*	spp						COI	
Labridae	*Halichoeres*	*marginatus*						COI	
Lateolabracidae	*Lateolabrax*	*maculatus*			COI		12S		
Leuciscidae	*Phoxinus*	*phoxinus*							
Leuciscidae	*Rhynchocypris*	*percnurus*			COI				
Leuciscidae	*Rutilus*	*rutilus*				COI			
Libridae	*Labroides*	*rubrolabiatus*						COI	
Liparidae	*Careproctus*	*colletti*						COI	
Macrouridae	*Coelorinchus*	*caelorhincus*				12S		COI	
Macrouridae	*Gadomus*	*longifilis*						COI	
Macrouridae	*Metaocephulus*	*cristatus*						COI	
Melanotaeniidae	*Telmatherina*	*bonti*	16S		16S			COI	

Monacanthidae	*Acreichthys*	spp						COI
Mugilidae	*Chelon*	*labrosus*	16S					
Mugilidae	*Mugil*	*cephalus*					12S	
Mugilidae	*Osteomugil*	spp						COI
Mugilidae	*Pseudomyxus*	*capensis*				COI		
Mullidae	*Upeneus*	*sulphureus*						COI
Myctophidae	*Lampanyctus*	*alatus*						COI
Nemacheilidae	*Nemacheilus*	*rueppelli*						COI
Nemacheilidae	*Pteronemacheilus*	*meridionalis*						COI
Nothobranchiidae	*Aphyosemion*	*herzogi*						COI
Nothobranchiidae	*Aphyosemion*	*franzwerneri*						COI
Nothobranchiidae	*Epiplatys*	*multifasciatus*				COI		
Nothobranchiidae	*Epiplatys*	*spilargyreius*						COI
Nothobranchiidae	*Fenerbahce*	*devosi*						COI
Nothobranchiidae	*Nothobranchius*	spp						COI
Nothobranchiidae	*Epiplatys*	*spilargyreiu*				COI		
Odontoceridae	*Odontocerum*	*albicorne*				16S		
Ophidiidae	*Brotulotaenia*	*nigra*						COI
Osmeridae	*Osmerus*	*eperlanus*				COI		
Osphronemidae	*Betta*	*splendens*	12S	12S				
Osphronemidae	*pseudosphromenus*	*cupanus*				COI		
Osteoglossidae	*Scleropages*	*formosus*				12S		12S & 16S
Oxudercidae	*Chaenogobius*	*annularis*						COI
Pangasiidae	*Pangasianodon*	*hypophthalmus*				COI		
Paralepididae	*Lestrolepis*	*intermedia*						COI
Paralichthyidae	*Paralichthys*	*brasiliensis*						COI
Paralichthyidae	*Pseudorhombus*	spp				COI		
Pempheridae	*Pempheris*	*poeyi*				COI		

Poeciliidae	*Poecillia*	*reticulata*			COI				
Polypteridae	*Erpetoichthys*	*calabaricus*				COI		COI	
Pomacentridae	*Stegastes*	*partitus*					16S		
Pseudochromidae	*Pseudoplesiop*	*revellei*				COI		COI	
Rivulidae	*Hypsolebias*	spp						COI	
Salmonidae	*Oncorhynchus*	*mykiss*				12S			
Salmonidae	*Salmo*	*trutta*				16S			
Scaridae	*Scarus*	*altipinnis*						COI	
Sciaenidae	*Johnius*	*borneensis*						COI	
Scianidae	*Johnius*	*carouna*						COI	
Scianidae	*Johnius*	*heteropis*						COI	
Scienidae	*Bairdiella*	spp						COI	
Scombridae	*Thunnus*	*albacares*					16S		
Serranidae	*Epinephelus*	*lanceolatus*						COI	
Serranidae	*Serranus*	*chionaraia*						COI	
Serranidae	*Variola*	*louti*						COI	
Siluridae	*Silurus*	*aristotelis*			12S				
Soleidae	*Solea*	*senegalensis*	COI & 12S	12S	12S	12S	12S		12S
Syngnathidae	*Hippocampus*	*denise*				COI			
Syngnathidae	*Hippocampus*	*comes*				16S			
Syngnathidae	*Nerophis*	*lumbriciformis*	16S				16S		
Syngnathidae	*Syngnathus*	*acus*				COI			
Tetrabrachiidae	*Tetrabrachium*	*ocellatum*				COI		COI	
Triglidae	*Bellator*	*loxias*				COI			
Valenciidae	*Valencia*	*hispanica*					12S		
Zenarchopteridae	*hemirhamphodon*	*pogonognathus*				COI			

