MORPHOMETRIC AND THE GENETIC VARIATIONS IN WILD POPULATIONS OF *OREOCHROMIS MOSSAMBICUS* AND ITS POTENTIAL HYBRIDS IN LIMPOPO PROVINCE, SOUTH AFRICA

BY

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DEDICATION

This masterpiece is dedicated to my wonderful grandparents, Abram and Melita Thosago. Ke sa leboga batswadi ba ka. May you continue to rest in peace. To Lesedi la mama le papa. We loved you dearly. You tore our hearts apart, but I know you will come back to us. See you soon my boy. A taste of your presence was priceless. Thank you for wiping our tears away, with your cute smile and sneeze. Your spirit will forever live on Dikobe Kganya Letsoalo. We loved you dearly. Rest my son.

To my latest oxygen Bornwise Adolf Kagiso Mashego, I dedicate this to you and our little king Lehumo Phaladi Molwantoa Mashego.

Psalm 103: 2-5

DECLARATION

I, Mahlatse Fiona Mashele, declare that MORPHOMETRIC AND THE GENETIC VARIATIONS IN WILD POPULATIONS OF *OREOCHROMIS MOSSAMBICUS* AND ITS POTENTIAL HYBRIDS IN LIMPOPO PROVINCE, SOUTH AFRICA thesis hereby submitted to the University of Limpopo, for the Master of Science degree in Aquaculture, is my own work in design and in execution, and that all the sources that I have used have been indicated and acknowledged by means of complete references and that this work has not been submitted before, for any other degree at any other institution.

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Mashele Mahlatse Fiona Date

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THESIS ABSTRACT

MORPHOMETRIC AND THE GENETIC VARIATIONS IN WILD POPULATIONS OF *OREOCHROMIS MOSSAMBICUS* AT ITS POTENTIAL HYBRIDS IN LIMPOPO PROVINCE, SOUTH AFRICA.

The current study presented a comprehensive analysis of *O. mossambicus* and its possible hybrids inhabiting four localities, with a focus on morphometric and genetic assessments to identify species and hybridization patterns. The morphometric analysis used discriminant analysis and principle component analysis to class the tilapia species and investigate which morphometric features contributed the most in differentiating among the four tilapia species. Genetic verification was conducted using mitochondrial DNA and nuclear DNA markers. The mitochondrial COI gene was amplified using the FishF primer, while the 5S gene for nuclear DNA was analyzed using the 5SA primer and analyzed using Bayesian inference and maximum likelihood. The morphometric analysis revealed the presence of four distinct species, *Oreochromis mossambicus*, *Oreochromis niloticus*, *Coptodon rendalli,* and *T. sparrmanii* alongside evidence of hybridization between *O. mossambicus* and *O. niloticus*. The genetic data corroborated the morphometric findings, confirming the existence of *O. mossambicus*, *O. niloticus,* their hybrids and *T sparrmanii*, however, *Coptodon rendalli* failed to amplify, due to poor amplicon quality. Hybridization occurring between the native *O*. *mossambicus* and the introduced *O. niloticus* showed a significant risk to the genetic diversity of *O. mossambicus*, as the introgression of genetic material from the exotic *O. niloticus* could lead to the erosion of unique genetic traits in the native species. The results also showed that the tilapia species in the study had the same nucleotides for the 5S gene and differentiation was only observed in the Non-transcribed spacer (NTS) part of the gene. The findings also highlighted the need for management strategies to mitigate the impact of hybridization and preserve the genetic integrity of *O. mossambicus,* noting significant differences between wild and cultured *O. mossambicus* species due to one mutation from a sample in Flagboshielo Dam. Conservation efforts should focus on monitoring hybridization rates and implementing measures to control the spread of *O. niloticus* within native ranges of native Oreochromis species. The genetic dilution of *O. mossambicus* by the exotic *O.*

niloticus emphasizes the urgent need for conservation actions to prevent the loss of genetic diversity in the native tilapia population.

CHAPTER 1

1. GENERAL INTRODUCTION

BRIEF HISTORY OF CICHLID CLASSIFICATION

Cichlids (Teleostei:Perciformes: Cichlidae) are from the family Cichlidae, in the order Chichliformes. Traditionally, cichlids were classed in a suborder, the Labroide, along with the wrasses (Labridad) in the order Perciformes, but molecular studies have contradicted this grouping (Wainwright *et al*., 2012). They rank among the most species-rich fish families, currently holding more than 1600 valid species taxa (Eschmeyer and Fong, 2012). The family cichlidae is generally found in fresh and brackish waters of South and central America, Madagascar, the eastern Mediterranean region, and parts of Arabia, india and Africa (Skelton, 2024). Turner *et al.,* (2001) noted that cichlids could count up to 3000 species, given their vast distribution. The African ciclids species are now classified as the subfamily Pseudocrenilabrine, making it the largest lineage in Africa with 165 genra and about 1.147 decribed species, which amounts to atleast two thirds of the described cichlids (Oliver, 2024 and Skelton, 2024). As a group, cichlids exhibit a similar diversity of body shapes, ranging from strongly laterally compressed species to cylindrical and highly elongated species. They tend to be medium-sized, ovate, and slightly laterally compressed (Stiassny *et al.,* 2007). They share a single key trait, which is the fusion of the lower pharyngeal bones into a single tooth-bearing structure (Helfman *et al.*,1997). Their dorsal and anal fins are each composed of spinous and soft-rayed sections and pelvic fins in a thoracic position, each comprising a spine and five branched rays (Skelton, 1993)

Certain features distinguish cichlids from other families in the Labroidei. They include (Raines and Pauly, 2006): a single nostril on each side of the forehead, no bony shelf below the orbit of the eye, divisions of the lateral line organ into two sections, one on the upper half of the flank and a second along the middle of the flank from about halfway along the body to the base of the tail (except in *Teleogramma* and *Gobiocichla*), a distinctively shaped otholith and the small intestine, left side exit from the stomach instead of its right side as in other Labroidei (Raines and Pauly, 2006).

Previously, there were several attempts to classify cichlids, resulting in the present system. They have included classifications by Boulenger (1915, 1916), who named 94

species during that time, basing their classification on dentition, squamation characters and fin meristics. Boulenger (1915, 1916) stated that this was a problem because dentition in certain species was subjected to variations, according to age (Dunz and Schliewan, 2013).

Hoedeman and De Jong, (1947) taxonomically formalized Regan's informal split which provided a supergeneric reclassification of African cichlids, into two significant groups, by introducing the subfamily *Tilapiine* for all African cichlids, based on additional characters, mainly the structure of the pharyngeal bones at the base of the skull, with a *Tilapia* type apophysis and the *Halochrominae* for the rest (Dunz and Schliewan 2013). Thys van den Audenaerde (1969), then followed about 50 years later with the first comprehensive published species level classification of African species of tilapia. Although Audenaerde (1969) referred to Regan (1920), Audenaerde (1969) did not consider the osteological characteristics described by Regan (1920). This led to the criticism of Regan and Hoedemans classification by Wickler's (1963), who stated that their classification was inconsistent with the distribution of ethological characters (Dunz and Schliewan 2013).

Trewaves (1973) laid the foundation of the current classification system. Trewaves (1973) amalgamated all the remaining species of Thys van den Audenaerdes (1969) (sections 1 and 2). comprising of exclusively substrate-brooding genera in a newly diagnosed genus, Tilapia without any further subgeneric division and mainly based on osteological characters and breeding behavior. Trewaves (1973) further elevated Thys van den Audenaerde's section 3 (comparison exclusively mouth-brooding genera) to full generic rank (Dunz and Schliewan 2013).

Poll 1986 adopted the definition of Trewaves, 1983 for Tilapiini, and added additional diagnostic characters. Stiassny (1991) provided the first clastic analysis of cichlids based mainly on morphological characters. Trewaves, (1983) identified two additional character states of the lower pharyngeal jaw, which she regarded as preliminary evidence for a monophyletic Tilapiine lineage, including *Danakilla*, *Iranocichla, Konia* (Trewavas, 1972), *Manyka* (Trewaves 1972), *Oreochromis, Pungu* (Trewavas, 1972), *Sarotherodon, Stomatepia* (Trewavas, 1962), *Tristramella* and *Tilapia.* The identification and classification of cichlids has continued until today and the ichthyology

of Southern African species is at an advanced stage, due to the pioneers who played a crucial role in obtaining it.

1.1. CICHLIDS IN SOUTHERN AFRICA

Paul Skelton, (1993) compiled a guide to freshwater fish in Southern Africa, highlighting some pioneers that assisted in fish identification. Skelton, (1993) noted that cichlids are the largest fish family in Africa with about 900 species described and many more still to be described. In Southern Africa, he noted 10 genera and 42 species. A simple key to cichlid genera is not always practical as many pertinent features are internal, skeletal, or behavioral. The genera in Southern Africa include *Tilapia, Oreochromis, Orthochromis, Thoracochromis, Pseudocrencilabrus, Hemichormis, Sargochromis, Pharyngochromis, Serranochromis* and *Chetia* (Skelton,1993). In South Africa, the genera *Tilapia* and *Oreochromis* are most prominent. The species from these two genera play a vital role in food security and as economic species (Munguti *et al.*, 2022).

1.1.1. Genus *Tilapia* (Smith,1840) (substrate spawners)

Tilapia (Smith 1840) is a genus of cichlid fishes endemic to freshwater habitats in South Africa. In the past, this was a vast genus including all species with the common name tilapia. These included nearly a hundred species of cichlid from the tribes, coelotilapine, coptodonine, heterotilapine, oreochromine, pelmatolapiine and tilapiine, but today most are placed in other genera (Dunz and Schliewen, 2013). In the past, *Oreochromis* (33 species Skelton, 2024) and *Sarotherodon* (13 species Froese and Pauly, 2018) were retained in the genus *Tilapia*, but these are currently treated as separate genera (Ford, 2019). The genus *Tilapia* was split, based on breeding behavior and feeding habits, into two subgenera: *Tilapia* (substrate spawners) and *Sarotherodon* 'brush toothed' (mouthbrooders). Later, the subgenus *Sarotherodon* was raised to a genus and further subdivided into two genera: *Oreochromis* and *Sarotherodon* based on whether parental females (*Oreochromis*), males (*Sarotherodon*) or both parental sexes (*Sarotherodon*) perform the mouthbrooding behaviour (Froese and Pauly, 2018). In 2013, a review of the group resulted in the removal of *Tilapia* species to the genera *Coelotilapia, Coptodon, Heterotilapia* and *Pelmatolapia.* (Dunz and Schliewen, 2013). The review was based

on both genetic (DNA sequencing) and morphological evidence, revealing that many of the species previously classified under *Tilapia* were more distantly related than previously thought. This helped clarify evolutionary relationships and resolve some confusion about species identification and classification. The removal of species from the Tilapia genus was necessary to create a more accurate and scientifically sound classification that reflects the evolutionary history of these fish. With these as separate genera, only three species remain *Tilapia* in Southern Africa (Skelton, 2024), out of four known species world wide (Ford, 2019 and Skelton, 2024). The species in Southern Africa include *T. guinasana, T. sparrmanii,* and *T. ruweti. Tilapia* baloni is native to the Congo River basin western Zambia. The genus is now restricted to substrate spawning species. *Tilapia* species are generally smaller than *Oreochromis* species and adults retain the distinctive "tilapia spot" (Skelton,1993). According to Skelton,(1993), *T. guinasana* is naturally endemic to Lake Guinas in Namibia. It has been introduced to Lake Otjikoto and several reservoirs in Namibia. *Tilapia sparrmanii* is distributed from the Orange River and KwaZulu-Natal south coast northwards to the upper reaches of southern Congo tributaries, Lake Malawi and the Zambezi system. It has been extensively translocated south of the Orange in the Cape (Skelton,1993). *Tilapi ruweti* is generally found in the Okavango Delta, Upper Zambezi, and southern tributaries of the Congo (Skelton,1993).

Characteristics used to differentiate species within the genus tilapia include dorsal spines, dorsal soft rays, body vertical bars, pelvic pigmentation as well as the shape of the caudal fin with the presence or absence of black blocks (Skelton.1993)

In the case of *T. guinasana* Skelton, 1993 noted that the critical identification factor was the dorsal spines, which were 12-14, with a varying color. For *Coptodon rendalli,* the dorsal spines were between 14-16, with 12-13 dorsal rays containing a body with 5-7 broad vertical bars and lightly pigmented pelvic.

In the case of *T. sparrmanii and T. ruweti,* they both have dorsal spines between 13-15, 9-12 dorsal soft rays, a body with 8-9 vertical bars, and heavily pigmented pelvic. However, *T. sparrmanii* has a truncated caudal fin, and it is not clearly marked with black blocks *T. ruweti* has a rounded caudal fin and clearly marked black blocks (Skelton,1993).

The genus Coptodon contains about 32 species of substrate-spawning austrotilapiine-like cichlids with bicuspid outer-row teeth on both jaws, a few broad vertical bars (which may be branched) on the body, and 16 rows of scales around the caudal peduncle. The genus is predominantly low African in distribution, occurring from Congo through to West Africa. There is one widespread in Southern Africa (Skelton, 2024). *Coptodon rendalli)* can be found in Cunene, Okavango, Zambezi system, east coastal rivers south to the Phongolo and coastal lakes to Lake Sibaya. *Coptodon rendalli* also occurs in estuaries in Mozambique and KwaZulu-Natal. *Coptodon rendalli* has been located in KwaZulu-Natal and the highveld region in the east Zaire basin (Lualaba) and Zambian Congo, Lakes Tanganyika, and Malawi (Skelton,1993).

1.2.2. Genus *Oreochromis* (Gunter, 1889) (arena-spawning maternal mouthbrooders)

Oreochromis is a large genus of Oreochromine cichlids, fishes endemic to Africa and the Middle East. Species in this genus and those in several other oreochromine and tilapiine genera, share the common name tilapia. Historically, most of these were in the genus *Tilapia* (Ford, 2019). *Oreochromis* contains more than 30 species worldwide, and several undescribed forms exist (Skelton, 1993). There are eight *Oreochromis* species in Southern Africa. The natives include: *O. mossambicus, O. placidus, O. shiranus, O. mortimer, O. andersonii,* and *O. marochir,* while the alien species include *O. niloticus* and *O. aureus,*. They are described as relatively large, deep-bodied, mouth brooding cichlids. They generally tolerate wide temperatures and salinity ranges (Welcomme, 1967; Farmer and Beamish 1969; Avella *et al*., 1993). They have fine teeth in several rows on the jaws, fine pharyngeal teeth, a high number of gill rakers, and long intestines (Skelton, 1993)

Oreochromis mossambicus is distributed from the East coastal rivers from the lower Zambezi system south to the Bushmans system, Eastern Cape, South Africa. It is naturally restricted to closed estuaries coastal reaches of rivers South of the Phongolo system, widely dispersed beyond this range to inland regions and southwest and west coastal rivers, including Namibia's lower Orange and rivers. It is introduced to tropical and warm temperate localities throughout the world. *Oreochromis mossambicus* occurs naturally in the Limpopo province (Skelton, 1993). *Oreochromis placidus*, is distributed along the coastal plain from lower Zambezi southwards to the Mkuze in

KwaZulu-Natal. Elsewhere, east coast rivers north to the Lukuledi in Tanzania (Skelton,1993). *Oreochromis shiranus* is occasionally found in Malawi's lower Shire, lower Zambezi system, Lake Malawi basin, upper Shire, and Lakes Chilwa and Chiuta (Skelton,1993). *Oreochromis mortimeri* is found in the Middle Zambezi system, including the Luangwa tributary (Skelton,1993). *Oreochromis niloticus,* is found along Cape Flats area, Western Cape, KwaZulu-Natal, and as a result of its global importance in warm-water aquaculture, is one of the most introduced species in the world (Ellender *et al*., 2014). It was widely spread in neighbouring Zimbabwe and Mozambique for aquaculture in the 1980s, and its subsequent escape from captivity and direct releases by anglers facilitated its invasion of the Inkomati and the Limpopo River system in South Africa (Weyl *et al*., 2020). It has invaded other eastern rivers in South Africa and Mozambique. The natural range includes the Nile basin, Rift Valley lakes, and certain West African rivers (Skelton,1993). *Oreochromis aureus* is found along the Cape Flats, Western Cape, and KwaZulu-Natal. for experimental purposes from Israel to the Jonkershoek Hatchery near Stellenbosch in 1959 and released into farm dams in the Lourens and Eerste River catchments in 1961 and 1962 to evaluate its potential to survive the Western Cape winter (Marr et al., 2018). The natural range includes Israel, lower Nile, Lake Chad, Niger, and Senegal rivers (Skelton,1993). *Oreochromis andersonii* is found in Cunene, Okavango, upper Zambezi and Kafue systems. It has occasionally been recorded from the middle *Zambezi* (Skelton,1993). *Oreochromis marochir,* is found along Cunene, Okavango, upper Zambezi and Kafue River, Lake Kariba and the Buzi River (possibly by translocation).

Characteristics used to differentiate amongst *Oreochromis* species include the number of anal spines, number of gill rakers, and caudal fin with or without prominent stripes; for breeding males, their dark throat and jaw can also aid in differentiation, while non-breeding adults with or without three spots on the body can be used as another distinction. Male genital papilla being simple or with a tassel is another character used to characterize *Oreochromis* species (Skelton,1993). The greatest challenge in the classification of *Oreochromis* species is hybridization among the sister species which gives rise to ambiguous features of the offspring (Shechonge *et al.*, 2018).

According to Skelton, (1993), the primary key for differentiation of *O. placidus* and *O. shiranus* is the presence of 4 anal spines. They are similar but have striking colors, with *O. placidus* adults olive-brown above and silvery below with sooty grey heads and

light spots and *O. shiranus* adults olive green with light cream or yellow ventral surfaces. In mature males, the margins of dorsal and caudal fins are bright orange (Skelton,1993). *Oreochromis mossambicus* usually has three anal spines, 17-20 gill rakers on the lower limb of the first arch, and breeding males have a white throat and enlarged jaw. *Oreochromis mortimeri* is similar to *O. mossambicus* with regards to anal spines and the number of gill rakers, but its breeding males have a dark throat and enlarged jaws (Skelton, 1993). *Oreochromis niloticus* has 20-27 on the lower limb of the first arch and prominent vertical stripes on the caudal fin. *Oreochromis aureus* is similar to *O. niloticus* in terms of the number of gill rakers, but the caudal fin does not have prominent strips (Skelton,1993). *Oreochromis andersonii* also does not have prominent stripes on the caudal fin as well as the juveniles and non-breeding adults with three spots on the body; breeding males are blue and silver with red-tinged fins, black throat, and its male genital papilla is simple (Skelton,1993). *Oreochromis macrochir* also does not have stripes on the caudal fin, and the juveniles and non-breeding adults without three spots on the body, head greenish with dark spots and vermiculation, and its male genital papilla has tassels (Skelton,1993).

1.2. IMPACTS OF INTRODUCED FISH SPECIES

Fish are moved for various reasons, including recreational angling, commercial harvesting, aquaculture, and occasionally for the pet trade (Marr *et al.*, 2018). The greatest driver of new species introductions include, economic benefits and biodiversity boosts. *Oreochromis niloticus* and *O. aureus* have both been introduced to South Africa for aquacultural purposes. *O. aureus* for its ability to withstand colder temperatures and *O. niloticus* for its fast growth (Marr, 2018). In Aquaculture hybrids of *O. niloticus* and *O. mossambicus* are known to produce bigger offspring than their parents with more desirable traits, such as a bigger fillet and higher food conversion ratio, which allows for maximum profits for farmers. They have also been introduced in some areas to assist in insect control as they eat mosquitos and other insects, especially in areas where the native populations are absent or declining. This helps in restoring balance in the ecosystem.

There are also negative aspects, of introduced species. Introduced species are recognized as a significant driver of biodiversity loss in aquatic environments (Clavero & Garcı´a-Berthou, 2005; Shechonge *et al.*, 2018), and many aquatic introduced

species have been associated with substantial economic and ecological impacts (Lowe *et al*., 2000; Pimentel *et al*., 2005). Freshwater environments are considered especially vulnerable to invasion (Sala *et al*., 2000; Cox & Lima, 2006). A key concern is the inability to reverse invasions and the subsequent impacts, and only a few species have ever been successfully eradicated from an aquatic environment after establishment (Hill & Sowards, 2015). The spread of these exotic species is predicted to continue as natural biogeographic barriers are overcome, either accidentally through unintended transport or deliberate introductions (Hulme, 2009). In South Africa, *O. mossambicus* was placed on the IUCN Red list of threatened species list due to the looming threat of its hybridization with the exotic *O. niloticus* species. The introduction of non-native fish species can reduce biodiversity and alter the local community in freshwater systems, causing one of the most harmful impacts on freshwater species in most regions of the world (Zengeya *et al*., 2013).

According to Ehrenfeld, (2010), the presence of such exotic species can alter habitats, affecting food chains with the introduction of new predators, affecting the ability of native species to compete for food and other limited resources, including habitat acquisition due to the lack of local controls (disease and predators) to keep populations of exotic species at a minimum. They can also introduce parasites and pathogens, that cause disease or parasitic infestations. Ekerette *et al*., 2018, noted the possible predation on eggs and fingerlings. Another problem with exotic species introduction is their tendency to hybridize with sister species, resulting in the decline and possible extinction of native species and reducing biodiversity (Shechonge *et al.,* 2018).

1.3. HYBRIDIZATION

Interspecific hybrid fish have been produced for aquaculture and stocking programs to increase growth rate, transfer desirable traits between species, such as faster growth, disease resistance, tolerance to a broader environmental condition, production of mono sex population to reduce early sexual maturity, and unwanted reproduction (Bartley *et al*., 2001). *Oreochromis* species are known for interspecific hybridization (Scribner *et al*., 2001).

In the natural environment, introduced *O. niloticus* has been documented as hybridizing with several species including *O. mossambicus* (D'Amato *et al*., 2007), *O. aureus* (Rognon & Guymard, 2003; Bakhoum *et al*., 2009), *O. andersonii (*Deines *et al.,* 2014), *O. macrochir* (Deines *et al*., 2014), and *O. esculentus* and *O. leucostictus* (Nyingi & Agne'se 2007; Angienda *et al*., 2011; Ndiwa *et al*., 2014). However, after decades of introduction and domestication of *O. niloticus*, they have highly adapted to a wide range of geographical locations and have shown phenotypic variations. This may be attributed to different environments (Turana *et al.,* 2006), hybridization between sister species, and introgression (Nyingi & Agne'se 2007, Chuhila, 2015).

A study by Scriber *et al.,* (2001) found that one in twenty suspected wild tilapia population individuals was a first-generation hybrid, suggesting interspecific natural hybridization. These results found in Scriber *et al.,* (2001) indicated the importance of combining morphometric and genetic data in identifying species. Costa-Pierce (2003) concluded that genetic markers were more effective in establishing the genetic composition of feral hybrids and in establishing the composition of mixed species in water bodies. It is, therefore, essential to retrieve phylogenetic analyses of species to regulate and control the species in a habitat, especially exotic species, using morphometric and genetic analysis (Gu *et al.,* 2016). Distinguishing between pure tilapia species and hybrids is vital for both farmed and wild populations (Syaifudin *et al*., 2019). This helps with the establishment of proper aquacultural management plans.

1.4. STUDY RATIONALE

Cichlid identification is a challenge. Identifying fish species is essential in developing optimal strategies for efficiently managing aquaculture species (Nyaku, 2023). Traditional morphometric analysis is one way of achieving this. It involves measuring a set of morphological characters or traits of the fish to determine its size, shape, and overall physical characteristics. The morphometric data collected can be used to identify and classify cichlid species (Ndiwa *et al*., 2016). Other means of identification include genetic analysis, which was proven more reliable, especially in the characterization of cichlids (Shechonge *et al.*, 2018 and Dunz and Schliewan, 2013). Cichlidae are native to Africa and the Southwestern Middle East (Canonico *et al.,* 2005, Bhassu *et al.,* 2004 and Trewavas, 1983) and form part of a group of economically important fish (Shechonge *et al.,* 2018). Tilapiines, are a major branch

of African cichlid fishes, highly prized in aquaculture and commercial and subsistence fisheries (Skelton, 1993). Syaifudin *et al*., (2014) noted that the identification of numerous native tilapia species, both wild and farmed, has become more difficult with the extensive introduction of exotic tilapia species. This is because they may hybridize, forming ambiguous features that make it harder to conclude which species the fish may be. Hybridization, either between species or between distinct populations of the same species, is a common natural phenomenon of evolutionary importance (Barton 2001; Seehausen 2004); however, the unnatural mixing of historically isolated taxa due to human-related activities has increased in recent years (Olden *et al*., 2004 and Zengeya *et al*., 2015). Such activities include habitat modifications or human-mediated introductions of non-native taxa (Rhymer and Simberloff 1996 and Copp *et al*., 2005). The unnatural mixing of historically isolated taxa has resulted in considerable changes to the global distribution of many taxa, often at the expense of regional and endemic native populations or species (Olden *et al.,* 2004, Copp *et al.*, 2005 and Gozlan *et al.,* 2010).

1.5. AIM

The study aims to improve the overall aquaculture practices in the Limpopo Province by identifying and documenting tilapia species found in four localities using morphometrical and molecular analyses, probing possible hybridization among sister species within the four localities and establishing genetic diversity indices of the native *O. mossambicus* in the localities. In this study *O. mossambicus* and its possible hybrids will be investigated, looking specifically at the presence and effects of exotic *O. niloticus.* Tilapia species will be collected and identified using both morphometric and genetic identification. *Oreochromis mossambicus* species will then be isolated and assessed to see if there are any morphometrical and genetic variations among the *O. mossambicus* species located in the four different localities and to determine if there are any changes in the historical demography among the *O. mossambicus* species within those localities.

1.6. THE OBJECTIVES INCLUDE:

i. To use traditional morphometrical features to identify tilapia species collected from the four localities.
ii. To determine genetic variation that might support the projected morphometrical variations between four localities using mitochondrial DNA and nuclear RNA markers.
iii. To investigate evidence of hybridization between *Oreochromis mossambicus* and *Oreochromis niloticus* in the four localities.
iv. To assess historical demography within *Oreochromis mossambicus* populations amongst the four localities.

1.7. HYPOTHESES

i. If traditional morphometric features are utilized, they can identify tilapia species collected from the four localities.
ii. If genetic variation is observed, they can support the projected morphometrical variations between the four localities.
iii. If there is mating/ spawning between *Oreochromis mossambicus* and *Oreochromis niloticus* species, then hybrids will be present.
iv. If changes are observed in the historical demography among the *Oreochromis mossambicus* populations, it could support some of the differences observed.

1.8. THESIS OUTLINE

CHAPTER 1: GENERAL INTRODUCTION

CHAPTER 2: LITERATURE REVIEW

CHAPTER 3: MORPHOMETRIC AND MERISTIC CHARACTER ANALYSIS OF TILAPIA SPECIES IN FOUR LOCALITIES IN THE LIMPOPO PROVINCE

CHAPTER 4: THE IDENTIFICATION OF TILAPIA SPECIES AND THEIR POSSIBLE HYBRIDS IN FOUR LOCALITIES, LIMPOPO PROVINCE.

CHAPTER 5: POPULATION GENETIC DIVERSITY OF *OREOCHROMIS MOSSAMBICUS* (PETERS, 1852) IN LIMPOPO PROVINCE

GENERAL DISCUSSION

REFERENCES

CHAPTER 2 : LITERATURE REVIEW

2.1. MORPHOMETRIC ANALYSES

Cichlids are diverse, and their morphological, behavioural, and ecological diversity has captivated biologists (Kornfield and Smith, 2000, Dunz and Schliewan, 2013). Over the last decades, cichlids have become a prime model system in evolutionary biology, especially in speciation research (Ford *et al.*, 2019). Aquaculture research is vastly invested in the "tilapia", i.e., members of the so-called tilapiine cichlid assemblage, because tilapiines gave rise to small species radiations (Schliewen and Klee, 2004), and they are of economic importance (Dunz and Schliewan, 2013). Studies have been conducted to identify tilapia and classify them, accordingly, noting their strengths and advantages in growing them to maximize production. These presented an opportunity to use morphometric characteristics as a form of segregation and differentiation. Within the South African context, there is little work done using morphometric and meristic characteristics to identify *O. mossambicus* and its possible hybrids. Technological advances have allowed for newer and more thorough genetic means of differentiation, leading to reorganizing and reclassifying some species (Ford *et al.*, 2019). Due to principles such as hybridization and introgression, these genetic techniques have proven more robust and reliable than morphometric identification (Syaifudin *et al*., 2019). In this study, morphometric features of tilapia species will be used to identify them and isolate O. *mossambicus* so that they are analyzed further for possible morphometric variations among the different populations. The same tilapia species will be subjected to genetic analysis to confirm the morphometric feature analysis among the tilapia species and to note the genetic diversity of the *O. mossambicus* species in the four localities where they are found.

The studies below indicated possible morphometric and meristic characteristics, that showed the most differentiation amongst single species being either *O. mossambicus* or *O*. *niloticus.* Most of the morphometric studies done are on *O.niloticus.* Only a few studies were undertaken on *O. mossambicus.* Herath *et al.*, (2014), looked at morphological variations in three *O. mossambicus* populations, using 12 morphometric characteristics. These included total length, standard length, body depth, preorbital length (POL), orbital diameter (OD), base length of anal fin (BLAF), caudal peduncle length (CPL), length of anterior end of dorsal fin to posterior end of

pelvic fin (ADPP), length of anterior end of dorsal fin to posterior end of anal fin (ADPA), pre anal length (PAL), head depth (HD) and caudal fin length (CFL). AMOVA, showed that characteristics regarding caudal fin length, pre-anal length, and distance from the anterior end of the dorsal fin to the posterior end of the pelvic fin were significantly different among locations ($p<0.05$). The pre-orbital length was significantly lower in Mawella Lagoon compared to the other two locations. The base length of the anal fin was significantly highest in Rekawa lagoon population, while higher head depth was recorded in the Mawella lagoon fish population. These differences in morphometric characters allowed the rejection of the null hypothesis that there was no morphological variation between the three *O. mossambicus* populations. They successfully proved differentiation amongst the *O. mossambicus* populations in the three locations. This study's limitation included omitting the use of meristic characterists which will be used in the current study. The studies that followed, looked at *O. niloticus* populations.

Hassanien *et al.*, 2011 looked at the multivariate analysis of morphometric parameters in wild and cultured *Oreochromis niloticus* used eight morphometric measurements, which included body length, standard length, head length, tail length, trunk length, body depth, body thickness, and head thickness. AMOVA, PCA, and DFA showed a low variability among populations. The study successfully showed morphological differences between subpopulations derived from a single gene pool isolated in separate sites for several decades, although bred in relatively similar environments. The study highlighted the efficiency of AMOVA, PCA and DFA, in the use of morphometric features to differentiate amongst tilapia populations. Those multivariate analyses were adopted in the current study. Unlike Jawad *et al*., 2020, Hassianien *et al*., 2011 did not use meristic characteristics.

Jawad *et al.*, (2020), examined body shape and meristic character variations in wild and cultured populations of *Oreochromis niloticus*. They used ten morphometric characteristics and five meristic characteristics. The morphometric characteristics included total length (TL), standard length (SL), head length (HL), eye diameter (ED), body depth (BD), pre-dorsal fin length (PreDFL),post dorsal fin length (PstDFL), pre anal fin length (PreAFL) and post anal fin length (PstAFL). All ten variables were significantly different ($P<0.001$). However, there were no significant difference among

meristic variables. This current study aims to find out if there are any morphometric and meristic variations in the *O. mossambicus* populations in the four localities.

Asmamaw and Tessema, (2021), looked at the morphometric variations in *Oreochromis niloticus* collected from three rift valley lakes in Ethiopia and used 12 morphometric characters that included total length (TL), standard length (SL), body depth (BD), preorbital length (POL), pre- pectoral length (PPCL), pre pelvic length (PPLL), pre dorsal length (PDL), pre anal length (PAL), Caudal height (CH), head length (HL), eye diameter (ED) and weight (W). Weight, total length, and preanal length were high in all populations. All the coefficients of variation of different morphometric characters were significantly different (P< 0, 05) between populations based on discriminant analysis. Function one showed that PAL, BD, ED, and W were the most influential variables. Discriminate analysis showed that morphometric differentiation between the three samples was mainly due to differences in TL, PDL, PPLL and ED, which were significantly higher in the Lake Langano population and PAL, BD, and W, which were higher in Lake Koka and Ziway populations. These morphometric variations may reflect differential habitat use.

