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DEPARTMENT OF ZOOLOGY

An investigation of the genetic integrity of *Oreochromis* species occurring in Nandoni and Albasini dams using the control region of mitochondrial DNA

MSc dissertation

By

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STATEMENT OF ORIGINALITY

I Vusi Besil Mboweni declare that this dissertation for the award of the MSc Degree in Zoology, of the University of Venda has not been previously submitted for a degree at this or any other university. All the reference material therein has been duly acknowledged.

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Date…10.06.2021……

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Date..10/06/2021……

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DEDICATION

This work is dedicated to my late grandparents, my late auntie, my older sister, and my mother who believe in me far more than I believe in myself. For putting their strength in me, forever dedicating themselves to my health and wellness. They truly love me.

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ABSTRACT

The genus *Oreochromis* represents a radiation of mouth-breeding Tilapiine fish inhabiting lakes and rivers throughout Africa. Due to incomplete reproductive isolation, most of the 31 species within this radiation can interbreed giving rise to fertile F1 hybrids. *Oreochromis mossambicus* is endemic to southern Africa but is now coming under threat throughout much of the sub-region because of the introduction of invasive *Oreochromis* species, which typically inhibit other parts of Africa. Due to their exceptional growth rates, invasive species were brought to South Africa for the aquaculture industry, and it is feared that they may have hybridized with or displaced *O. mossambicus.* This study aims to determine, using genetics, the extent of invasion of non-native *Oreochromis* species into Nandoni and Albasini dams of the upper Levubu in Limpopo South Africa. It is suspected that *O. niloticus* entered the River after the creation of Albasini Dam, which is upstream of Nandoni Dam. Therefore, it is predicted that typically *O. niloticus* mtDNA haplotypes will be observable in Nandoni, but that their frequency should be much lower upstream in Albasini Dam. I collected 141 samples from both dams, amplified and sequenced the control region of mitochondrial DNA. I then reconstructed networks and phylogenies with our samples combined with the downloaded samples from which I was able to determine the magnitude of *Oreochromis* invasion into the upper Levubu. Surprisingly, not one of the sequenced samples possessed a haplotype that clustered with *O. mossambicus* reference samples. However, I was able to identify two invasive species within the upper Levubu: *O. andersonii* in both Albasini and Nandoni dams and *O. niloticus* in Nandoni Dam. *Oreochromis andersonii* has high genetic diversity and with evidence of demographic expansion based on results from its mismatch distribution and Bayesian skyline plot. These results provide insights into the events that led to the invasion of foreign *Oreochromis* species to the Levubu system. A genetic signal for a demographic

expansion might have been caused by *O. andersonii* haplotypes being in the system before the "big flood" in the year 2000, with a re-introduction into Nandoni after the flood from a different source. This could explain why some haplotypes of *O. andersonii* are present in both dams and some are only present in Nandoni Dam. *Oreochromis niloticus* on the other hand, has low genetic diversity in Nandoni Dam compared to downloaded samples, and was probably introduced only once, and may have undergone a demographic bottleneck.

From these results, it is clear that *O. mossambicus* has been all but replaced by non-native *Oreochromis* in the upper Levubu. Hybridization or total replacement of *O. mossambicus* may have also occurred in another river system across its native range. *O. mossambicus* is better adapted to poorer eutrophic conditions and, most importantly it is well adapted to high salinity. Therefore, a strategy conserving genetically diverse *O. mossambicus* population in the lower reach of the river system, where there is higher salinity, like the lower Changane river, could be most appropriate for this species. The estuarine swamps could then become a refuge for *O. mossambicus* within its native range.

Keywords: Mitochondrial DNA, Haplotypes, *Oreochromis* species, hybridization, Nandoni dam, Albasini dam, Levubu river.

1. INTRODUCTION

1.1. The Genus *Oreochromis*

Oreochromis is one of three genera in the family Cichlidae, commonly referred to as tilapia. Like other cichlid fish, the African genus *Oreochromis* has diversified over time from a common ancestor that existed over 5.3 million years ago (Carnevale *et al*. 2003). The genus consists of some 31 species (Trewavas 1983), and they are differentiated from other *Tilapia* by their breeding behaviour as only the females care for the young (Trewevas, 1983). *Oreochromis* a monophyletic group (Meyer *et al*. 1990; Schliewen 1994) that employs the common cichlid reproductive strategy of mouthbrooding, which protects young from predation until they are larger and more self-sufficient (Trewevas 1983; Luna 2012). All *Oreochromis* species are found in Africa, naturally distributed in different rivers and countries across the continent.

Owing to their recent common ancestry, reproductive isolation mechanisms among *Oreochromis* species are incomplete, and several species can hybridize without necessarily incurring a fitness disadvantage (D'Amato e*t al.* 2007; Trewevas 1983; Wolfarth and Hulata 1981). Some scientists suggest that incomplete reproductive isolation among wild populations might play a role in facilitating or reinforcing adaptive radiations and the evolution of many phenotypically distinct species in a short space of time (Seehausen 2004; Rakotoarivelo *et al.* 2019; Masello *et al*. 2019). Gene flow (introgression) between two taxa can establish a third hybrid taxon within a hybrid zone (Allendorf *et al*. 2001; D'Amato *et al*. 2007; Masello *et al*. 2019). Introgression between recently separated species is probably widespread in tilapia (D'Amato *et al*. 2007; Hey *et al*. 2004). Therefore, evolution through hybridization appears to have endowed cichlid fish with the ability to rapidly produce different phenotypes (Salzburger *et al*. 2002). However, although *Oreochromis* species appear not to be reproductively isolated, the extent of their hybridization in

the natural environment is poorly documented (D'Amato *et al.* 2007; Rognon and Guyomard 2003).

On the other hand, hybridization between phenotypically different taxa through human intervention could be detrimental to the native species. This is because hybridization with native species might result in the loss of the genetic integrity of native species completely or they might only exist as a hybrid (invader X endemic) just like the case in Victoria Lake, where *O. variabilis* was wiped out after the introduction of *O. niloticus* and *O. mossambicus* (D'Amato *et al*. 2007; Welcomme 1967.) A similar situation happened in a Madagascan lake where *O. macrochir* was completely replaced by *O. niloticus* in only 10 years (Firmat et al 2013; Daget and Moreau 1981). Most importantly the role the endemic species plays in the ecosystem would be compromised.

Tilapia fish have become important aquaculture species, responsible for over 1.5 million metric tons of food worldwide, second to only carp and salmon (Fessehaye 2006). Tilapia grows fast and can reach sexual maturity at around 2-3 months, which is very good in aquaculture making them a great source of food. Skelton (1993) suggested that tilapia species are well utilized in commercial and subsistence fisheries. He also stated that the Tilapiine is also greatly valued as angling species. Among the *Oreochromis* species, the Mozambique tilapia (*O. mossambicus* Peters 1852) and the Nile tilapia (*O. niloticus* Linnaeus 1758), are among the most important for aquaculture (Romana-Eguia *et al*. 2004). *Oreochromis* species, have been introduced throughout the world and have established so well in the natural ecosystem outside their native range. (D'Amato *et al*. 2007). Their extralimital establishment is usually linked to individuals either escaping from aquaculture facilities or the deliberate introduction of individual fish in non-native river systems (Van der Waal 2002).

In Africa, where many river systems contain their species of *Oreochromis* (Figure 1), the introduction of *O. niloticus* in systems outside its natural distribution range has resulted in hybridization with native tilapia (Bezault *et al*. 2011; Eknath and Hulata 2009). Fish that escaped from aquaculture farms, illegally introduced for angling and introduced as biological control agents are now recognized as the leading threat to the conservation of native *Oreochromis* in Africa (D'Amato *et al*. 2007). This created an advantage that benefitted aquaculture greatly, because the newly introduced species was able to act as a biological control for aquatic weeds, diatoms, algae, macro-invertebrates tadpoles and even small fish (D'Amato *et al*. 2007; Wohlfarth and Hulata 1981; Trewavas 1983; de Moor and Bruton 1988; Russell *et al*. 2012; Arthington and Bluhdorn 1994; Komarkova and Tavera 2003). Hybrids tend to grow larger than their parental species, which is good for anglers and great for commercial business. The bigger the fish, the better the price (Skelton 1993).

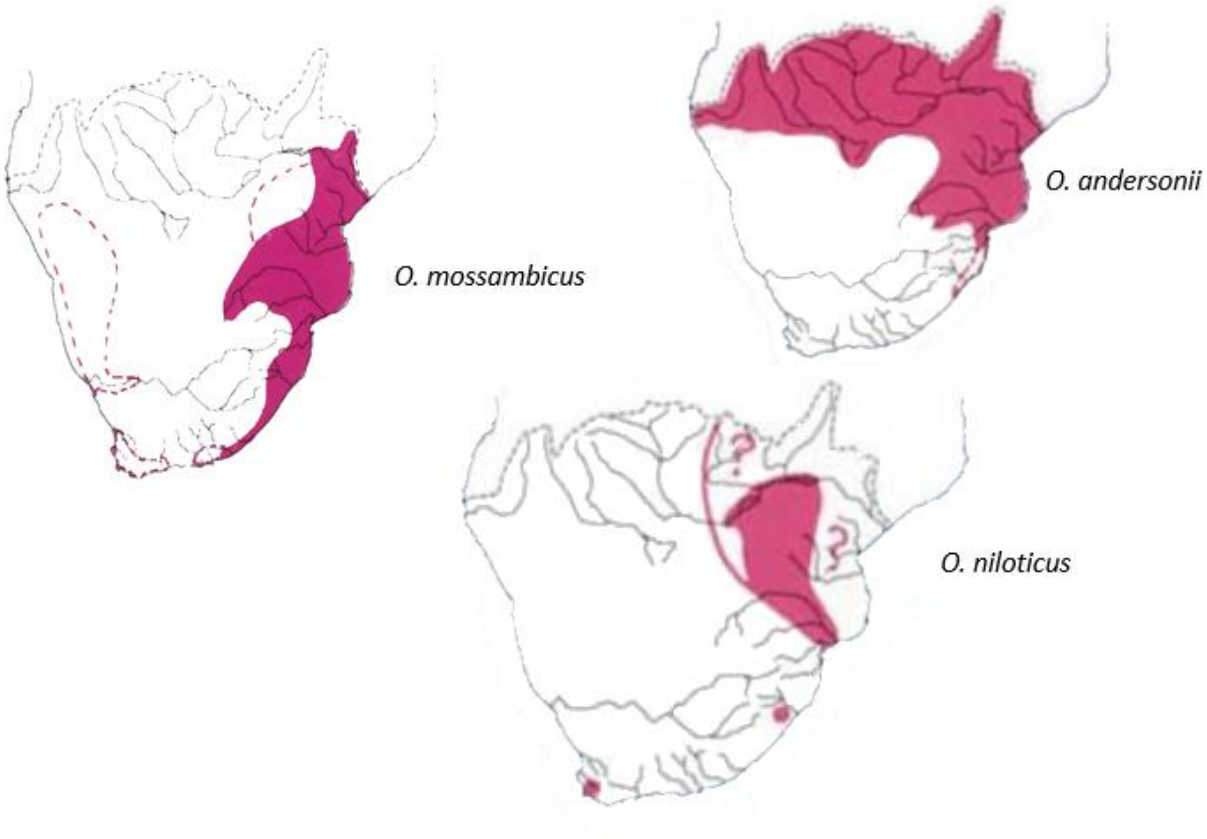


Figure 1. Map showing the approximate extant distributions of, *O. andersonii*, *O. mossambicus* and the distribution of *O. niloticus* in southern Africa. The map was obtained from (Skelton 1993)