6.5 Discussion

In this study, I employed a multimarker approach using primer cocktails targeting COI, 12S rRNA, and 16S rRNA to assess the efficacy of eDNA metabarcoding for detecting freshwater fish in South Africa, with a focus on detecting the vulnerable Mozambique tilapia (*Oreochromis mossambicus*) in the KwaZulu-Natal Province. Our findings provide valuable insights into the potential applications and limitations of eDNA metabarcoding in monitoring freshwater fish species in the region. One significant outcome of our study is the successful detection of *O. mossambicus* as well as the introduced *O. niloticus* using the COI marker. While COI primers were effective in detecting *Oreochromis* species, the 12S rRNA and 16S rRNA markers did not perform as well for these specific species. However, the combination of all three markers proved useful for broader community assessment.

Our findings indicated that these three marker sets identified both overlapping and distinct species assemblages across the seven sites studied. This suggests that a multimarker approach provides a more comprehensive understanding of community dynamics and the applicability of the eDNA method. Therefore, while COI primers are particularly effective for monitoring *O. mossambicus* populations, integrating multiple markers enhances the overall detection and monitoring of various other fish species. While eDNA metabarcoding serves as a potent tool for identifying vulnerable and invasive species, alongside providing insights into broader fish community assessments (Shaw et al. 2016, Belle et al. 2019, Alam et al. 2020, Sahu et al. 2023). However, for studies focused on pinpointing specific species presence or absence across numerous sites, targeted eDNA methods like quantitative polymerase chain reaction (qPCR) or Single Nucleotide Polymorphism (SNP) panels may offer a more cost-effective solution (Melville et al. 2017, Forsdick et al. 2021, Tremblay et al. 2022). In this study, I effectively used metabarcoding to detect the vulnerable *O. mossambicus* and invasive *O.*

niloticus, while also identifying additional fish species. Moving forward, to enhance efficiency, future research solely dedicated to detecting specific vulnerable or invasive species over larger areas may benefit from an integrated approach. This would involve initial screening using metabarcoding for comprehensive community assessment, followed by confirmation using targeted qPCR or SNP panels.

In addition to the findings gained from this study, it is essential to consider complementary techniques that can further improve species-level assignments. One promising avenue is the integration of advanced sequencing technologies into the monitoring toolkit. While our study focused on specific markers (COI, 12S rRNA, and 16S rRNA), exploring methods such as shotgun metagenomics or targeted amplification could significantly enhance taxonomic resolution. Shotgun metagenomics provides a more comprehensive genetic profile by sequencing all DNA in a sample, allowing for finer-scale discrimination between closely related species (Wang et al. 2021, Jin et al. 2023). This is especially important for *Oreochromis* species that have undergone recent evolutionary radiation (Downing et al. 2011, Schroeter et al. 2020, Nikolic et al. 2023). This approach holds great potential for addressing the challenges encountered in our study, where higher taxonomic identifications were prevalent due to incomplete reference databases.