Mahmoud and Hassan, 2019, undertook a similar study, however they used more morphometric characteristics. They looked at the use of discriminant analysis as a tool for the characterization of *Oreochromis niloticus* and used 19 characteristics that included, standard length (SL), body weight (BW), head length (HL), eye diameter (ED), head width (HW), snout length (SnL), pre maxillary peduncle length (PPL), caudal peduncle length (CPL) anal fin base length (AFB) laryngeal depth (LAD), cheek depth (CD), Caudal peduncle depth (CPD), body depth (BD), interorbital width (IOW), pre pectoral distance (PRV), preanal distance (PRA), pre dorsal distance (PRD), pre pelvic distance (PRP) and dorsal fin base (DFB). DA successfully separated *O.niloticus* from Kosti lagoon based on LAD, HW, CPD, PPL, and PRD. This study utilized 19 morphometric characteristics, and the most useful ones were incorporated into the current study. The limitations of both studies were the omission of the use of meristic characteristics, to classify the *O. niloticus* species.

Other studies were conducted to note differences amongst two or more tilapia species. These studies were able to identify and classify tilapia species using morphometric and meristic characteristics.

Azua and Akogwu, 2017 looked at the variation in the morphometry measurements of two tilapia fish species from the lower Benue River. They examined the morphometry of *Tilapia zille* and *O. niloticus* using seven morphometrical characteristics. These included body weight, standard length, total length, dorsal fin length, caudal fin length, head length and body width. The correlation analysis between the morphometry of *T. zille* was significant between head length and total length only. However, correlation analysis between morphometry of *O. niloticus* revealed a significant positive correlation between standard length and body weight, total length and body weight, standard length and total length, dorsal fin length and body width and dorsal fin length only. Azua and Akogwu (2017) were thus successful in concluding that the two species are different, with different morphological features used in their identification. The limitation in this study was that they did not use meristic features and they used only 8 morphometric characteristics to achieve their results. This showed that even with fewer morphometric features, species differentiation using morphometry was possible. The current study will however employ 18 morphometric characters and ultimately note the characteristics that are most useful for differentiation amongst the tilapia species found.

Hassan and Mahoud, (2021) looked at the variability of morphometry of three species *O. niloticus, Sarothedon galilaeus,* and *Coptodon zille* from the Nile and its tributaries in Sudan using 19 characteristics, including total length, body depth (BD), head length (HL), head width (HW), interorbital width (IOW), snout length (SnL), lower jaw length (LJL), pre maxillary pedicle length (PPL), cheek depth (CHD), eye diameter (ED), lachrymal depth (LAD), anal fin base (AFB), pre anal distance (PRA), pre pectoral distance (PRP), pre pelvic distance (PRV), caudal peduncle length (CPL), caudal peduncle depth (CPD). Their results showed that CDF and SCDF analysis showed that in factor 1, PRD, SL, HW, SnL, PP, HL, LAD, AFB, IOW, W, PRA, PRV, and BD were the most influential morphometric traits in differentiating amongst the tilapia species. They were thus successful in classifying and separating the fish species. This study used more morphometric characteristics but did not use any meristic features to

note if there were also differences in them. The current study will answer if there are differences in meristic features among those tilapia species.

Samaradivakara *et al.,* 2012, used more morphometric and meristic characteristics. They looked at morphological variation of four tilapia populations in selected reservoirs in Sri Lanka using 20 morphometric variables and 14 meristic variables. The morphometric characteristics included: body weight (BW), total length (TL), standard length (SL), body depth (BD), head length (HL), head depth(HD), snout length (SnL), base length of dorsal fin (BDF), posterior end of the dorsal fin to the dorsal origin of the caudal fin (PDDC), the dorsal origin of the caudal fin to ventral origin of the caudal fin (DCVC), the ventral origin of the caudal fin to insertion of the anal fin (VCIA), length of the anal fin (AF), base length of the anal fin (BA), origin of the anal fin insertion of the pelvic fin (OAIP), length of pelvic fin (LP), posterior end of the dorsal fin to insertion of the anal fin (PDIA), posterior end of the dorsal fin to origin of the anal fin (PDOA), origin of the dorsal fin to insertion of the pelvic fin (ODIP), caudal peduncle length (CL) and caudal peduncle depth (CD). The canonical discriminant function coefficients obtained for morphometric data showed that for factor 1, SL, BD, PDDC LA, and ODIP were the most influential for differentiation. They found that each of the four populations was highly isolated. Plots of the morphometric measurements' canonical discriminant functions 1 and 2 showed a complete separation between wild populations and the broodstock. Individuals from the four locations were well separated and differentiated along the first function.

This study will use 18 traditional morphometric characteristics to identify and classify tilapia species. These were shown to be the most effective at differentiation of tilapia species. They include; PAL-pre-anal length, BD-body depth, LCP-length of caudal peduncle, PPL-pre pectoral length, CFL- caudal fin length, PFL-pectoral fin length, SNL-snout length, ED-eye diameter, DCP- depth of caudal peduncle, HL- head length, SL-standard length, W- weight, LDFB- length of dorsal fin base, UJL- upper jaw length, LJL- lower jaw length, LAFB- length of anal fin base, TL- total length, PPECL-pre-pectoral length, PPELL- pre-pelvic length. Meristic features will be used for differentiation as they have proved vital in differentiating amongst different tilapia species (Skelton, 1993). The current gap is the lack of information on the tilapia species in the four localities in Limpopo, and it is not known which characteristics will

be best to use for the differentiation amongst the tilapia species within the four localities and between the *O. mossambicus* species within the four localities. This will be done by discriminant analysis (DA). The condition factor of the fish will be analyzed to indicate whether the variations will be due to environmental conditions or other factors such as genetics.

Variables to note and consider then using morphometric analysis include traditional morphometrics, which involves measuring various body parts, considering the lengths (total length, standard length, head length, fin length, etc), depths (body depth, caudal peduncle depth, etc), widths (body width, head width) and proportions (i.e. ratios calculated from the previous measurements, e.g. head length/ total length) and meristic counts, which involve counting specific features such as number of fin rays, number of scales along the lateral line, number of gill rakers, etc. The main advantages are their relative simplicity to analyse and cost effectiveness, where data is collected with basic tools and analysed with readily available software. They were found to be useful for field identification and an integrative approach of combining other methods such as genetics or ecology provided a more comprehensive understanding of species boundaries and variations. The disadvantages included environmental influences, such as temperature and food availability that can lead to variations within species. The other issue was subjectivity as the placement of measuring tools may vary slightly between observers or even by one observer especially if sample sizes are numerous. The other problem with regards to tilapia identification included the limited resolution were morphometrics was sometimes not able to distinguish between closely related species or populations with subtle differences. Data analysis could be complex requiring statistical expertise and the appropriate software. These are some of the things to look out for when morphometrics are analysed.

2.2. MOLECULAR PHYLOGENETICS

Molecular phylogenetics is a relatively new research area usually associated with organismal, evolutionary, and taxonomic studies. Genetic studies on tilapia species focused on reconstructing phylogenetic relationships between species (Feresu-Shonhiwa & Howard, 1998) while some focused-on tilapia population genetics (De Silva, 2004, Yaqub *et al*., 2019, Mojekwu *et al*., 2020, Nascimento *et al*., 2023).

Due to its quick variation, rapid evolution, and absence of recombination, mtDNA has been employed extensively in genetic studies, taxon categorization, phylogenetic evolution research, and population studies to date (Kilawati *et al.,* 2023). Hassanien *et al.,* (2011) noted that the management of aquaculture genetics, including that of tilapia, should include several measures, such as keeping suitable records of the genetic resources and the various ecosystems in which they are found, categorizing and ordering these resources to assess genetic variation and conservation potential, determining direct and indirect economic potential of the resources, and utilizing them in sustainable genetic improvement schemes.

Tilapia species characterization should be done using molecular approaches, which will also help to establish phylogenetic relationships between different species (Dunz and Schliewan 2013, Shechonge *et al.,* 2018, Ford *et al*., 2019). Studies involving Cytochrome Oxidase type I (COI) Mt DNA, 16S ribosomal DNA (rDNA), 5S (rDNA), 12S (rDNA), 18S (rDNA), and ribulose-biphosphate carboxylase are suitable for identification (Hebert *et al*., 2003 and Bhattacharya *et al.,* 2016). While mitochondrial DNA (mtDNA) analysis has been used to distinguish tilapia species, it is limited in analyzing hybridization and introgression (Syaifudin *et al*., 2019). This is because mtDNA only shows the maternal strain of the DNA sequence, and a hybrid consists of two different sister species. Hence, in this study, ribosomal DNA primer (5S) will also be compared with (FishF) primer to note possible hybrids.

Anbarasi *et al*., (2015) looked at bar coding profiling and intra-species variation within the barcode region of two tilapia species *Oreochromis mossambicus* and *Oreochromis niloticus*. They tested the efficiency of the COI gene in identifying the species and demonstrated its intraspecies variations within the barcode region. Anbarasi *et al.*, (2015) successfully found significant alignments with maximum identity ranges of 99% to 100%. Anbarasi *et al.*, (2015) concluded that even though their present results confirm that the COI gene could be potentially used for barcoding at species-level

identification, further research is needed in describing the divergence of sequences in a broader sense. A study by Mohammed-Geba *et al*., (2017), looking at the use of COI gene to identify various haplotypes, found a unique haplotype of Nile Tilapia, *Oreochromis niloticus* thriving in Egyptian freshwater and brackish water lakes. Mohammed-Geba *et al*., (2017) successfully identified phylogenetic interrelationships among these haplotypes indicating the main lineages in the study and around the world. A newly identified haplotype with natural tolerance to different salinity was identified from both lakes. This study will highlight if there are any new haplotypes or genetic variations in the *O. mossambicus* species within the four studied locations. Both studies highlighted the efficiency of COI gene, which was ultimately used in this study.

Yaqub *et al.*, (2019), collected samples of tilapia species from different aquatic systems of Pakistan, and Cytochrome Oxidase subunit I (COI) of mitochondrial genes were investigated to clarify molecular characterization and find out phylogenetic relationships of *O. mossambicus.* The results from the sequence analysis of the genes showed no significant divergence among all the specimens studied. The species of *O. mossambicus* was confirmed and separated from other species of the family Cichlidae with the help of a molecular approach using the mtCOI sequence. When comparing the *O. mossambicus* species from Pakistan with samples from Bold and Genbank, the highest sequence divergence based on COI of studied sequences was 0.082%, which was against the tilapia sequence obtained from Egypt, and the lowest divergence was 0.003% against Indian Mozambique tilapia. Thus, they concluded that the mtDNA COI gene is an effective barcoding marker for the precise identification and phylogeny of exotic Mozambique tilapia. Dailami *et al*., (2021) looked at the DNA barcoding of tilapia fish from Merauke, Papua and Malang, to identify tilapia fish existing in Indonesia based on nucleotide composition, Polymorphic sites, haplotype grouping, nucleotide blast, and phylogenetic tree analysis was done using the COI gene (Cytochrome c oxidase I). Dailami *et al.*, (2021) successfully identified the tilapia species. In this study ribosomal DNA was also utilized for the identification of tilapia species.

The ribosomal DNA markers used to date have limitations, the most important being the small number of markers available that distinguish between tilapia species (e.g., species-specific alleles have been found using allozymes, but only for a few loci) (Mojekwu *et al.*, 2020). A combination of two markers proved more effective in

distinguishing hybrids from pure breeds if there were no sequences for comparison from Genbank or BOLD. This report will use two markers, mtDNA COI and nuclear 5s ribosomal DNA, for species identification and differentiation, as well as identifying possible hybrids amongst the fish species. The mutation rate established by the mtDNA is higher than that of nuclear genes (Lawless *et al.,* 2020). The mtDNA's high mutation rate and negligible genetic recombination cause regional variation within and between species, making it the most widely used marker for estimating genetic diversity among species (Galtier *et al.,* 2009).

The results from Syaifudin *et al*., (2019) 's findings, when they utilized the COI marker to differentiate amongst ten tilapa species using various markers, indicated that the gene tree separated the *Tilapia* genus from the other two genera, however *Sarotherodon* and *Oreochromis* were not clearly separated. The largest group consisted of most of the *Oreochromis species* i.e. *O. niloticus, O. mossambicus, O. karongae, O. u. hornorum, O. andersonii and O. macrochir* (the last two were not separated from each other within this group). However*, O. aureus* and some *O. niloticus* were in a group with *S. galilaeus*, while *S*. *melanotheron* was in a separate group from *S. galilaeus*. West African *O. niloticus* exhibited COI haplotypes typical of *O. aureus*, although nuclear markers clearly indicated the differences between these two species.

Syaifudin *et al*., (2019) also found that unlike the ddRADseq-based analyses, the COI gene tree did not clearly separate the *Sarotherodon* and *Oreochromis* genera (in contrast to the ddRADseq-based analyses, in which all three genera were separated). In addition, the COI sequence data did not separate *O. andersonii* and *O. macrochir* or West *African O. niloticus* from *O. aureus.* Although nuclear markers (allozymes) showed distinct separation between *O. aureus* and *O. niloticus* in West African populations, identical mtDNA sequences were detected in both species. Syaifudin *et al*.'s (2019) study indicated an apparent differentiation between these two species at the nuclear DNA level. Considering that the COI gene tree is based on a single maternally inherited locus, it is unsurprising that it did not have the depth of the trees constructed from multiple nuclear DNA markers. Syaifudin *et al*, (2019) highlight the need to utilize more than one marker; hence 5S ribosomal DNA was also used to identify species and possible hybrids in conjunction with mtDNA COI.

Hybridization poses an identification problem due to ambiguous morphometric features (Bradbeer *et al*., 2019). Hybridization is of particular concern in cases where one of the species is a threatened species. For example, in North America, Kodric-Brown and Rosenfield, (2004) noted that vulnerable endemic Pecos pupfish *Cyprinodon pecosensis* (Echelle & Echelle 1978) have hybridized with the invasive sheepshead minnow *Cyprinodon variegatus* (Lacepe'de 1803) with potentially no pure populations remaining due to the apparent vigour of hybrid individuals. Hybridization has been invoked as a potential driver of biodiversity loss in the tilapiine cichlid fish of the genus *Oreochromis* (Blackwell *et al*., 2021, Bradeer *et al.,* 2019 and Deines *et al.,* 2014). The studies agreed that hybridization between sister species leads to biodiversity loss.

Bbole *et al*., (2014) found that putative hybrids between *Oreochromis niloticus* and *Oreochromis andersonii* cannot be distinguished from parental species based on morphometric or meristic traits. They further noted differences in the morphological characteristics of tilapia species, which are consistent with their classification as different tilapia species. According to Ehrenfeld, (2010), the presence of such exotic species can alter habitat, affecting food chains with the introduction of new predators, affecting the ability of native species to compete for food and other limited resources, including habitat acquisition due to the lack of local controls (disease and predators) to keep populations of exotic species in check. They can also introduce "pest species" that cause disease or parasitic infestations. Ekerette *et al*., (2018), noted the possible predation on eggs and fingerlings. Another problem with introducing exotic species is their tendency to hybridize with sister species, resulting in the decline and possible extinction of native species and reducing biodiversity. Nowadays, some of these hybrids are commonly produced in aquaculture farms, but mainly to benefit from both the good growth of *O. niloticus* and the best tolerance at low temperatures of *O. aureus*. While hybridization is helpful for aquaculture, it can have a negative impact on fish populations.

Shechonge *et al.*, (2018) investigated the genetic and morphological consequences of stocking invasive tilapia species in two water bodies in central Tanzania. Schechonge *et al.*, (2018) screened individuals at 16 microsatellite loci and quantified morphology using geometric morphometrics and linear measurements. In both the

Mindu and Kidatu systems, Schechonge *et al.*, (2018) identified evidence of hybridization between indigenous Wami tilapia (*Oreochromis urolepis*) and the introduced Nile tilapia (*Oreochromis niloticus*) or blue-spotted tilapia (*Oreochromis leucostictus*). At both sites, purebred individuals could largely be separated using geometric morphometric variables, with hybrids occupying a broad morphospace among the parental species. Schechonge *et al.*'s (2018) data demonstrated that the gene pools and phenotypic identity of the indigenous *O. urolepis* have been severely impacted by the stocking of the invasive species. This study will note possible hybridization between sister species, and the possible impacts and repercussions of hybridization in the localities where sister species were found to hybridize will be examined.

To identify genetically if a tilapia is a hybrid, both COI and 5S markers are used as the 5S marker codes for the paternal strain of the fish genes, where both the COI and 5S gene will indicate if it is a pure breed or with varying parents from sister species. Alves-Costa *et al.*, (2006) 's paper aimed to discuss the nucleotide sequence and genome organization of 5S rDNA in *O. niloticus, T. rendalli,* and in the hybrid *O*. *urolepis hornorum x O. mossambicus*. The obtained results on 5S rDNA in the tilapiine and the reviewed data on 5S rDNA strongly supported that a dual 5S rDNA system seems to be of general occurrence in teleost fish genome. Their results further highlighted that PCR amplification of the genomic DNA of *Oreochromis niloticus, Tilapia rendalli* and the hybrid *O. urolepis hornorum* x *O. mossambicus*, with the set of primers 5SA and 5SB, generated just one band of approximately 500 bp. Sequences ranged from 475 to 505 bp, including a 120 bp coding region (5S rRNA gene) and a variable NTS (non-transcribed spacer). Alves-Costa *et al.*, (2006) noted the occurrence of two distinct 5S rDNA sequence types or subfamilies. Alves-Costa *et al.,* (2006) found that the 5S RNA genes are highly conserved, even among non-related taxa, both concerning length and nucleotide sequence, whereas the NTS evolves more rapidly. Alves-Costa *et al.*, (2006) concluded that "the 5S marker was a suitable genetic marker candidate to be applied in the assessment of identification of fish species, strains and hybrids of economic or ecological importance". It was therefore chosen and utilized in this study due to its reported efficiency. The identification of these native and exotic species and their possible hybrids in the four localities in Limpopo Province will indicate the current genetic diversity in those localities.

2.3. RESEARCH PROBLEM

It is important to know exactly which species are in our waters so that plans and mitigating factors can prevent such scenarios in our Limpopo water systems. This study will use mtDNA and ribosomal DNA to identify possible hybrids, as suggested by previous authors. The study will note the genetic diversity of *Oreochromis mossambicus* in four localities and if hybridization is taking place, which could contribute to the reduction in genetic diversity and possible extinction of the species if measures are not taken. Therefore, the objective of this study is to use morphometric and molecular approaches to characterize and identify tilapia species occurring in four localities in Limpopo Province and further investigate the genetic structure and diversity of *Oreochromis mossambicus* in these localities using the mitochondrial COI marker and nuclear 5s rRNA markers.

CHAPTER 3: MORPHOMETRIC AND MERISTIC CHARACTER ANALYSIS OF TILAPIA SPECIES IN FOUR LOCALITIES IN THE LIMPOPO PROVINCE.

3.1 INTRODUCTION

3.1.1 TRADITIONAL MORPHOMETRIC AND MERISTIC ANALYSIS

Morphometric and meristic analysis provides a non-expensive and statistically robust means of species identification (Oponda *et al*., 2017). It describes morphology based on length measurements, ratios or angles (Webster and Sheets, 2010). Morphometric methods remain the simplest and most direct way among methods of species identification (Chuhila, 2015).

Syaifudin *et al*., (2019) noted that the identification of many native tilapia species, both wild and farmed, has become more difficult due to the extensive introduction of exotic tilapia species. This could be due to ambiguous morphometric features caused by hybridization. Besides hybridization, Georgakopoulou *et al.,* (2007) stated that environmental factors affect body shape. Temperature is one of the most critical environmental factors influencing body shape (Eagderi *et al*., 2015). For example, cold water can lead to the creation of a slim body, which could be a response to low-temperature effects on the physiological rates of the individuals due to changes in muscles and bone growth patterns (Campinho *et al*., 2004), and changes in kinematic viscosity of the water in which fish move in (Sfakianakis *et al*., 2011). Therefore, environmental factors that fish experience significantly influence their phenotype (Zamani-Faradonbe, 2021).

3.1.2. TILAPIA SPECIES IN SOUTH AFRICA AND THEIR DISTINGUISHING FEATURES.

In Southern Africa morphometric features have been used to identify tilapias. The genus *Tilapia, Oreochromis* and *Coptodon* which are present in South Africa with their prominent distinguishing features.

Tilapia genus once included all the tilapiine species in Southern Africa, but now it is restricted to the substrate spawners. A firm pair-bond relationship is observed between spawning fishes and both parents guard the eggs and possess a prominent “tilapia spot” (Skelton,1993). The species present in South Africa include:

T. sparrmanii – Vlei Kurper or banded tilapia – is the most widespread species in South Africa. It has a deep body and a blunt snout, but it is unsuitable for aquaculture as it does not grow longer than 12cm. It is the most cold-tolerant of indigenous tilapia and can survive at 6°C for short periods. It will live through the Highveld winter and has been widely trans-located through inter-basin river transfer scheme. Juveniles have characteristic light “bubbles” behind the tilapia mark on the soft dorsal fin. It attains 230mm standard length (Skelton, 1993). It is similar to *O. mossambicus* but has a distinct tilapia spot also in adults. Its color varies, but it is predominantly deep olive green with 8-9 dark vertical bars on the body, 2 bars between the eyes, with a dark spot on the gill cover surrounded by iridescent green or blue scales along the lower jaw (Skelton,1993)

Coptodon rendalli: was formerly in the genus *Tilapia* and it was changed into the *Copotodon* genus. The species was described in 1897 by Boulenger G.A. as *Chromis rendalli* and later classified as a member of the genus *Tilapia* subgenus *Coptodon*. The subgenus *Coptodon* was elevated to a genus in early 2013 (Dunz and Schliewen, 2013). Its body is typically deep, its head profile convex, and its mouth protruding with prominent bicuspid teeth. Mature specimens are olive green to brown, often with scattered blue scales, 5-7 dark olive broad vertical bars on the body and a clear tilapia spot and a bright red throat and chest, the extremities of the soft dorsal, anal and lower half of the caudal fin vary from yellow to red. Juveniles are recognized by the rounded head, beak-like mouth, the few broad body bands, pelvic without pigment, and barred pattern of soft dorsal fin. The red breast tilapia, is restricted to water over 13°C in winter. Females have red chests and up to nine vertical bands. Males have white chests. Redbreast tilapia often have a distinct two-tone caudal fin colouration, red at the base and pale above, with no spotting. It attains 400mm standard length (Skelton,1993).

Oreochromis (mouthbrooders) are relatively large, deep-bodied, mouthbrooding cichlids that are economically important fish. They are generally tolerant of wide temperature and salinity ranges. *Oreochromis mossambicus:* the Mozambique bream, or blue kurper, is the best-known tilapia species (Moyo and Rapatsa, 2021). It is deep-bodied and occurs in the warmer parts of South Africa in east-flowing rivers and dams. Juveniles are silvery, with 6-7 vertical bars, and three spots along the flank. Adults are silvery olive to deep blue grey, dorsal and caudal fins with red margins (Skelton,1993).

It has also been widely translocated to the Western Cape and Namibia. Its limitations include relatively slow growth, and early maturity. It attains about 400mm standard length (Skelton, 1993).

Oreochromis placidus: is similar to *O. mossambicus* but a smaller species, *O. placidus* is found in the Mkuze swamps and the northeastern lowlands extending into Mozambique. It has a different breeding coloration and four anal fin spines compared with the three of *O. mossambicus.* It attains 300mm standard length (Skelton,1993).

The Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) (*Cichlidae; Teleostei*), is a widespread species used in tropical aquaculture (Moyo and Rapatsa, 2021). It has a deep-bodied, compressed shape. This species is bronze to brownish-grey dorsally and laterally and white ventrically. It has a truncated caudal fin with many thin black bands and a pinkish-red posterior margin, which distinguishes it from *O. mossambicus* and *O. placidus*. Their scales are large, with a prominent lateral line that runs from the gill cover to the tail. It has distinctive, regular, vertical stripes on the caudal fin, with variable coloration. The iridescent spots in the caudal fin identify the species. Adult fish can reach up to 500cm in body length and up to 4.3 kg body weight (Skelton,1993).

Nile tilapia can be found in canals, creeks, rivers, and lakes, that usually have aquatic vegetation. Natural populations of these fish occur in Africa, and the species *O. niloticus* has been introduced to almost every tropical country in the world for aquaculture purposes (Nyingi *et al.,* 2009). Among tilapia species, *O. niloticus* has the fastest growth rate of approximately 1000g in 12 months compared to other *tilapia,* such as *O. mossambicus*, which would grow about 400g in 12 months (Lind *et al.*, 2015). *O. niloticus* is preferred for its faster growth and bigger size, with a relatively longer life span, giving it an edge above other tilapia species, including *O. mossambicus* (Zengeya *et al.*, 2012 and Nyirenda, 2017). As a result of *O. niloticus* plasticity, the species has been able to successfully establish itself in extreme environmental conditions such as temperate winter conditions (Peterson *et al*., 2005; Grammer *et al*., 2012), hot springs (Trewavas 1983; Nyingi *et al*., 2009; Ndiwa *et al*., 2014) and saline waters (Schofield *et al*., 2011). Legislation drafted in some Southern African countries prohibits the culturing of *O. niloticus*, however, its culturing is widespread (Moshobane, 2020, Moyo and Rapatsa, 2021). However it is permitted in some areas for aquaculture in South Africa (Moshobane, 2020).

O. niloticus- Nile tilapia has been introduced to South Africa for aquaculture as it has the potential to grow large and rapidly. However, only the most carefully selected strains perform well; many inbred and stunted populations do poorly. Nile tilapia and the genetically improved Nile tilapia GIFT strains are used by more than 80% of tilapia farmers. They readily hybridize with indigenous species (Zengeya *et al.*, 2015 and Nyirenda, 2017).

Species such as *O. andersonii* (three-spot tilapia), *O. aureus* (Israeli tilapia) and *O. macrochir* (green-head tilapia) are non-native to South Africa, but have reportedly been introduced in certain places (De moor, 1996, D'Ameto *et al*., 2007, Marr *et al.,* 2018, Mboweni, 2020). *O. andersonii* has considerable aquaculture potential and is used in Zambia (Moyo and Rapatsa, 2021 and Basiita *et al*., 2022). *O. niloticus* and *O. andersonii* grow far better than the native *O. mossambicus.*

Morphometric and meristic characteristics have been utilized to differentiate among different tilapia species in South Africa. However, meristic characters were less useful than the morphometrical characters, when comparing morphological variations in the same species. This is because they remain consistent in number once the fry has been established and are not affected by environmental changes or other physical changes that might occur within the surroundings of the fish's habitat. This is supported by studies by Murta, (2000) for Horse mackerel and Munasighe & Thushari, (2010) for shrimp. However, they have been used for tilapia identification in South Africa. Different tilapia species often exhibit variations in their meristic features. These differences while sometimes subtle, are good in differentiating different tilapia species, especially when combined with other morphological species. With regards to population variations, even within a single tilapia species, different populations may show slight variations in meristic features due to genetic adaptations to their specific environments. These variations can be used to identify and track different stocks, which is important for fisheries management and conservation. Meristic data contribute to a broader morphological study that aim to understand diversity and the evolution of tilapia species. By analysing meristic features alongside other morphological and genetic data, researchers can gain insights into relationships between different tilapia species. Therefore, six meristic characteristics, the number of dorsal spines and rays, anal spines and rays, lateral line scales as well as the number of gill rakers will be analyzed.

In this study 18 morphometric characteristics will be used to differentiate and identify amongst tilapia species. These will include recommended characteristics from authors including Hassanien, *et al.,* 2011 who used 8 morphometric characteristics, Herath *et al.,* 2014 who used 12 morphometric characteristics, Mahmoud and Hassan, 2019 who used 19 morphometric characteristics, Samaradivakara *et al.,* 2012 who used 20 and Asmamaw and Tessema, 2021 who used 12 morphometric characteristics. Only the most useful (18) characteristics for tilapia identification were used. They were identified as the features that had a statistical significance of above 0.900 when tested in PCA or as stated by the authors who tested the characters. Therefore this study will note the most useful morphometric characteristics for the identification of tilapia in Southern Africa and identify tilapia species in four localities in Limpopo Province.

3.2. SAMPLING SITE MAP

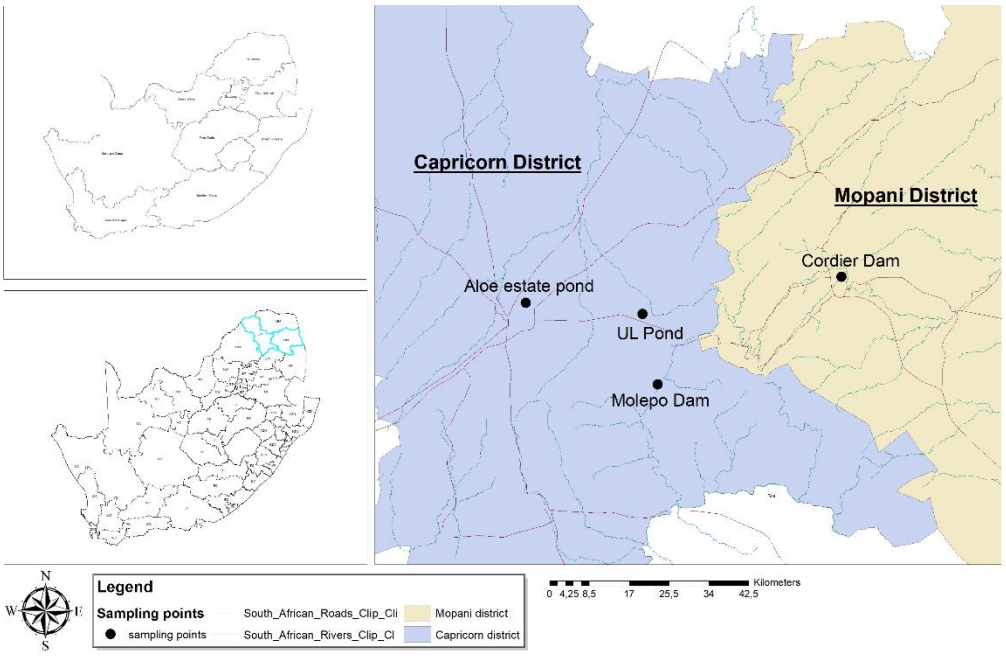


Fig 3.1. Four sites under investigation. Aloe estate pond (also known as Mall of the North Pond), UL Pond situated at University of Limpopo, Molepo Dam (All within the Capricorn District) and Cordier Dam in the ZZ2 farmstead in Mopani District.

3.2.1. STUDY SITES

3.2.1.1 CORDIER DAM

Cordier Dam, a private earthen Dam situated in Mooketsi along the ZZ2 plantations (23.64212S, 30.04179E) stemming from the Middle Letaba River (Fig 3.1). This Dam was reconstructed in 2016 and stocked with fish from the Middle Letaba River has a maximum depth of 10 meters and 18 meters on the widest part of the dam, with a capacity of 1.8 million cubic meters.

3.2.1.2. MOLEPO DAM

Molepo Dam is an earthen Dam situated in Ga-Ramphere next to Ga-molepo under the care of the Department of Water Affairs (24.034752S, 29.803396E) (Fig 3.1.). The Dam was completed in 1987, has a maximum depth of 22m and 45 meters wide on the widest part of the dam, with a capacity of 4.52 million cubic meters (Tracks4Africa). The dam is fed by the Mphogodima River. It is a relatively small dam that is used for recreational swimming, fishing, livestock drinking and grazing around its banks as well as camping and occasional festivals.

3.2.1.3. UNIVERSITY OF LIMPOPO POND

University of Limpopo Pond is a small earthen pond that is situated in the University (23.8888S, 29.73866E) (Fig 3.1.). It is a small recreational pond that was set up for recreational purposes. Students are currently using it to collect fish for research where applicable. It was constructed and stocked in 1976 with fish from the Turfloop Dam.

3.2.1.4. MALL OF THE NORTH POND

Mall of the North Pond is a private pond situated next to Mall of the North inside Aloes Estate (23.8688918S, 29.4971057E) (Fig 3.1.). It was constructed for recreational purposes. This pond was chosen because the owner knows he stocks pure *O. mossambicus* species. It was built four years ago.

3.3. SAMPLING PROCEDURE AND THE DETERMINATION OF WATER QUALITY PARAMETERS.

Tilapia fish species were collected from the various ponds and dams using a seine net of 10mm and 30mm, 2mx4m in length. Sampling was done twice, in March and November 2022. About 20 fish were collected at each locality per sampling month, making it 40 fish per locality. The fish were transported to the Aquaculture Research Unit and stored in labelled tanks in a recirculating system. Daily, they were fed fish pellets at 10% of their body weight until the start of the project. Water quality parameters and temperature were monitored daily to ensure optimum living conditions for the fish. Dissolved oxygen (mg/L), pH, and salinity (ppt) were measured at the four localities using a YSI multi-probe meter. Water samples were collected in 1L sample bottles at the surface of the water column for laboratory analysis of phosphorus, nitrate, and nitrogen/ammonia.