1.2. *Oreochromis mossambicus* (Peters 1952)

1.2.1. Native distribution of *O. mossambicus*

The Mozambique tilapia is native throughout eastern southern Africa (Figure 1), from the lower Zambezi River upstream to Cahora-Bassa Dam (Van der Waal 2002), as well as in the Limpopo river system and all the eastward flowing rivers to the south at least as far south as the Bushmen's River in the Eastern Cape. The *Mozambique tilapia*, *O. mossambicus*, is classified

as ‘‘near-threatened’’ on the IUCN Red List because of its hybridization with the widely introduced *O. niloticus*. Throughout their range, they prefer slow-flowing ponds, in both permanent and yearly streams, and avoid fast, rolling waves and other areas of rapidly flowing water (Gaigher 1973). The habitat of *O. mossambicus* was conducted by Gaigher (1973) in the Limpopo river system for the populations at that time.

1.2.2. Morphology and reproduction of *O. mossambicus*

Van der Waal 2002 observed that *Oreochromis mossambicus* has a deep body accompanied by a long dorsal fin with 10-13 rays and spines. The fish has a dull yellow-green with weak banding patterns along the body (Froese and Pouly 2007). The head is often dark as the body, however, it can sometimes be light silver, to brownish. In some cases, it can appear yellowish to blue-grey and even black. It has three unclear spots in a horizontal row on flanks, with six or seven unclear vertical bands on the body. The dorsal and anal fins are not prominently high as in Nile tilapia, but the anal and dorsal spines are well developed and thick. The eyes are yellow to dark brown, never red. The caudal fin has spots but never in vertical lines. The caudal fin and especially the dorsal fin edges are normally red. Males in a spawning (breeding) dress are dark to black with a white chin. The mouth is enlarged and a concave head profile (Van der Waal and Bills 2000; Skelton 1993).

This species generally reaches about 38 cm in length (Trewevas 1983); however, a maximum size of 48.2 cm has been reported at Nyamiti pan in KwaZulu-Natal, South Africa (Bruton and Allanson 1974). This fish can reach sexual maturity within 2-3 months at the length **of** about 15.2-17.9 cm (Luna 2012)

Oreochromis mossambicus displays a polygamous complex spawning system (Russell *et al*. 2012; Fryer and Iles 1972). Territorial males dig a circular nest with their mouth on a soft substrate (Bruton and Boltt 1975; Russell *et al*. 2012). Dominant males guard and defend their nests from other males and sneakers (males that are of intermediate rank and low ranking) while using the nest to attract females during spawning. Sneaker males display a light-dark colouration and temporarily occupy a territory for a short time (minutes or seconds), to court females when the owner is absent. The sneaker males intrude nest during a spawning episode and try to remain near the female while simultaneously exhibiting quivering behaviour, a behavioural pattern usually related to sperm release (Oliveira and Almada 1998b). the mating tactics displayed by sneakers be sued by the same individual at different times. These tactics linked to male social status, it was also clear that only dominant male establishes and defend territories. Sneakers behaviour is common in males of intermediate rank that attempted to establish territory without success. However, sneaking is predominantly performed by males of low rank (i.e. subordinates or non-territorial males) (Oliveira and Almada 1998b). The females will lay eggs normally when the temperature exceeds 23° (Russell et al, 2012; Arthington and Milton 1986), and are then externally inseminated by males (either dominant or sneakers). The female then broods the fertilized eggs for 20-22 days in her mouth (Russell *et al*. 2012; Fryer and Iles 1972; Baerends and Baerends van Room 1950; Bruton and Bolt 1975). The females belligerently safeguard her brood (Russell *et al*. 2012).

1.2.3. Suitability of *O. mossambicus* for aquaculture

The species favours freshwater, but unlike most other *Oreochromis*, is also able to inhabit estuaries and saltwater, withstanding a high saltwater content and can acclimatize to salinities up to double-strength saltwater (Yamaguchi *et al*. 2018; Fiess *et al*. 2007; Trewevas

1983) and low temperatures of below 10°C with a lower fatal limit of 9.5°C in controlled environments (Shafland and Pestrak. 1982*).* *O. mossambicus* can also tolerate low dissolved oxygen levels of < 1 mg L^{-1} (milligram per litre), for a brief period and can boost their oxygen intake by 'gulping' air at the surface of the water (Maruyama 1958). Philippart and Ruwet (1982) suggested some species of tilapia can thrive in swampy water bodies where depleted O_2 concentration can occur frequently. *O mossambicus* can withstand drought and can thrive in a high salt environment (D'Amato *et al*. 2007).

This species was attractive for aquaculture because of its ability to tolerate areas that have limited riparian cover and can survive in poor conditions (Arthington *et al*. 1983). This species has been reported to eat terrestrial vegetation, aquatic macrophytes, diatoms, algae, periphyton, planktons, macro-invertebrates, tadpoles, and small fish (Russell *et al*. 2012; Arthington and Bluhdorn 1994; Komarkova and Tavera 2003). *O. mossambicus* is also a generalist in terms of diet requirement, reproduce frequently with the advantage of maternal care. (Pérez *et al*. 2006). And most impressively *O. mossambicus* could withstand ammonia concentrations of 3 mg L-1 without any significant adverse impact on food uptake or growth (Sampath et al. 1991).

The feeding strategies of the species depend on the food source, available and can include filter-feeding, scavenging, cannibalism, grazing, etc. *O. mossambicus* also can resort to alternate food resources by swiftly modifying their braincase and teeth structure to suit convenient food types (Pérez *et al*. 2006). These advantages allow *O. mossambicus* to adapt and use poor aquatic systems (Pérez *et al*. 2006). It also provides them with a competitive advantage over their relatives and other freshwater fish species as they are usually not repelled by eutrophication or algal blooms, which can be lethal to other species (Arthington *et al*. 1983).

1.2.4. Non-native distribution of *O. mossambicus*

O. mossambicus has also become widely distributed around the world. Although it was originally valued as an ornamental fish and biological control agent (Canonico *et al*. 2005), its value as an aquaculture species was quickly recognized, resulting in worldwide range expansion through this utilization (Russell *et al*. 2012; Arthington *et al*. 1984; Philippart and Ruwet 1982). The species can be found in supermarkets across the world. However, the fact that it can tolerate a wide range of ecological conditions made *O. mossambicus* a model invader. The species has been included in the Global Invasive Species Database (2006) and has invaded five continents, with established populations in 94 countries (Fish base 2010).

1.3.*Oreochromis niloticus* (Linnaeus 1758)

1.3.1. Native distribution of *O. niloticus*

Oreochromis niloticus has the largest natural dispersal among *Oreochromis* genus, including the entire Nilo–Sudanian province (from Senegal to Nile basin and Nile delta), the Ethiopian Rift Valley (Bezault *et al*. 2011; Boyd 2004; Moralee *et al*. 2000). However, in southern Africa, *O. niloticus* has established territories in the cape flats area, Western Cape, Kwazulu-natal and widely distributed in Zambia and Zimbabwe. This species has been introduced to southern Africa from Israel around 1955 as an aquaculture species (Skelton 1993) and today has invaded several rivers in southern Africa (Van der Waal and Bills 1997). The successful establishment of *O. niloticus* can be ascribed to their capability to thrive in poor environmental conditions, which allows them to colonize an extensive range of habitats, such as from man-made dams, rivers, brackish water, lakes, and alkaline pools with hot springs (Bezault *et al*. 2011; Trewavas 1983).

1.3.2. Morphology and reproduction of *O. niloticus*

Oreochromis niloticus is described as a deep-bodied fish with rounded scales, of silver colour with olive/grey/black body bars (Skelton 1993). The Nile tilapia males usually turn reddish during the breeding season (Picker and Griffiths 2011). Borders and caudal fin might be red, but not sharply edged red. It has a dark green to silvery grey head and body with a vertical prominent bar with a dorsal fin that is never a red colour. They usually have 8 clear vertical bands on the body and caudal-fin base. The dorsal fin spines are long, very thick, and pointed. The dorsal and anal fins are relatively high compared to body depth. Eyes are typically red-coloured, they have clear bars on the tail that can be 2-22 layers and more (Skelton 1993; Van der Waal 2000).

Males in their breeding dress appear pinkish. Unlike *O. mossambicus* does not have an enlarged mouth and the concave profile of the snout (Van der Waal 2002). The breeding strategy is the same as in *O. mossambicus* ((Russell *et al.* 2012; Skelton 1993; Arthington and Milton 1986; Fryer and Iles 1972; Brutton and Boltt 1975). The species is said to have an average length of about 20cm (Bwanika *et al*. 2004). At the estimated age of 9 years, a recorded specimen of *O. niloticus* weighed 3.65Kg reaching a length of 62 cm (FAO 2012).