6.5 Conclusions

In conclusion, this study contributes valuable insights into the application of eDNA metabarcoding for freshwater fish monitoring in South Africa. The successful detection *of O. mossambicus* and *O. niloticus* using the COI marker highlights the method's potential for targeted species monitoring. However, given that these species were only detected at one site, despite being known to be present in other locations. This underscores the need for further refinement and optimisation of the eDNA metabarcoding method to improve detection rates and coverage for these focal species. In contrast, the 12S rRNA and 16S rRNA markers did not detect these species, underscoring their variable performance in this context. However, the multimarker approach proved highly efficient in detecting a diverse array of fish species across all sampled sites. The study findings also emphasise the critical challenge of incomplete reference libraries, necessitating collaborative efforts to expand and improve databases on platforms such as GenBank and BOLD. Enhancing these resources is essential for accurate species identification and a comprehensive understanding of freshwater fish communities in the region. This study establishes a valuable foundation for future applications of eDNA metabarcoding in aquatic ecosystem monitoring and conservation efforts. By overcoming current limitations and refining methodologies, eDNA metabarcoding has the potential to significantly enhance our ability to protect and manage freshwater biodiversity in South Africa and beyond.

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CHAPTER 7

General discussions and conclusions

The global concern over the vulnerability of freshwater ecosystems to anthropogenic stressors is well-established (Revenga et al. 2005, Dudgeon 2019, Belle et al. 2019, Lima et al. 2023), with specific emphasis on water-scarce regions like South Africa (Dallas and Rivers-Moore 2014, Govender et al. 2022). South Africa, recognised for its high species richness and endemism, faces significant threats to its freshwater biodiversity resulting from pollution, invasive species, water extraction, water management regimes, aquaculture, and overexploitation (Dudgeon et al. 2006, Nel et al. 2011, Dallas and Rivers-Moore 2014, O'Brien et al. 2019, Desai et al. 2021, Evans et al. 2022). These stressors contribute to a decline in genetic diversity and population viability of freshwater species (Lande 1998, Crook et al. 2015, Machado et al. 2022). Hence, there is an urgent need for heightened attention to effective monitoring, identification of vulnerable areas, and prioritisation of species for conservation and management efforts (Fierro et al. 2019, O'Brien et al. 2019, Tsoupas et al. 2022). This research project addressed this critical knowledge gap by conducting a comprehensive genetic assessment of *Oreochromis mossambicus* across three South African provinces (Limpopo, Mpumalanga, and KwaZulu-Natal). This study employed microsatellite markers and eDNA metabarcoding to gain critical insights for improving regulatory frameworks and management practices for *O. mossambicus* populations in South Africa. These advancements aimed to enhance the conservation of this species and promote the sustainable development of aquaculture in the region. The research focused on investigating the genetic diversity and population structure of this vulnerable fish species within three provinces (Limpopo, Mpumalanga, and KwaZulu-Natal) in South Africa. Additionally, it examined potential threats from introduced species (*O. niloticus* and *O. aureus*) and developed innovative monitoring methods.

7.1 Research Findings

In **Chapter 2**, this research investigated the genetic makeup of wild Mozambique tilapia (*O. mossambicus*) populations across Limpopo, Mpumalanga, and KwaZulu-Natal provinces in South Africa. Using microsatellites, the study identified 15 distinct genetic clusters, indicating a decline in genetic diversity. Human activities, particularly dam construction and water management practices, were identified as potential factors contributing to this decline. The findings of this chapter emphasise the need for improved water management strategies that consider the genetic distinctiveness of *O. mossambicus* populations. The proposal is to create dedicated *O. mossambicus* Management Areas (OMAs) where populations with similar genetic makeup are grouped. This approach will help conserve existing genetic diversity and ensure the long-term survival of vulnerable fish species.

Chapter 3 examined the genetic diversity of farmed *O. mossambicus* populations. The analyses showed a strong genetic similarity between these farmed *O. mossambicus* and their wild counterparts. This suggests these farmed fish likely originated from local wild stock, potentially making them suitable for restocking purposes if needed, as they share a similar genetic makeup. However, an exception emerged, the uMphafa ponds *O. mossambicus*. Unlike others, this population displayed a distinct genetic signature, and its origin remains unclear.