Phosphorous was determined according to the spectroquant 1.14848.0001. In this method, phosphate ions in solution acidified with sulfuric acid, reacts with molybdate ions to form molibdofosforic acid which was reduced by ascorbic acid phosphomolybdic blue (PMB) which was determined photometrically. This method is analogous to ISO 6878/1-2005 method and is used to determine othophosphates and total phosphorous (Habibah *et al.,* 2018).

Nitrate was determined according to spectroquant nitrogen test 1.14537.0001. In this method, organic and inorganic nitrogen compounds are transformed into nitrate

according to Koroleff's method by treatment with oxidizing agent in a thermoreactor. In concentrated sulfuric acid, this nitrate reacts with a benzoic acid derivative to form a red nitro compound that is determined photometrically (Merck KGaA, 2024).

Nitrogen\ammonia were determined according to spectroquant ammonium-test 1.14752.0001/1.14752.0002/1.00683.0001 method. The method is based on the principle that in a strongly alkaline solution ammonium nitrogen is almost entirely present as ammonia, which reacts with a chlorinating agent to form monochloramine. This in turn reacts with thymol to form a blue indophenol derivative that was then determined photometrically at 630nm wavelength. The method is analogous to EPA 350.1, APHA 4500-NH3 F, ISO7150-1, and DIN 38406-5 (Merck KGaA, 2024).

3.4. ANALYSIS OF MORPHOMETRIC FEATURES

The fish were placed in 2phenoxyethanol to relax the fish momentarily. They were tagged individually with a number for identification during analysis, then identified and characterized using 18 traditional morphometrical and six meristic characters (Fig 3.2), taking note of the following: total length, standard length, body depth, pre-dorsal length, pre-pectoral length, pre-pelvic length, pre-anal length, depth of caudal peduncle, length of caudal peduncle, head length, eye diameter, snout length, upper jaw length, lower jaw length, caudal fin length, pectoral fin length, length of dorsal fin base, length of anal fin base, as well as, the number of dorsal spines and ray, anal spines and rays, lateral line scales as well as the number of gill rakers in the tilapia individuals. All these measurements were taken with a Viener caliper to the nearest 0.01mm. The fish were then weighed using an electronic scale. However, to avoid possible biases produced by size effects on the morphometric variables, all morphometric characters were standardized by the formula ACi = log OCi – [β * (logTLi–logMTL)] (Claytor & Mac Crimmon, 1987) where ACi is the adjusted logarithmic character measurements of the i^{th} specimen, OCi is the unadjusted character measurement of the i^{th} specimen, β is the common within-group regression coefficient of that character against total length after the logarithmic transformation of both variables and TLi is the total length of the ith specimen; and MTL is the overall mean total length.

Statistical analysis of the morphometric characteristics of *Tilapia* species found in the two dams (Cordier Dam and Molepo Dam) and two ponds (Mall of the North Pond and University of Limpopo Pond) was then performed. DA (discriminate analysis) was used to classify the identified and measured fish, while PCA was used to identify the morphometric and meristic characteristics which contributed the most differentiation of the tilapia species. After identification, *Oreochromis mossambicus* species were isolated and a one way ANOVA was performed on the samples to find out if there were morphometric variations present among the *O. mossambicus* species in the different locations. The data was then subjected to DA (discriminative analysis) and PCA, using XLSTAT, 2020 and SPSS, 2020 respectively to identify and classify the tilapia species and PCA was used to determine which characteristics accounted for the most variation among the tilapia species.

Finally, the identified *O. mossambicus* species were subjected to One-way ANOVA to determine if there were any differences in morphometric characteristics among the same species of fish in different localities. SPSS, 2020 version was used to analyze the data. The dependent variables were the morphometric characteristic measurements, while the independent variable was the locality, 1 for Mall of the North Pond, 2 for University of Limpopo Pond and 3 for Cordier Dam. The condition factor was also calculated to check the wellbeing of the fish, aiding in discussing possible differences if any were present.

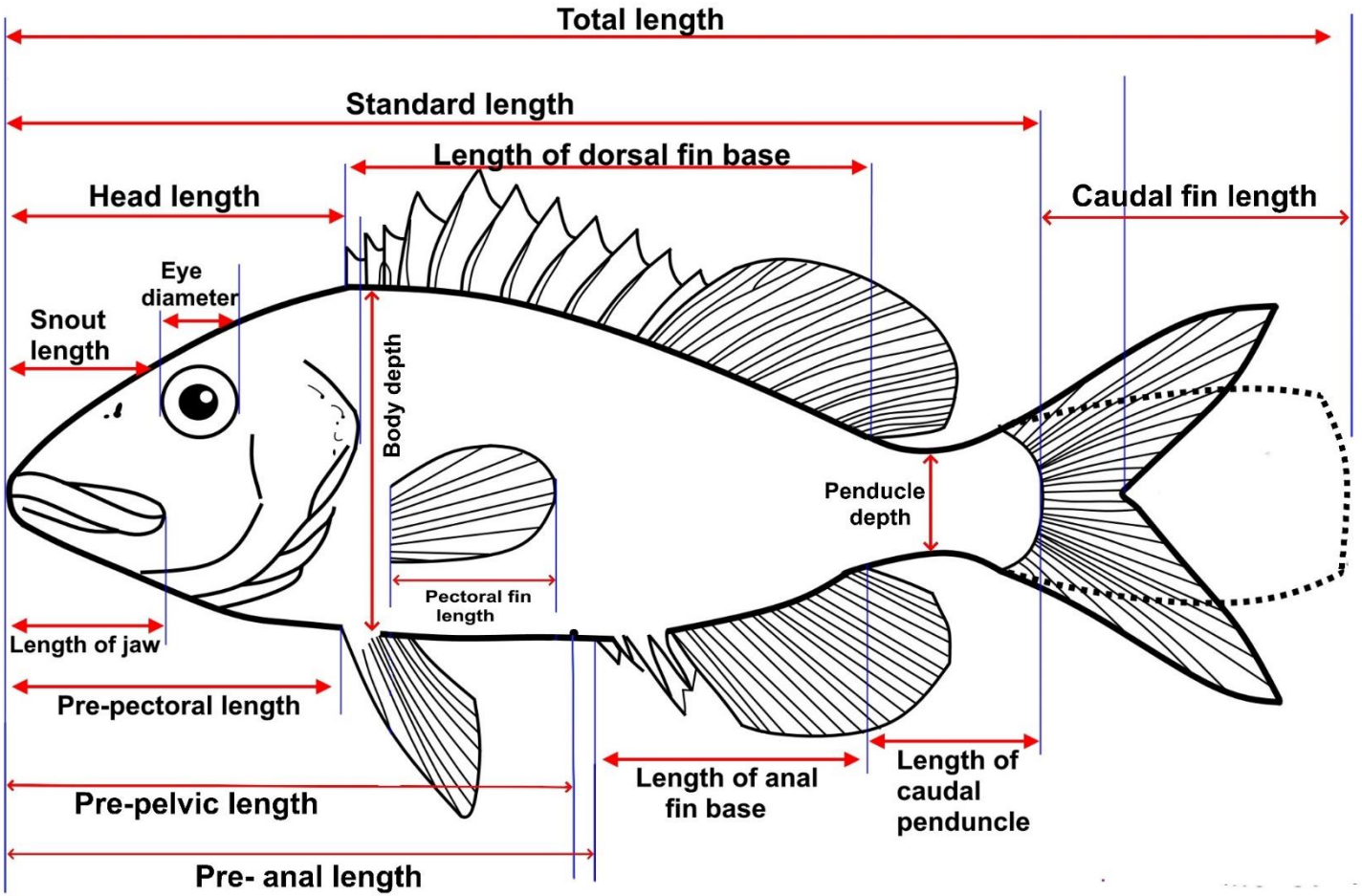


Fig 3.2: Measured morphometric features of the tilapia species.

3.5. RESULTS

3.5.1. WATER QUALITY PARAMETERS

Table 3.1: Water quality parameters of the four localities under investigation.

Water quality	Cordier Dam	Molepo Dam	Mall of the North	University of Limpopo
Temperature (°C)	**31.5 ± 1.06[a]**	**22.8 ± 1.04[b]**	**23.1 ± 0.26[b]**	**24.7 ± 1.01[b]**
Dissolved Oxygen (mg/l)	**10.80 ± 1.84[a]**	**10.31± 1.48[a]**	**9.37 ± 0.68[a]**	**10.36 ± 0.14[a]**
pH	**5.93 ± 1.1[a]**	**7.42 ± 0.59[b]**	**7.35 ± 0.52[b]**	**7.17 ± 0.61[b]**
Salinity (ppt)	**0.17 ± 0.06[a]**	**0.30 ± 0.08[b]**	**0.27 ± 0.03[b]**	**0.06 ± 0.02[c]**
Phosphorus (mg/l)	**0.53 ± 0.07[a]**	**0.78 ± 0.04[a]**	**3.61 ± 0.07[b]**	**0.93 ± 0.06[a]**
Nitrate (NO^-_3-N) (mg/l)	**5.0 ± 0.16[a]**	**3.9 ± 0.03[b]**	**5.0 ± 0.09[a]**	**3.7 ± 0.05[b]**
Nitrogen/Ammonia NH^-_3 H (mg/l)	**0.08 ± 0.02[a]**	**0.10± 0.02[a]**	**0.03 ± 0.03[a]**	**0.12 ± 0.01[a]**

Water quality parameters were relatively similar in the four localities under investigation (Table 3.1); however, the temperature at Cordier Dam was statistically different from the other sites, as it is in the warmer low veld region, pH was statistically different in Codier Dam, which is expected owning to the higher water temperatures. The salinity levels at the University of Limpopo were the most statistically different from the other sites, Cordier Dam was also statistically different from Molepo Dam and Mall of the North Pond. Phosphorus levels were statistically different in the Mall of the North Pond due to runoff from fertilizers on the farm property. Nitrate levels were statistically different at the University of Limpopo Pond and Molepo Dam, while Nitrogen/Ammonia levels were not statistically different at all the four sites.

DISCRIMINANT ANALYSIS (DA) RESULTS

3.5.2. CORDIER DAM.

Discriminant analysis was done by comparing the individuals at Cordier Dam (Fig 3.3). fish were prior identified as *O. niloticus, O. mossambicus,* and their possible hybrids. Two spheres were formed; however, they overlapped due to possible hybrids. The overlapping possible hybrids were individual: 894M (Fig 3.4), 908M (Fig 3.5), 912M (Fig 3.6), 910M (Fig 3.7), 895N (Fig 3.8), 900N (Fig 3.9), and 896N (Fig 3.10). Individuals 893M (Fig 3.11) and 905M (Fig 3.12) were identified as *O. mossambicus* however their DA placement was in the *O. niloticus* sphere.

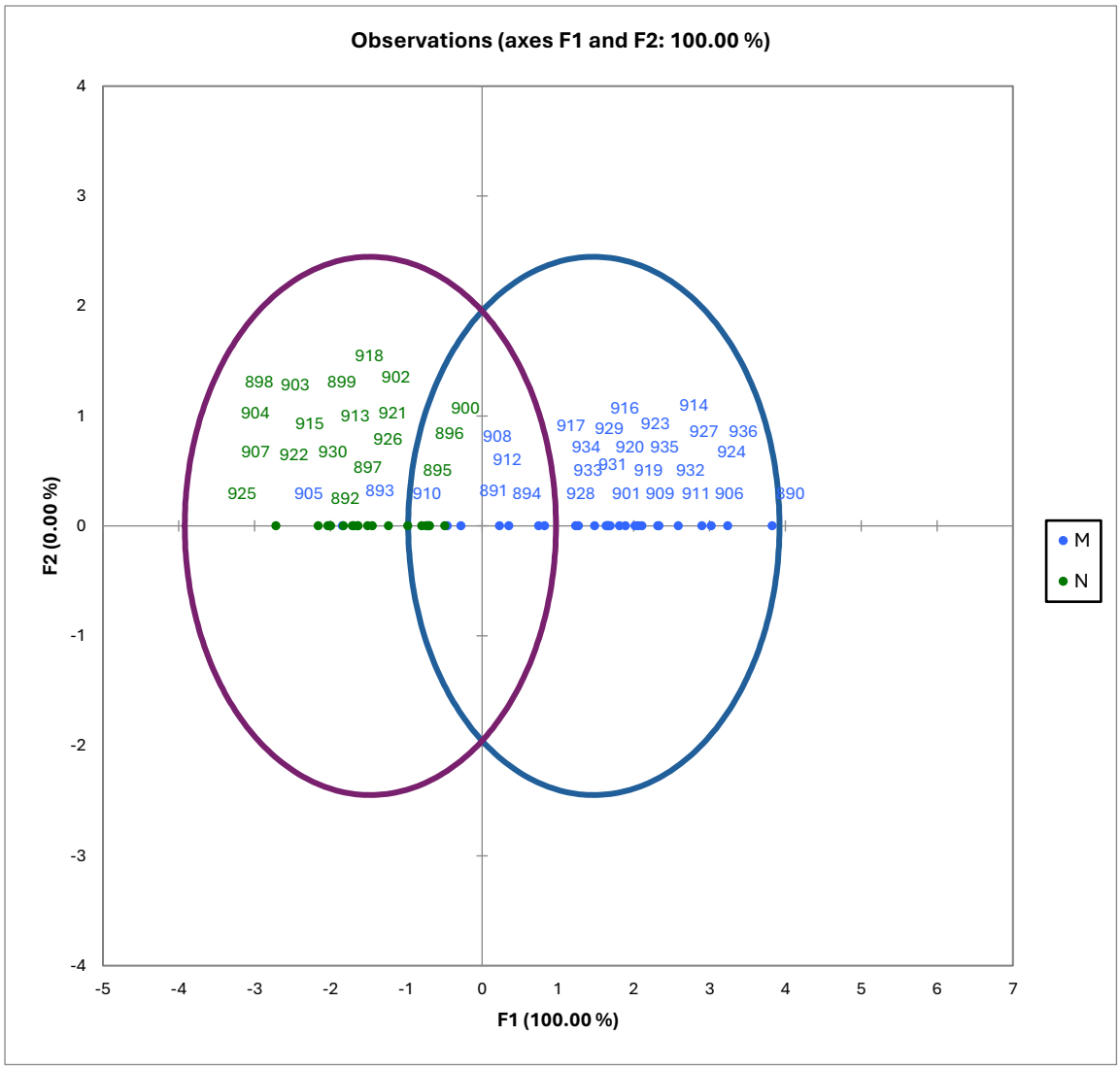


Figure 3.3. Discriminant analysis between *O. mossambicus* and *O. niloticus,* with their possible hybrids, where the green numbers identified *O. niloticus* species, and the blue

numbers indicated *O. mossambicus* species. M- indicates *O. mossambicus* species and N- indicates *O. niloticus* species. The fish in the overlap could be possible hybrids.

The DA results generated a confusion metrics to show the percentage of fish that where possibly correctly identified according to discriminant analysis. (Table 3.2).

Table 3.2. Confusion metrics generated by discriminant analysis for Cordier Dam.

The results showed that 85.71% of the *O. mossambicus* were correctly identified as two of the suspected *O. mossambicus* were placed in the *O. niloticus* sphere (Fig 3.3) while the rest were possible hybrids.

from \ to	M- O. mossambicus	N- O. niloticus	Total	% Correct
M- O. mossambicus	20,14286	3,357143	23,5	85,71%
N- O. niloticus	0	23,5	23,5	100,00%
Total	20,14286	26,85714	47	92,86%

Below are the pictures of the fish that overlapped *O. mossambicus* and *O. niloticus* spheres in the DA metrics analysis, showing that they are possible hybrids.

Figure 3.4. Individual 894M morphometrically identified through its phenotypic features. Due to the lack of prominent bands on the caudal fin, it was identified as *O. mossambicus*, but the discriminant analysis found that it was a possible hybrid, placing it in the overlap.

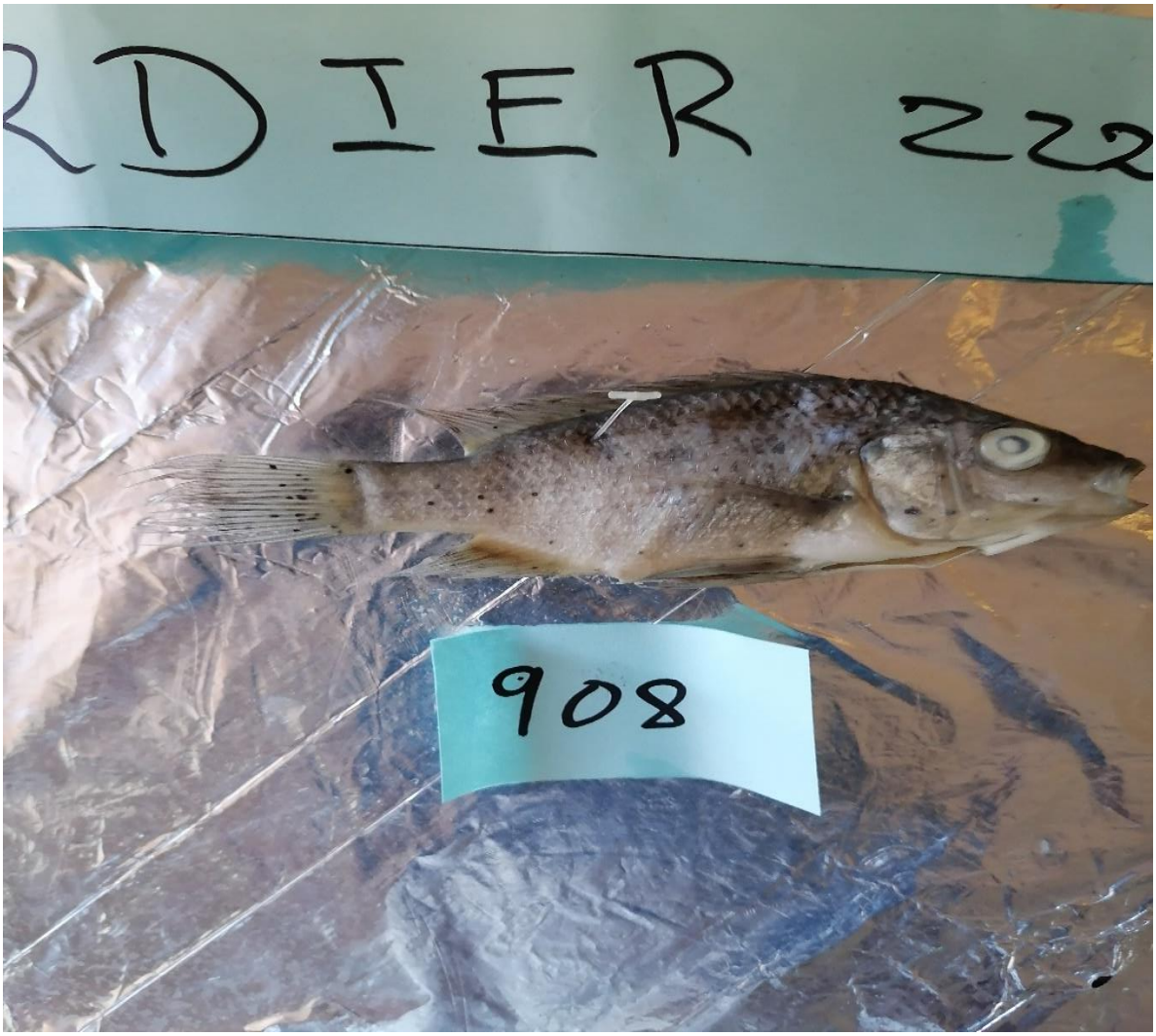


Figure 3.5. Individual 908M morphometrically identified through its phenotypic features. Due to the lack of prominent bands on the caudal fin, it was identified as *O. mossambicus* but the discriminant analysis found that it was a possible hybrid, placing it in the overlap.

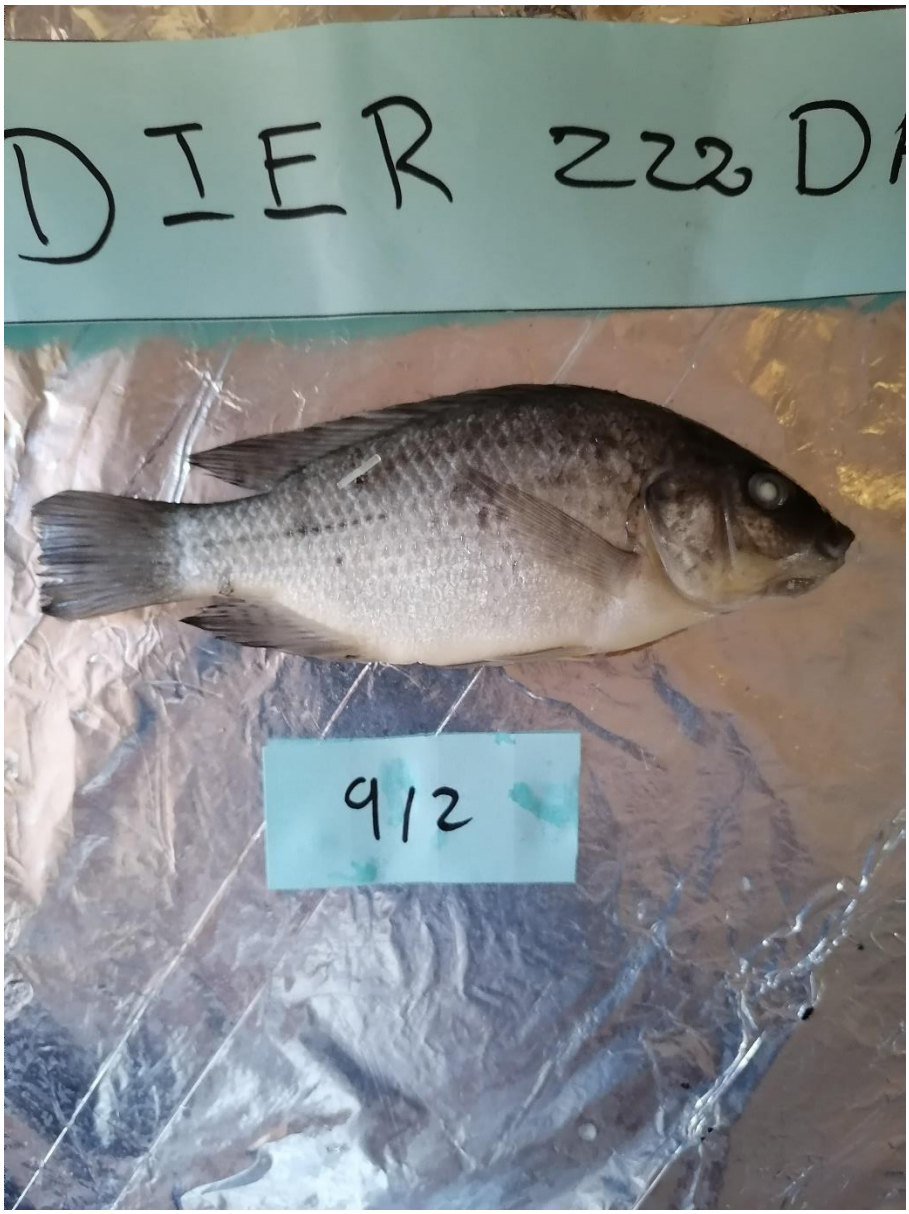


Figure 3.6. Individual 912M morphometrically identified through its phenotypic features. Due to the lack of prominent bands on the caudal fin, it was identified as *O. mossambicus* but the discriminant analysis found that it was a possible hybrid, placing it in the overlap.

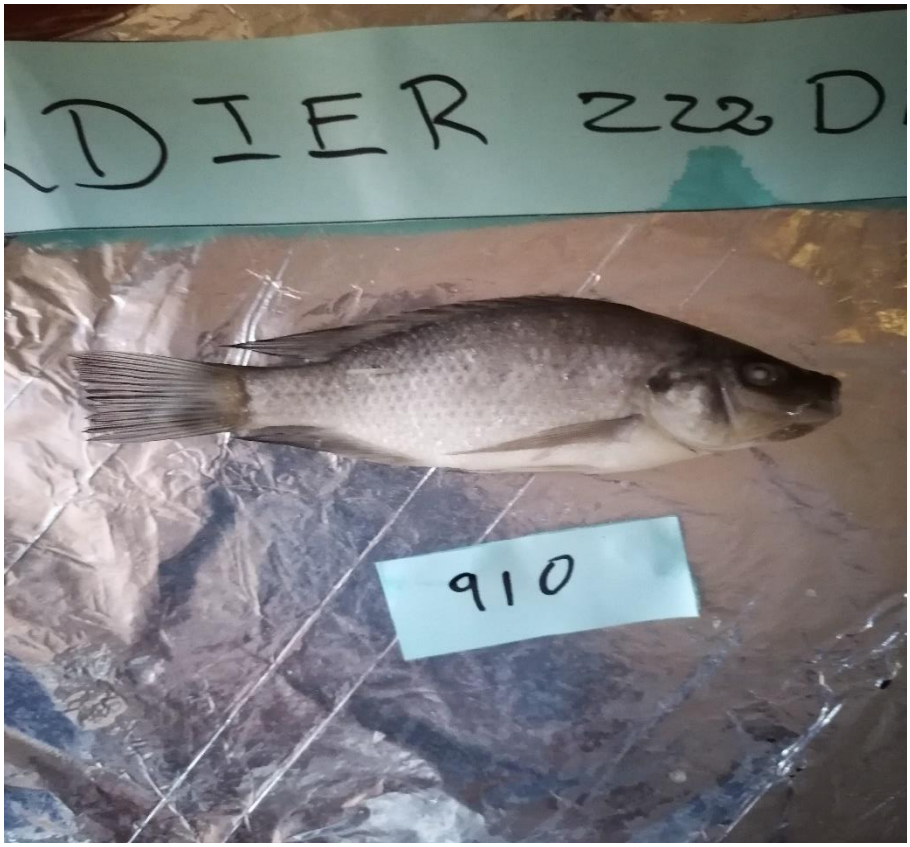


Figure 3.7. Individual 910M morphometrically identified through its phenotypic features. Due to the lack of prominent bands on the caudal fin, it was identified as *O. mossambicus* but the discriminant analysis found that it was a possible hybrid, placing it in the overlap.

Figure 3.8. Individual 895n morphometrically identified through its phenotypic features. Due to the presence of prominent bands on the caudal fin, it was identified as *O. niloticus* but the discriminant analysis found that it was a possible hybrid, placing it in the overlap.

Figure 3.9. Individual 900N morphometrically identified through its phenotypic features. Due to the presence of prominent bands on the caudal fin, it was identified as *O. niloticus* but the discriminant analysis found that it was a possible hybrid, placing it in the overlap.

Figure 3.10. Individual 896N morphometrically identified through its phenotypic features. Due to the presence of prominent bands on the caudal fin, it was identified as *O. niloticus* but the discriminant analysis found that it was a possible hybrid, placing it in the overlap.

Further analysis showed that some of the individuals were incorrectly identified as they showed a different placement in the DA analysis spheres. Below depicts individuals phenotypically described as *O. mossambicus* but their discriminant analysis placement was in the *O. niloticus* sphere, meaning they are also possible hybrids.

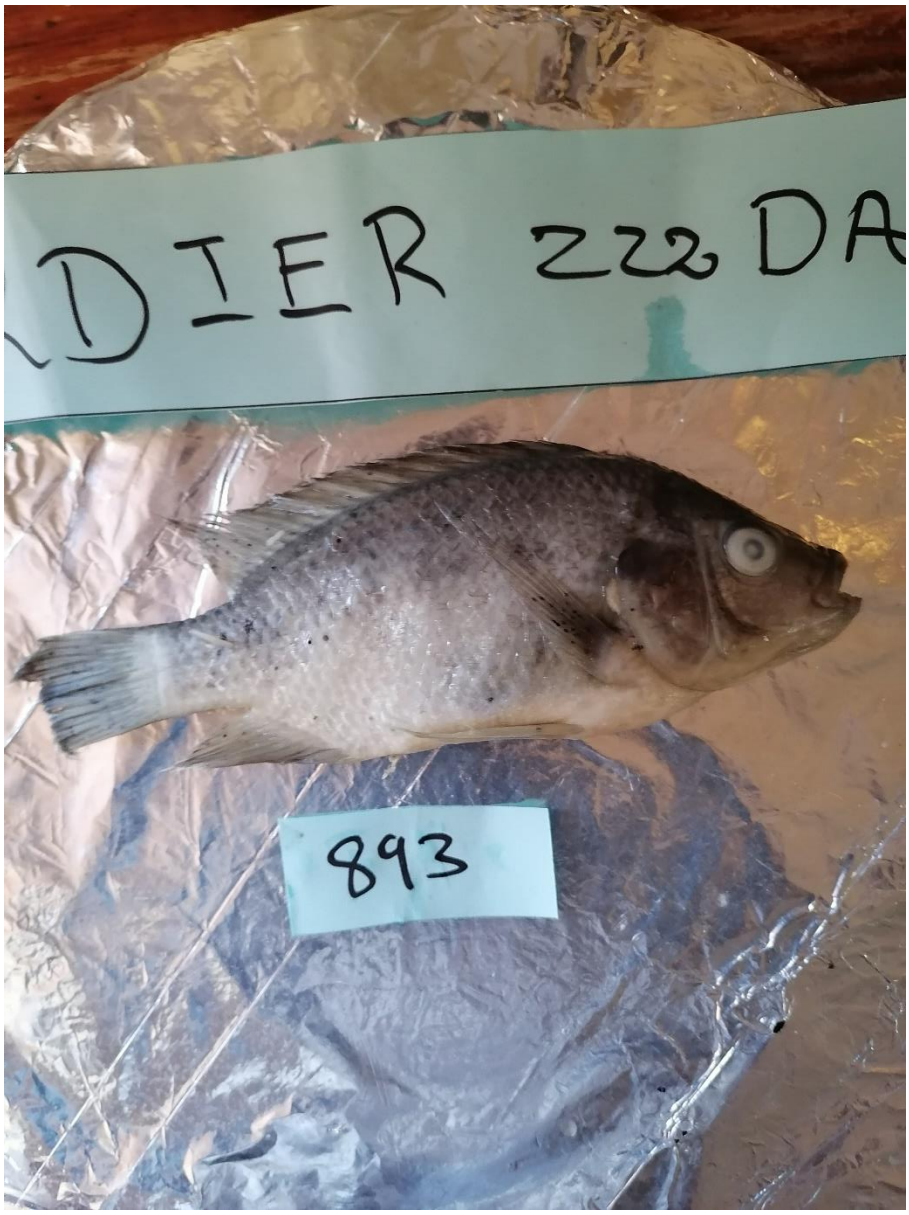


Figure 3.11. Individual 893M had no obvious bands on the caudal fine and was thus morphometrically identified as *O. mossambicus* however its DA placement was in the *O. niloticus* sphere. This means that it can be a potential hybrid.

Figure 3.12. Individual 905M had no prominent bands on the caudal fin and was thus morphometrically identified as *O. mossambicus;* however, its DA placement was in the *O. niloticus* sphere. This means that it can be a potential hybrid.