1.3.3. Suitability of *O. niloticus* for aquaculture

Oreochromis niloticus lives in freshwater habitats of northern and western river basins of Africa and can only tolerate salinities of up to 25 parts per thousand (ppt) (Yamaguchi *et al*. 2018; Trewavas 1983; Watanabe *et al*. 1985). *Oreochromis niloticus* is an omnivorous fish that feeds on aquatic plants, bacterial films, invertebrates, periphyton, detritus, benthic fauna, phytoplankton, including other fishes and eggs. (FAO 2012). This fish is a filter feeder. However, it can be a cannibal if the opportunity presents itself and can graze depending on the food source

(GISD 2012). Nile tilapia has a life span of over 10 years (GISD 2012) and their growth is only limited by factors such as predation, water temperature, and food availability (Kapetsky and Nath 1997). They reach peak growth between 28-36°C while growth becomes poor when the temperature decrease. The survival limits are between 11 and 46 °C. They can tolerate low levels of oxygen (as low as 3-4mg/L) (Boyd 2004). This fish matures at the age of 5-6 months in aquatic ponds (FAO 2012).

Britz *et al.* (2009) reported that South Africa had only one tilapia farm in 2008, which was a pilot project. In 2009 and 2010, South Africa was estimated to produce about 10 tons of tilapia (DAFF 2012 a).

1.3.4. Non-native distribution of *O. niloticus* in South Africa.

Nile tilapia was initially introduced in South Africa in 1959, since then, they have been set free into dams of the Western Cape and Kwazulu-Natal, to nourish largemouth bass (Van Schoor 1966). Introductions into dams since 1980 in most areas of southern Africa were followed by fish escaping and establishing populations in rivers. The outcome is that *O. niloticus* has populated Limpopo, and Inkomati river systems (Picker and Griffiths 2011). Picker and Griffiths (2011) and Kleynhans *et al.* (2005) showed that the species is present in at least 12 of the ecoregions of South Africa which includes the Limpopo Plains, Soutpansberg, Waterberg, Western Bankenveld, Bushveld Basin, Natal Coastal plains, South Eastern uplands, and North Eastern Coastal Belt.

Hybridization of *O. niloticus* with native *O. mossambicus* is a possibility that could threaten the existence of *O. mossambicus* genetically (*O. mossambicus* may only exist as hybrids) in its natural range. D'Amato *et al.* (2007) suggested that hybridization could reduce or destroy

the adaptive ability of *O. mossambicus* to drought, endurance to low temperatures, and adaptation to high salt environments.

1.4.*Oreochromis andersonii* (Castelnau 1861).

1.4.1. Native distribution of *O. andersonii*

Oreochromis andersonii, also known as the three-spot tilapia is naturally native to Cunene, Okavango, upper Zambezi, and Kafue river system and has been occasionally recorded from the middle Zambezi (Skelton 1993; Trewavas 1983).

1.4.2. Morphology and reproduction of *O. andersonii*

Oreochromis andersonii is a small-mouthed cichlid, where the adults are blue-grey and the juveniles are silver in colour with 8-9 irregular thin bars. This fish has three distinctive mid-lateral spots on the body. The breeding adults are almost always blue-grey with light scale borders giving a mesh effect outward appearance. The flexible fins (dorsal and anal) are blue-grey with light sports, while the edges of the dorsal and anal fins are always vivid red (Skelton 1993). Breeding males are always blue-black with silver mesh, maroon flash on top of the head. The outer dorsal and caudal fins have an intense red colour. This fish normally has 11-14 thick dorsal spines and 31-35 lateral line scales. They also have 21-27 gill rackers on the bottom lip of the very first-gill arch (Skelton 1993).

Breeding behaviour is the same as that of *O. niloticus* and *O. mossambicus* and *O. andersonii*. This breeding behaviour is very common in tilapia. (Skelton 1993; Russell *et al*. 2012; Arthington and Milton 1986; Fryer and Iles 1972; Bruton and Boltt 1975). This similarity in breeding behaviours may also help facilitate interspecific hybridization.

1.4.3. Suitability of *O. andersonii* for aquaculture

The three-spot tilapia is a detritivore (Skelton 1993; Trewavas 1983). Large adults fish also eat insects and invertebrates (Thorstad *et al.* 2003). It can tolerate fresh brackish water and prefers slow-flowing or standing water such as in pools, backwaters, and floodplains lagoons. Adults normally prefer deep open water, while small fish have to stay inshore amid vegetation (Skelton 1993). *Oreochromis andersonii* typically grows up to 50 cm in total length and can reach a maximum weight of 3,2 Kg. The largest recorded specimen weight 3,09 Kg and was reported in Zimbabwe while the largest in South Africa was reported to weigh 1.97 Kg. the fish can live for 7-9 years (Skelton 1993).

1.4.4. Non-native distribution of *O. andersonii*

Oreochromis andersonii was reported to have been introduced in the 1970s into the Shashe Dam in Botswana, in the upper Limpopo river system (Firmat *et al.* 2013; de Moor and Bruton 1978) and the middle and lower reaches of the Limpopo River (Firmat *et al*. 2013; D'Amato *et al*. 2007). This might be the reason why we have *O. andersonii* in Levubu River system. It should not be surprising that fish can easy swim upstream against the current to establish new territories. The building of Nandoni Dam in 2005 might have favoured the invasive species greatly, hence they are abundant today. However, since Albasini Dam was built in 1952, *O. andersonii* might have used the dam as proper breading ground, hence the haplotypes found in Albasini Dam are also found in Nandoni Dam.

1.5.Human-mediated hybridization of *Oreochromis* species in the Levubu system

As detailed in the previous section, because of their amenability to aquaculture practices, species of the genus *Oreochromis* have been introduced in areas outside their native range for several reasons. This has resulted in the species colonizing new habitats and hybridizing with the native species of the same genus (Trewavas 1983; D'Amato *et al*. 2007). Hybrids of *O. niloticus* from South Africa and *O. mossambicus* from Lake Albert (Avault and Shell 1968) *were* reported by Mires (1977) in pond and aquaria in Alabama. They discovered that the hybrid's tolerance to low temperature is greater than that of its parents (Pruginin 1965; Iversen 1968; Trewavas 1983).

Albasini Dam (coordinates (-23. 10499958 30. 1246995012) is located near the town of Elim, outside Louis Trichardt, Limpopo South Africa, and is the first dam on the Levubu River (Figure 2). From here, the river flows eastwards for 42 km, reaching the much larger Nandoni Dam (-22. 98666272 30. 604497582), which lies 16 km southeast of Thohoyandou, Limpopo Province South Africa. The dam was constructed in 1952, so any later downstream introduction of invasive species, such as *O. andersonii*, could not have affected the species composition in the dam.

Nandoni Dam was constructed in 2005, and any downstream introduction could have reached this part of the river system. Fouché *et al.* (2010) reported that tilapia specimens thought to be *O. mossambicus*, collected from Nandoni Dam (Figure 2) in the Levubu river system displayed what could be regarded as *O. niloticus* characteristics. This included red eye colour and faint vertical lines on the caudal fin. Hybrids of this species are very difficult to identify

morphologically, as they tend to resemble one or other parent species (D'Amato *et al*. 2007). The observations by Fouché *et al.* (2010) could be an indication that *O. niloticus* is present in Nandoni Dam and that hybridization between *O. niloticus* and *O. mossambicus* may already have taken place. There is no study that published the *Oreochromis* species of Albasini Dam, therefore it is assumed to be populated by *O. mossambicus* because it forms parts of its recorded natural range.

For this project, it was important to sample *Oreochromis* in both dams to investigate whether the fish in both dams were genetically similar and whether potential introductions into Nandoni Dam have any consequences for fish in Albasini Dam.

Figure 2. Map showing the sampling area. Nandoni and Albasini dams are on the upper Levubu river system in northern South Africa. The Levubu river flows from the west side of Albasini Dam towards the east into the Nandoni Dam. The distance between the two dams is about ~41,5 kilometres. The entire system was once inhabited by the Mozambican tilapia (*O. mossambicus*). The two thick blue arrows indicate the direction of the river flow

1.6. Identifying *Oreochromis* hybrids

Morphological identification is problematic as hybrids tend to resemble either parent species (Moralee *et al*. 2000). The process of identifying these includes counting dorsal fin spines, anal fin spines, presence of spots on the body of the fish, number of bars on the side of the

body, caudal fin bars and gill rakers on the lower part of the first-gill arch (Moralee *et a*l. 2000; Van der Waal and Bills 2000).

Another way to identify hybrids is by using genetic analysis. The common genetic markers in such cases are mitochondrial DNA (mtDNA) and nuclear microsatellite loci (nDNA). In eukaryotes, cells possess multiple mitochondria that are required for the energy requirements of the cell, since mitochondria evolved through endosymbiosis, thus allowing aerobic respiration in proto-eukaryote cells (Margulis 1970). The best evidence for an endosymbiotic origin is that mitochondria have their DNA, separate from the nuclear genome. The mitochondrial DNA is about 16500 bp, circular encoding 37 genes; 13 of which encodes for the polypeptide of the Oxidative Phosphorylation System (OXPHOS). The mtDNA possesses 22 tRNAs and 2 rRNAs (Sato and Sato 2013; Garesse and Vallejo 2001), required for translation by mitoribosomes within the matrix (Gupta *et al*. 2015; Gray and Doolittle 1882; Taanman 1999). Mitochondrial DNA possesses 4 main portions: D-loop or control region, rRNA, tRNA and the cording region (Anderson *et al*. 1982; Gupta *et al*. 2015). Mitochondrial DNA is also inherited independently of nuclear DNA, from the mitochondria of the oocyte hance it is maternaly inherited (Sato and Sato 2013; Giles *et al*. 1980; Birky 1995; Ankel-Simons and Cummins 1996).

Being only maternally inherited, mtDNA may not seem like the ideal marker for the identification of hybrids, as a nuclear marker might seem more appropriate, because nuclear DNA is a copy from both parents. However, some properties make mtDNA more favourable, in addition Oreochromis genus form a monophyletic clade (Meyer *et al*. 1990; Schliewen 1994) therefore, mtDNA is good tool in identifying hydrids.. Nuclear microsatellites are difficult and

expensive to isolate (Selkoe and Toonen 2006). Microsatellites being nuclear in origin are present in only one copy per cell, so it is far less common than mtDNA in DNA extractions (Glenn and Schable 2005). Most importantly, microsatellite data sets are usually species and project-specific (Glenn and Schable 2005). So, allele profile data from one project or laboratory cannot be readily used in another study from the same species. This means that a study of *Oreochromis* in South Africa cannot make use of previously published *Oreochromis* microsatellite data from other studies, which poses a serious limitation for genetic studies. On the other hand, mtDNA sequences are directly comparable regardless of study or laboratory. There are several *Oreochromis* species mtDNA datasets available for comparison. The high copy number of mtDNA, also makes it far less difficult to isolate and amplify mtDNA than nuclear DNA (Mengel-From *et al*. 2014; Chang and Clayton 1985).