The study also discovered that some individuals from the Thukela River shared the same genetic cluster as the uMphafa ponds' *O. mossambicus*. This finding presents two possibilities, one scenario suggests that fish from the genetically distinct uMphafa ponds might have escaped and established themselves in the Thukela River. This escape could pose a threat to the wild Thukela

River *O. mossambicus* if interbreeding occurs. Alternatively, the Thukela River *O. mossambicus* could be the historical source of the uMphafa ponds' *O. mossambicus*. More investigation is needed to determine the origin and possible historical connection between these locations. This discovery underscores the critical importance of tracking the source of farmed fish. Introducing genetically different individuals into the wild through escapes can disrupt the genetic integrity of wild populations. The study emphasises the need for responsible management practices in aquaculture. This includes using native *O. mossambicus* for farming and implementing stricter biosecurity measures to prevent escapes and potential interbreeding with wild populations.

Chapter 4 assessed the presence of genetic material from introduced tilapia species (*O. niloticus* and *O. aureus*) in farmed and wild *O. mossambicus* populations. While no evidence of interbreeding with introduced species was found in farmed populations, some wild populations showed signs of genetic introgression (from *O. niloticus*), suggesting potential interbreeding in the past. The study recommends using native *O. mossambicus* for aquaculture and implementing stricter biosecurity measures to prevent future hybridisation events between farmed and wild populations. This will help maintain the genetic integrity of wild *O. mossambicus*.

Chapter 5 reviewed the availability of DNA barcode reference libraries for freshwater fish species in South Africa, emphasizing their crucial role in accurately identifying species using environmental DNA (eDNA) metabarcoding. This technique analyzes environmental samples for genetic material, making reliable reference libraries essential. The review highlighted a significant gap in comprehensive reference libraries for native fish species, particularly for the 12S rRNA gene. Building accurate and reliable reference libraries for markers used in eDNA studies is vital, as it will alleviate issues related to inaccurate species assignments in the databases, thereby enhancing the precision of eDNA-based species identification.

Chapter 6 applied eDNA metabarcoding for tilapia detection. While the study successfully distinguished *O. mossambicus* from *O. niloticus* using COI markers, achieving accurate species identification for all species remained a challenge likely due to limitations in reference databases. The study emphasises the need to improve the completeness of reference databases for more effective eDNA metabarcoding in monitoring fish populations.

7.2 Performance of microsatellites in *O. mossambicus* population genetics: limitations and future research directions

The utilisation of microsatellite markers has revolutionised the measurement of genetic diversity in fish species crucial for aquaculture and fisheries (Hall 2001, Crispo et al. 2011, Mather et al. 2018, Kariuki et al. 2021, Ramya and Behera 2023, Wenne 2023). These markers are particularly valuable for assessing genetic variation in *Oreochromis* spp. populations (Hassanien and Gilbey 2005, D'Amato et al. 2007, Saju et al. 2010, Simbine et al. 2014, Richmond 2018, Anane-Taabeath 2019). Additionally, they successfully identify hybridisation between species, reconstruct demographic history, and assess the potential effects of inbreeding, bottlenecks, social structure, dispersal, and reproductive behaviour on genetic structure (Svåsand et al. 2007, Bezault et al. 2012, Mather et al. 2018, Kariuki et al. 2021, Ramya and Behera 2023).

In this study, the application of microsatellite markers provided critical insights into the genetic diversity and population structure of both farmed and wild *O. mossambicus* in South Africa, particularly in KwaZulu-Natal, Limpopo, and Mpumalanga. The results indicated varying levels of genetic diversity across populations, highlighting the complexity and variability of genetic structure within and between these populations. Additionally, the microsatellites were able to detect the presence of genetic material from introduced species, suggesting their capability in identifying potential hybridisation and detecting hybrids. However, there were various levels of missing data in some populations, which posed a challenge for comprehensive analysis. Moreover, the microsatellite markers did not perform well for *O. aureus*, indicating a limitation in evaluating possible hybridisation with this species. This complexity and these limitations underscore the necessity of integrating these findings with more advanced molecular technologies.