3.5.3. MOLEPO DAM

Discriminant analysis showed two species *C. rendalli* and *T. sparrmanii* (Fig 3.13). Individual 960 (Fig 3.14) was phenotypically incorrectly identified and DA analysis placed it in the correct sphere of *C. rendalli* while it was incorrectly identified as *T. sparrmanii.* Individual 956 (Fig 3.15) was placed outside the *T. sparramanii* sphere. The Molepo Dam contained more *T. sparrmanii* (40) compared to *C. rendalli (5).*

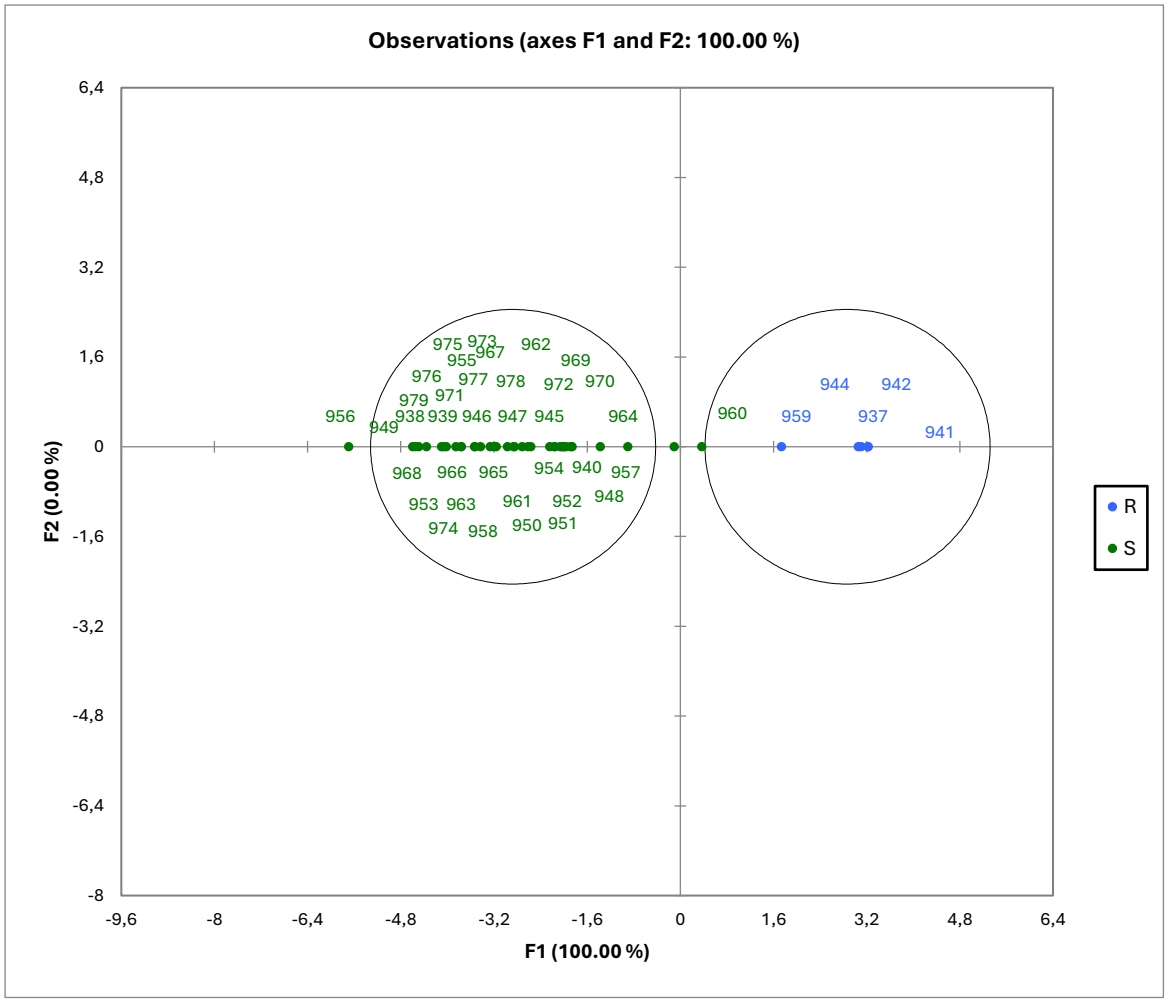


Figure 3.13. Discriminant analysis between *C. rendalli and T. sparrmanii*, where the green numbers identified *T. sparmanni* individuals, and the blue numbers indicated *C. rendalli* individuals. R- indicates *C. rendalli* individuals and S- indicates *T. sparrmanii. niloticus* individuals.

The DA results showed two distinct spheres indicating two species being *C. rendalli* and *T. sparrmanii.* The individual 944 was initially identified as *T. sparrmanii* was found in the *C. rendalli* sphere. It could have been incorrectly identified as *T. sparrmanii* while they were *C. rendalli.* This highlights the effectiveness of the discriminant

morphometric analysis in classing and correctly identifying fish species. Individual 956 was placed outside both spheres, indicating that it was possibly another species, possibly *O. mossambicus.*

The DA results generated a confusion metrics to show the percentage of fish that where possibly correctly identified (Table 3.3).

Table 3.3. Confusion metrics generated by discriminant analysis for Molepo Dam.

The matrix showed that all the *C. rendalli* were correctly initially identified but 2.70% of the *T. sparrmanii* were not correctly identified.

Confusion matrix for the estimation sample:				
from \ to	R- C. rendalli	S- T. sparrmanii	Total	% correct
R- C. rendalli	21	0	21	100,00%
S- T. sparrmanii	0,5675676	20,432432	21	97,30%
Total	21,567568	20,432432	42	98,65%

The individual initially phenotypically identified as *T. sparrmanii* was a juvenile *C. rendalli* individual.

Figure 3.14. Individual 960 which was incorrectly identified as *T. sparrmanii* instead of *C. rendalli.* The discriminant analysis placed it correctly in the *C. rendalli* sphere.

The individual below was placed on the outskirts of the *T. sparrmanii* sphere.

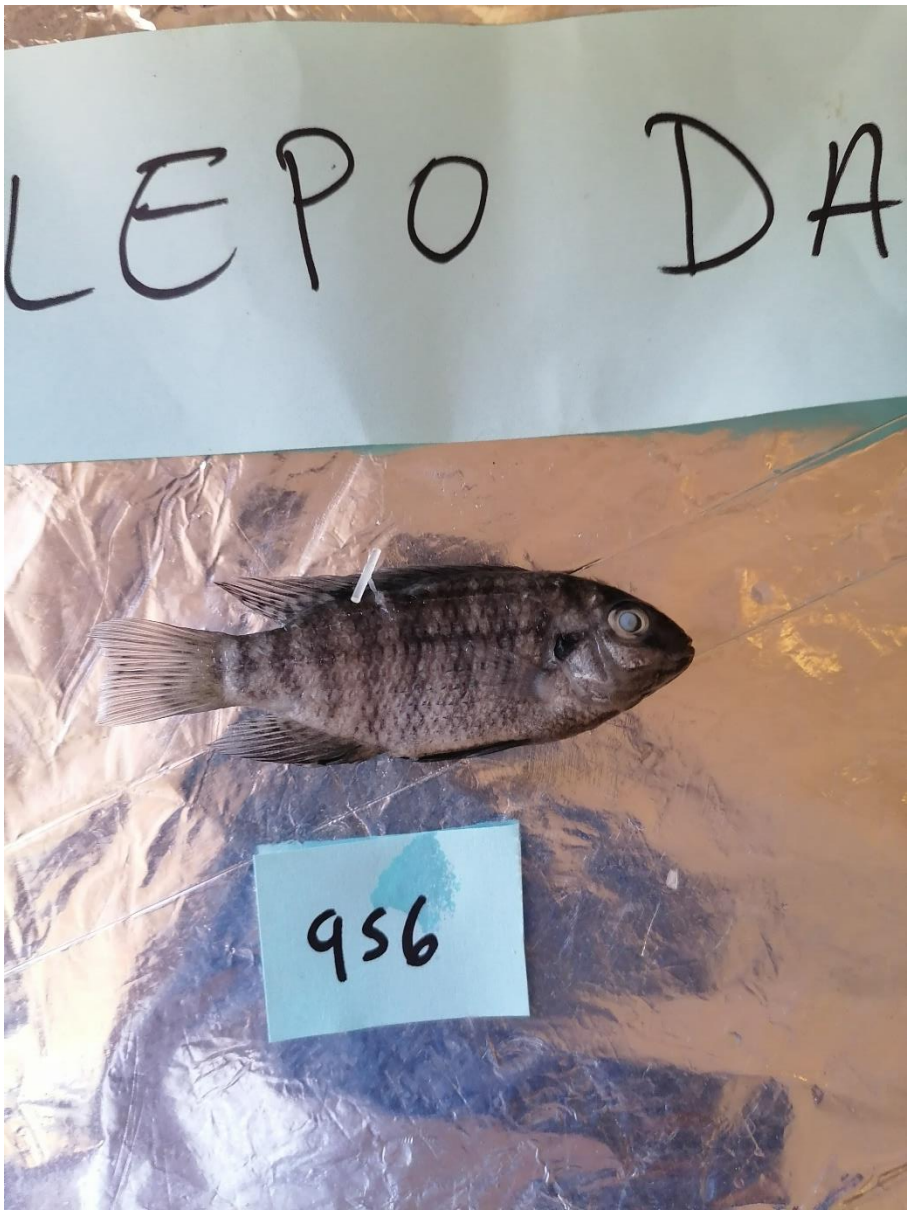


Figure 3.15. Individual 956 which was placed outside the *T. sparrmanii* sphere. Due to its prominent tilapia spot, it can be concluded that it is *T. sparrmanii* and not *O. mossambicus*.

3.5.4. MALL OF THE NORTH POND

The individuals at the Mall of the North Pond all gathered in the *O. mossambicus* species (Fig 3.16) except 873 (Fig 3.17), 877 (Fig 3.18), 874 (Fig 3.19), 866 (Fig 3.20), and 868 (Fig3.21) which were outside the *O. mossambicus* sphere. The *O. niloticus* individuals 922 and 921 were added for differentiation for the DA test to run. It can thus be concluded that the species at the Mall of the North Pond are all *O.mossambicus*.

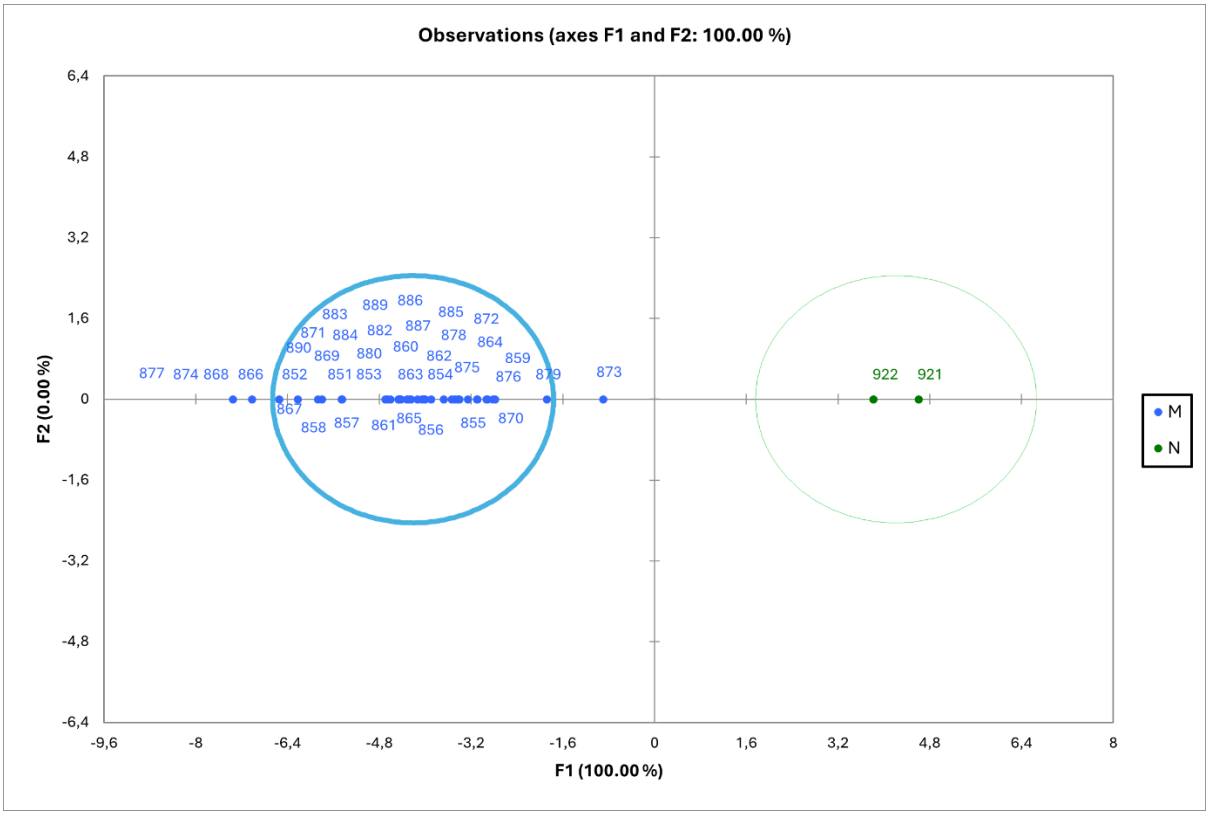


Figure 3.16: The DA analysis figure showing *O. mossambicus* from Mall of the North Pond and *O. niloticus* samples added from Cordier Dam to allow the system to run, as it requires a minimum of two different species for the analysis to compute. M-depicts *O. mossambicus* individuals while N-depicts *O. niloticus* individuals.

The DA results generated confusion metrics to show the percentage of fish that were possibly correctly identified. (Table 3.4). The confusion matrix shows that none of the individuals were incorrectly identified.

Table 3.4. Confusion metrics generated by discriminant analysis for Mall of the North Pond.

The confusion matrix showed that none of the fish were incorrectly identified.

from \ to	*M- O. mossambicus*	*N- O. niloticus*	Total	% correct
M- *O. mossambicus*	20	0	20	100,00%
N- *O. niloticus*	0	20	20	100,00%
Total	20	20	40	100,00%

Below are the individuals that were placed outside the *O. mossambicus* sphere. The figures below show that the individuals were also *O. mossambicus* and could have been incorrectly measured at some characteristics leading to their displacement outside the *O. mossambicus* sphere.

Figure 3.17. Individual 873M is placed outside the discriminant analysis sphere next to the *O. mossambicus* sphere.

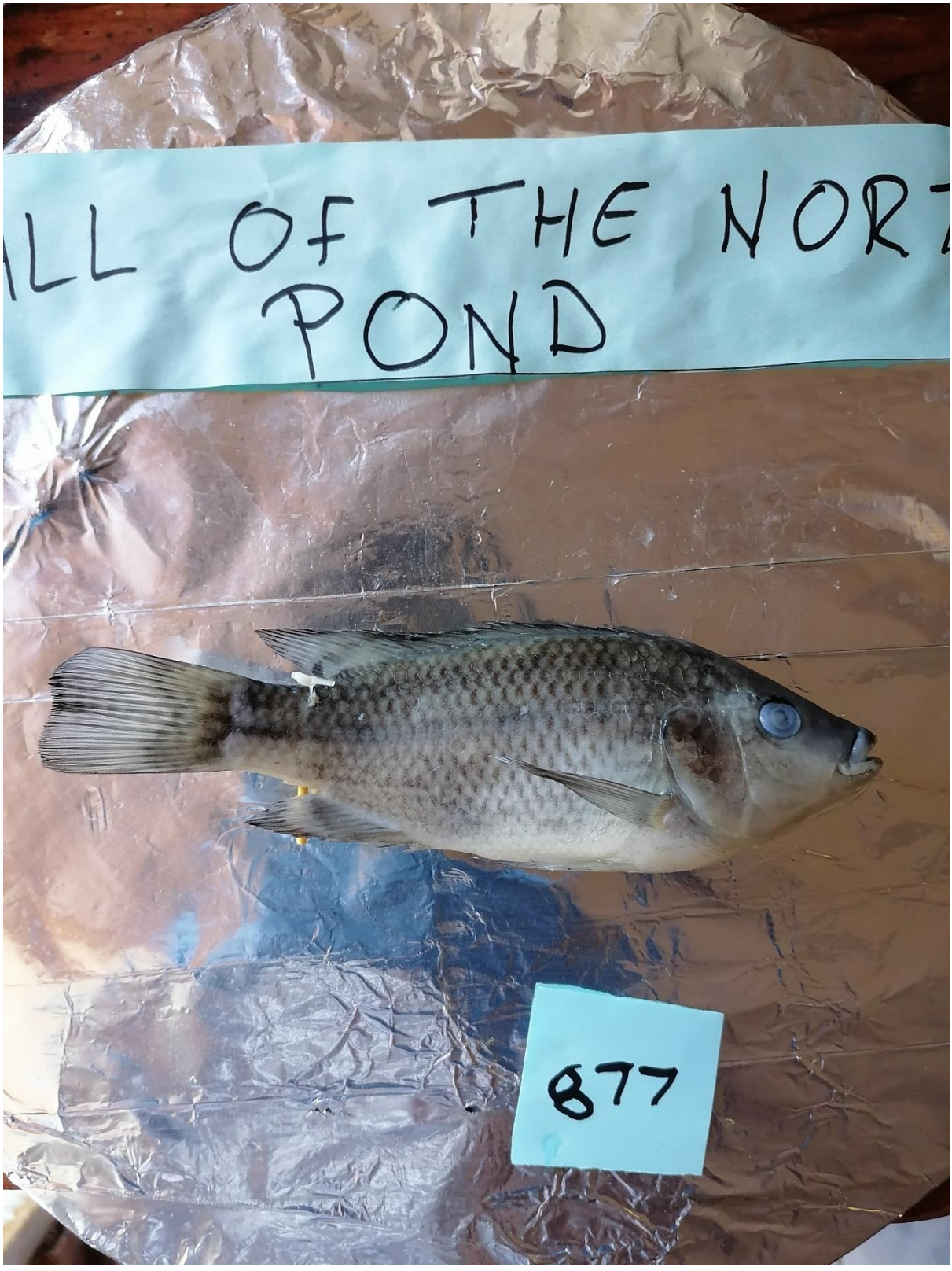


Figure 3.18: Individual 877M is placed outside the discriminant analysis sphere next to the *O. mossambicus* sphere.

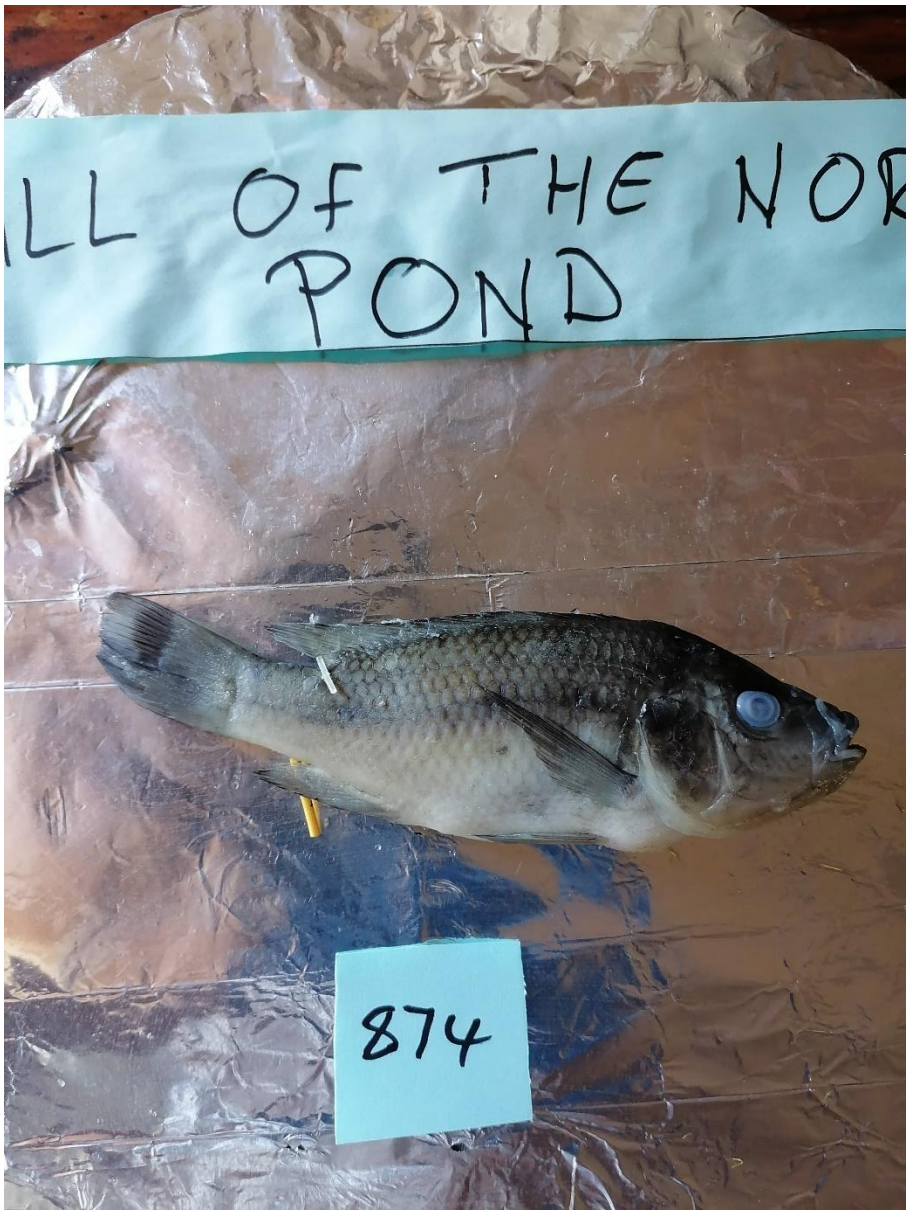


Figure 3.19: Individual 874M is placed outside the discriminant analysis sphere next to the *O. mossambicus* sphere.

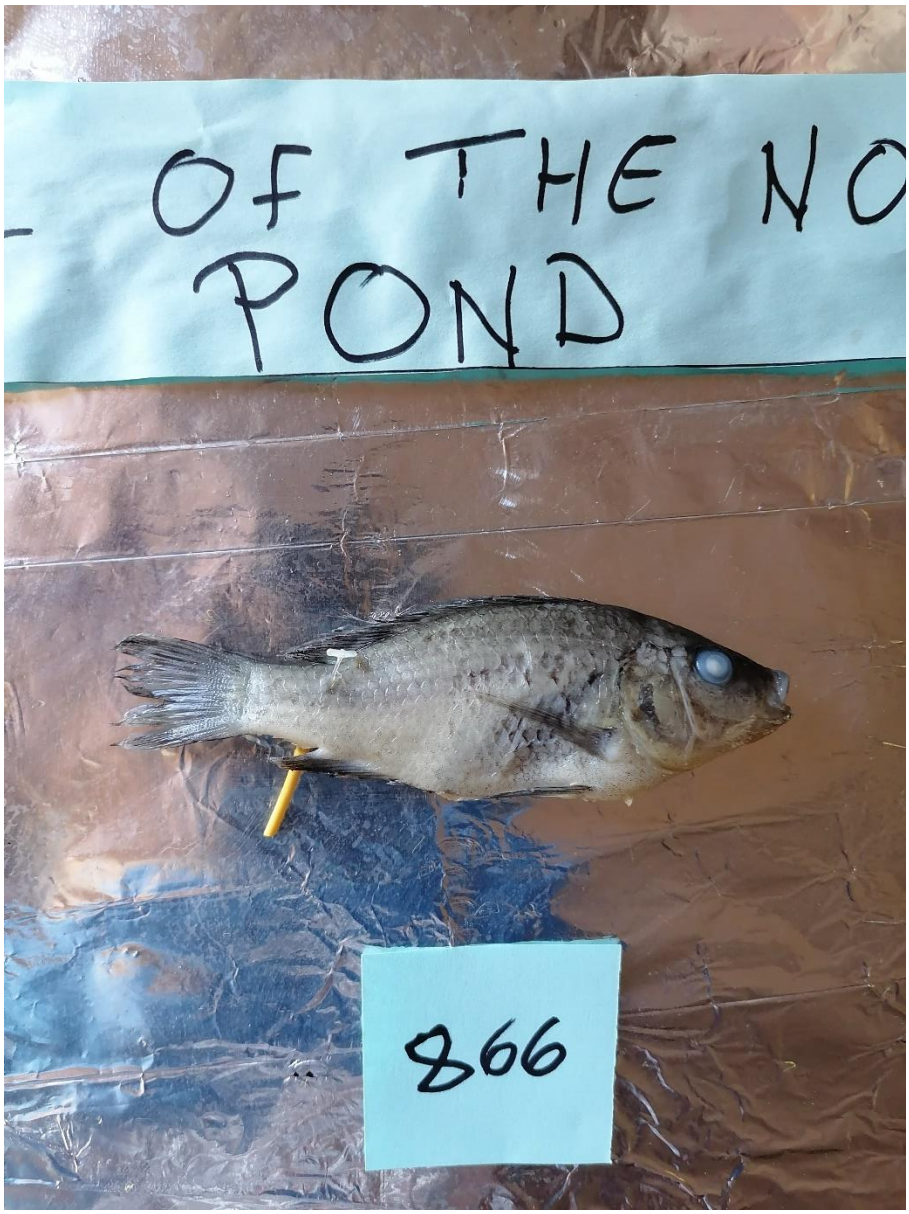


Figure 3.20: Individual 866M is placed outside the discriminant analysis sphere next to the *O. mossambicus* sphere.

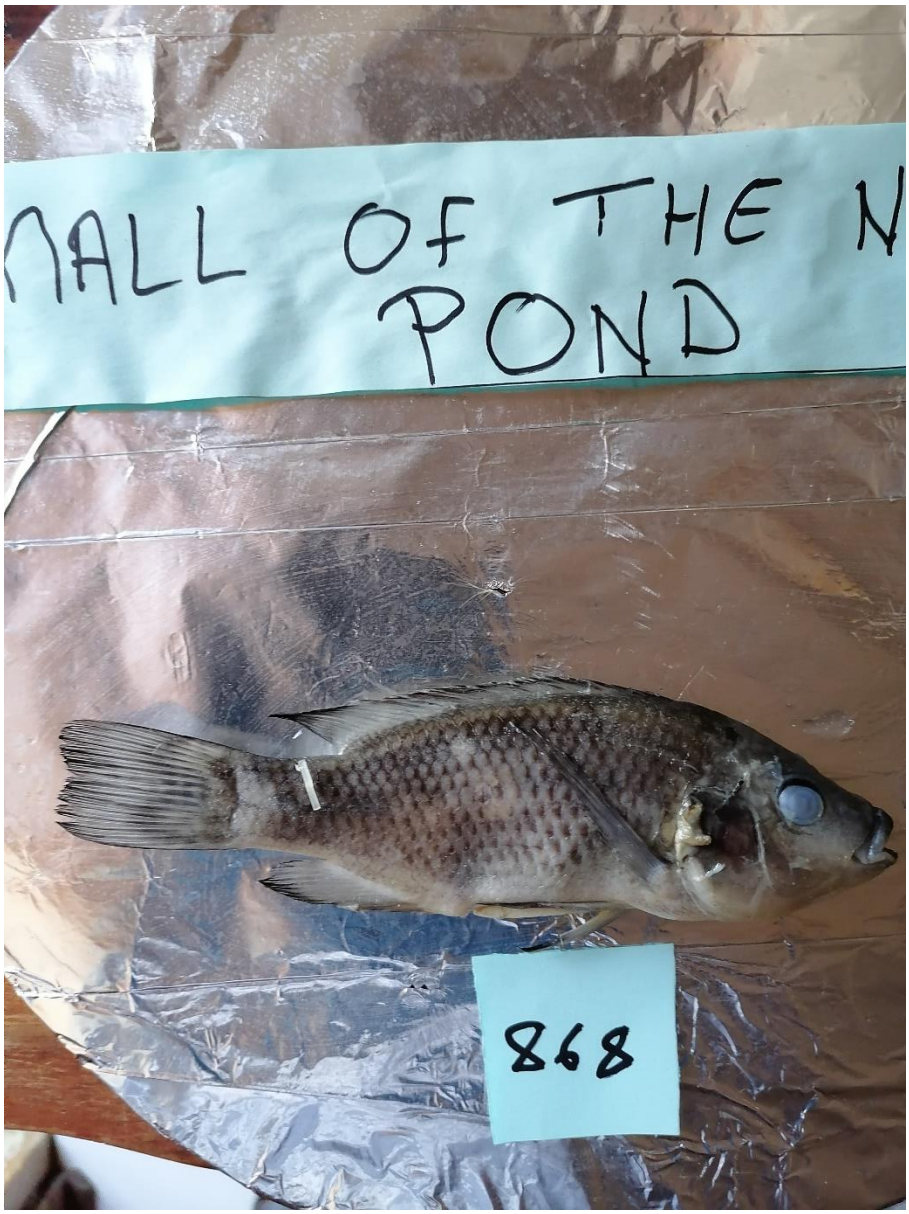


Figure 3.21: Individual 868M is placed outside the discriminant analysis sphere next to the *O. mossambicus* sphere.

3.5.5: UNIVERSITY OF LIMPOPO POND

DA showed that indeed the University of Limpopo Pond contained one species (*Oreochromis mossambicus)* with two *O. niloticus* samples being added so that the program could generate a result (Fig 3.22). Four samples (Fig 3.23), (Fig 3.24), (Fig 3.25), and (Fig 3.26) were on the outer skirts of the blue sphere.

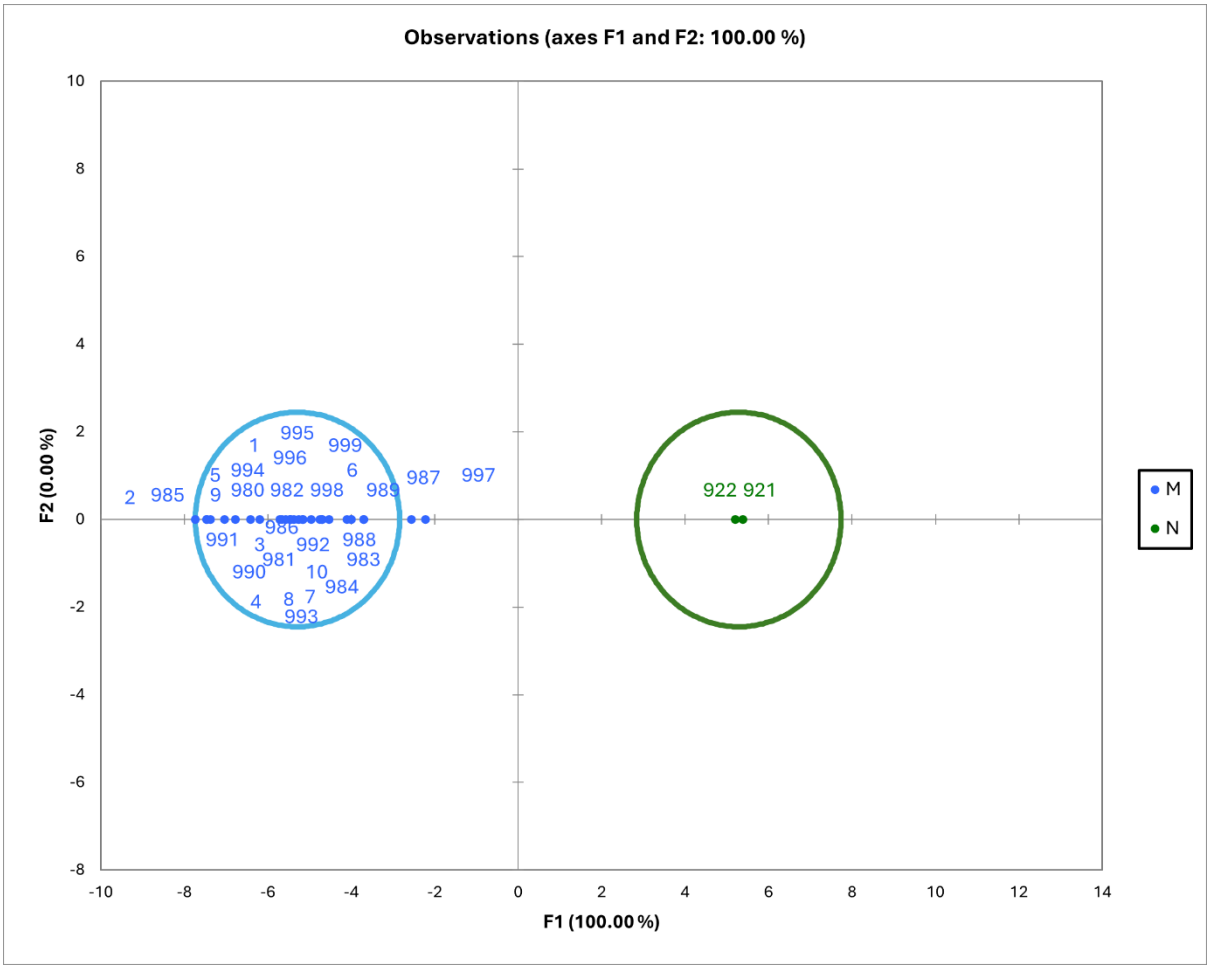


Figure 3.22: The DA analysis figure showing *O. mossambicus* from the University of Limpopo Pond and *O. niloticus* samples added from Cordier Dam to allow the system to run, as it requires a minimum of two different species for the analysis to compute. M-depicts *O. mossambicus* individuals, while N-depicts *O. niloticus* individuals.

The DA results generated confusion metrics to show the percentage of fish that were possibly correctly identified. (Table 3.5).

Table 3.5: Confusion metrics generated by discriminant analysis for University of Limpopo Pond.

The confusion metrics showed that the pond had only one species of *O. mossambicus*. All of them were correctly identified.

from \ to	M- O. mossambicus	N- O. niloticus	Total	% correct
M- O. mossambicus	16	0	16	100,00%
N- O. niloticus	0	16	16	100,00%
Total	16	16	32	100,00%

The figures below depict the fish that were on the outer side of the spheres.

Figure 3.23: Individual 987 was placed outside the discriminant analysis sphere next to the *O. mossambicus* sphere.