1.7. Previous studies on *Oreochromis.*

Previous studies found that hybridization of introduced *Oreochromis* with native species is a great concern for the conservation of the native species (D'Amato *et al*. 2007; Van der Waal and Bills 2000). This hybridization makes it extremely difficult to find pure native species, such as *O. mossambicus* in rivers like the upper Limpopo. (D'Amato *et al*. 2007; Moralee *et al*. 2000). In the study by Firmat *et all*. 2013, they found that specimens of *O. andersonii* which were introduced to upper Limpopo river drainage (Botswana) in 1973 and *O. niloticus* which was released into the Limpopo system (Zimbabwe) have expanded and invaded deep into the lower Limpopo. This was evident by the presence of their haplotypes in lower Limpopo drainage. The presence of the *O. niloticus* and *O. andersonii* haplotypes in hybrids means hybridization has been taking place for some time in Mozambique rivers that are part of the natural range, which is the

natural range of *O. mossambicus*. A genetic study by Moralee *et al*. (2000) was able to identify at least five hybrids that had alleles of both parent species (*O. niloticus* and *O. mossambicus*). It is extremely challenging to identify morphologically as hybrids tend to look like one of the parent species. This reality pushes future researchers to rely more on genetic studies than traditional morphological studies. These early studies (Moralee *et a*l. 2000; Van der Waal and Bills 2000; Trewavas 1983; Avault and Shell 1968; Mires 1977; Skelton 1993) also provide an important repository for DNA sequences, which can be downloaded and compared to new samples from anywhere in Africa.

The species-specific monophyletic clades identified from mtDNA in the studies of African cichlids species (Meyer *et al*. 1990: Schliewen 1994) are potentially very useful in hybrid detection. The mouthbrooding genera *Oreochromis*, *Sarotherodon*, *Iranocichla* and *Tristramella* formed a monophyletic group, hereafter named "Oreochromini", after the most species-rich genus within this group *Oreochromis* (Schwarzer *et al*. 2009). The study by Klett and Meyer (2002) found that the Lake Tanganyika radiation appears to be monophyletic concerning tilapiine lineages, in this study, all represented members of the genus *Oreochromis*, which includes *O. tanganyikae*, an endemic species from Lake Tanganyika, form a strongly supported monophyletic group. In a hybrid situation, the hybrids will have the mtDNA of their mothers, but when these mothers belong to an extralimital species, they will possess the mtDNA typical of the invading species. In this way, the presence of non-native DNA in a population can be determined, and this presence will be used in this study as a measurement of the level of hybridization in the population.

1.8.Research questions.

Based on the information above, I now ask the following research questions:

- Do both *O. niloticus* and *O. andersonii* occur in Nandoni and Albasini dams in

- the Levubu river in northern South Africa? And has hybridization occurred between the two species and the native *O. mossambicus*?
- Did the flood in 2000 have an impact on the system in terms of introducing new species or even new haplotypes?
- Are the haplotypes found in the Albasini Dam the same as the haplotypes found in the Nandoni Dam?

1.9. Hypothesis

The assumption is that *O. niloticus* escaped from a fish farm that was in the Levubu farm settlement. Since the farm was located in between Albasini Dam and Nandoni Dam, the fish most likely went downstream with the water and could not swim upstream and into Albasini Dam. The biggest challenge is the high wall of Albasini Dam, a fish can't cross it and enter the dam. This left the idea that *O. niloticus* haplotypes would be present in Nandoni Dam downstream. We predict therefore that *O. niloticus* mtDNA haplotypes will be observable in Nandoni, but that its frequency should be much lower upstream in the nearby Albasini Dam. If somehow the *O. niloticus* managed to enter Albasini Dam, it would be important to compare the Albasini haplotypes with the downstream haplotypes.

1.10. Aims and objectives

The aims of the study are to:

1. Collect *Oreochromis* samples from Nandoni and Albasini dams in the Upper Levubu river, Limpopo Province, South Africa.

2. Determine, the phenotype of the collected specimens.

3. Determine, using the mtDNA control region, whether non-native *Oreochromis* species haplotypes occur in these two dams, using haplotypes available online for comparison.

4. To determine the past demography of *Oreochromis* clades using Bayesian skyline plots

5. To compare non-native haplotypes with phenotype information to determine whether hybridization has occurred.

2. MATERIALS AND METHODS

2.1. Sample collection

Samples (whole fish or fin clips) were collected with fleets of multifilament experimental gill nets in Nandoni and Albasini dams. Nandoni Dam, which is situated in the Vhembe district, Limpopo Province, South Africa (Figure 2). Nandoni Dam is part of the Levubu River Government Water Scheme (LWGS) (DWAF 2001), and Albasini Dam is part of the Levubu Irrigation Scheme. The two dams are approximately 42 km apart. One hundred and forty-one (141) specimens were collected in total from both dams. Ninety-three (93) specimens were collected from the Nandoni Dam and 48 were collected from the Albasini Dam (see Table 1 below). Samples (fin clips) (Ex, Ex4, Ex5, Ex6, Ex7, Ex8 and Ex9) were collected using fleets of multifilament experimental gill nets. Each fleet consist of five individual nets, each 30 cm long and 1.8 m deep with the following stretched mesh sizes: 28, 45, 73, 93, and 181 mm, these were used in limnetic zones. At the littoral site, fish were collected using narrow traps, seine nets and composite gill nets with 5 m sections of different sizes: 16, 28, 48, 57, 73 and 99 mm stretched mesh sizes. Some of the samples were bought from fishermen who uses similar nets to ours, but much larger and are for commercial use. The date, coordinates, and names of the sample collection have been tabulated below in TABLE 1.

The rest of the 362 samples were downloaded from GenBank and have been provided in TABLE 2, the Accession number, Species, Site/country of origin, Wild /aquaculture, base pairs (Bp), sample number and references.

Table 1: Details of the sample collection at different locations at different times

Samples	N	Location	Site country	Date	site
Ex4 (1-5, 7, 8, 11, 13 -19)	15	S22°59'20.00" E30°31'14.33"	Nandoni Dam, South Africa	2010/10/21	Littoral zone
Ex1 (1-3,5, 16, 17-20)	18	S23°06'25.43" E30°06'49.98"	Albasini Dam, South Africa	2015/04/05	Littoral zone
Ex8 (4, 10, 24)	3	S22°34'19.41° E30°34'19.41"	Nandoni Dam, South Africa	2010/09/15	Littoral zone
2014 (1-4, 8, 12, 15, 16-18, 20, 22, 26, -28, 30, 35, 43, 45)	19	S22°59'50.69" E30°31'24.79"	Nandoni Dam, South Africa	2010/03/11	Littoral zone
Ex5 (2, 5, 7, 8, 11, 12, 16-19, 21, 22)	11	S22°58'59.47" E30°35'41.89"	Nandoni Dam, South Africa	2010/12/01	Littoral zone
Ex6 (1)	1	S22°59'50.57" E30°32'43.61"	Nandoni Dam, South Africa	2010/08/04	Littoral zone
Ex7 (2, 4, 5, 8 – 10)	6	S22°34'19.41° E30°34'19.41"	Nandoni Dam, South Africa	2010/09/15	Littoral zone
Ex8 (1, 3, 9, 14, 17 -21, 23)	10	E22°58'57.24" E30°33'52.40"	Nandoni Dam, South Africa	2011/04/13	Littoral zone
Ex9 (5, 6, 11)	3	S22°59'50.57" E30°32'43.61"	Nandoni Dam, South Africa	2011/06/20	Limnetic zone
Ex12 (1, 2, 5, 7, 12, 13, 23)	7	S22°58'28.68" E30°35'22.68"	Nandoni Dam, South Africa	2016/11/03	Limnetic zone
Ex12 (3, 18, 43, 53, 55, 64)	6	S23°06'12.53" E30°05'17.38"	Albasini Dam, South Africa	2016/11/03	Limnetic zone
Ex12 (85)	1	S22°59'52.85" E30°31'28.78"	Nandoni Dam, South Africa	2017/10/30	Limnetic zone

Ex12 (15, 58, 73, 75, 79)	5	S23°05'51.35" E30°04'09.95"	Albasini Dam, South Africa	2017/12/18	Littoral zone
Ex5 (23)	1	S22°58'59.47" E30°35'41.89"	Nandoni Dam, South Africa	2010/12/01	Littoral zone
23	1	S23°05'51.35" E30°04'09.95"	Albasini Dam, South Africa	2016/11/03	Limnetic zone
Nan (1 -4, 7, 20, 30, 33, 35, 37, 42 – 45, 70, 73)	16	S22°59'52.85" E30°31'28.78'	Nandoni Dam, South Africa	2017/10/30	Limnetic zone
9, 15, 16, 18, 20,	5	S23°05'51.35" E30°04'09.95"	Albasini Dam, South Africa	2016/11/03	Limnetic zone
ALB (1-9, 11, 12, 14, 15)	13	S23°05'51.35" E30°04'09.95"	Albasini Dam, South Africa	2017/10/02	Littoral zone

Table 2: Details of the downloaded samples

Accession number	Species	Site, country of origin	Wild /aquaculture	Base pairs(Bp)	N	Reference
AF296466,	*O. mossambicus*	Mozambique	N/A	375	1	Nagl *et al*. 2001
EU430997-9	*O. mossambicus*	N/A	N/A	998, 898	3	Chang *et al*. 2008
KY587515.1	*O. mossambicus*	N/A	N/A	407	1	Chang *et al*. 2008
KY587510-11	*O. mossambicus*	N/A	N/A	407	2	Chang *et al*. 2008
KY587500-09	*O. mossambicus*	N/A	N/A	407	10	Chang *et al*. 2008
KY587495.-99	*O. mossambicus*	N/A	N/A	407	4	Chang *et al*. 2008
KY587492.1	*O. mossambicus*	N/A	N/A	407	1	Chang *et al*. 2008
KY587489.1	*O. mossambicus*	N/A	N/A	407	1	Chang *et al*. 2008
KY587487.1	*O. mossambicus*	N/A	N/A	407	1	Chang *et al*. 2008