To complement and improve the data generated in the present study, it will be crucial for future research to incorporate additional molecular markers, such as mitochondrial DNA (mtDNA), single nucleotide polymorphisms (SNPs), and shotgun metagenomics. These advanced techniques can provide more detailed and comprehensive insights into the genetic structure and diversity of *O. mossambicus* populations. Mitochondrial DNA can help verify the maternal lineage and provide information on historical population dynamics (Avise et al. 1987, Kivisild 2015, Jaisamut et al. 2023). SNPs offer a high-resolution tool for detecting fine-scale genetic variations and hybridisation events (McCarroll et al. 2008, Melville et al. 2017, Forsdick et al. 2021). Shotgun metagenomics can provide a broader genomic perspective, enabling the identification of genomic regions associated with adaptation and fitness (Douglas and Langille 2019, Tremblay et al. 2022). By integrating these advanced molecular tools with microsatellite data, we can achieve a more robust and accurate understanding of the genetic landscape of *O. mossambicus* populations, ultimately enhancing conservation and management strategies in South Africa.

7.3 Environmental DNA (eDNA) for monitoring *Oreochromis* spp.

Recently, the adoption of more advanced molecular technologies, such as DNA barcoding and metabarcoding methods, for fish assessment and monitoring has become increasingly prevalent (Shaw et al. 2016, Belle et al. 2019, King et al. 2022). Specifically, the utilisation of environmental DNA (eDNA) metabarcoding, leveraging high-throughput sequencing, proves to be a non-invasive, time-efficient, and cost-effective tool for monitoring freshwater fish communities, detecting invasive species, and mapping the distribution of both rare and abundant species in freshwater ecosystems (Shaw et al. 2016, Belle et al. 2019, Alam et al. 2020, Rishan et al. 2023). The rapid collection and processing of eDNA samples make this method suitable for enhancing species detection, monitoring, and conservation efforts (Mardis 2011, Shaw et al. 2016, Miya et al. 2020, Sahu et al. 2023).

Our study also explored the use of eDNA metabarcoding to detect *O. mossambicus* and the introduced *O. niloticus* in KwaZulu-Natal. This method proved to be a valuable tool for identifying fish species within the study sites, offering a non-invasive and efficient approach to biodiversity monitoring. The results indicated the presence of these species at only two out of the seven sampling locations using the COI marker, demonstrating eDNA's effectiveness in detecting both native and invasive species. However, several challenges were identified with the eDNA approach. A primary issue was the incomplete reference databases for native fish species, which impeded accurate species identification and limited the resolution of our results. Most species identified were only classified at the genus or family level, further complicating the analysis. Additionally, the presence of closely related species with recent evolutionary divergence complicated species-level identification using standard markers (COI, 12S rRNA, and 16S rRNA). These limitations highlight the need for more comprehensive reference libraries and improved markers to enhance the accuracy and reliability of eDNA metabarcoding in biodiversity studies.

To improve the efficacy of eDNA methods, it is crucial to enhance and expand reference databases, ensuring comprehensive coverage of native species. Efforts are needed to build and curate these databases, incorporating genetic information from a wide range of taxa. Additionally, integrating advanced sequencing technologies, such as shotgun metagenomics, can provide a more comprehensive genetic profile of the sampled communities, allowing for finer-scale discrimination between closely related species. Moreover, adopting a multi-marker approach by adding other markers and combining mitochondrial and nuclear markers can improve the accuracy of species identification.

Improving the sampling strategy is also essential, targeting more suitable habitats for the target species and collecting samples from multiple localities within a river can enhance the detection probability and accuracy of eDNA methods. Continuous refinement of eDNA methodologies, coupled with robust reference databases, advanced molecular techniques, and optimised sampling strategies, will significantly enhance the monitoring and conservation of freshwater fish species, including *O. mossambicus*, in South Africa and beyond.