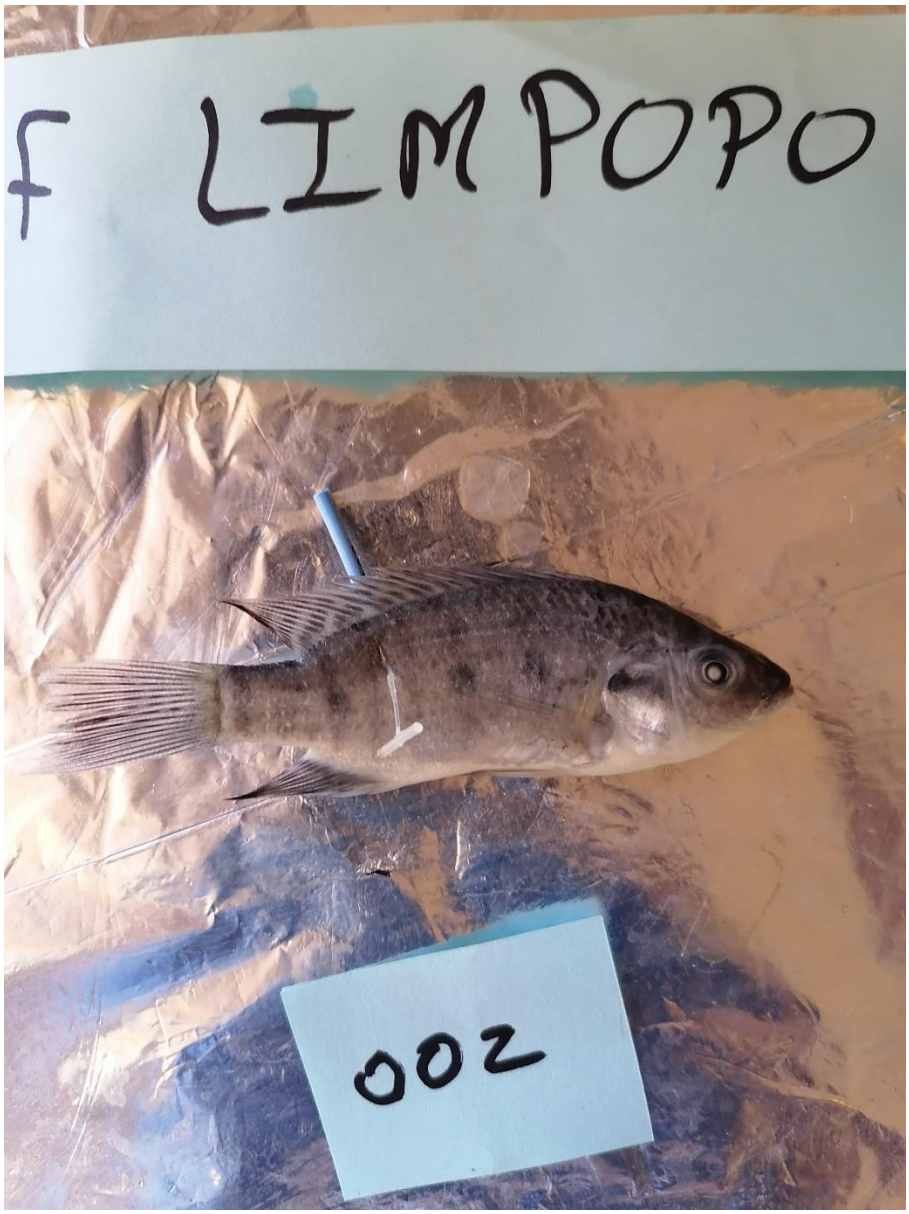


Figure 3.24: Individual 002 was placed outside the discriminant analysis sphere next to the *O. mossambicus* sphere.

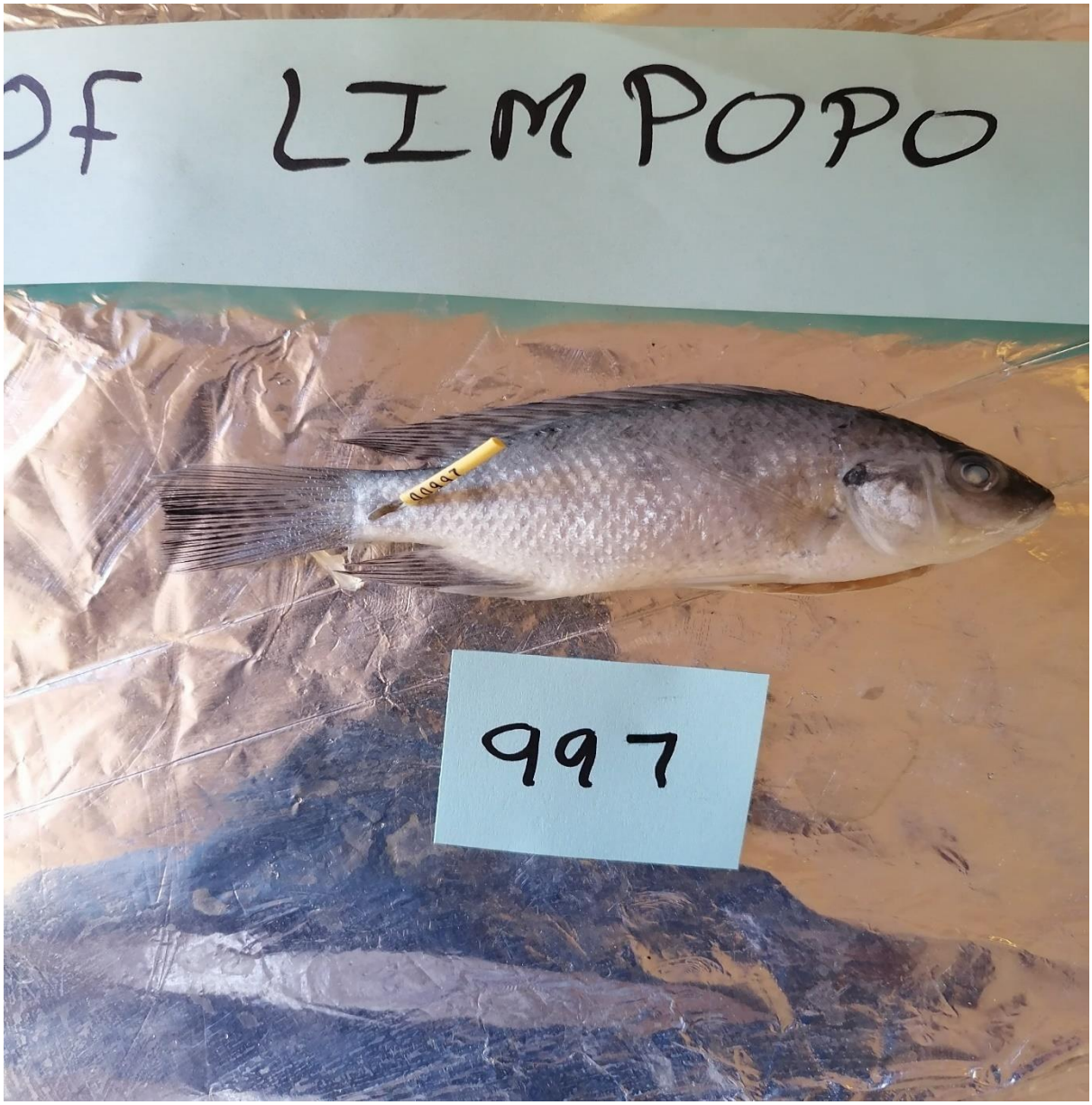


Figure 3.25: Individual 997 was placed outside the discriminant analysis sphere next to the *O. mossambicus* sphere.

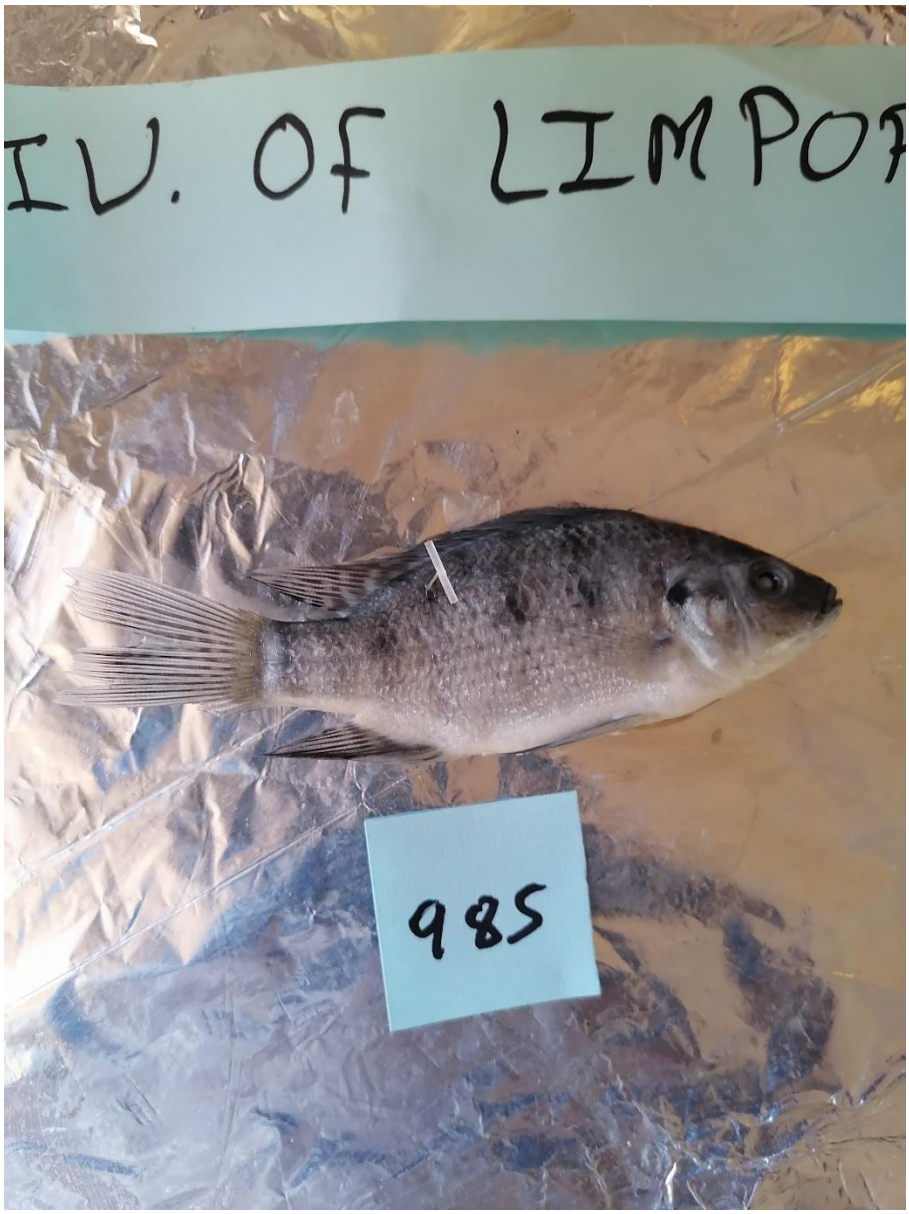


Figure 3.26: Individual 985 was placed outside the discriminant analysis sphere next to the *O. mossambicus* sphere.

3.5.6. MERISTIC CHARACTERISTICS

Meristic characteristics were also used to differentiate between the four tilapia species (Fig. 3.27). The meristic characteristics were able to differentiate amongst the four tilapia species into 4 distinct groups, grouping all the same species. They were separated into *C. rendalli, T. sparrmanii, O. mossambicus* and *O. niloticus*. This shows the effectiveness of meristic characters in the identification of various tilapia species. The three localities where *O. mossambicus* was present shared a sphere, however the differences in the number of gill rakers in the fish at different locations accounted for their slight separation.

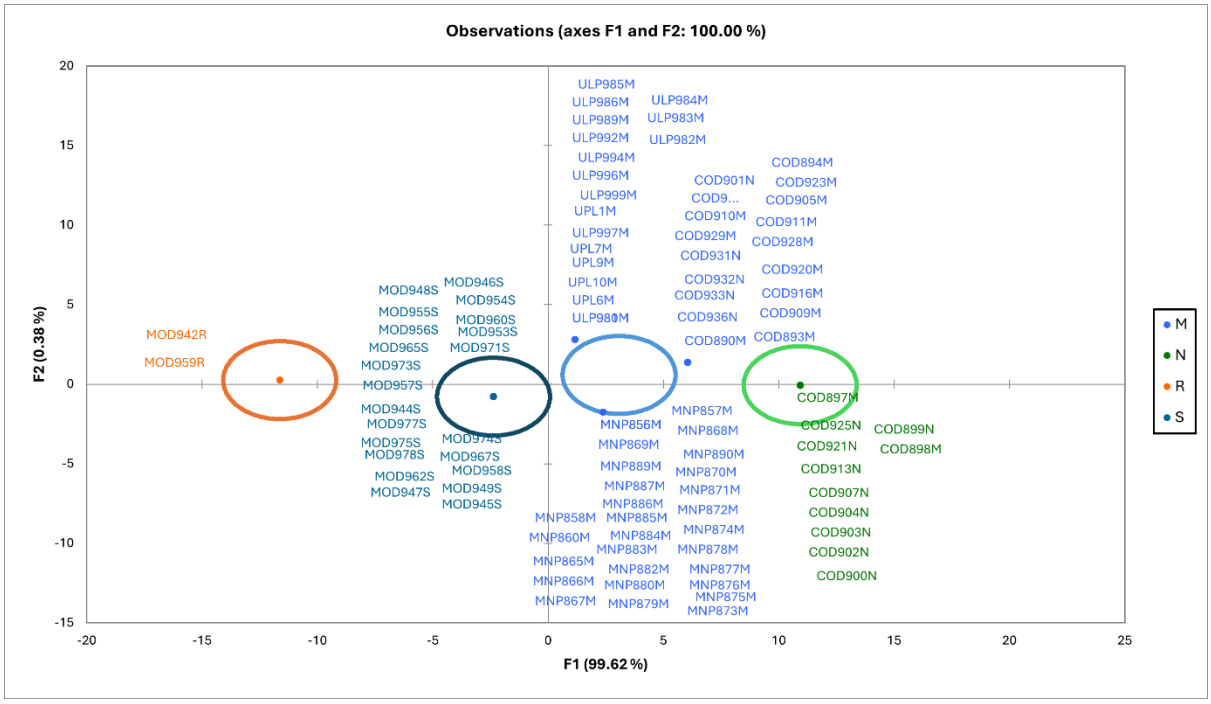


Figure 3.27: Meristic characteristics discriminant analysis taking into account the number of dorsal spines and rays, anal spines and rays, lateral line scales as well as the number of gill rakers in the tilapia individuals.

Key: M-depicts *O. mossambicus* (Sky blue color), N- depicts *O. niloticus* (Green color), R- depicts *Coptodon rendalli* (Orange color) and S depicts *Tilapia sparrmanii* (navy blue color). MOD- depicts individuals from Molepo Dam, ULP depicts individuals from University of Limpopo Pond, MNP- depicts individuals from Mall of the North Pond and COD depicts individuals from Cordier Dam.

3.5.7. PRINCIPAL COMPONENT ANALYSIS (PCA).

All the tilapia species were put together and analysed on SPSS to see which morphometric characteristics played a greater role in differentiating amongst the tilapia species. The principal component analysis showed that: The first two-axis PCA ordination explained 57.21% of the variation amongst the individual tilapia fish species (Table 3.6). Axis 1 explained 46.16% of the correlations between the variables and factors in the biplot. Axis 1 had a strong positive loading for total length (TL), standard length (SL), body depth (BD), Head length (HL), upper jaw length (UJL), length of dorsal fin base (LDFB), and Weight (W) (Table 3.7.). The second axis explained 11.05% of the correlations between the variables and the factors in the bioplot. Axis 2 had a strong positive loading for caudal peduncle length (LCP) (Table 3.7). Factors with strong positive loading had a value of more than 0.900.

Table 3.6: Eigenvalues values of the correlation matrix between (morphological characteristics) and the (tilapia species).

	F1	***F2***	***F3***	***F4***
Eigenvalues	*8,309*	*1,989*	*1,123*	*1,071*
Variability (%)	*46,159*	*11,052*	*6,239*	*5,952*
Cumulative (%)	***46,159***	***57,211***	***63,450***	***69,403***

Seven characteristics total length, standard length, body depth, head length, upper jaw length, length of dorsal fin base, and weight accounted for the most variation among the tilapia species.

Table 3.7. Correlation matrix between variables (morphometric characteristics) and factors (tilapia species).

	Axis1	***Axis 2***
	F1	F2
TL- total length	0.978	-0.001
SL- standard length	0.979	0.003
BD- body depth	0.978	0,016
PPECL- pre pectorial length	0.037	-0.018
PPELL- pre pelvic length	0.559	-0.037
PAL- pre anal length	0.771	-0.018
LAFB- length of anal fin base	-0.018	0.032
LCP- length of caudal peduncle	0.040	0.997
DCP-depth of caudal peduncle	0.054	0.678
CFL- caudal fin length	0.040	0.727
HL- head length	0.960	0.001
ED- eye diameter	0.021	0.036
SnL- snout length	0.112	0.004
ULJ- upper jaw length	0.929	0.018
LJL- lower jaw length	0.734	-0.043
PFL- pectorial fin length	0.607	-0.012
LDFB- length of dorsal fin base	0.983	-0.012
W- weight	0.926	-0.024

Three prominent clusters were identified on the biplot, where the length of the caudal peduncle (LCP), the caudal fin length (CFL), and the depth of the caudal peduncle (DCP) clustered together these were prominent in factor 2, showing little use in differentiation amongst the four tilapia species. Length of the anal fin, eye diameter, snout length, and Pre-pectoral length clustered around the center point of the biplot indicating that they are the same in all four species and cannot be used for

differentiation of the four species, while the rest of the characteristics total length (TL), Standard length (SL), Pre-pelvic length (PPELL), Pre-anal length (PAL), lower jaw length (LJL), weight (W), upper jaw length (UJL), Length of Dorsal fin base (LDFB), Pectoral fin length (PFL), Head length (HL) and body depth (BD) showed the greatest use in differentiation among the four tilapia species.1

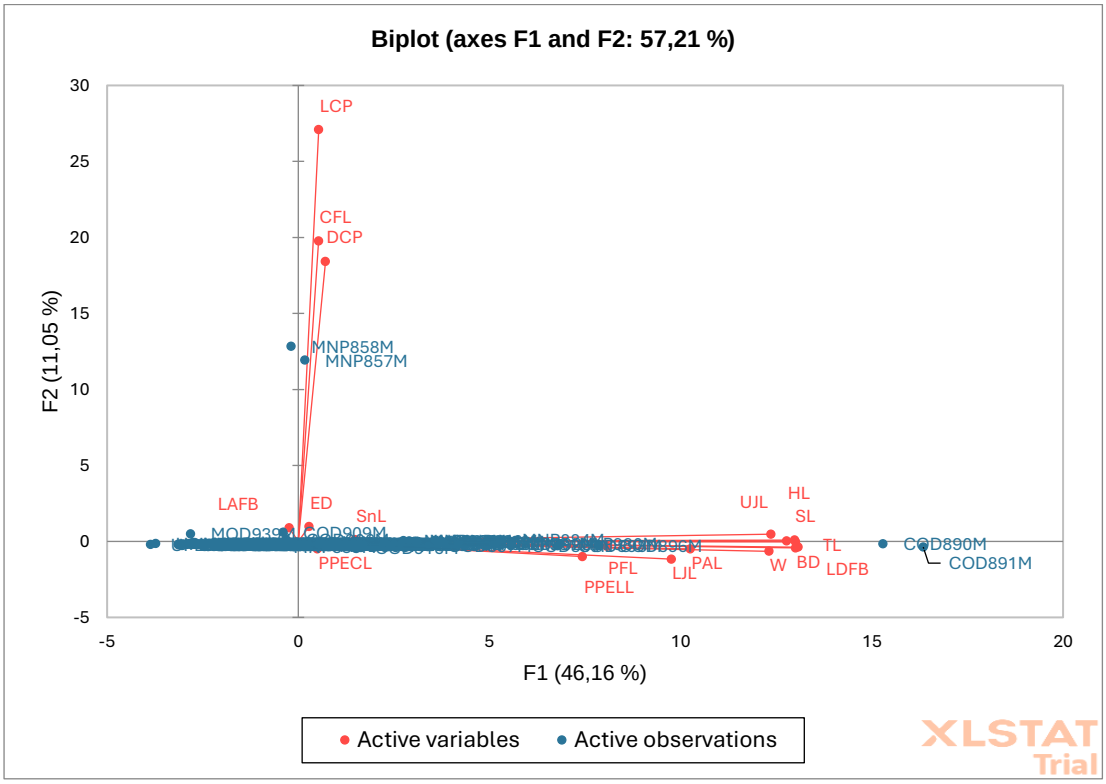


Figure 3.28. PCA biplot indicates the correlation between the morphometric characteristics and the fish species.

KEY: PAL-pre-anal length, BD- body depth, LCP- length of caudal peduncle, PPL- pre pectoral length, CFL- caudal fin length, PFL- pectoral fin length, SNL-snout length, ED-eye diameter, DCP- depth of caudal peduncle, HL- head length, SL-standard length, W- weight, LDFB- length of dorsal fin base, UJL- upper jaw length, LJL- lower jaw length, LAFB- length of anal fin base, TL- total length, PPECL-pre-pectoral length, PPELL- pre-pelvic length.

3.5.8. Analysis of variance (ANOVA)

After morphometric identification of the tilapia species, *Oreochromis mossambicus* species were isolated and a one-way ANOVA (Table 3.8) was performed on the samples to find out if morphometric variations were present among the *O. mossambicus* species in the different locations. Only 3 locations out of the four contained *O. mossambicus* species. These were the Mall of the North Pond, the University of Limpopo Pond, and some individuals within the Cordier dam. The table below presents the mean and standard error within the locations for the various characters and the post hoc analysis indicated in superscripts. Measurements with different superscripts in each row are significantly different from each other($P<0.05$).

Table 3.8: AMOVA and posthoc Turkey analysis of *O. mossambicus* between three locations, where it was found.

	Mall of the North	**University of Limpopo**	**Cordier dam**
TL- total length	14.6 ± 0.5^{a}	11.41 ± 0.6^{b}	12.78 ± 0.8^{ab}
SL- standard length	11.14 ± 0.4^{a}	8.75 ± 0.5^{b}	10.19 ± 0.7^{ab}
BD- body depth	3.93±0.2	3.24±0.2	3.72±0.3
PPECL- pre pectoral length	1471±9.6	3.58±0.2	4.09±0.3
PPELL- pre pelvic length	7.35 ± 0.2^{ab}	5.34 ± 0.3^{a}	6.29 ± 0.4^{b}
PAL- pre anal length	5.71 ± 0.3^{a}	6.12 ± 0.4^{ab}	6.96 ± 0.5^{b}
LAFB- length of anal fin base	2.10±0.6	1.91±0.3	1.69±0.1
LCP- length of caudal peduncle	7.61±4.3	1.09±0.1	1.53±0.2
DCP- depth of caudal peduncle	5.62±3.7	1.24±0.9	1.71±0.4
CFL- caudal fin length	9.35±6.3	2.35±0.1	2.59±0.1
HL- head length	4.01 ± 0.1^{a}	$3,24\pm0.2^{b}$	3.69 ± 0.3^{ab}
ED- eye diameter	0.72±0.02	0.57±0.03	3.31±2.7
SnL- snout length	2.47±1.9	0.43±0.1	0.45±0.4

UJL- upper jaw length	1.53±0.1	1.29±0.1	1.18±0.2
LJL- lower jaw length	1.11±0.1	0.98±0.1	1.10±0.3
PFL- pectorial fin length	3.41±0.1	2.95±0.2	3.19±0.2
LDFB- length of dorsal fin base	5.68±0.2	4.78±0.3	5.22±0.4
W- weight	51.77±5.4	33.93±7.6	50.46±19.8

One way ANOVA results indicate that characters regarding total length, standard length, pre-pelvic length, and head length were significantly different among the different locations.

A post hoc test utilizing Turkey HSD (Table 3.8) indicated morphometric variations in total length, standard length, pre-pelvic length, pre-anal length, and head length amongst the three locations. Total length, standard length, pre-anal length and head length were significantly different between the Mall of the North Pond and the University of Limpopo Pond while there were no significant differences between the two ponds with Cordier Dam. Pre pelvic length exhibited significant differences between Mall of the North Pond and the University of Limpopo Pond as well as between Mall of the North and Cordier Dam while no significant differences were noted between the University of Limpopo Pond and Cordier Dam. Characters regarding body depth, pre-pelvic length, length of anal fin base, length of caudal peduncle, depth of caudal peduncle, caudal fin length, eye diameter, snout length, upper jaw length, lower jaw length, pelvic fin length, length of dorsal fin base and weight exhibited no significant differences among the different locations.

3.5.9. DISCRIMINANT ANALYSIS OF THE *O. MOSSAMBICUS* IN THE THREE LOCALITIES

Discriminant analysis was done on the three populations of *O. mossambicus* to see if there are morphometric variations amongst the *O. mossambicus* species. They clustered according to their various location with prominent overlap amongst them (Fig. 3.29). This indicates that they are somewhat similar, although some characteristics have differences. The average condition factors of the localities, Mall of the North Pond (1.634), University of Limpopo (1.650), and Cordier Dam (1.602) showed no significant differences ($P<0.05$) at 0.427 among the fish in the localities. These results show that the variations were due to other factors such as genetics (Which will be confirmed in Chapter 5) or anthropogenic as they were living in environmental conditions that were relatively the same.

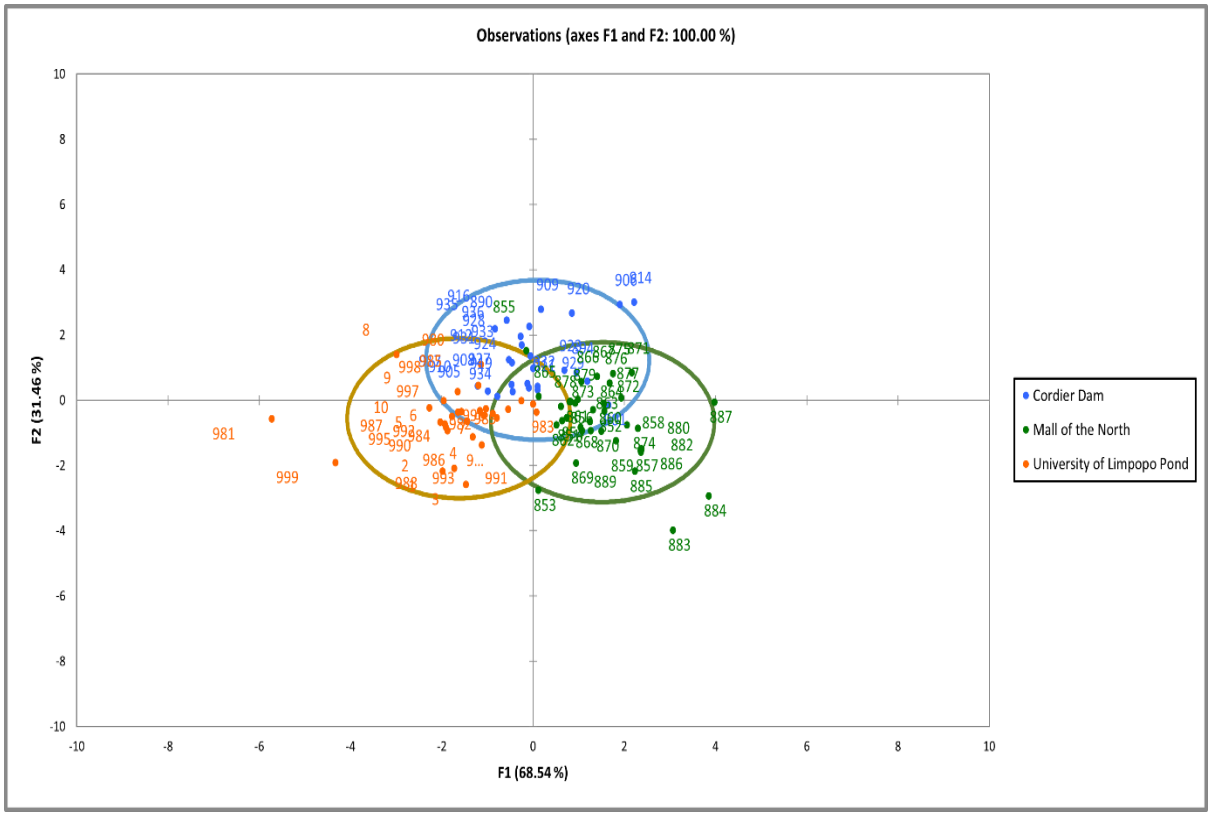


Figure 3.29: The discriminant analysis of *O. mossambicus* in three localities it was found. The blue sphere depicts *O. mossambicus* from Cordier Dam, green sphere from Mall of the North Pond and orange sphere from University of Limpopo Pond.

3.6. DISCUSSION

Morphometric and meristic features have been used in the identification of tilapia species (Barriga-Sosa *et al*., 2004, Naeem and Salam, 2005). Fish identification is important in stock selection especially in aquaculture to maximize profits, where certain fish species are favored for their faster growth and weight gain. Although morphometric and meristic features have successfully identified tilapia species, limitations have been noted. These were seen in the results of Cordier Dam (figure 3.3) which was shown to contain primarily two species of fish, according to physical observation and guidance from the Skelton guide. These were found to be *O. niloticus* and *O. mossambicus*. However discriminant analysis (Fig 3.3) showed a possibility of hybrids of *O. mossambicus* and *O. niloticus* species. This proves that *O. niloticus* has found its way into Cordier Dam and *O. mossambicus* is at risk of loss of genetic diversity due to hybridization (Todesco *et al.,* 2016), despite it being prohibited for distribution within Limpopo Province (Moshobane *et al*., 2020). It is however allowed for facilities with permits (Wilson and Kumschick, 2024).

The phenotypic observation could not decipher possible hybrids among the fish species. Cordier Dam presented some individuals, that were phenotypically identified as *O. mossambicus* yet they possessed *O. niloticus* features or vice versa (Fig 3.4 to 3.10). Hybrids can take any phenotypic form and characteristics of either parent (Barton 2001), increasing the confusion factor in morphometric identification (Fig 3.11-3.12). Figure 3.11 and figure 3.12 presented another limitation to hybrid identification as discriminant analysis concluded that the two individuals had *O. niloticus* morphometric characters however, their phenotype was of *O. mossambicus* individuals where they lacked the prominent bands on their caudal fin to constitute them as *O. niloticus* species. Phenotypic observation is therefore inappropriate for hybrid identification but can be used effectively in pure tilapia species. This was seen in identifying *O. mossambicus* species in the Mall of the North Pond (Fig 3.16) and the University of Limpopo Pond (Fig 3.22).

Cultivation of tilapia has been hindered by factors that include difficulties in species identification due to hybridization among sister species. Molepo Dam discriminate analysis results (Fig 3.13) yielded *C. rendalli* and *T. sparrmanii s*pecies within the Dam. Discriminant analysis showed two distinct spheres with no overlap between the two

species. This indicates that morphometric measurements of these two species can be used to classify the two fish species. Individual 960 was confirmed to be *C. rendalli as* shown by the discriminant analysis. It was initially incorrectly identified as *T. sparrmanii*. This highlights the efficiency of DA and morphometric measurements in the classifying of these tilapia species. This implies that morphometric analysis can be successfully used in classifying and identifying these two species. Where there is no hybridization amongst the tilapia species morphometric and meristic characteristics can therefore be used for the identification and classification of tilapia species. Barriga-Sosa *et al.,* 2004 was also successful in classifying two tilapia species using discriminant analysis. Meristic characters were also employed to support the discriminant analysis data.

Meristic traits are commonly used to differentiate species populations (Sedaghat *et al*., 2012). In this study, they were also subjected to discriminant analysis. They were successful in identifying and classifying the four tilapia species in the four localities (Fig 3.26). Meristic characteristics are determined by the response of the fish to short-term environmental factor variations during embryonic development (Samaradivankara *et al.,* 2012). These could result in wide variations among members of the same and even different year classes of a single stock of fish (Lindsey, 1988). This was seen in the three *O. mossambicus* populations (Fig 3.26) which had variations in the number of gill rakers. According to Skelton, 1993, there is a possible range for the number of gill rakers and other meristic characters such as dorsal spines and rays, in *most tilapia* species The University of Limpopo Pond *O. mossambicus* population had the highest number of gill rakers (26). Matsumota and Kohda, 2001 noted that variations in the diet of the fish could contribute to variations in gill raker numbers. Jawad *et al.,* 2020, and Fagbuaro, 2015, found no variations in the meristic characteristics of the *O. niloticus* and *T. zillii* populations in their studies.