KY587485.1	*O. mossambicus*	N/A	N/A	407	1	Chang *et al*. 2008
KY587479-81	*O. mossambicus*	N/A	N/A	407	3	Chang *et al*. 2008
AY833436, 41, 42, 54, 59, 73, 92	*O. mossambicus*	Olifant River, Limpopo basin, South Africa	Wild	378	7	D'Amato *et al.* 2007
AY833457- 58	*O. mossambicus*	Amatikulu Kwazulu Natal, South Africa	Farm	378	2	D'Amato *et al.* 2007
AY833455-56	*O. mossambicus*	Velley Farm, South Africa	Farm	378	2	D'Amato *et al.* 2007
AY833447-53	*O. mossambicus*	Research station on the Shire River system at Kasinthula, Malawi	Wild	378	7	D'Amato *et al.* 2007
AY833448.1	*O. mossambicus*	Sucoma, lower Shire river System, Malawi	Wild	378	1	D'Amato *et al.* 2007
AY833445-46	*O. mossambicus*	Makathini research station, Pongola River, Natal, South Africa	Wild	378	2	D'Amato *et al.* 2007
AY833440-44	*O. mossambicus*	Pongola/Usutu River at Ndumu, South Africa	Wild	378	5	D'Amato *et al.* 2007
AY833437-43	*O. mossambicus*	Boesmans River, South Africa	Wild	378	7	D'Amato *et al.* 2007
AY833438-39	*O. mossambicus*	Nick James Farm, South Africa	Farm	378	2	D'Amato *et al.* 2007
JQ907486-507	*O. mossambicus*	Mozambique	N/A	378	22	Firmat *et al.* 2013
KY587474.1	*O. mossambicus*	N/A	N/A	407	1	Sowah and Seki 2017
KU180640	*O. mossambicus* X *O. niloticus*	Daze, Tanjiang river, Guangdong Province, China	N/A	433	1	Gu *et al*. 2016

KU180639	*O. mossambicus X O. niloticus*	Muzhou, Xijian River, Preal River, Guangdong Province, China	N/A	422	1	Gu *et al*. 2016
KU180621-23, 25	*O. mossambicus* X *O. niloticus*	Maoming, Meihuajiang River, Jianjiang River, Guangdong Province, China	N/A	433	4	Gu *et al*. 2016
KU180541-43, 46, 53, 89	*O. mossambicus* X *O. niloticus*	Huizhou, Dongjiang River, Preal River, Guangdong Province, China	N/A	433	6	Gu *et al*. 2016
KU180569-70, 72, 74	*O. mossambicus* X *O. niloticus*	Zhaoqing, Xijiang River, Preal River, Guangdong Province, China	N/A	433	4	Gu *et al*. 2016
KU180534	*O. mossambicus* X *O. niloticus*	Wuzhou, Xijiang River, Preal River, Guangxi Province, China	N/A	433	1	Gu *et al*. 2016
KU180590-91	*O. niloticus*	Huizhou, Dongjiang River, Preal River, Guangdong Province, China	N/A	433	2	Gu *et al*. 2016
KU180622, 24, 26	*O. niloticus*	Maoming, Meihuajiang River, Jianjiang River, Guangdong Province, China	N/A	433	3	Gu *et al*. 2016
EF016715-23	*O. niloticus*	Senegal River (Saint Louis population)	N/A	367	9	Nyingi *et al.* 2009
KU180609-10, 14	*O. niloticus*	Chaozhou, Hanjiang River, Guangdong Province, China	N/A	433	3	Gu *et al*. 2016

KU180643-45	*O. niloticus*	Daze, Tanjiang river, Guangdong Province, China	N/A	433	3	Gu *et al*. 2016
KU180631-38	*O. niloticus*	Muzhou, Xijian River, Preal River, Guangdong Province, China	N/A	433	8	Gu *et al*. 2016
KU180580-82, 86-88	*O. niloticus*	Huazhou, Jianjiang River, Guangdong Province, China	N/A	433	6	Gu *et al*. 2016
KU180544-45, 47-52, 54-56	*O. niloticus*	Haikou, Nandujiang River, Hainan Province, China	N/A	433	11	Gu *et al*. 2016
EF016680-96	*O. niloticus*	Lake Turkana (North Island population)	N/A	367	17	Nyingi *et al.* 2009
EF016671-79	*O. niloticus*	Lake Albert (Butiaba Bay population)	N/A	367	9	Nyingi *et al.* 2009
KX757698	*O. niloticus*	Lake Baringo	N/A	374	1	Nyingi *et al.* 2009
KX757700-04, 89-91,93,95, 98	*O. niloticus*	Lake Baringo (Robertson Camp)	N/A	367	11	Nyingi *et al.* 2009
FJ664199-08, 31-42	*O. niloticus* X *O. mossambicus*	N/A	N/A	926	22	Li *et al.* 2009
AY833474-80	*O niloticus* X *O. mossambicus*	Dam at Limpopo River, South Africa	Wild	381	7	D'Amato *et al.* 2008
KX757683-84	*O. niloticus*	Lake Albert (Butiaba Bay population)	N/A	367	2	Nyingi *et al.* 2009
HM017914-19	*O. niloticus*	N/A	N/A	335	5	McKinna *et al.* 2010
EU430996.2	*O. niloticus*	N/A	N/A	1001	1	Chang *et al.* 2008
KY587508-09, 12-14	*O. niloticus*	N/A	N/A	407	5	Sowah and Seki 2017

KY587490-91, 93-94	*O. niloticus*	N/A	N/A	407	4	Sowah and Seki 2017
KY587482-84, 86,88	*O. niloticus*	N/A	N/A	407	5	Sowah and Seki 2017
KY587475-78	*O. niloticus*	N/A	N/A	407	4	Sowah and Seki 2017
KJ746025.1	*O. niloticus*	Loboi Swamp (Lake Bogoria Hotel Spring population)	N/A	367	1	Nyingi *et a*l. 2009
JQ907514.1	*O. niloticus*	Mozambique	N/A	378	1	Firmat *et al.* 2013
EF016709-14	*O. niloticus*	Suguta River, Kenya	N/A	364	6	Agnese and Nyingi 2006
EF0167697, 99-01, 03-08	*O. niloticus*	Lake Baringo (Robertson Camp)	N/A	363	10	Agnese and Nyingi 2007
AY833487-91	*O. niloticus*	Central Luzon State University, Philippines	Farm	378	5	D'Amato *et al.* 2007
AY833470-73, 82-86	*O. niloticus*	Nile River, Northern Nile River, Egypt	Wild	381	9	D'Amato *et al.* 2007
AY833466-69,	*O. niloticus*	Dam at Limpopo River, South Africa	Wild	381	4	D'Amato *et al.* 2007
AY833474-80	*O niloticus* X *O. mossambicus*	Dam at Limpopo River, South Africa	Wild	381	7	D'Amato *et al.* 2008
KU180641-2	*O. aureus*	Daze, Tanjiang river, Guangdong Province, China	N/A	433	2	Gu *et al*. 2016
HU180586-87	*O. aureus*	Huazhou, Jianjiang River, Guangdong Province, China	N/A	433	2	Gu *et al*. 2016
KU180586	*O. aureus*	Zhaoqing, Xijiang River, Preal River, Guangdong Province, China	N/A	433	1	Gu *et al*. 2016

KU180573	*O. aureus*	Zhaoqing, Xijiang River, Preal River, Guangdong Province, China	N/A	433	1	Gu *et al*. 2016
KU180535. 37-39	*O. aureus*	Wuzhou, Xijiang River, Preal River, Guangxi Province, China	N/A	433	4	Gu *et al*. 2016
AF328851	*O. leocosticus*	Mozambique	N/A	375	1	Nagl *et al*. 2001
KX757689, 94	*O. leocosticus*	Mozambique	N/A	375	2	Nagl *et al*. 2001
EF016702.1	*O. leocosticus*	Mozambique	N/A	375	1	Nagl *et al*. 2001
KX757686, 88, 92, 96-97, 99	*O. spilurus*	Lake Baringo (Robertson Camp)	N/A	370	6	Chuhila *et al*. 2016
EU431000.2	*O. spilurus*	N/A	N/A	1002	1	Chang *et al*. 2008
KX757685.1	*O. spilurus*	Lake Baringo, Kenya	N/A	370	1	Chuhila *et al*. 2016
AF296480-85	*O. esculentus*	Mozambique	N/A	370	6	Nagl *et al*. 2001
AJ237399.1	*O. esculentus*	Mozambique	N/A	370	1	Nagl *et al*. 2001
AF296489-90	*O. amphimelus*	Mozambique	N/A	442	2	Nagl *et al*. 2001
AF296492.1	*O. jipe*	Mozambique	N/A	446	1	Nagl *et al*. 2001
JQ907508-13	*O. andersonii*	Mozambique	N/A	381	6	Firmat *et al.* 2013
AF296487-88	*O. andersonii*	Okavango, Botswana	N/A	446	2	Nagl et al. 2001
KC811358.1	*O. andersonii*	South Africa	N/A	387	1	Zengeya *et al*. 2013
KC811367.1	*O. andersonii*	South Africa	N/A	387	1	Zengeya *et al*.2013
KC811363.1	*O. andersonii*	South Africa	N/A	387	1	Zengeya *et al*. 2013
KC811359.1	*O. andersonii*	South Africa	N/A	387	1	Zengeya *et al*. 2013
KC811370.1	*O. andersonii*	South Africa	N/A	387	1	Zengeya *et al*. 2013