7.4 Sustainable aquaculture practices informed by genetic data

The data from my PhD thesis chapters on the genetic diversity and population structure of *O. mossambicus* in South Africa offers significant implications for sustainable aquaculture practices. By identifying genetically distinct populations and assessing their relationships with farmed stocks, the study underscores the importance of genetic diversity management in aquaculture. This includes selecting appropriate broodstock to enhance resilience and disease resistance. Furthermore, the findings support conservation breeding efforts aimed at preserving unique genetic lineages, particularly in regions like uMphafa ponds, while advocating for stringent biosecurity measures to prevent genetic contamination from introduced species such as *O. niloticus* and *O. aureus*. The genetic similarity observed between certain farmed populations and their wild counterparts also suggests the potential for using aquaculture facilities as sources for restocking efforts, thereby contributing to fisheries management and food security goals.

While the present study provides valuable insights, future research can be significantly strengthened by expanding its scope. To gain a more comprehensive picture of the genetic diversity of farmed *O. mossambicus* across South Africa, a broader sampling effort encompassing a larger number of aquaculture facilities, particularly from provinces beyond KwaZulu-Natal and Mpumalanga, would be beneficial. This wider geographic coverage would allow for a more robust assessment of potential regional variations. Additionally, tracing the origins of broodstock used in different facilities would provide valuable information on potential genetic bottlenecks or founder effects within farmed populations. This information is crucial for developing sustainable broodstock management strategies.

7.5 Recommendations for developing South Africa *Oreochromis* aquaculture:

In light of the GIFT tilapia being hybrids of *O. mossambicus*, *O. niloticus*, and *O. aureus* (Etherington et al. 2022), the present research recommends several strategies for developing sustainable *Oreochromis* aquaculture in South Africa. Leveraging hybrid vigor in breeding programs could enhance productivity and disease resistance, crucial for sustainable aquaculture (Etherington et al. 2022, Kashyap et al. 2024, Thakur et al. 2024). However, conservation efforts should prioritise maintaining pure *O. mossambicus* stocks to safeguard against genetic dilution from hybrids and ensure the species' long-term viability. Regular genetic monitoring of both farmed and wild populations is essential to detect and mitigate any genetic introgression from hybrid species. This proactive approach helps prevent unintended genetic consequences in aquaculture and natural ecosystems, promoting a balanced approach to food security and biodiversity conservation.

7.6 Directions for ensuring long-term management and conservation of *O. mossambicus* in South Africa:

To ensure the future longevity of *O. mossambicus*, it is vital to expand genetic monitoring efforts across different regions and populations. Collaborative research efforts among aquaculture practitioners, conservationists, and researchers are crucial for sharing data and insights, and fostering collective efforts to safeguard *O. mossambicus* populations and their genetic diversity. These efforts aim to mitigate potential genetic issues arising from hybridisation with introduced tilapia species and ensure the resilience of *O. mossambicus* populations in South Africa's freshwater ecosystems. In addition to genetic monitoring, it is essential to consider how water management strategies influence the current genetic population structure observed in wild *O. mossambicus* populations. The prevalence of impoundments and restricted free-flowing sections in river systems can contribute to the observed decline in genetic diversity. Anthropogenic activities such as water extraction, pollution, and habitat modification can further impact the genetic resources of *O. mossambicus*. The information generated from our study can guide policymakers and conservation frameworks in incorporating the effects of anthropogenic activities and water management strategies on genetic resources. By recognising the impact of these factors, conservation efforts can be more effectively tailored to protect and enhance the genetic diversity of *O. mossambicus*. This approach will ensure the sustainable management of water resources and the long-term viability of O. mossambicus populations in South Africa.