Morphometric features were better in the identification of tilapia species as compared to meristic characteristics, because all tilapia species share some meristic characteristics and, some meristic characteristics differed in the same species such as the number of gill rakers, and dorsal spines (Skelton, 1993), in this case for *O. mossambicus*. This can create a challenge in classifying as it may evoke incorrect placement of tilapia species. This was seen in the differences in the number of gill rakers in the *O. mossambicus* species. It is thus suggested that both morphometric

and meristic characters are used together to verify the classification of tilapia species. Species such as *O. niloticus* and *O. aureus* have similarities in meristic characters. This includes *O. shiranus* and *O. placidus, and/or O. mossambicus and O. mortimeri* (Skelton,1993) in the tilapia subfamily. Their differences are generally phenotypic, which can pose a challenge if only meristic characteristics are used for identification. Principle component analysis (PCA) was employed to see which characteristics were most significant in differentiating among the tilapia species.

The eigenvalues for four components of PCA (F1-F4) amounted to 69.403 % which is well below the required limits for PCA to apply. This was due to the close similarities shared among the species.

When comparing the discriminant analysis and PCA results, PCA showed that all the species were relatively similar in morphometrical characteristics were only 4 samples were outliers (MNP858M, MNP857M, COD890M and COD891) on the biplot (Figure 3.28). DA did not highlight anything peculiar about these four individuals (Fig 3.3 and Fig 3.16) as they were placed within the spheres of analysis, which shows that they were correctly measured. So it is not immediately clear why they are outliers in this regard. PCA (Table 3.7) and (Fig 3. 27) showed that Total Length, Standard Length, Body Depth, Head Length, Upper Jaw Length, Length of Dorsal Fin Base, and Weight had the strongest positive correlation (>0.900) for differentiation among the four tilapia fish species, and these characteristics could be utilized for effective differentiation among the four tilapia species.

Total length, standard length, body depth, and weight are indeed used in the differentiation of tilapia species because the tilapia species of the same age are known to grow at different lengths with varying weights. This is seen in the growth proficiency of *O. niloticus* which is favored for its faster growth compared to the other tilapia species, hence its use worldwide in aquaculture (Skelton, 1993, Eknath and Hulata, 2009, De Verdal *et al.,* 2014 and Munguti *et al*., 2022). Tilapia like *T. sparrmanii* and *C. rendalli* are overlooked as great aquaculture species because of their smaller size and slower growth respectively (Skelton, 1993, Weyl and Hetch, 1998). The length of the dorsal fin base (LDFB) varied in all four tilapia species and showed the highest value of 0.983. This feature is one of the features used even in phenotypic differentiation. The dorsal fin is used to stabilize the fish during movement thus

preventing rolling and assisting in sudden turns. Since the tilapia species are of different lengths and weights, the dorsal fin will have to vary to compensate for its effective functions on that specific species. Therefore the dorsal fin of *T. sparrmanii* cannot be the same length as that of *O. niloticus* of the same age.

Upper jaw length and head length also showed a high correlation of over 0.900. It was expected that they too would yield a strong positive correlation for differentiation amongst the four tilapia species as their head profiles are not the same and are used for their phenotypic differentiation. *Coptodon rendalli* is well recognized by its convex head and beak-like protruding mouth, while the head profile of *O. mossambicus* is straight in juveniles and females, and concave in mature males (Skelton, 1993). The presence or absence of stripes on the caudal fin of *O. niloticus* was key in the main differentiation of the species.

The length of the Caudal Peduncle had the strongest positive correlation in factor 2 (0.997), indicating that the length of the caudal peduncle was similar or the same in all four tilapia species. All four different species overlapped on the discriminant analysis plot, indicating morphometric similarities among all the tilapia fish species. Samaradivakara, *et al.,* (2012), noted that morphometric characteristics could be susceptible to environmental factors. In their study standard length, body depth, posterior end of the dorsal fin to the dorsal origin of the caudal fin, and origin of the dorsal fin to insertion of the pelvic fin had the strongest correlation in factor 1. The findings of Samaradivakara, *et al*., (2012) indicated the existence of localization of tilapia fish had occurred according to the morphometric characters of the fish. A comparison of *O. niloticus* species in three localities was investigated to note if there are any morphometric variations between the tilapia of the species. Morphometric variations were found amongst the tilapia species in the different locations.

Variation in body form has important fitness consequences in fish both in cultured and wild species (Gulliet *et al*., 2003). Morphological differentiation between fish populations in different localities/ habitats may not be related to genetic differentiation alone but by the inclusion of environmental factors or their interactions (Kara *et. al.,* 2011). Morphological variability of fish was reported to be an important adaptive strategy for populations experiencing inconsistent environmental conditions (Scheiner, 1993). Environmental factors, on the other hand, can produce phenotypic plasticity,

which is the capacity of a genotype to produce different phenotypes in different environmental conditions (Scheiner, 1993). This was evident in this study where *O. mossambicus* species of three localities were seen to have differences in their morphometric characteristics (Fig 3.27).

The differences among the *O. mossambicus* species (Table 3.8), showed that there were indeed differences in the morphological characteristics of the individuals of the same species. The fish in Mall of the North Pond exhibit significant differences in certain body lengths (total length, standard length, and pre-pelvic length) compared to those in the University of Limpopo Pond and Cordier Dam. This suggests that ecological factors at the Mall of the North Pond may be influencing these specific body dimensions. Diet could be the differentiating factor in this case because the *O. mossambicus* at the University of Limpopo Pond and Cordier Dam are not fed any commercial pellets and rely solely on natural food from the pond and dam respectively. However, the lack of significant differences in most other body dimensions indicates that, despite variations in a few specific measurements, the overall body shape and size of the fish are relatively consistent across the three locations. In essence while everything else is consistent in all three locations, total length, standard length, pre-pelvic length, pre-anal length and head length vary in the *O. mossambicus* populations of these three localities. Ikpeme *et al,* 2017, Mahmoud and Hassan, 2019 also noted variations in *O. niloticus* species from different regions. This shows that this phenomenon is not unique to *O. mossambicus* species only. A study by Herath *et al.*, (2014) looking at the morphological variations in three *O. mossambicus* populations using 12 morphometric characteristics, when analysed using ANOVA, showed that characters regarding caudal fin length, pre-anal length, distance from the anterior end of the dorsal fin to the posterior end of pelvic fin were significantly different among locations ($P<0.05$). These morphometric differences in the three *O. mossambicus* populations allowed for the rejection of the null hypothesis that there was no morphological variation between the three *O. mossambicus* populations. Their work agrees with the findings of this study.

In conclusion, tilapia identification is complex due to factors such as hybridization among sister species and plasticity or phenotypic variations in the same species. In this study hybridisation between *O. niloticus* and *O mossambicus* was noted. It was found that the phenotype of hybrids can indeed take the form of either parent making

it difficult to deduce the species, thus requiring genetic probing for conclusive evidence of the species in identification for effective record keeping in stock delimitation.

4. THE GENETIC IDENTIFICATION OF TILAPIA SPECIES AND THEIR POSSIBLE HYBRIDS IN FOUR LOCALITIES, IN LIMPOPO PROVINCE, SOUTH AFRICA.

4.1 INTRODUCTION

Tilapia are the second most cultured non-cyprinid species (FAO, 2021). Most of the tilapia cultured are *Oreochromis. niloticus* (Nyirenda, 2017, Moyo and Raphatsa, 2021). *Oreochromis niloticus* is the preferred species because of its fast growth rate compared to other tilapia species (Schawrzer *et al*., 2009). This has led to its widespread introduction in different countries (Nyingi *et al*., 2009, Lind *et al*., 2015). However, this introduction has resulted in extensive hybridization with other tilapia species particularly *Oreochromis mossambicus* (Peters 1852) in South Africa (D'Amato *et al*., 2007, Zengeya *et al.,* 2013), *Oreochromis aureus* (Steindachner 1864) in West Africa (Rognon & Guymard, 2003; Bakhoum *et al*., 2009), *Oreochromis andersonii* (Castelnau 1861) and *Oreochromis macrochir* (Boulenger 1912) in Zambia (Deines *et al*., 2014), and *Oreochromis esculentus* (Graham 1928) and *Oreochromis leucostictus* (Trewavas 1933) in Kenya (Nyingi & Agne`se 2007; Angienda *et al*., 2011; Ndiwa *et al.*, 2014). Poor hatchery management practices have led to hybrid introgression (Amoussou, 2017). The phylogenetic status of tilapia has become complex, and identification of tilapia species is now difficult (D'Amato *et al*., 2007, Syaifudin, 2015, Barman *et al*., 2018, Dailami *et al.,* 2021). *Oreochromis niloticus* has established viable populations in the Limpopo River (D'Amato, 2007). It has spread outside the Limpopo River and is now found in different Limpopo sub-catchment areas (Zengeya *et al*., 2013, 2015).

Oreochromis mossambicus is native to the Limpopo Province. However, the phylogenetic status of *O. mossambicus* in these ecosystems has not been adequately investigated. The few phylogenetic studies by D'Amato, *et al*., 2007, and Zengeya *et al*., 2015 undertaken in South Africa noted the looming threat of further genetic encroachment of *O. mossambicus* by *O. niloticus* and called for further investigation inland in streams, dams, and ponds to see the extent of hybridization, however, none of the studies have genetically identified the tilapia species we have in our four localities and the phylogenetic status of wild and cultured *O. mossambicus* populations in those localities is yet to be investigated.

Different identification methods were chosen in this study because of the complex genetic status of tilapias. Cytochrome oxidase subunit I (COI) is essential in the identification of tilapia species (Ferguson *et al.,* 1995, Herbert *et al*., 2003, Pentinsaari, *et al.,* 2016), however it cannot effectively identify hybrids. 5S was therefore employed to identify possible hybrids by comparing both marker phylogenetic results and verifying them with a haplotype TCS analysis.

4.2 OBJECTIVES

4.2.1 To identify tilapia species and possible hybrids in four localities, using mitochondrial DNA cytochrome oxidase subunit I (COI) and ribosomal DNA (5S rDNA) markers.

4.2.2 To identify possible hybrids amongst the tilapia species in the four localities.

4.3 MATERIALS AND METHODS

4.3.1 STUDY AREA REFER TO CHAPTER 3

4.3.2 DNA EXTRACTION, PCR, AND SEQUENCING

Approximately thirty fish per locality were collected using seine nets and a cast net, with specimens humanely euthanized with an overdose of 2-phenol ethanol and subsequently preserved in 70% ethanol. The Genomic DNA was extracted from small body muscle tissue biopsies using the NucleoSpin®Tissue kit (Macherey–Nagel, Düren, Germany) extraction kit following the manufacturer's instructions. The quality of the DNA was estimated using 1% agarose gel electrophoresis at 100 volts for 30 minutes and viewed under UV light. The quantity of the DNA was estimated using a spectrophotometer (NanoDrop, Thermo Fisher Scientific Inc., USA). The extracted DNA was then stored at −20˚C until it was used for PCR.

The polymerase chain reaction (PCR) was used to amplify the targeted mitochondrial DNA and nuclear DNA regions by employing the primer pair FishF1: 5′-TCAACCAACCACAAAGACATTGGCAC-3′ and FishR1: 5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′ (Ward *et al.*, 2005) for the cytochrome oxidase *c* subunit I region and 5SA: 5′-TACGCCCGATCTCGTCCGATC-3′ and 5SB: 5′-CAGGCTGGTATGGCCGTAAGC-3′ (Komiya and Takemura, 1979) for the 5S rDNA region. Only DNA aliquots with a concentration between 1.7- 2.0 were used for the

polymerase chain reaction. The PCR amplification reaction was set to a volume of 30 µl, containing eight ng/µl of template genomic DNA, one µl of forward and one µl of reverse primers, 12 µl of Taq Master Mix, and eight µl nuclease-free water. PCR cycling was performed in the GeneAmp® PCR system (9700 thermal cycler, USA) using an initial denaturation step at 95°C for 15 min, followed by 36 cycles of denaturation at 94°C for 45 s, optimum annealing temperatures (54.4°C for COI and 59°C for 5S rDNA) for 45 s, and extension at 72°C for 1 min and a last extension step at 72°C for 10 min. PCR products were checked on 1% agarose gel electrophoresis against a DNA ladder for 30 min at 120V and visualized under ultraviolet light, as stipulated in Ekerette *et al*., (2018). The DNA ladder was used as a reference to ensure that the correct size fragments were amplified at 658 bp for COI and 420 bp for 5S rDNA. The successful PCR amplicons: 61 COI and 45 5S rDNA with clear bands, were sent for sequencing at Inqaba Biotechnical Industries, South Africa, using the forward primers. The remaining samples were not used due to poor PCR amplicon production.

The primers were selected for their known efficiency in identification, and the 5S primer was instrumental in hybridization identification. Several genetic applications have been used for identification, but mitochondrial DNA stands out as the best (Ferguson *et al.,* 1995, Herbert *et al*., 2003, Pentinsaari *et al.,* 2016). This is proven by the vast Barcode of Life catalogue containing 325 737 barcode sequences of formally described species of flora, fauna, fungi, and protists (www.boldsystems.org, 25 April 2024). The Barcode of Life contains sequences extracted from mitochondrial Cytochrome *c* oxidase subunit I gene (COI), which has proved to be the most effective for identification, including for cichlids where records of identified Cichlidae were 11 254 barcoded sequences with 969 identified species (www.boldsystems.org, 25 April 2024); hence it was used in this study.

Cytochrome *c* oxidase subunit I gene (COI) has a higher mutation rate than single-copy nuclear DNA (Ferguson *et al*., 1995). It is maternal in inheritance and lacks recombination, making it ideal for identification at around 658 bp (Herbert *et al.,* 2003). This means that the Cytochrome *c* oxidase subunit I gene (COI) can be used to organize individuals into maternal lineages, even after hybridization has occurred (Ferguson *et al*., 1995); this makes it ideal for identification. Nuclear DNA is used to

test for hybridization and determine if interbreeding has occurred. In this study, 5S ribosomal DNA was utilized.

Ribosomal RNA (rDNA) genes are organized as two distinct muti-gene classes consisting of tandemly arrayed repeats composed of hundreds to thousands of copies (Martins *et al*., 2000 and Alves-Costa, 2006). The 5S part of ribosomal DNA consists of a highly conserved coding sequence of 120 base pairs (bp) (Figure 4.1), which is separated from each transcriptional unit by a variable non-transcribed spacer (NTS) (Pendas *et al*., 1994, Alves-Costa, 2006 and Martins *et al.,* 2004). A dual 5S ribosomal RNA gene system (Type I and Type II) was described for vertebrates, including fish and amphibians (Komiya *et al.,* 1986). The 5SA and 5SB primers cut for both the 5S gene and the NTS segment, giving a sequence of about 490 base pairs (Martins and Galetti, 2001).

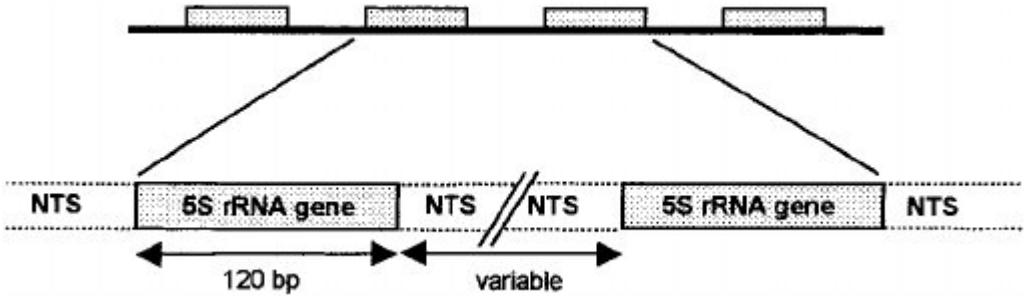


Figure 4.1: Arrangement of higher eukaryotic 5S rRNA genes intercalated with non-transcribed DNA segments (NTS) (Martins and Galetti, 2001).

4.3.3. OUTGROUP SELECTION

The outgroup was selected based on close relativeness and the availability of sequences for the chosen primers in the GenBank nucleotide database. However, due to the paucity of 5S rDNA reference sequences in Genbank, *Symphysodon sp.* was the only closest relative of tilapia species with both COI and 5S rDNA sequences available in GenBank (Table 4.1), and therefore, it was chosen as an outgroup.

4.3.4 PHYLOGENETIC ANALYSES

A total of 61 COI sequences were received from Inqaba, of which 17 were from the University of Limpopo Pond, 18 were from Mall of the North Pond, 21 were from Cordier Dam, and five were from Molepo Dam. For 5S rDNA, a total of 45 sequences were received from Inqaba, four from Molepo Dam, 15 from Cordier Dam, 12 from the University of Limpopo Pond, and 14 from Mall of the North Pond. All sequences were manually checked, and primer sequences and ambiguous bases were removed using Chromas V2.6.5. All cleaned sequences were run against the NCBI GenBank database using nucleotide blast to identify the sequences at the species level. Additionally, sequences of tilapia species from previous studies for each marker were downloaded from GenBank and used as reference sequences (Table 4.1). They were chosen because they were the closest identified match on NCBI BLAST species regarding e-values (e-values maintained at 0.0 for COI and 5S from 0.0 to 6e-178 at more than 96% similarity percentage identification). A total of 102 COI sequences (61 from the current study and 41 from Genbank) comprising *O. mossambicus, O. niloticus, O. aureus, T. sparrmanii and C. rendalli (previously T. rendalli*) from the current study and GenBank, respectively were combined.

Meanwhile, 5S rDNA comprised 79 sequences (45 from the current and 34 reference sequences from GenBank). The combined sequences were then aligned using CLUSTAL W, as illustrated by Thompson *et al*. (1994), and trimmed to equal length (580 bp for COI and 470 bp for 5S rDNA) in BioEdit v7.2.6 (Hall, 1999). The best-fitting substitution model for each dataset was identified using the correct Akaike information criteria (AIC) (Akaike, 1973) in jModeltest v2.1 (Darriba *et al.,* 2012), which was shown to be GTR+I+G for both COI and 5S rDNA. All sequences from the current study were submitted to GenBank to obtain the accession number (Table 4.1). All the hybrids seen in the overlap of chapter 3 (Figure 3.3.) failed to amplify due to poor amplicons. Unfortunately this study does not contain these sequences, however they will be added in a future paper after they are rerun.

4.3.4.1. Phylogenetic tree construction

Phylogenetic trees were constructed individually based on the COI and 5S rDNA sequences. The trees were constructed for each locality separately and then for all localities together for each marker. The Bayesian inference (BI) was used to infer the

phylogenetic relationship among the COI and 5S rDNA sequences in MrBayes v3.2. (Ronquist *et al*., 2012). Each analysis consisted of two runs for 10 million generations, sampling each chain every 1000th generation using default parameters (Ronquist *et al*., 2012). The first 25% of the trees are discarded as burn-in. A 50% majority-rule consensus tree was then retained from each run as part of the output, and nodes with posterior probability ≤0.95pP were regarded as poorly resolved. Tracer v1.6 (Rambaut *et al*., 2014) was used to visualize the convergence of the runs (ESS>200); trees where ESS<200 were considered poorly resolved. Maximum likelihood (ML) trees were also constructed using RAxML with 1000 bootstrap replications, followed by a search for the best-scoring ML tree and default parameters (Trifinopoulos *et al.*, 2016). Bootstrap values ≥75% were considered as well supported. A general time-reversible model with GTR+I+G was used throughout the maximum likelihood analysis. The final trees were visualized in Fig Tree v1.43 (Rambaut, 2016).

Table 4.1: List of localities where samples used in the current study were collected, their respective number of sequences (*N*), GenBank accession numbers, reference sequences, and their country of origin.

Species	*N*	Sample ID	GenBank accession number		Origin	Reference
			5S rDNA	COI		
Oreochromis mossambicus	9	C47, C3, C35, C40, C46, C19, C27, C28, C22 respectively	PP296916-PP296923	-	Cordier Dam	Current study
Oreochromis mossambicus	14	MP12, MP35, MP1, MP5, MP13, MP20, MP28, MP29, MP30, MP34, MP6, MP15, MP8 respectively	PP296926-PP296939	-	Mall of the North	Current study
Oreochromis mossambicus	13	UL1, UL29, UL12, UL8, UL14, UL15, UL17, UL21, UL22, UL23, UL 25, UL26, UL28 respectively	PP296940-PP296952	-	University of Limpopo Pond	Current study

Species	*N*	Sample ID	GenBank accession number		Origin	Reference
			5S rDNA	COI		
Oreochromis mossambicus	6	-	GU075907-GU075912		China	Zhu *et al.,* 2010
Oreochromis urolepis hornorum	6	-	GU075913-GUO75918	-	China	Zhu *et al*., 2010
Oreochromis ***niloticus***	2		AF176504-AF176505		Canada	Martins *et al.,* 2000
Oreochromis niloticus	3		AF176349, AF176500-AF176501		Canada	Martins *et al*., 2000
Oreochromis niloticus	4		AY945239-AY945242		Brazil	Alves-Costa *et al*, 2006
Oreochromis niloticus	6		AY945256-AY945261		Brazil	Alves-Costa *et al*, 2006
Oreochromis niloticus	6	C5, C18, C6, C7, C11, C10	PP296952-PP296955, PP296957-PP296958		Cordier Dam	The current study
Tilapia sparrmanii	4	Mo3, Mo28, Mo35, Mo36	PP296959-PP296962		Molepo Dam	The Current study
Tilapia rendalli	6		AY945234-AY945238, AY945253		Brazil	Alves-Costa *et al*., 2006
Symphysodon sp.	2	-	KP715280-KP715281		China	Yu., 2015 unpublished
Oreochromis mossambicus	13	C17, C19, C21, C22, C23, C25, C28, C31, C35, C39, C44, C45, C46, C12 respectively	-	PP264662-PP264674 PP267919	Cordier Dam	Current study
Oreochromis mossambicus	17	MP12, MP35, MP6, MP7, MP8, MP10, MP13, MP15, MP16, MP18, MP20, MP22, MP26, MP29, MP34, MP37, MP38 respectively	-	PP264645-PP264661	Mall of the North Pond	Current study
Oreochromis mossambicus	18	UL12, UL29, UL1, UL3, UL4, UL6, UL8, UL9, UL10, UL14,	-	PP264627-PP264644	University of Limpopo Pond	Current study

Species	*N*	Sample ID	GenBank accession number		Origin	Reference
			5S rDNA	COI		
		UL20, UL21, UL22, UL23, UL25, UL26, UL27, UL28 respectively				
Oreochromis mossambicus	3		-	MZ269461, MZ269466-MZ269467	Mozambique	Ferrari *et al.*, 2022; Ferrari *et al.,* 2021
Oreochromis mossambicus	2		-	OL306350, MK497065	South Africa	Chakona, 2021; Mojekwu *et al.*, 2021 both unpublished
Oreochromis mossambicus	3		-	MT418253, MT418256, OQ286133	Panama	Diaz-Ferguson, 2020; Diaz-Ferguson *et al.*, 2023 both unpublished
Oreochromis mossambicus	1	-	-	MK497156	Zimbabwe	Mojekwu *et al.*, 2019
Oreochromis mossambicus	1	-	-	ON604293	Madagascar	Vences *et al.*, 2022
Oreochromis niloticus	7	C29, C6, C7, C10, C18, C24, C27 respectively.	-	PP267919-PP267924, PP267926	Cordier Dam	Current study
Oreochromis niloticus	3		-	MK497139, MK497137-MK497138	South Africa	Mojekwu *et al.*, 2021. unpublished
Oreochromis niloticus	4		-	LC052672, LC487083, MG428623, KM438538	Egypt	Zein and Ezzalregal, 2015. unpublished
Tilapia sparrmanii	5	Mo33, Mo12, Mo14, Mo15, Mo40 respectively	-	PP273156, PP277040-PP277043	Molepo Dam	Current study
Tilapia sparrmanii	6		-	HQ567378, HQ567374, HM914632, HQ567347, HQ567373, HQ567377	South Africa	Van der Bank and Greenfield, 2010 unpublished
Coptodon rendalli	2		-	HQ959715, HQ959717	South Africa	Van der Bank and Greenfield, 2010 unpublished
Coptodon rendalli	8		-	JN989243-JN989250	Brazil	Pereira *et al.*, 2013

Species	*N*	Sample ID	GenBank accession number		Origin	Reference
			5S rDNA	COI		
Oreochromis aureus	3		-	KM438527-KM438529	Egypt	Syaifudin *et al*., 2014
Oreochromis aureus	5		-	MF817703-MF817707	South Africa	Marr *et al*., 2017

4.3.5. HAPLOTYPE NETWORK ANALYSIS

Haplotype networks were constructed using the COI locus (as the fastest-evolving mitochondrial marker) to support the COI identification analysis of the phylogenetic tree. All 61 COI sequence alignments were performed by MEGA X (Kumar *et al*., 2018) and trimmed to equal length in the FaBox online toolbox (Villesen, 2007). Haplotype networks were constructed using DnaSP v.6 (Rozas *et al*., 2017) and TCS in POPART (Clement *et al*., 2002).

4.4. RESULTS

4.4.1. Phylogenetic analysis, all locations combined COI and 5S analysis and tree.

Bayesian Inference (BI) and Maximum Likelihood trees in both markers (COI and 5S rDNA) generated similar topologies; therefore, only the BI trees are presented. COI marker recovered five well-supported clades (Clade A-E: Fig. 4.3) separated into different species and was congruent with morphological identification except where hybrids are concerned. Clade A comprised *O. mossambicus* reference sequences from GenBank, sequences from the cultured earthen pond systems at UL Pond and Mall of the North Pond, and some sequences from the wild Cordier Dam population. There was no genetic difference between the cultured and wild *O. mossambicus* individuals in all three locations with *O. mossambicus* species. Its sister clade B comprises *T. sparrmanii* reference sequences and sequences from Molepo Dam. The rows and the columns represent the comparison of the 5 species with its self and each other. A genetic divergence of 0.02 was noted amongst the *T. sparrmanii* population. It is also worth noting that, within clade B, sample Mo40 exhibited evidence of genetic divergence (0.02) (Table 4.2) from the rest of the individuals in that clade, which means it was genetically different by 0.02 compared to its sisters meaning, it had altered or different codons or mutations.

Table 4.2. The genetic diversity of Mo40 compared to the other test *Tilapia sparrmanii* individuals

	1	2	3	4	5
PP273156 *Tilapia sparrmanii* Mo33F		*0.00*	0.00	0.00	0.01
PP277040 Tilapia sparrmanii Mo12F	0.00		0.00	0.00	0.01
PP277041 *Tilapia sparrmanii* Mo14F	0.00	0.00		0.00	0.01
PP277042 *Tilapia sparrmanii* Mo15F	0.00	0.00	0.00		0.01

PP277043 Tilapia sparrmanii Mo40F	0.02	0.02	0.02	0.02	

Clade C, comprised of *O. niloticus* reference sequences and a few sequences from wild Cordier Dam. Sister clades D and E comprising of *C. rendalli* and *O. aureus*, respectively were sister to the other three clades. Based on the COI tree, there is no *O. aureus* species in any of the test localities. It must be noted that in chapter 3 *C. rendalli* was found in Molepo Dam, but due to poor quality of the DNA amplicons those *C. rendalli* samples couldn't be analysed, due to poor amplicon quality, hence none were noted for DNA identification and verification on the phylogenetic trees, hence only the genbank sequences are on the tree. The phylogenetic tree further highlighted the relationship between tilapia species, where *O. mossambicus* was sister to *T. sparrmanii*, *C. rendalli* showed sister relationship with *O. aureus* while *O. niloticus* was placed at the centre of the tree. The groupings showed occurrence of three tilapia species in the four test localities. The results further show that *O. mossambicus* is the most widespread tilapia species in Limpopo Province, as it was found in three of the four localities except for Molepo Dam. On the other hand, *O. niloticus* and *T. sparrmanii* were only found in Cordier Dam and Molepo Dam, respectively.

4.4.2 ALL FOUR LOCATIONS COMBINED COI PHYLOGENETIC ANALYSIS AND TREE

The Bayesian Inference posterior significant probability values are above the node (#), and the maximum likelihood significant bootstrap values are below the node (*). BI ≤ 0.95pP is considered well-supported (#). ML Bootstrap values ≥75% are considered well resolved (*). UL depicts *O. mossambicus* cultured samples from the University of Limpopo Pond, MP depicts cultured *O. mossambicus* individuals, and C depicts wild *O. mossambicus* and *O. niloticus* individuals from Cordier Dam. (Mo) depicts wild *Tilapia sparrmanii* individuals from Molepo Dam.

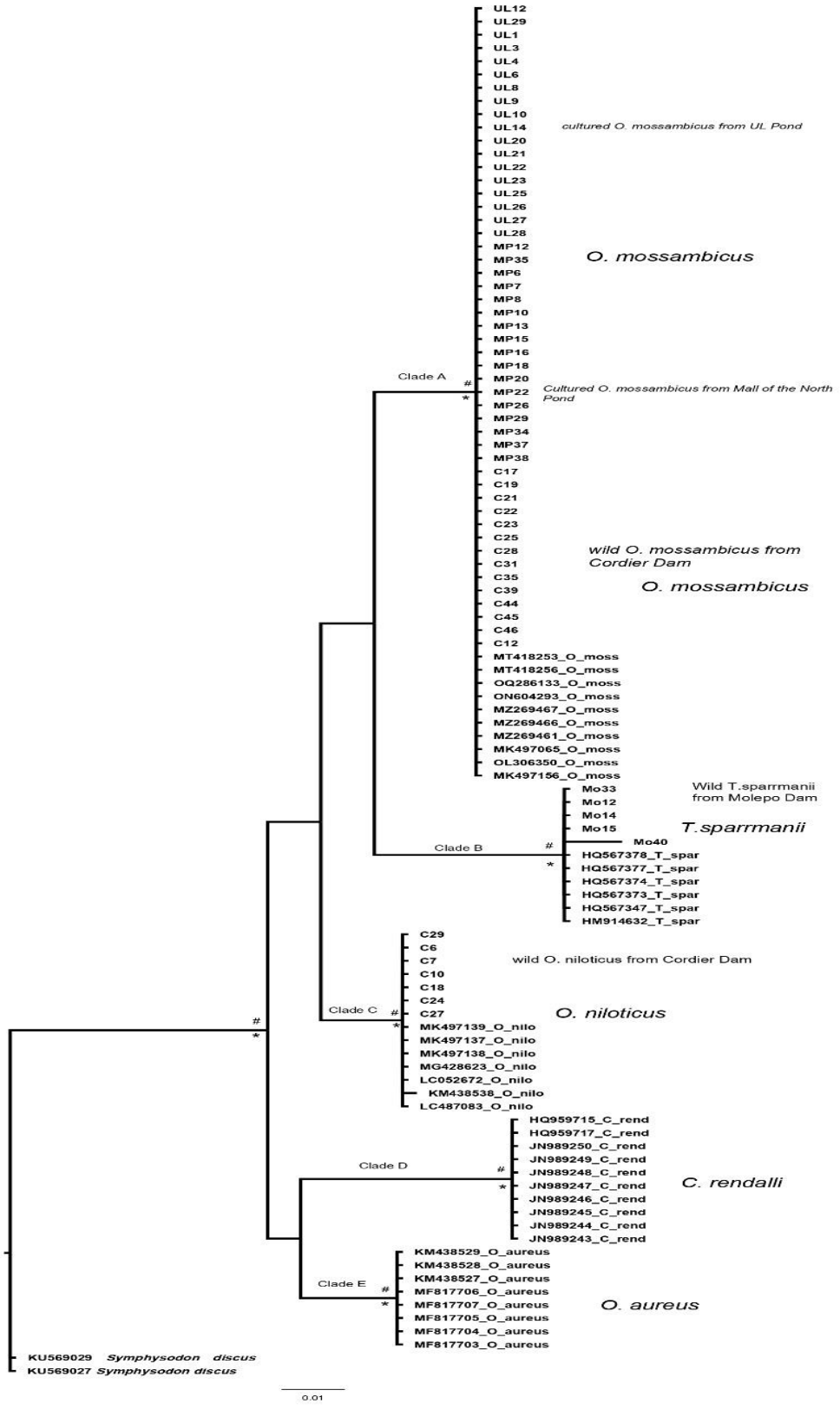


Figure 4.2: COI Bayesian Inference tree of tilapia species included in the current study,showing clades recovered by both BI (#) and ML (*) analyses.

4.4.3. ALL LOCATIONS COMBINED 5S ANALYSIS AND PHYLOGENETIC TREE

Nuclear species BI and ML trees were similar in topology (Fig 4.4); hence, only the BI tree is presented. Sequences ranged in size from 450-500bp, including a 120bp coding region (5SrRNA gene) and a variable Non-transcribed spacer (NTS) gene, which formed part of the 5S sequences. The occurrence of two distinct 5S rDNA sequence types, hereafter referred to as 5S rDNA type I and 5S rDNA type II classes that, were detected in the analysed tilapia individuals. The analyses of the whole 5S rDNA sequences and using the non-transcribed spacers reflected the existence of the two classes. On the other hand, the analysis of just the 5S rRNA gene did not reflect the two classes clearly discriminated by the Non-transcribed spacer (NTS) variations. The two 5S rDNA classes coexisted within the tilapia individuals and were not differentiated between individuals. The 5S rDNA coding region was quite conserved among the tilapia individuals and between the two 5S rDNA types.