KC811368.1	*O. andersonii*	South Africa	N/A	387	1	Zengeya *et al.* 2013
KC811361.1	*O. andersonii*	South Africa	N/A	387	1	Zengeya *et al.* 2013
KC811360.1	*O. andersonii*	South Africa	N/A	387	1	Zengeya *et al.* 2013
AY833494-95, 98, 500, 502	*O. andersonii*	Dam at Limpopo River, South Africa	Wild	387	5	D'Amato *et al.* 2007
AY833492-93	*O. andersonii*	Olifant River	Wild	387	2	D'Amato *et al.* 2007
KC811365.1	*O. andersonii*	South Africa	N/A	387	1	Zengeya *et al.* 2013
KC811369.1	*O. andersonii*	South Africa	N/A	387	1	Zengeya *et al.* 2013
KC811364.1	*O. andersonii*	South Africa	N/A	387	1	Zengeya *et al.* 2013
KC811374.1	*O. andersonii*	South Africa	N/A	387	1	Zengeya *et al.* 2013
KM658973.1	*O. variabilis*	Kisii, Kenya	N/A	16626	1	Kinaro and Xue 2014
AF015006.1	*O. malagarasi*	N/A	N/A	527	1	Mayer *et al.* 1998
AJ237401.1	*O. malagarasi*	N/A	N/A	481	1	Seegers *et al.* 1998
AF296463.1	*O. malagarasi*	N/A	N/A	449	1	Nagl *et al.* 2001
AF296494.1	*O. urolepis*	N/A	N/A	449	1	Nagl *et al.* 2001
AF296467.1	*O. urolepis*	N/A	N/A	447	1	Nagl *et al.* 2001
AF296505.1	*T. rendalli*	Zimbabwe	N/A	447	1	Nagl *et al.* 2001
KJ925075.1	*T. rendalli*	N/A	N/A	372	1	Kide *et al.* 2016
AF296503.1	*T. rendalli*	Zimbabwe	N/A	372	1	Nagl *et al.* 2001

2.2.Morphological identification

Initially, species were identified based on morphological and morphometric traits observed on-site using (Skelton 1993). This included looking at the colour of the fish and its through, enlarge jaws, caudal fins and the presence of sport on the body. A detailed identification key for the three species most likely to be encountered in southern Africa was used to perform the morphological identification. These details are provided below in Table 2.

Table 3: Detailed characteristics of the morphological identification of *Oreochromis* species. The traits were modified from (Skelton 1993; Van der Waal 2000).

Characters	*O. niloticus*	*O. mossambicus*	*O. andersonii*
Colour	Silver in colour with olive/grey/black body bars.	Silvery olive to deep blue-grey.	Blue-grey.
Body Bars	8 vertical bars on the body.	6-7 vertical bars on juveniles.	8-9 irregular thin bars on the body.
Dorsal fin spines	12-14	11-13	11-14
Gill rakers	20-26	16-20	21-27
Caudal fin	No red border in the dorsal or caudal fins. But has distinctly vertical black stripes.	Dorsal and caudal fins with a red border, without vertical bars.	Dorsal and caudal fins are almost always red, without any vertical bars.
Anal fin spines	9-11	9-12	11-13
Literal line scale	20-34	30-32	31-35
Breading male colour	Blue-black with silvery mesh. The maroon flush on top of the head. Outer dorsal fins and caudal fins have an intense red colour.	Deep greyish black with a white lower head and throat.	Red flush to the head, lower body, dorsal and caudal fins.

2.3.Genetic Identification

2.3.1. Isolation of DNA

Two different procedures were used to extract DNA, because of the price change of the first kit. The first 112 samples were extracted using a blood and tissue kit from QIAGEN. The protocol was as follows: fins or muscle tissues (about 25 mg) were cut and placed in 2 ml microcentrifuge tubes separately. One hundred and eighty microliters (180 µl) of digestion buffer was added to each tube containing a sample. Twenty microliters (20 µl) of proteinase K was added to the sample and mixed by vortexing. The sample was then incubated at 56^0C until the tissue was completely digested. Two hundred microliters (200 µl) of AL buffer (lysis buffer) and 200 µl of 99 % ethanol was added to the sample respectively, the samples were thoroughly mixed by vortexing. The mixture was pipetted into a mini spin column in a 2 ml collection tube and centrifuged at 8000 rpm for 1 minute. The flow-through was discarded and the spin column was placed in a new 2 ml collection tube. Five hundred microliters (500 µl) of wash buffer 1(AW1) were added to the sample and centrifuged at 8000 rpm for 1 minute and the flow-through was discarded. The spin-column placed in a new 2 ml collection tube. Five hundred microliters (500 µl) of wash buffer 2 (AW 2) were added into the sample and centrifuged at full speed for 3 minutes and the flow-through was discarded. The spin column was placed in a new 2 ml microcentrifuge tube. Twenty microliters of elution buffer were added, and the sample was incubated at room temperature for 1 minute. The DNA was eluted with buffer AL at 8000 rpm for 1minute and stored in a freezer for later use.

The second protocol was an adapted high salt method (Ajanabi and Martinez 1997) (used to extract 29 samples). The procedure is as follows: About 25 mg of a muscle sample was placed in a 2 ml tube. Four hundred microliters (400µ) of sterile homogenizing buffer (0.4 M NaCl,

10 mM Tris-HCl at pH of 8.0 and 2 mM (EDTA pH 8.0) and 40 µl of 20% SDS 2% final concentration were added respectively. Eight microliters (8 µl) of proteinase (400 µg/ml final concentration) was added to the sample and incubated until the tissue was completely digested. Three hundred microliters (300 µl) of 6 M NaCl (NaCl saturated H_2O) were added and mixed for 30 seconds at minimum speed by vortexing. The sample was then centrifuged at 12000 rpm for 30 minutes. The supernatant was transferred to a new clean tube. Three hundred microliters (300 µl) of isopropanol was added to the sample, mixed by vortexing and incubated at -20^{o}C for 1 hour. The sample was then centrifuged for 20 minutes at 12000 rpm. The pellet was washed with 70% ethanol, dried and resuspended in 100 µl double distilled water (dd$H_2$0). The DNA was stored in the freezer.

The concentration of all extracted DNA was checked with a NanoDrop™ Spectrophotometer. All concentrations were adjusted to give a final concentration of 25 ng/µl for the Polymerase Chain Reaction (PCR) step.

2.3.2. DNA amplification

The control region of the mitochondrial DNA was amplified with primers: L19 (5'-TGTAAAACGACGGCCACTCCACTAGCTCCCAAAGCTA-3') (Bernatchez *et al*. 1992) and H16498 (5'-CAGGAAACAGCTATGACCCCTGAAGTAGGAACCAGATC-3') (Meyer *et al*. 1990). Twenty microliter PCR volume was performed containing 4 µl of 25ng DNA extract, 5 µl of 5x One Taq standard reaction buffer, 0.4 µl of 10 uM each primer, 0.1 µl of 10 uM dNTPs, 0.1 µl of One Taq DNA Polymerase [0.025U/ µl] (New England Bio Labs), and 9.5 µl PCR water. The thermocycler conditions were, initial denaturation for 7 min at 95^{o}C, 35 cycles with 40 s at 95^{o}C, 45 s at 53^{o}C and 45 s at 72^{o}C, followed by a final extension for 5 min at 72^{o}C. The PCR

products were verified using 1 % gel via electrophoresis and the rest of the positive products were used in cycle sequencing.

2.3.3. DNA sequencing

Sequencing was performed using a Big Dye Direct Sequencing Master mix. An amount of 0.5µl Big dye direct sequencing Master mix was pipetted into a PCR tube, followed by 1 µl of M13 tagged L19 forward primer (only a forward primer was used per sequencing cycle) and 0.5 µl of PCR. Then 10 µl of the PCR products were pipetted into the mix to make a final volume of 13 µl. The mixture was then run on a thermocycler under the following conditions: the initial step holds at 37^{o}C for 15 min, followed by the next hold at 80^{o}C for 2 min and another one at 96^{o}C for 1min. This was followed by 25 cycles of denaturation at 53^{o}C for 5 sec. the final step was to hold the sequenced DNA at 4^{o}C which completes the first part of sequencing. The sequenced products were sent to INQABA biotec to be read.

2.3.4. Sequence alignment.

The sequences were checked by eye for mistakes and double peaks. If a sequence traces had errors, the PCR product was sequenced again. The sequence was cleaned, trimmed and aligned with Clustal W (Thompson *et al*. 1994)), together with 362 sequences downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) that highly matched our sequences during BLAST searches (Basic Local Alignment Search Tool) in BioEdit software version 7.2.6. (Hall 1999). These closely related downloaded sequences were from the following species: *O. niloticus* (203), *O. mossambicus* (91), *O. aureus* (10), *O. leucosticus* (4), *O. spilurus* (8), *O. esculentus* (7), *O. amphimelus* (2), *O. jipe* (1), *O. andersonii* (27), *O. variabilis* (1), *O. malagarasi* (3), *O. urolepis*

(2) and *T. rendalli* (3) (*T rendalli* was used as an outgroup) (D'Amato *et al*. 2007; Agnese *et al*. 2014; Nyingi *et al*. 2009; Nagl *et al.* 2001; Kide *et al*. 2016; Sowah and Seki 2017; Ndiwa *et al*. 2014; Li *et al*. 2009; Chuhila *et al*. 2009; Gu *et al.* 2016 and Chang *et al.* 2008). The final control region DNA alignment contained 1122 base pairs sequences, trimmed to 456 base pairs in length. At this stage, the alignment was saved as a fasta file.

2.4. Data analysis

2.4.1. Genetic structure.

To investigate genetic structure that might show reticulate relationships, a median-joining network was produced in POPART (Population Analysis with Reticulate Trees; Leigh and Bryant 2015). Each species was assigned a particular colour in a network tree produced and another per location sampled. Three networks were produced, the first network consisted of 80 haplotypes produced from 503 samples (141 from this study and 362 downloaded samples) to verify the relationships amongst the species involved.