7.7 Contribution to managing invasive species.

The present study has significantly contributed to developing effective strategies for managing invasive species, particularly *O. niloticus* and *O. aureus* in South African waters. By utilising microsatellite markers and eDNA metabarcoding, the research has advanced methods for early detection and assessment of genetic impacts from introduced species. These methodologies provide a foundation for risk assessment frameworks tailored to invasive species management, enhancing our ability to monitor and mitigate genetic threats to native *O. mossambicus* populations. Given the findings on possible hybridisation and the presence of genetic material from the two introduced species, the study highlights the importance of continuous monitoring of introduced species and the genetic integrity of native populations. The detection of possible hybridisation events underscores the need for improved detection methods and regular genetic assessments to prevent the potential loss of native genetic diversity. The integration of eDNA methods for early detection of these species shows promise, but further refinement and validation are necessary to improve accuracy and sensitivity. The study's findings and methodologies offer transferable insights for managing other invasive species in aquatic ecosystems, supporting broader conservation efforts across different regions and species. Implementing regular eDNA monitoring can provide an efficient and non-invasive tool for early detection of invasive species, allowing for timely management actions. Additionally, combining eDNA methods with traditional genetic analysis techniques can enhance the accuracy of species identification and the assessment of genetic impacts.

To further improve detection and monitoring, it is recommended to explore advanced molecular techniques such as shotgun metagenomics, which can provide comprehensive genomic data and improve the resolution of species identification. Continuous collaboration among researchers, policymakers, and conservation practitioners is essential to develop and implement effective monitoring and management strategies, ensuring the protection of native species and the integrity of aquatic ecosystems.

7.8 Enhancing the study: addressing limitations and future directions.

Beyond highlighting its immediate contributions, this study holds broader implications for freshwater fish conservation, aquatic taxa management, and freshwater ecosystem sustainability. By integrating genetic diversity assessments with eDNA metabarcoding and microsatellite marker analyses, the research provides a template for monitoring and managing other freshwater fish species facing similar genetic and conservation challenges. The methodologies developed, particularly in eDNA metabarcoding and genetic monitoring, can be adapted to assess and manage diverse aquatic taxa beyond *Oreochromis* species, enhancing biodiversity conservation efforts globally.

The study's findings underscore the necessity for adaptive water management strategies and policies that integrate genetic data into conservation planning and aquaculture management. Recommendations include revisiting aquaculture policies to prioritize genetic integrity, enhancing biosecurity measures, and promoting sustainable practices that minimize genetic introgression from introduced species. This approach not only supports sustainable aquaculture but also strengthens resilience against environmental changes and invasive species impacts in freshwater ecosystems. However, the study acknowledges several limitations. These include the need for more extensive spatial and temporal sampling across the entire distribution range of *O. mossambicus* to capture regional genetic variations comprehensively. Furthermore, while microsatellite markers provide valuable insights, integrating more advanced genomic technologies like SNP genotyping and whole-genome sequencing could enhance resolution and accuracy in genetic studies of *Oreochromis* species. Addressing these shortcomings will be crucial for fulfilling the study's objectives and improving the robustness of genetic monitoring and conservation strategies.

Looking forward, the study calls for greater collaboration among researchers, policymakers, and local communities to overcome barriers to implementing genetic-based management practices effectively. These barriers may include resource constraints, technological limitations, and the need for capacity building in genetic monitoring and conservation genetics. By addressing these challenges and fostering knowledge exchange, there is potential for widespread uptake of the study's results to inform evidence-based policies and practices for safeguarding freshwater fish biodiversity and ecosystem health globally.

7.9 Conclusions and management implications

This study underscores the critical importance of genetic diversity management and conservation for *O. mossambicus* in South Africa. It highlights the significant genetic variability present among both wild and farmed populations, emphasizing the need for careful stewardship to preserve this diversity. Sustainable aquaculture practices can leverage locally adapted genetic stocks of *O. mossambicus* to enhance food security while minimizing the risk of genetic introgression from non-native species. The findings advocate for integrating genetic data into water management policies, particularly in refining management areas to protect genetically distinct populations effectively. Methodologically, the study advances the use of microsatellite markers and eDNA metabarcoding for monitoring and detecting introduced species, paving the way for future genomic applications in conservation genetics. Looking ahead, continued genetic monitoring and the application of advanced genomic tools are crucial for informing adaptive management strategies aimed at safeguarding *O. mossambicus* populations across their distribution range in South Africa and beyond.

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