In contrast, the NTSs were highly differentiated between the two types and quite conserved within the same type (fig 4.5). The results showed that all the *O. mossambicus* test individuals from all three localities were type II species, as well as the majority of *O. niloticus* test individuals and one *T. sparrmanii* individual. The second major clade comprised of type I individuals with three *T. sparrmanii* individuals from Molepo Dam and two *O. niloticus* individuals from Cordier Dam.

Tilapia species from GenBank were identified by their country of origin, and test individuals were identified with C- for Cordier Dam, showing *O.mossambicus* and *O. niloticus* individuals, MP for mall of the North *O*. *mossambicus* individuals, UL for *O.mossambicus* individuals and Mo for Tilapia sparrmanii individuals from Molepo Dam. BI ≤ 0.95pP is considered well-supported (#). ML Bootstrap values ≥75% are considered well resolved (*).

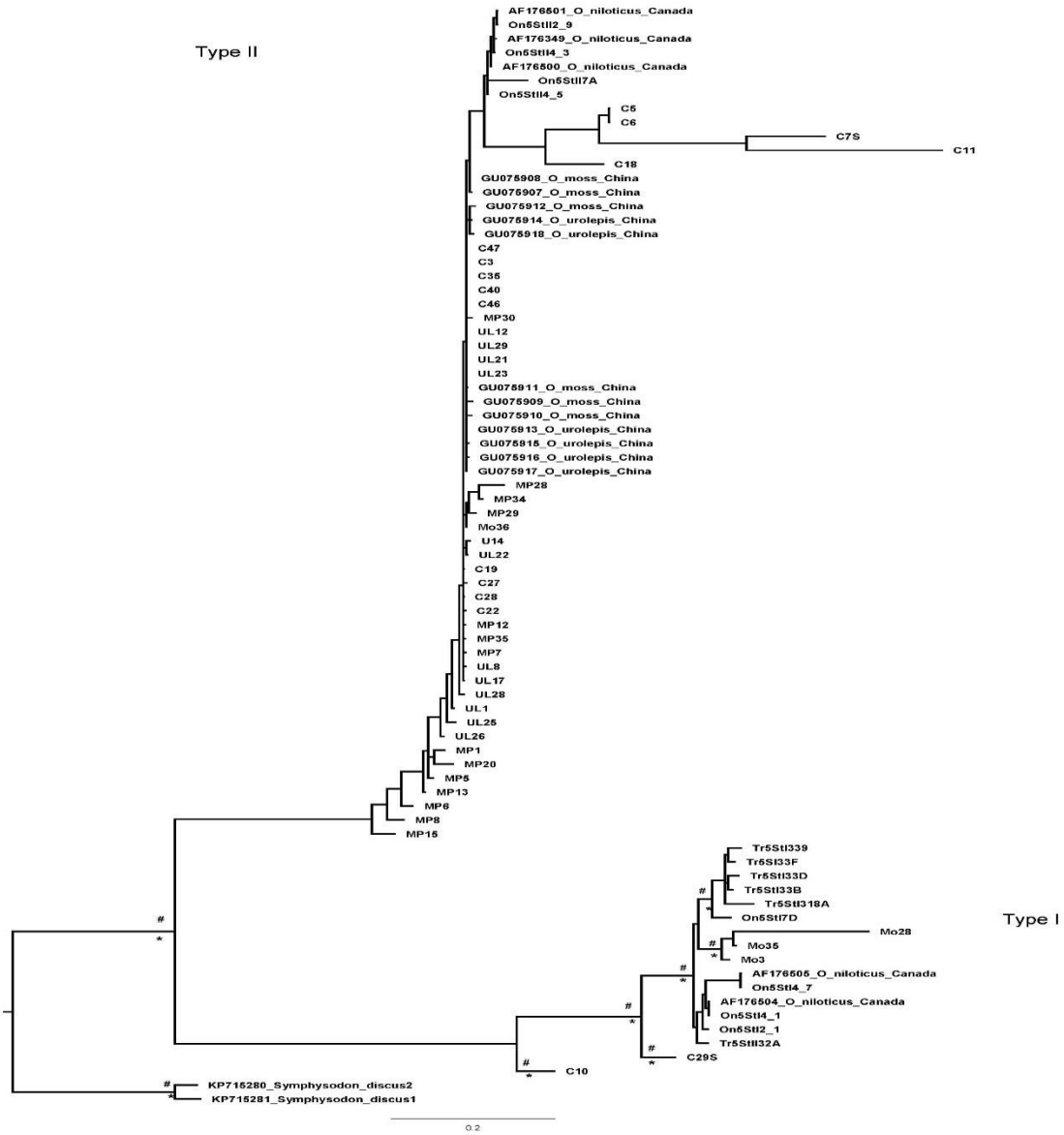
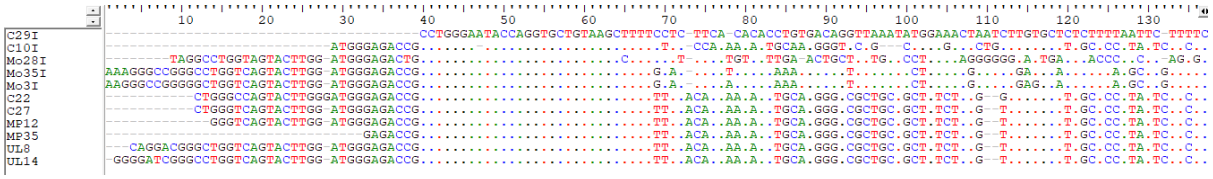


Figure 4.3: 5S Bayesian and maximum likelihood tree of all four localities combined.

4.4.4. THE COMPARISON OF 5S AND NTS BASE PAIRS (bp) IN IDENTIFYING TILAPIA SPECIES

The 5S gene was represented in the figure below, depicted by the first 70 base pairs. The rest of the 5S bases are found from base 460, which is not depicted here to make the total 120 base pairs. All the tilapia species investigated (*O. mossambicus, O. niloticus,* and *T. sparrmanii*) presented no differences in nucleotides apart from one nucleotide (65) at Mo28. This shows that the 5S gene is conserved for all tilapia species and similar. The variation amongst the tilapia species is seen in the NTS part of the gene fig.4.6.



5S gene **Non-transcribed spacer (NTS)**

Figure 4.4: The first 70bps of the 5S gene comparing all three species of the same type I class tilapia.

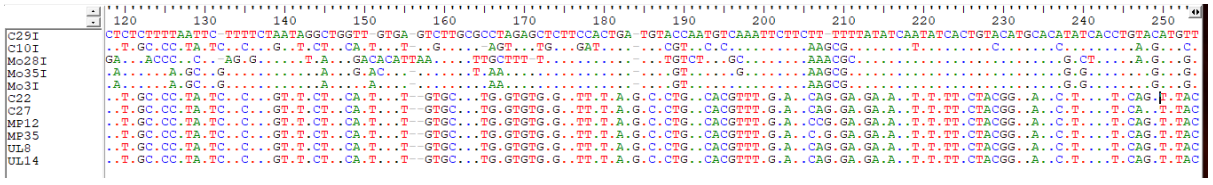


Figure 4.5 The Non-transcribed spacer (NTS) base pairs from 121 to 250 comparing all the test type I individuals represented in all localities.

4.4.5 IDENTIFYING POSSIBLE HYBRIDS.

Additionally, the phylogenetic analyses revealed evidence of hybrids in Cordier Dam. For example, sample C5 was morphometrically identified as *O. mossambicus* based on morphological characters, Fig 4.7, however, the phylogenetic tree based on the 5S rDNA sequences recovered it as *O. niloticus*. Sample C5 could not be identified based on the COI region due to poor amplicon quality obtained from PCR for verification.

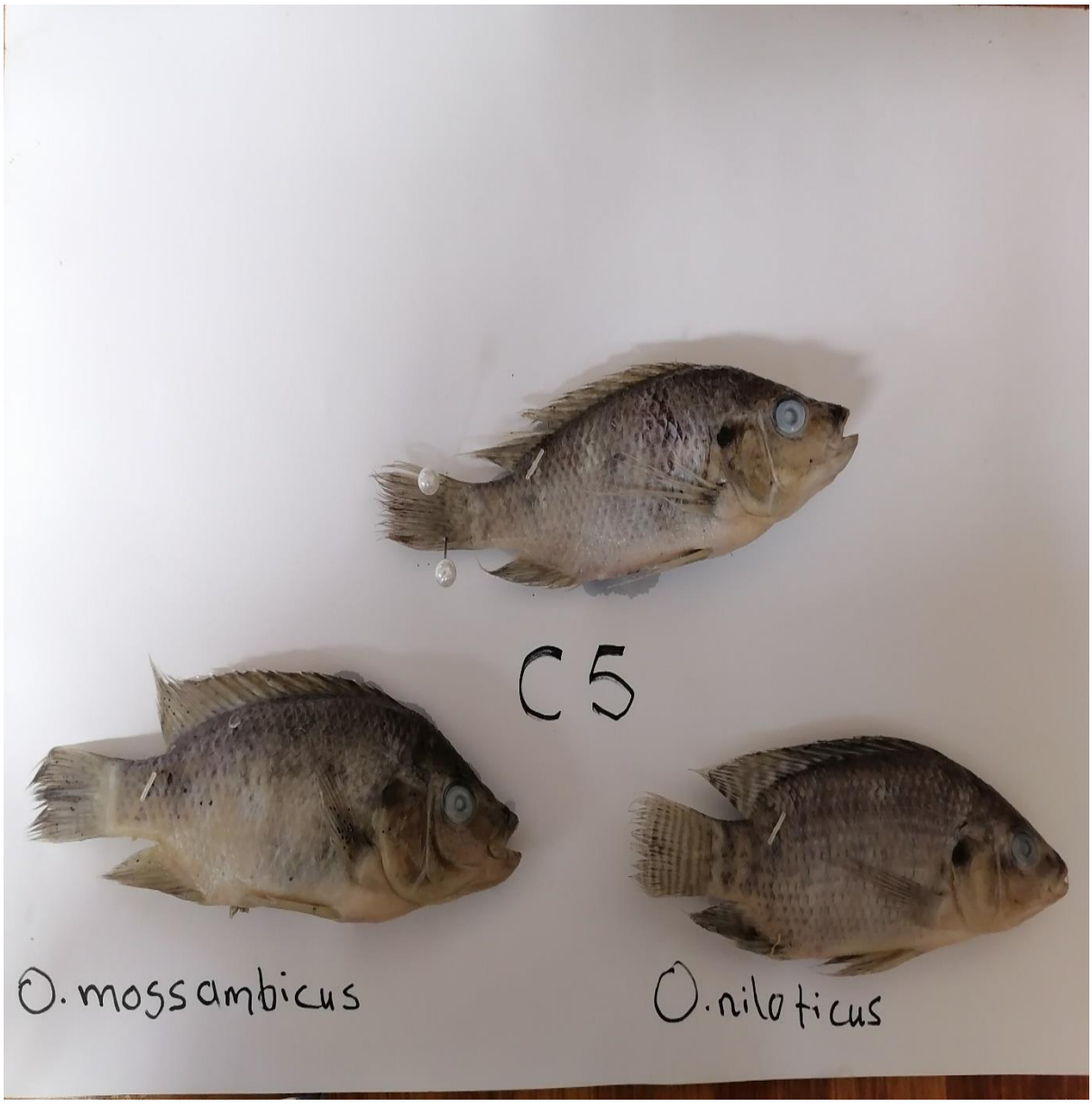


Fig 4.6:. Individual C5 was morphometrically described as *O. mossambicus,* but 5S primer identified it as *O. niloticus,* indicating a possible hybrid. Underneath C5 lies pure *O. mossambicus* and *O. niloticus* samples for comparison from the same dam as C5.

Morphometrically, individual C27 (Fig 4.8) was described as *O. mossambicus;* however, phylogenetically, sample C27 was identified as *O. mossambicus* based on the COI sequences (Fig 4.9) and as *O. niloticus* based on 5S rDNA sequences (Fig 4.10).

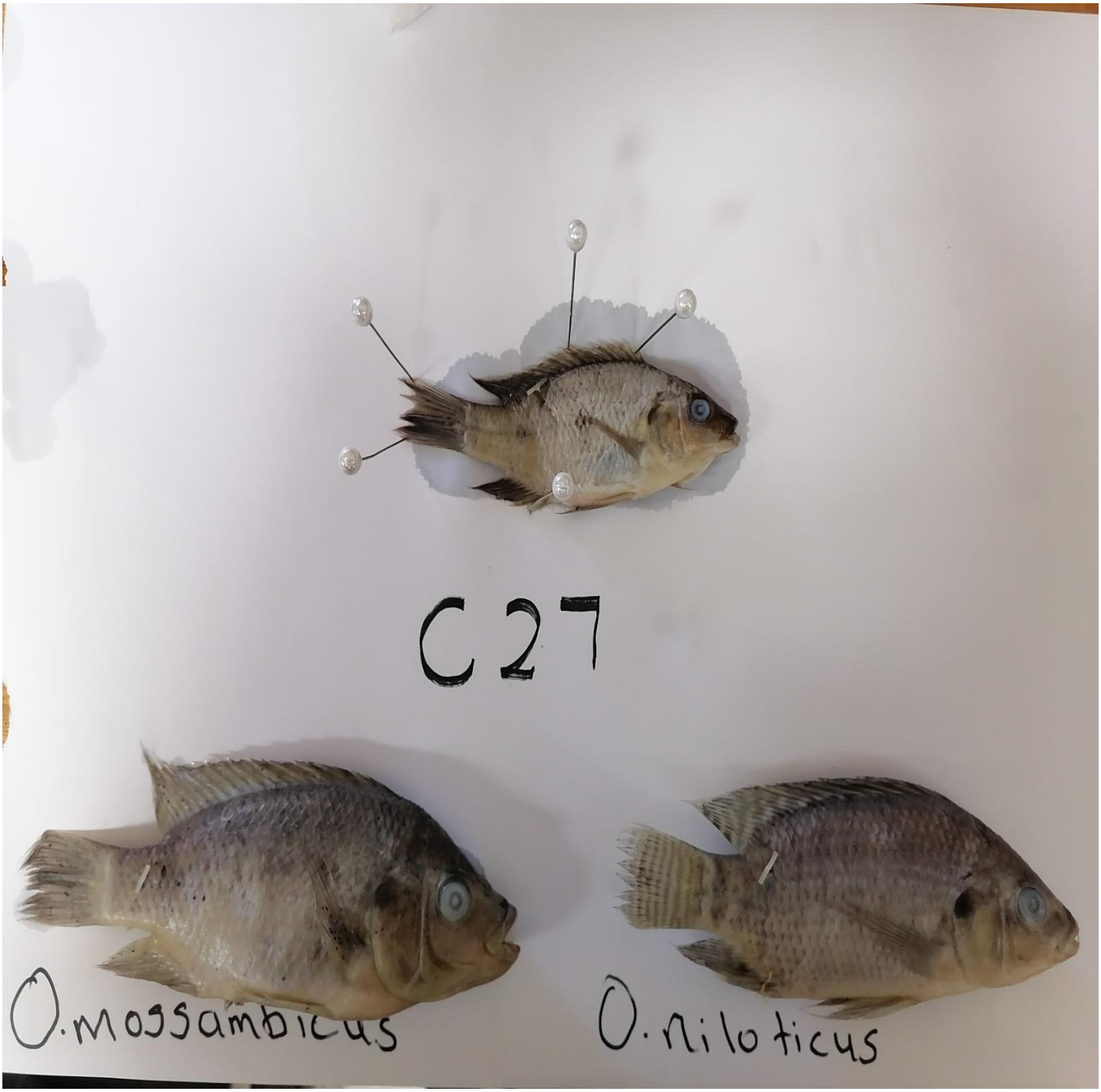


Figure 4.7: Individual C27 that was morphometrically described as *O. mossambicus* but turned out to be a hybrid. Below C27 lies *O. mossambicus* and *O. niloticus* individuals for comparison with the hybrid.

The genetic pairwise distance (Fig 4.8.1) based on the COI marker between the hybrid and *O. mossambicus* was 0.0839 and the hybrid and *O. niloticus* was 0.0313. The pairwise distance was done because the phenotype showed that it is *O. mossambicus* it was however genetically closer to *O. niloticus.*

Table 4.3. Nucleotide pairwise distance C27 COI marker.

Pairwise distance between possible hybrid C27 compared with pure *O. niloticus* and pure *O. mossambicus* individuals

	1	2	3
C27F FishF1		0.0070	0.0112
C29F *O. niloticus*	0.0313		0.0092
C19F *O. mossambicus*	0.0839	0.0556	

The phylogenetic trees below show the placement of sample C27 as analysed by the COI and 5S primers respectively. The COI phylogenetic tree depicting sample C27 as *O. niloticus.* This shows that its maternal strain was from an *O. niloticus* donor. BI ≤ 0.95pP is considered well-supported (#). ML Bootstrap values ≥75% are considered well resolved (*).

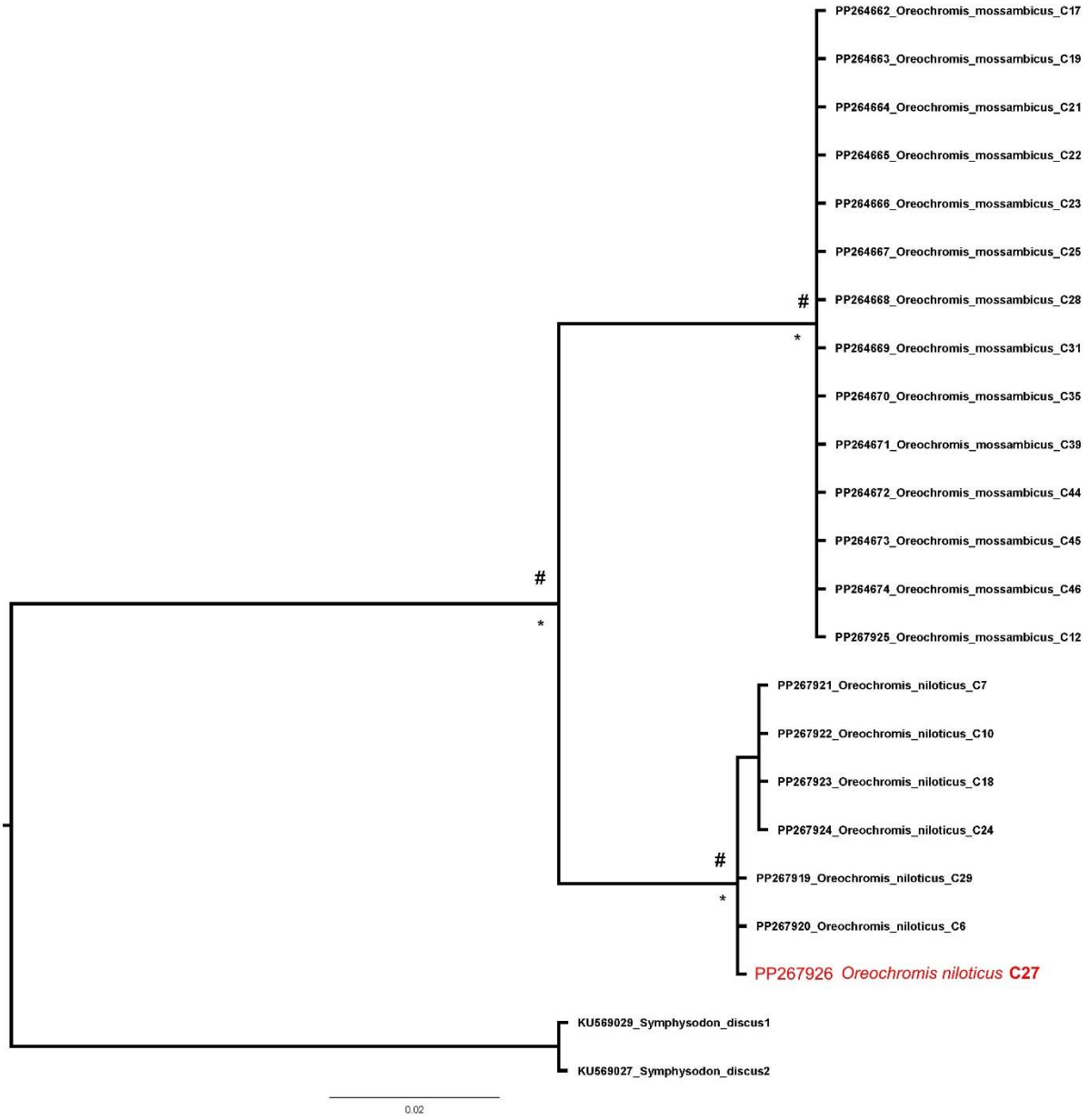


Fig 4.8: COI tree depicting BI and ML results of species from Cordier dam marked with C-. Sample 27 was identified as *O. niloticus,* on the COI tree.

5S phylogenetic tree depicting sample C27 as *O. mossambicus.* This shows that its paternal strain was from an *O. mossambicus* donor.

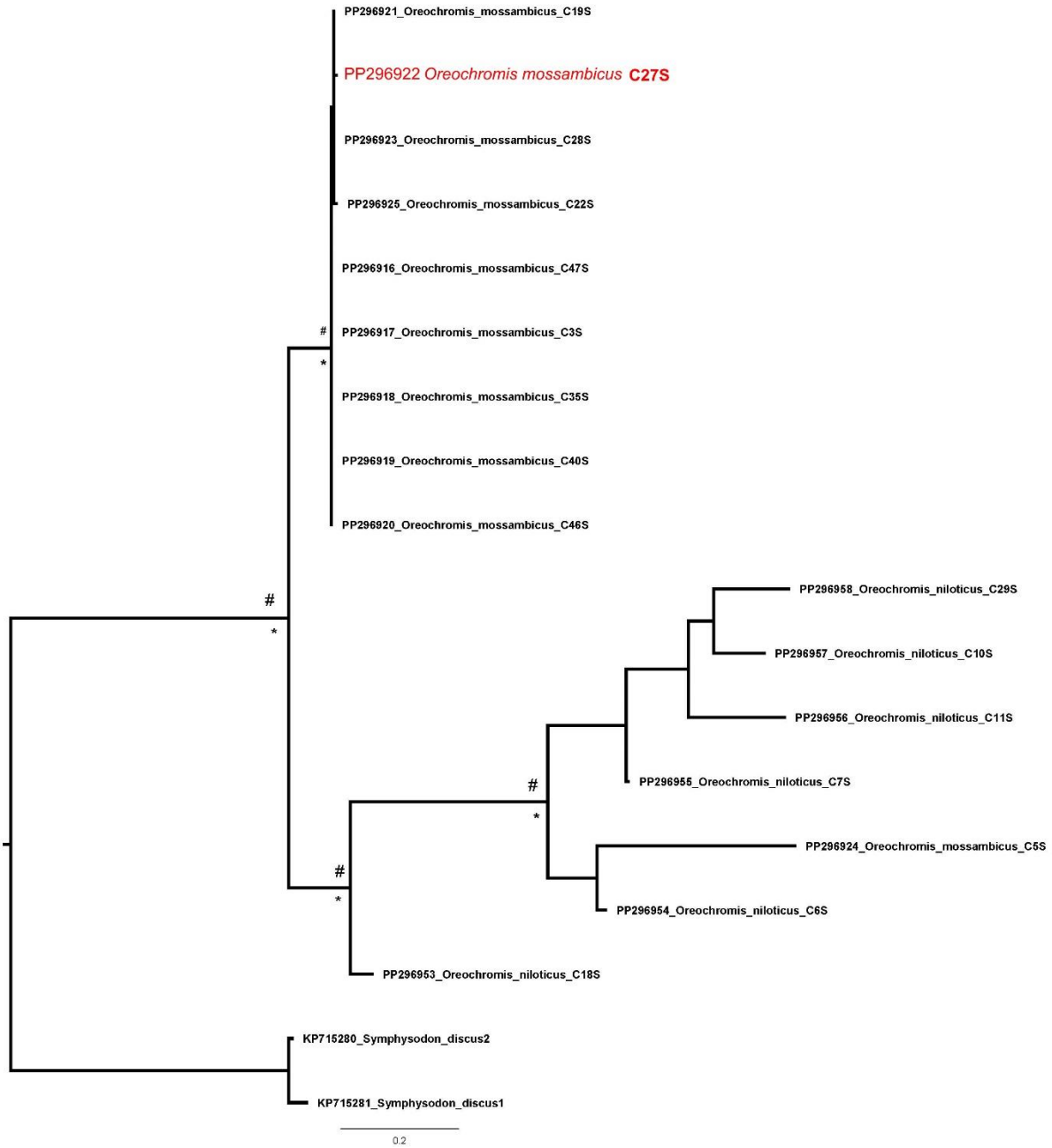


Fig 4.9: 5S tree depicting BI and ML results of species from Cordier dam marked with C-. Sample 27 was identified as *O. mossambicus,* on the 5S tree.

Genetic pairwise distance fig (4.10.1) based on the 5S marker between the C27 hybrid and *O. mossambicus* was 0.0177, and hybrid and *O. niloticus* was 0.1501. The pairwise distance was done because the phenotype showed that it is *O. mossambicus.* 5S showed that sample C27 was closer to *O. mossambicus,* which agrees with the phenotypic observation.

Table 4.4. Nucleotide pairwise distance (C27 5S).

Genetic distance depicting differences amongst 5S results for sample C27 compared with pure *O. mossambicus* and *O. niloticus*

	1	2	3
C27 hybrid		0.0062	0.0171
C22 *O. mossambicus*	0.0177		0.0169
C6 O. *niloticus*	0.1501	0.1481	

The genetic pairwise distance based on the 5S marker between the C5 hybrid and *O. mossambicus* was 0.2283, and hybrid and *O. niloticus* was 0.000. The pairwise distance was done because the phenotype showed that it is *O. mossambicus*.

Table 4.5 Nucleotide pairwise distance (5S C5 difference between possible hybrid and parents).

Genetic distance depicting differences amongst 5S results for sample C5 compared with pure *O. mossambicus* and *O. niloticus*

	1	2	3
C5 possible hybrid		0.0177	0.0000
C22 *O. mossambicus*	0.2218		0.0177
C6 *O. niloticus*	0.0000	0.2218	

4.6. HAPLOTYPE TCS NETWORK

TCS (Templeton, Crandal, and Sing) network spanned four haplotypes unique to the species (Figure 4.11). Haplotype one and two, separated by six mutational steps, were unique to individuals identified as *T. sparrmanii*. Furthermore, haplotype one was unique to Mo40. Haplotype three and four were unique to *O. mossambicus* and *O. niloticus* respectively. The TCS Fig 4.11 also indicated the location where the three identified species were found. Haplotypes one and two were found in Molepo Dam, which were identified as *T. sparrmanii*, haplotype four, which was identified as *O. niloticus* was found in Cordier Dam only, while haplotype three, which was identified as *O. mossambicus,* which was the most abundant haplotype was found in three locations, Cordier Dam, University of Limpopo Pond and Mall of the North Pond.

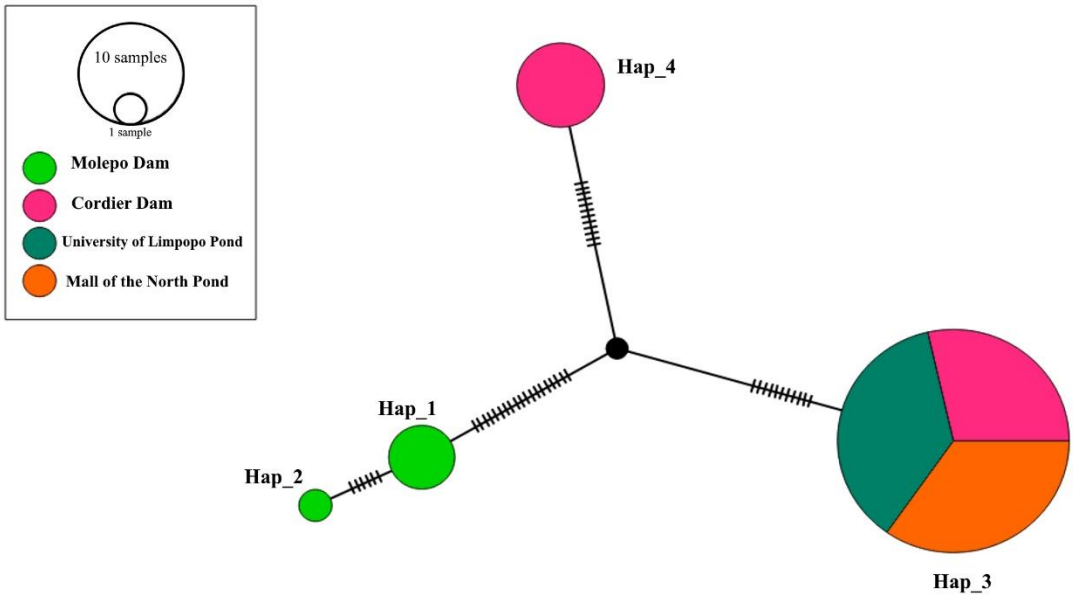


Figure 4.10. Haplotype TCS network based on the COI dataset indicates the different haplotypes found at various locations. Dashes indicate mutational changes between haplotypes. Each circle represents a haplotype, and its size is proportional to its frequency.

4.7. DISCUSSION

This study presents a phylogenetic analysis for identifying tilapia species and the presence of tilapia hybrids in Limpopo Province, South Africa, based on the mitochondrial (COI) and nuclear (5S rDNA) regions. Phylogenetic analysis revealed that all fish species were well clustered according to their taxonomic level. The COI tree showed that there were three tilapia species within the four localities, namely, *O. mossambicus, O. niloticus,* and *T. sparrmanii,* where *O. mossambicus* was in all localities except Molepo dam, due to the decrease in *O. mossambicus* populations at Molepo dam. These results were consistent with those species' morphometric results (Chapter 3). However, we observed some differences in the morphometric and genetic characteristics of the hybrid species (C27 (916) & C5 (894)).

The COI (Fig 4.3) tree showed that the wild and cultured *O. mossambicus* contained the same genetic makeup with no genetic divergence. This shows the conservation of the COI gene and its ability to resist mutation in different ecological (temperature fluctuations) and anthropogenic factors (feeding diet); hence, it was a successful marker for identification (Herbert *et al.,* 2003; Pentinsaari *et al*., 2016).

To further analyse genetic characteristics and prove possible hybrids, rDNA (5S) sequences were analysed. Several authors have considered the usefulness of 5S ribosomal DNA sequences as phylogenetic or population markers, aiding in the identification of species (Pendas *et al.,* 1994 and Martins, 2004). Alves-Costa *et al*., (2006) cautioned that special attention must be exercised, mainly in phylogenetic interpretations, since the 5S ribosomal DNA family might show a complex organization with the presence of paralogous sequences in the genome (Martins and Galetti, 2001).

The results revealed that two distinct 5S rDNA classes (type I and type II) were characterized by distinct NTSs and base substitutions in the 5S rRNA gene. Different 5S rDNA classes have been observed in several fish species (Martins *et al.,* 2000; Alves-Costa, 2006; Zhu *et al.,* 2010). However, there are often two 5S gene fragments in bony fish, such as in *Carassius auratus* and *Cyprinus carpio* (Martins and Galeti, 2001). Thus, possessing two 5S rDNA classes seems to be a general trend for organizing these sequences in the genomes of fish (Qin *et al*., 2010). A dual 5S ribosomal RNA gene system (Type I and Type II) was described for vertebrates, including fish and amphibians (Komiya *et al.,* 1986). The presence of type I and type

II genomic 5S ribosomal DNA class has been documented for several groups of teleost fish, suggesting that such a pattern could be a common characteristic among fishes (Komiya *et al.*, 1986). The obtained results from Alves-Costa *et al*., (2006) concluded that, with regards to 5S ribosomal DNA in the tilapia and their reviewed data on 5S ribosomal DNA, it strongly supported that two types of 5S ribosomal DNA system (Type I and Type II) to be of general occurrence in teleost fish genome. Nuclear rDNA has proved instrumental in the identification of species, especially cichlids. Ford *et al.,* (2019) found it to be more robust as compared to other genes.

Researchers have proposed that the genomes of diploid likely experienced a polyploidization process, and during the process, the variations and reorganizations of the genomes may have resulted in the appearance of new 5S rDNA (Cao *et al*., 2024). Zhu *et al.,* (2010) reported that 5S ribosomal DNA in *O. mossambicus*, *O. urolepis hornorum,* and their hybrids exhibited more of the type I class and less of the type II. This agrees with the findings of this study as all the test *O. mossambicus* species were Type I class. Owing to the low intra-specific and high inter-specific variability of NTS domains, the 5S rDNA is a valuable molecular marker for fish species (Qin *et al*., 2010).