Model selection was performed in JModelTest (Darriba *et al*. 2012) to find the best fitting substitution model for the mtDNA control region under the Bayesian information criterion. To safeguard against over parametrization by averaging the likelihood over all included parameters, the Akaike information criterion (AIC) was performed. The best likelihood scores were obtained for the GTR (General Time Reversible). The fasta file was converted into a nexus file using a format converter in the HIV database (Foley *et al*. 2018). Then phylogenetic reconstruction was done using Bayesian Evolutionary Analysis Sampling Trees (BEAST). In order to obtain the time value for tree branching patterns, a fossil calibration of 6 million years was used for the genus *Oreochromis.* This was the most important specimen that could be attributed to the

genus Oreochromis. (Carnevale *et al*. 2003; Krijgsman *et al*. 1999). The final Bayesian tree was contracted from five (5) independent runs of 100 million generations and the final tree was opened with Figtree v1.4.3. (http://tree.bio.ed.ac.uk/software/figtree/). Each run had one Monte Carlo Markov Chains (MCMC) with topologies sampled every 10,000 generations. To estimate a mutation rate, we used a strict clock model with the base substitution rate estimate of 0.324 changes per site per million years (SE 0.0139) of Genner *et al* (2010). To see if the effective sample size (ESS) had reached >200, the program Tracer v1.6 (Rambauti *et al*. 2014) was utilized. A burn-in of 20% generation was discarded in each simulation.

2.4.2. Genetic diversity.

To understand the population genetic diversity in our study area, haplotype diversity (Hd), nucleotide diversity (π) and standard deviation of haplotype diversity (SD-HS) were calculated in DnaSP v6.12.03 (Librado and Rozas 2009) for each species in its location to see if the population was growing or decreasing. However, this analysis could be performed for 4 sequences or more. The high haplotype diversity would suggest that the population is increasing, and the lower haplotype diversity would suggest that the population is small and might have been produced from fewer haplotypes. Summary statistics were also calculated for all species separately using all available data, to look at the haplotype diversity of the world population. The genetic diversity of a species is highly impacted by the number of samples used; therefore, it is fair to acknowledge that *T. rendalli* as an outgroup was represented by a few samples.

2.4.3. Demography at sampling sites.

Tajima's D (T's D) (Tajima, 1989), Fu and Li'sF*(FLF), Fu and Li's D (FLD) and Fu's Fs statistics (Fu, 1997) were used for inferring demography. Demographic changes in each species were also inferred from the observed mismatch distribution for each population per sampling site and species. If there are invasive species in our study area, we expect a unimodal increase in the number of pairwise differences among sequences, which will show up as a population expansion. Bayesian skyline plot BSP (Drummond *et al.* 2005) was used to estimate changes in effective population size. To plot population changes over time, Skyline plot uses the coalescent properties on gene trees, however, the inferred population size could be biased due to population decline over time if the site is genetically structured (Ho and Shapiro 2011; Heller *et al*. 2013). Our BSP was set to use a strict clock model with the base substitution rate estimate of 0.324 changes per site per million years (SE 0.0139) (Genner *et al*. 2010; Dieleman *et al*. 2018). The MCMC length was set at 100 million to achieve an effective sample size (ESS) and the mixing of Markov chains.

3. RESULTS.

3.1.Morphological identification based on traits.

The 141 samples obtained from Albasini and Nandoni dams in the upper Levubu were identified using the traits provided in Table 2. In Albasini Dam, 41 were incorrectly identified as *O. mossambicus* and were later reidentified with genetics and found to be carrying *O. andersonii* mtDNA. Five were correctly identified as *T. rendalli.*

In Nandoni Dam, 73 were identified as *O. mossambicus*, (only one sample of *O andersonii* Ex12 23) from the Nandoni Dam was correctly identified), meanwhile, all the samples carrying a mtDNA of *O. andersonii* were misidentified as *O. mossambicus*) 19 were correctly identified as *O. niloticus*, and the last one was correctly identified as *T. rendalli*.

Table 4: Summarised results, showing the samples identified using morphological characters V/S mtDNA.

Samples	Location	Morphological identification	mtDNA identification
Nan (1-9, 11, 12, 14, 15), Ex8 (4,10,24)	Nandoni Dam	*O. niloticus*	*O. niloticus*
23, Ex12(15, 58, 73, 75, 79)	Albasini Dam	*T. rendalli*	*T. rendalli*
Ex5 (23)	Nandoni Dam	*T. rendalli*	*T. rendalli*
Ex12 (85)	Nandoni Dam	*O. niloticus*	*O. aureus*
Ex1(all), Ex12 (18, 43, 53, 55, 64)	Albasini Dam	*O. mossambicus*	*O. andersonii*
Ex4 (all), EX5 (2, 4, 8, 11, 12, 14, 16-19, 21, 22), Ex6 (1), Ex7 (all), Ex8 (1, 3,9, 14, 17-21, 23, (Ex9 (5, 6, 11), Ex12 (1-3, 5, 7, 12, 13, 23,) and 2014 (all)	Nandoni Dam	*O. mossambicus*	*O. andersonii*

3.2.Genetic structure.

Three hundred and sixty-two (362) sequences were downloaded from GenBank and aligned with 41 of our newly obtained sequences. The total alignment contained 80 unique haplotypes. *O. mossambicus* haplotypes were separated into three clusters (cluster 1-2 and 6, Figure 3). One *O. mossambicus* cluster (Cluster1) was distantly related to other species, Cluster 2 was more closely related to *O. andersonii* and *O. niloticus*, and cluster 6 was mainly comprised of

O. andersonii. O andersonii haplotypes also formed two clusters (Cluster 5 and 6). Haplotypes of *O. spilurus, O. variabilis, O. leucosticus, O. urolepis, O. aureus,* were found inside and between Cluster 6 and 1. The *T. rendalli* haplotypes clustered at the root of the network but closer to Cluster 3 (*O. niloticus*).

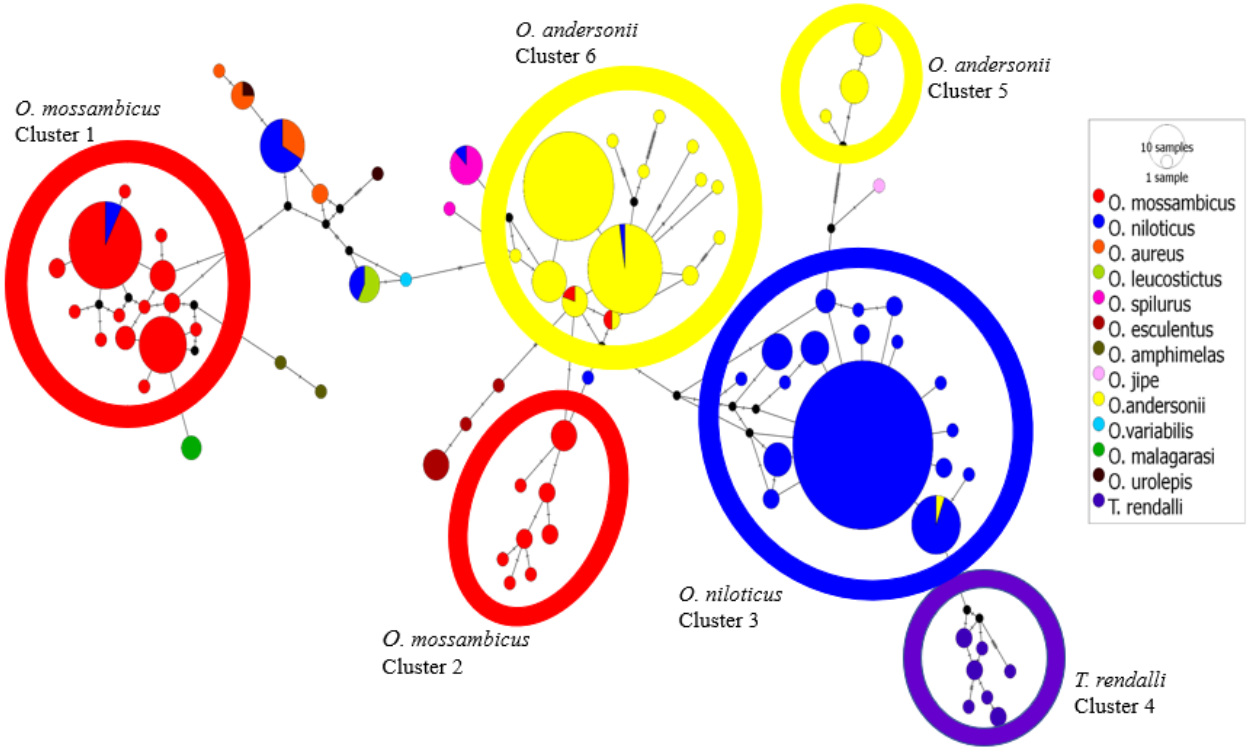


Figure 3. Median-joining networks of 80 haplotypes constructed based on 456 bp of the mitochondrial control region. Each circle and its colour represent a haplotype. Each haplotype was assigned a particular colour, for example, *O. niloticus* was assigned a blue colour, *O. andersonii* has given a yellow colour, O. mossambicus was given a red colour etc.

The network was simiar to the Bayesian phylogenetic tree (Figure 4), which confirm the two non-sister clades of *O. mossambicus*. *O. andersonii* haplotypes also fell into two clades, but these were monophyletic. *O. niloticus* was found to have evolved much earlier than both *O. mossambicus* and *O. andersonii*. *T. rendalli* has been taken out as an outgroup taxon and it shows that this branch evolved much earlier than the *Oreochromis* branch.