Alves-Costa (2006) noted that the NTS part of the gene contributed to vast differentiation. The results showed that the NTS portion was highly differentiated (Fig 4.6), and differences could be seen among the *O. mossambicus, O. niloticus,* and *T. sparrmanii* species that were investigated in both classes. This agrees with Alves-costa's (2006) work and Zhu *et al*., (2010). 5S primer is, therefore, efficient for tilapia identification. 5S was able to show more differentiation in nucleotides than the COI gene. This can help in noting possible hybrids and possible introgression.

With regards to proving the presence of hybrids in the Cordier Dam where both *O. niloticus* and *O. mossambicus* are present, the results based on COI and 5S DNA phylogenetic analyses (Fig. 4.9 and 4.10) showed that isolate C27 from Cordier Dam was a hybrid of *O. niloticus* (female parent) and *O. mossambicus* (male parent). Phylogenetic analysis for COI revealed that C27 were closely clustered with their female parent. However, their phenotype was closer to the male parent for C27 and closer to the female parent for C5. This implies that phenotypic selection is random for each possible offspring.

Different morphometric characteristics for the hybrid between the tilapia species have been reported. However, in this study, individual C27, based on morphometrics, was identified as *O. mossambicus*. The phenotypic features of both C27 and C5 were those of *O. mossambicus*; however, the DNA results indicated the presence of *O. niloticus* in one parent. The genetic distance between C27, the hybrid and the pure *O. mossambicus* was 0.0839 for COI, 0.017 for 5S, 0.0313 for *O. niloticus* COI, and 0.1501 for 5S. This result showed a closer genetic distance to *O. mossambicus*. This accounts for the stronger phenotypic morphology of the hybrid, which looks more like *O*. *mossambicus* than *O. niloticus*.

The Haplotype TCS network (Figures 4.11 and 4.12) spanned four haplotypes unique to the taxon unit, although *T. sparrmanii* showed two unique haplotypes. Therefore, the haplotype network successfully inferred congruence with the phylogenetic tree. *T. sparrmanii* was shown to have one sample, Mo40, which was genetically divergent from the other *T. sparrmanii* test individuals. This was clearly shown by the differences in the two haplotypes for *T. sparrmanii,* accounting for the 0.02 genetic distance between the Mo40 and the other *Tilapia sparrmanii* test samples. It is not immediately clear why Mo40 differs from the other test samples. This needs further genetic probing and sample analysis to investigate whether the change was due to ecological or genetic factors.

In conclusion, three species in the four localities were investigated, and hybrids between *O. mossambicus* and *O. niloticus* were found in Limpopo Province. COI is most efficient in tilapia identification, however, on its own, it cannot efficiently identify hybrids, especially if they are first-generation hybrids where the COI gene has not changed. 5S, in conjunction with the NTS region, can identify hybrids as the genes evolve much quicker, showing changes in nucleotide diversity. Cordier Dam showed hybridization between *O. mossambicus* and *O. niloticus.* This implies that hybridization is taking place in this locality, posing the threat of loss of genetic diversity in *O. mossambicus,* which is now hybridizing with *O. niloticus*. This was seen in studies by Daget and Moreau, 1981, Weyl, 2008 and Marr, 2017 where entire populations of native tilapia species were decimated due to hybridization among sister species. *O. mossambicus* populations are therefore under threat in Cordier Dam due to hybridization with *O. niloticus* species.

CHAPTER 5: POPULATION GENETIC DIVERSITY OF *OREOCHROMIS MOSSAMBICUS* (PETERS, 1852) IN LIMPOPO PROVINCE

5.1. INTRODUCTION

Genetic diversity in *O. mossambicus* refers to the variety of genetic characteristics within and between populations of *O. mossambicus*. This diversity is vital for the health, adaptability, and survival of *O. mossambicus*, particularly in response to environmental changes and disease pressures (Pauls *et al.*, 2013, Pavlova *et al.*, 2017, Willougby *et al.*, 2018, Yamamichi *et al.,* 2023). This study investigated the genetic diversity within and between four locations: the cultured populations from the University of Limpopo Pond and the Mall of the North Pond and, wild populations from Cordier Dam and, Genbank samples from Flagboshielo Dam. Flagboshielo Dam was added in this chapter to compare two ponds (cultured population) and two dams (wild population) because Molepo Dam had no amplified *O. mossambicus* sequences. Genetic diversity is caused by factors such as mutations within DNA structures, gene flow through migration of *O. mossambicus* between populations, genetic drift caused by random changes in allele frequencies, and natural selection prompted by environmental pressures that lead to the survival of certain traits over others (Booy, 2000, Verma, 2017 and Yamamich *et al.,* 2023).

Genetic diversity aids in the adaptability of *O. mossambicus* to withstand ecological challenges such as harsh weather, new diseases, and changes in water quality parameters that are below or above optimum. Protecting the genetic diversity of wild tilapia populations is important for ecosystem health and biodiversity (Jump *et al*., 2009; Parsons *et al*., 1996 and Pauls *et al*., 2013). Inbreeding and loss of wild populations due to anthropogenetic habitat destruction contribute to a decrease in genetic diversity. Invasive species can exploit this vulnerability, out-competing native species that lack genetic diversity to resist their impacts (Rhymer and Simberloff, 1996, Barton, 2001, Olden *et al.,* 2004, Vava and Primmer, 2006). *Oreochromis mossambicus* populations are said to be possibly decreasing due to invasive tilapia species such as *Oreochromis niloticus,* which is outbreeding it, into possible extinction due to hybridization among the two sister species (D'Amato, *et al.*, 2007 and Zengeya *et al*., 2015). It must be noted that there are several other factors that can contribute to the decrease in *O. mossambicus* populations. Anthropogenic influences also reduce

the genetic diversity of the fish populations, potentially affecting the population's evolutionary ability and persistence in the habitat (Barasa *et al.*, 2016).

This study will note the genetic diversity status of *O. mossambicus* within the four localities and whether *O. mossambicus* remains threatened in the localities under investigation. This will be achieved by analysing haplotype diversity, nucleotide diversity, and raggness/ mismatch test to estimate genetic diversity, by using the TCS haplotype network to infer genetic relationships and distribution patterns of haplotypes in the cultured and wild populations, by using Fu's D and Tajima's D tests to determine the neutrality and population size change as well as employing AMOVA to review the genetic structure of the wild and cultured populations.

5.2 OBJECTIVES

- To determine the genetic diversity of *Oreochromis mossambicus* in Limpopo Province.
- To investigate the genetic structure of *Oreochromis mossambicus* in Limpopo populations.

5.3 MATERIALS AND METHODS

5.3.1 Study area, refer to Chapter 3

5.3.2 DNA extraction, PCR. Sequencing and Baysian phylogenetic analysis refer to Chapter 4.

5.3.3. Genetic diversity of wild and cultured *O. mossambicus*.

The quality of the sequences generated from the current study (see Table 1 in Chapter 4), along with GenBank sequences from Flagboshielo Dam (Accession no MK497125-MK497131, Mojekwu *et al*., 2019), were analyzed in BioEdit (Hall, 1999), confirming the presence of variable sites by manual inspection of the chromatograms. Sequence alignment was performed by MEGA X (Kumar *et al.,* 2018) and trimmed to equal length in the FaBox online toolbox (Villesen, 2007).

The degree of genetic diversity was estimated by the number of haplotypes (H), the number of segregating sites (S), haplotype diversity (Hd), nucleotide diversity (π) and

riggness/ mismatch test for all four populations, using DnaSP version 6 (Rozas *et al*., 2017).

5.3.3.1. TCS haplotype network analysis

To examine the relationship among the haplotypes in the population of *O. mossambicus*, the sequence data for the TCS haplotype Network (Clement *et al*., 2002) were exported from the DnaSP v6.12.03 program and for subsequent haplotype network analysis in the PopART (Leigh and Bryant, 2015) program.

5.3.3.2. Neutrality and population size change test

Statistical tests like Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) were used to determine whether mutations are selectively neutral or not. Arlequin version 3.5 (Exoffer and Lischer, 2010) was used to perform the analyses, which involved running 1000 simulations under a selective neutrality model. The population size change was exported from the DnaSP v6.12.03 program to show the population expansion model.

5.3.3.3. AMOVA genetic structure analysis.

The study estimated genetic diversity within and among populations using analysis of molecular variance (AMOVA). Conventional F-statistics (Fst) were calculated with 10,000 permutations using Arlequin version 3.5 (Exoffer and Lischer, 2010). This analysis was accomplished by treating all populations as a single group to determine the degree of interspecific genetic diversity among and within the populations and by grouping the populations according to wild (Cordier Dam and Flagboshielo Dam) and cultured (University of Limpopo Pond and Mall of the North Pond).

5.4. RESULTS

5.4.1. Genetic diversity and haplotype network analysis

The Hd values for all populations varied from 0.000 (Cordier Dam, UL Pond, and Mall of the North Pond) to 0.28571 for Flagboshielo Dam (Table 5.1). The total value of haplotype diversity (Hd) was 0.0364, and the total value of nucleotide diversity (π) was 0.21856 (Table 5.1). Additionally, among all populations analysed, Flagboshielo showed the highest genetic diversity (0.28571) and nucleotide diversity (0.0061), while the Cordier Dam, University of Limpopo Pond, and Mall of the North Pond showed the

lowest (0.00). All the locations compared showed only one segregating site from Flagboshielo Dam.

Table 5.1. Genetic diversity and raggness/ mismatch values for the 56 sequences of *O. mossambicus* of mtDNA COI

	Cordier Dam	University of Limpopo	Mall of the North	Flagboshielo Dam	Total
Sample size	14	18	17	7	**56**
Haplotype	0	0	0	0.29	**0.04**
Nucleotide	0	0	0	0.01	**0.22**
Number of	0	0	0	0.29	**0.04**
Number of	1	1	1	2	**2**
Number of	0	0	0	1	**1**
Riggness test	0	0	0	0.27	**0.86**

5.4.2. RIGGNESS/ MISMATCH TEST

The Raggness test showed a change of 0.27 for *O. mossambicus* from Flagboshielo Dam, while the samples from the other three sites showed no change (Table 5.1). When all four locations were combined, the Riggness test showed a change of 0.86.

5.4.3. HAPLOTYPE ANALYSIS SUPPORTING GENETIC DIVERSITY VALUES.

TCS (Templeton, Crandall, and Sing) haplotype network spanned two haplotypes, representing the distribution pattern of haplotypes from different *O. mossambicus* populations in the four cultured and wild locations (Fig. 5.1). Haplotype 1 was a shared haplotype for all populations. Flagboshielo population showed one singleton haplotype with one mutation as haplotype 2.

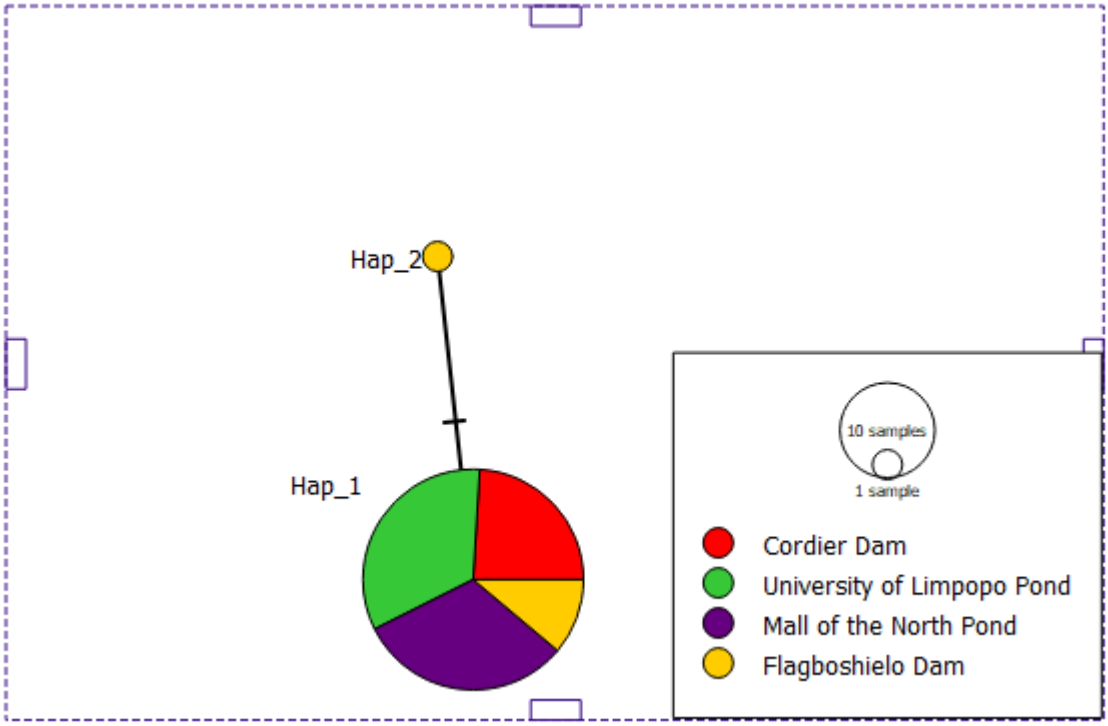


Figure 5.1: A TCS haplotype network of *Oreochromis mossambicus*. The size of the circle indicates the relative frequency of the corresponding haplotype, and the colors represent the corresponding population. The branches' black lines point to the mutational changes between two haplotypes.

5.4.4. Demographic history and neutrality

Fu's D test was found to be -1.05 (P > 0.10) for Flagboshielo Dam and all four populations combined were -1.87 (P > 0.10) (Table 5.1). There was no polymorphism for the University of Limpopo, Mall of the North, and Cordier Dam populations. The results were similar also for Tajima D's test where Flagboshielo Dam was -1.01(P > 0.10) and all populations were -1.09 (P > 0.10) with no polymorphism noted for the three test populations (Table 5.1). Both tests (Tajima (D) and Fu's Ds) were not significant from zero (P > 0.10) (Table 5.1).

Table 5. 2. Fu's and Tajimas D tests results from specimens of *O. mossambicus* obtained from four localities.

	Cordier Dam	University of Limpopo	Mall of the North	Flagboshielo Dam	Total
F	0	0	0	-1.05 (P > 0.10)	**-1.8**7 (***P* > 0.10**)
T	0	0	0	-1.01 (P > 0.10)	**-1.09** (***P* > 0.10**)

5.4.5. AMOVA population genetic structure

Analysis of Molecular Variance (AMOVA) of all four populations in different locations was accessed to determine the population's genetic structure (Table 5.2). The results of the AMOVA showed that the highest percentage of variation (91.05) was found within populations for all locations. In contrast, a lower percentage of variation (-3.12) was found among groups for all populations, as indicated by the differences among populations at P <0.001 (Table 5.2).

Table 5.3. AMOVA genetic structure analysis sequences of cultured (Mall of the North Pond and University of Limpopo Pond) and wild (Cordier Dam and Flagboshielo Dam) specimens of *O. mossambicus.* **P < 0.001.

Analysis	Source of variance	df	Sum of squares	Variance component	% Total of variance	Significance
All locations (wild and cultured)	Among groups	1	0.03	-0.001	-3.12	**Fct = -0.03****
	Among populations	2	0.09	0.002	12.07	**Fsc = 0.17****
	Within populations	51	0.86	0.017	91.04	**Fst = 0.09****
	Total	**54**	**0.98**	**0.02**	**100**	

The F_{ST} value per paired population significantly differed from zero among groups (F_{ST} = 0.09; P < 0.001), indicating a significant genetic structure among the populations. The F_{ST} values were used to determine differentiation, whether it was due to drift, inbreeding, or adaptive population structures. Within populations, Fct = -0.03 P < 0.001

measured the proportion of genetic variation that attributed to differences among groups of the cultured and wild populations. Among populations, Fsc = 0.17 $P < 0.001$ measured the proportion of genetic variation that contributed to differences among populations within groups.

5.5. DISCUSSION

In this study, the genetic diversity and genetic structure of *O. mossambicus* was defined using the COI dataset. The results indicate no genetic diversity of *O. mossambicus* populations from the Cordier Dam, University of Limpopo Pond, and the Mall of the North Pond, where haplotype diversity (Hd)= 0, nucleotide diversity (π)= 0,000 and raggness/ mismatch test 0. The University of Limpopo Pond and Mall of the North Pond populations are isolated ponds initially stocked with a small number of broodstock with no genetic variation amongst the parents and no genetic flow from outside fish populations. These results were thus expected. The *O. mossambicus* in the three test locations experienced similar environmental and water quality factors (Table 3.1), which can account for their similarities in genetic diversity. The University of Limpopo Pond was constructed Forty-eight years ago. This is not enough time for the COI gene to differentiate. The *O. mossambicus* from Mall of the North Pond is four years old, which is also not enough time for differentiation to occur unless an abrupt event such as disease or other anthropogenic events occurs. In this case, the results were due to a population bottleneck and inbreeding. These results agreed with those of Fatsi *et al*., 2020 who also found a low haplotype diversity but a high nucleotide diversity in their study looking into the population genetics of wild tilapia in Japan.

The same is true for Cordier dam, which is 19 years old and where the COI gene of pure *O. mossambicus* did not change due to ecological disruptions. Cordier Dam, on the other hand, due to possible genetic flow with other *O. mossambicus* individuals from the Middle Letaba River and the possible noted hybridization from the Cordier Dam, the pure *O. mossambicus* individuals still showed no genetic variation in the *O. mossambicus* investigated. However, differences have been noted in fish that were found to be hybrids in Cordier Dam, as the change is generational and abrupt. Crozier and Hutchings, (2013) found that it generally took 15 generations or at least 60 years for genetic change to be noted in a population due to environmental stressors. Hoffmann and Willi, (2008) indicated that adjusting through phenotypic plasticity and

adapting through evolutionary change are ways for species survival if moving to more suitable habitats is not possible. Stockwell (2003), noted that fast or slow rates of changes in evolutionary structures need to be interpreted relative to the time interval over which they are measured or as positive or negative deviations from the overall time scale trend. According to Stockwell, (2003), the range of evolutionary rates for different taxa over the same number of generations was rather large. In essence, the time required for change to occur in the phenotypic and genetic structure of tilapia will also vary. This disputes the findings of Zianada *et al.,* (2009) and Crozier and Hutchings, (2013), who found that it took 15 generations or at least 60 years in *Cynotilapia afra* and sockeye salmon (*Oncorhynchus nerka*) respectively for genetic change to be noted.

With the presence of *O. niloticus* in the Cordier dam, the genetic diversity of the pure *O. mossambicus* was not altered in the pure samples investigated. For this chapter, only samples considered pure *O. mossambicus* were analyzed, and those that were hybrids were not included because they are no longer pure and possess DNA from other species which will bring changes in the genetic structure of that individual. This is why there was no genetic diversity in Cordier Dam, even though it contained hybrids. There was, however a reduction in the number of pure *O. mossambicus* species due to hybridization between *O. niloticus* and *O. mossambicus,* which is directly detrimental to the population size of *O. mossambicus* species in the Province. For now, *O. mossambicus* remains the most abundant fish species in the localities investigated, but as time goes by, it runs a risk of being displaced by *O. niloticus* and its hybrids. Genetic drift is therefore a reality where hybridization has occurred. This was seen in studies by Daget and Moreau, (1981), Arthur *et al*., (2010), Cononico *et al.*, (2005), Martins *et al*., (2010), Champneys *et al*., (2021) and Stauffer, (2022), where native populations were displaced by exotic populations.

The Flagboshielo individuals with a haplotype diversity Hd= 0, 29, nucleotide diversity of (π) = 0, 01, and raggness/ mismatch test =0.27 showed the highest genetic diversity among the sampled populations, because there are possibly multiple haplotypes in this population. Flagboshielo Dam, which exhibited only one segregating site, shows that this location contributed to the genetic variation observed. Flagboshielo Dam was constructed in 1987 (37 years). However, it must be noted that the *O. mossambicus* population has always been there in the Olifants River system, meaning the population

is much older than 37years. One individual from Flagboshielo Dam showed one mutational difference compared to other individuals from the four localities, giving the only haplotype difference among the four locations. What accounts for the single mutational change in Flagboshielo Dam is not immediately apparent. However, anthropogenic activities around the Dam can probably bring about a change in genetic diversity. These anthropogenic activities include, subsistence and commercial farming where the main pollutant is run off from fertilizers and pesticides. Urbanization and development are leading to increased sewage and waste deposal around the dam. Increased runoff also adds to the pollutants washed into the dam. While there is limited mining activities directly around Flagboshielo Dam, it is however located down stream the Loskop Dam which is affected by mining activities. Activities such as boating, tourism and fishing can contribute to disturbing the aquatic life and pollution due to oil and fuel spills and litter that can be disposed into the dam. Adapting to environmental and ecological changes is crucial for the survival of all species (Lecaudey *et al*., 2019). Since Flagboshielo Dam is highly impacted by anthropogenic activities, that may also account for the haplotype variation. This indicates *O. mossambicus's* adaptability; hence, it can thrive in most areas where it is translocated. Hence its placement on the list of the most invasive species in the world. Natural or human-mediated factors, can affect intra- and interspecific genetic diversity (Radosavljevic *et al*., 2015). Studies by Willie *et al*., (2006), Mendez *et al.,* (2014), Meester *et al.*, (2018), all agree that larger populations are typically buffered from the effects of genetic drift and larger populations tend to maintain high levels of genetic variation. This was seen in the Flagboshielo population which has a relatively larger population and genetic variation. This is due to the mutational process which in turn increases the adaptive capacity of the population.

The overall combined haplotype diversity Hd= 0. 04 and nucleotide diversity π= 0. 22 are influenced by the high diversity at Flagboshielo Dam. The overall values are, however, low because three out of the four locations have no diversity. Regarding genetic diversity, the genetic diversity of Flagboshielo Dam may be crucial for the conservation of the species as higher genetic diversity is associated with greater adaptability and resilience to environmental changes (Booy *et al.*, 2000 and Paula *et al*., 2013). In their study, Fatsi *et al*., (2020) also noted a low haplotype diversity and a high nucleotide diversity value for the *O. mossambicus* populations.

TCS haplotype network analysis provides insight into the genetic relationships and distribution patterns of haplotypes in the study populations of *O. mossambicus* in the four locations, supporting the genetic diversity results. Haplotype one, shared by all populations, shows that it is the most common and widespread haplotype across both cultured and wild populations, suggesting a common ancestral haplotype. This indicates that mitochondrial lineages have evolved slowly over time in different locations. Fatsi *et al*., (2020) noted that the level of genetic diversity in a population varies based on the primer being examined. COI is highly conserved (Herbert *et al*., 2003, Pentinsaari, *et al.,* 2016) and can account for the low genetic diversity amongst the *O. mossambicus* species. According to Aminisarteshnizi and Moyo, (2024) the presence of shared haplotypes is attributed to gene flow among populations, in this case, a common ancestor is shared among the fish in those localities. Haplotype 2, a Singleton haplotype found in the Flagboshielo wild population, with one mutation distinguishing it from haplotype 1 indicates a recent genetic variation or a mutation event that has not spread to the other populations. The shared haplotype 1 across all locations shows a lack of extensive genetic divergence among the populations. In this case, it is due to historical connectivity and a limited evolutionary time for divergence.

The unique haplotype 2 in Flagboshielo indicates some level of genetic differentiation and suggests that this population may be experiencing unique evolutionary, anthropogenic pressures or historical events leading to this divergence. The sample size is however too small to conclusively make declarations. The emergence of haplotype 2 at Flagboshielo Dam suggests that there is potential for future genetic diversity if such mutations continue to arise and spread. The TCS haplotype network analysis shows that most populations of *O. mossambicus* share a common haplotype, indicating genetic homogeneity. There is a unique haplotype in Flagboshielo Dam that represents a current mutation. Studies by Hickerson *et al*., (2010) and Fatsi, (2020) showed that a relatively low haplotype diversity, together with high nucleotide diversity values, indicate the genetic relationship between haplotypes, suggesting an active ongoing expansion and diversification causing the evolutionary establishment of independent lineages from ancestral populations of considerably small effective population sizes, this is depicted by the haplotype network, which also supports the haplotype and nucleotide diversity results.

The results of Fu's D test and Tajima D test offer insight into the demographic history and neutrality of the *O. mossambicus* populations in the four local locations. They indicate an excess of recent mutations, which suggests population expansion and a negative Tajima D's value suggests an excess of low-frequency polymorphism also indicating population expansion. However, the P-value (P>0.10) suggests that this result is not statistically significant. This was due to frequent population expansion from small number of parental brood stock. A negative Fu and Tajimas D test results were also seen in the study by Mboweni, (2020) in *Oreochromis* species.

The absence of polymorphism was noted in the University of Limpopo Pond, Mall of the North Pond and Cordier Dam due to the lack of genetic diversity. This is due to the possible population bottleneck and limited genetic flow, especially in the two ponds University of Limpopo Pond and Mall of the North Pond. Limited parental stock and high fecundity during a short space of time contributed to this. This leads to the Founders effect (limited founders i.e. if a new population is established by a small number of individuals (founders), even if those individuals are highly fecund, the genetic diversity of the new population will be limited to the genetic variation present in the founders) and/or bottle necking (if a population experiences a drastic reduction in size, even if the surviving individuals are highly fecund, the genetic diversity of the population will be reduced because many genetic variations will be lost during the bottle neck event). The ponds are enclosed, therefore there is no genetic flow in these populations, and they are at great risk due to low genetic variability which can affect their adaptability and resilience. Efforts must be made to increase genetic diversity in populations with no observed polymorphism. Translocating genetically diverse fish of the same species is a good way to achieve this.

The demographic history and neutrality tests suggest that *O. mossambicus* populations studied are not experiencing significant demographic changes or strong selection pressures. The lack of genetic diversity in some of the populations at the University of Limpopo Pond, Mall of the North Pond, and Cordier Dam is a concern that needs to be addressed through conservation efforts that aim to enhance genetic variability and monitoring of population health. The combination of negative Fu's Ds and Tajima's D suggests an increase in population size with a lack of rare alleles. This highlights that Ponds can be utilized as sanctuaries to propagate pure *O. mossambicus* species for conservation of the species.

AMOVA provides insight into the genetic structure of *O. mossambicus* populations across different locations. The results indicated that within populations variation was more than among populations, which shows that the majority of the genetic variation occurs within individual populations rather than between them (Fang *et al*., 2022). This indicates that there is substantial genetic diversity within each population (Chen *et al*., 2006). In this case, Flagboshielo provided the most change as there was no polymorphism in the other 3 localities. This is a positive sign for the adaptive potential and resilience of different populations. These results were similar to the results of Mashaphu *et al*.,(2024) in *Oreochromis mossambicus*.

The variation among groups presented a negative value for among groups variation suggesting that there is no significant genetic differentiation between the groups of populations (cultured and wild). This shows that the observed differences are less than what was expected, which occurred due to the lack of true differentiation (Naro-Maciel *et al.*, 2011). The low and negative percentage of variation among groups suggests that the genetic differences between the cultured Group One (Mall of the North Pond and University of Limpopo Pond) and the wild population group 2 (Cordier Dam and Flagboshielo Dam) are minimal (Fatsi *et al*., 2020). This indicates a recent common ancestry (Ending and Meuwissen, 2001). Therefore within population diversity has the most genetic diversity and conservation efforts should be put in place to maintain and enhance this within population diversity (Van Dyke, 2008). The significant (P< 0, 001) Fst value indicated a moderate genetic differentiation amongst the populations. This shows a moderate genetic differentiation, which suggests that, while there is some genetic differentiation among the populations, it is not extremely high. This is probably due to the limited gene flow, genetic drift, and local adaptation (Angienda *et al.,* 2011 and Dilytė, 2020).

In conclusion, the *O. mossambicus* species showed little variation in genetic diversity and genetic structure in all locations in Limpopo, showing a common ancestor amongst the individuals. It is, however, able to adapt when the need arises. This is shown in the Flagboshielo Dam population, subjected to anthropogenic pollution and activities such as overfishing and irrigation. For conclusive population demographics on *O. mossambicus,* other mtDNA genes should be considered as COI, which is highly conserved and does not readily show changes in genetic diversity, especially in first-generation offspring. The implications of this study show that the COI gene, due to its

conserved nature, does not provide good results for comparing population demographics. In this regard, other mtDNA genes, such as D-loop, should be considered. Most importantly, this highlights the conservation potential of the COI gene and its effectiveness as an essential identification gene. *Oreochromis mossambicus* populations in the four locations are at risk of a possible decrease in numbers if a new ecological, pathological, or anthropogenic activity or shift is to occur. However, the individual at Flagboshielo Dam has shown that the *O. mossambicus* adapts to possible change when necessary.

6. GENERAL DISCUSSION

This study addressed the contemporary problem of the identification of a tilapia species, *O. mossambicus.* This species is mostly widely cultured species in South Africa, however the identification of *O. mossambicus* is now a major challenge in Southern Africa. This is attributed to its hybridization with *Oreochromis niloticus* (D'Amato *et al*., 2007, Zengeya et al., 2013) and *Oreochromis aureus* (Marr, 2018). Its ability to hybridize with sister species has made morphometric identification of *O. mossambicus* difficult.

However, this introduction has resulted in extensive hybridization with other tilapia species particularly *Oreochromis mossambicus* (Peters 1852) in South Africa, *Oreochromis aureus* (Steindachner 1864) in West Africa (Rognon & Guymard, 2003; Bakhoum *et al*., 2009), *Oreochromis andersonii* (Castelnau 1861) and *Oreochromis macrochir* (Boulenger 1912) in Zambia (Deines *et al*., 2014), and *Oreochromis esculentus* (Graham 1928) and *Oreochromis leucostictus* (Trewavas 1933) in Kenya (Nyingi & Agne`se 2007; Angienda *et al*., 2011; Ndiwa *et al*., 2014).

This study has clearly shown that morphometric features that were used by Skelton, (1993) to identify, may no longer be useful now. It is thus suggested that the morphometric and meristic features that were used to identify *O. mossambicus* in this study, be adopted as the point of reference. Proper identification of *O. mossambicus* is important for fish farmers. It has been noted that the different hybrids of *O. mossambicus* with its sisters species do not have the same growth rate as the pure *O. mossambicus* species. Hybrids between *O. mossambicus* and *O. niloticus* give a skewed sex ratios of mostly male, which are prefered due to their faster growth (Wohlfarth, 1994 and Fuentes- Silva, 2013) and a higher growth potential (Wohlfarth, 1994). However hybrid vigor will be lost with second generation (F2) or third generation (F3) (Bentsen and Gjerde, 1994). It is therefore important to establish research centres where pure stocks of pure *Oreochromis mossambicus* can be kept, to maintain genetic purity of the species.

Climate change is likely to exacerbate the problem of morphometric and meristic identification of *O. mossambicus*. It is projected that the temperature in South Africa will rise by 2°C by 2050 (Ngepah *et al*., 2022). This will affect the morphometric and meristic features used in this study. The analysis of variance carried out showed that

the morphometric and meristic features differed between localities. This highlights that with climate change, the identification of *O. mossambicus* may become more complicated in the future. Fish husbandry practices at different farms also affect morphometric and meristic features, further compounding the problem of the misidentification *O. mossambicus*.

The mitochondrial DNA identified three species, *O. mossambicus, O. niloticus* and *T. sparrmanii.* It further highlighted the limits of morphometric analysis because several *O. mossambicus* were misidentified. However mitochondrial DNA (COI) did not identify hybrids. The hybrids were identified by comparing mitochondrial DNA with nuclear DNA. Genetic probing thus offers a much more accurate identification of *O. mossambicus* and its hybrids. However, the challenge associated with genetic analysis is that it is expensive and thus not a sustainable method of identification for developing countries like South Africa.

This study also showed the limitations of the COI gene. The COI gene has proved to be very conserved showing little variation amongst individual nucleotides. It therefore proves its efficiency as an identification gene for pure species, however where hybrids are concerned other mitochondrial DNA genes like D-loop must be used as they are less conserved compared to the COI gene. The 5S primer showed that the 5S gene is not sufficient for tilapia species identification. It showed that the 5S gene is the same in all tilapia species and the variation comes from the non-transcribed spacer (NTS). The combination of 5S and non-transcribed spacer (NTS) showed more differentiation among the species and highlighted differences in the nucleotides of the individuals. Therefore the nuclear DNA is better at showing hybrids and possible introgression as compared to COI mitochondrial DNA.

It is therefore recommended that more studies be under-taken with fish from different localities in South Africa because one of the major challenges with *O. mossambicus*, fish farmers have, is poor fingerling quality and this may be attributed to introgression. The sourcing of good quality fingerlings is now a major challenge in South Africa (Moyo and Rapatsa, 2021), thus one of the key implications of this study is that the aquaculture potential of *O. mossambicus* will decrease because of the wide spread hybridization with *O. niloticus* taking place within Southern Africa.

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