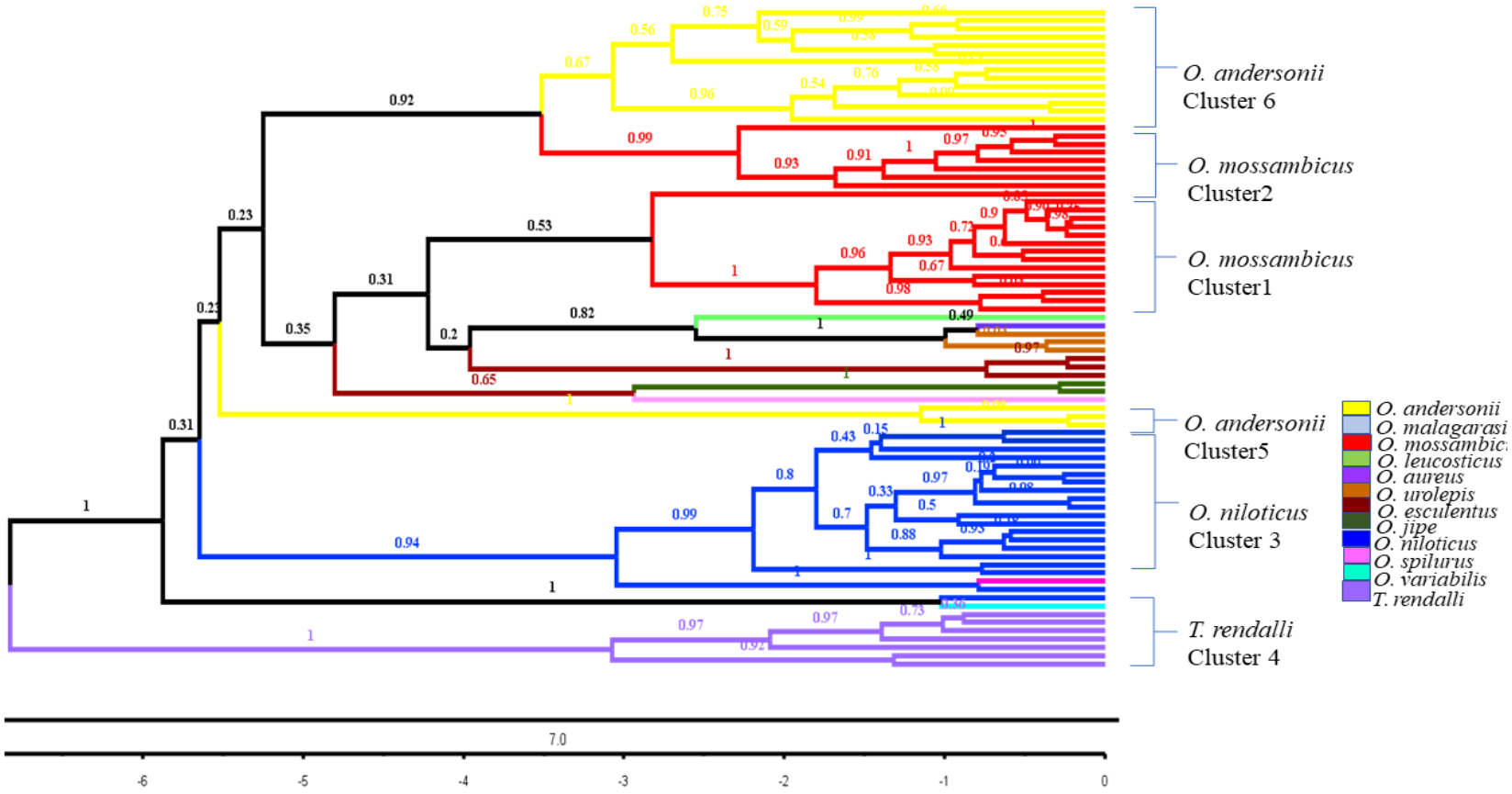


Figure 4: Bayesian phylogenetic tree, species (*O. mossambicus, O. niloticus, O. andersonii, O. aureus, O. leucosticus, O. spilurus, O. esculentus, O. amphimelus, O. jipe, O. variabilis, O. malagarasi, O. urolepis*, and *T. rendalli*) as an outgroup based on the control region of mitochondrial DNA.

3.3.Occurrence of haplotypes (O. *niloticus* and *O. andersonii*) in Nandoni and Albasini dams.

The median-joining consist of 80 haplotypes of which 9 are from Albasini Dam in grey and 10 from Nandoni Dam in black. In our samples, we found 11 haplotypes of *O. andersonii*, 7 haplotypes from Albasini cluster 6. Three haplotypes found in both dams nested in cluster 6, four (4) haplotypes in Nandoni Dam. The last 1 haplotype of *O. andersonii* from Nandoni is situated in cluster 5. The 2 haplotypes of *O. niloticus* are positioned in cluster 4. Lastly, 5 more haplotypes of *T rendalli* have been observed, where 4 are from Albasini and 1 from Nandoni Dam situated in cluster 4.

These results are highly surprising, and not a single typical *O. mossambicus* haplotype (neither Cluster 1 nor Cluster 2) was detected in all our samples. Instead, we found haplotypes of *O. niloticus* and *O andersonii*. The sighting of these two invasive species had never been reported in the Levubu system. What makes this situation worse is that there are several haplotypes per species, meaning that their populations are expanding quicker than expected.

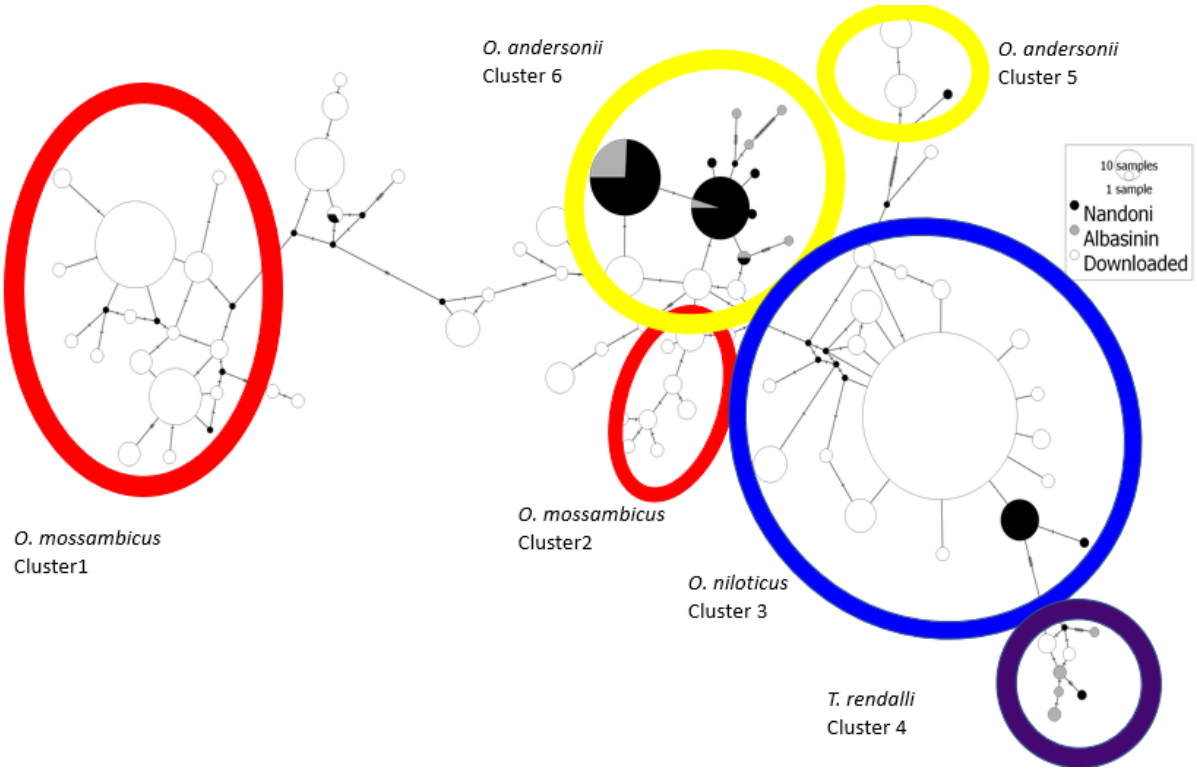


Figure 5: Median-joining networks of 80 haplotypes constructed based on 456 bp of the mitochondrion control region. This network shows haplotypes from Nandini Dam in black and haplotypes from Albasini Dam in grey.

3.4. Genetic diversity at study sites.

Genetic diversity parameters were calculated for all clusters identified in the genetic structure analyses (Table 3). The haplotype diversity of *O. niloticus* was found to be 0.96 out of 19 samples from the Nandoni Dam with a nucleotide diversity of 0.01, compared to 0.91 and 0.03 for the 203 downloaded *O. niloticus* samples. The haplotype diversity of *O. andersonii* was also found to be at 0.88 in Nandoni Dam and 0.85 in Albasini Dam with both having the nucleotide diversity of 0.03, compared to a haplotype diversity of 0.99 and nucleotide diversity 0.28 for downloaded *O. andersonii*. Although no *O. mossambicus* haplotypes were found in our

data set, downloaded sequences had a haplotype diversity of 0.91 and a nucleotide diversity of 0.05. The haplotype diversity of *T. rendalli* from the Albasini Dam was found to be 1.00 with the standard deviation of haplotype diversity of 0.08.

Table 5: Location of the samples and the summary statistics of genetic diversity.

species	Locality	N	H	Hd	SD-Hd	π
O. niloticus	Nandoni	19	14	0.96	0.03	0.01
O. niloticus	Downloaded	203	47	0.91	0.01	0.03
O. andersonii	Nandoni	73	26	0.88	0.03	0.03
O. andersonii	Albasini	41	13	0.85	0.03	0.03
O. andersonii	Downloaded	27	22	0.99	0.01	0.28
O. mossambicus	Downloaded	91	51	0.91	0.03	0.05
O. aureus	Downloaded	10	7	0.91	0.08	0.01
O. leucosticus	Downloaded	4	1	0.00	0.00	0.00
O. spilurus	Downloaded	8	3	0.68	0.12	0.00
O. esculentus	Downloaded	7	7	1.00	0.08	0.07
O. amphimelus	Downloaded	2	2	1.00	0.50	0.00
O. jipe	Downloaded	1	1	1.00	0.00	0.00
O. malagarasi	Downloaded	3	3	1.00	0.27	0.01
O. urolepis	Downloaded	2	2	1.00	0.50	0.11
O. variabilis	Downloaded	1	1	1.00	0.00	0.00
T. rendalli	Albasini	6	6	1.00	0.09	0.08
T. rendalli	Downloaded	3	3	1.00	0.28	0.00

Note

N: numbers of samples, **H**, Number of haplotypes, **Hd**: haplotype diversity, **SDHd**: standard deviation, π: nucleotide diversity.

3.5. Demography at study sites.

Demography was estimated using a variety of methods. Tajima'S D and Fu's Fs were negative for both *O. andersonii* and *O. niloticus* species found in the Nandoni Dam, but only Tajima's D was negative for Albasini Dam (Table 4). Furthermore, mismatch distributions (Figures 13 (II)) were unimodal for only one test (Nandoni Dam *O. andersonii*, Figure 7 (III)) and multimodal for the other tests. Bayesian skyline plots (Figurs9 (II)) suggest that the population of *O. niloticus* from the Nandoni Dam declining through evolutionary time, with no demographic information over the recent timeframe when introductions to the Levubu occurred. While the downloaded samples showed stable BSPs. BSP also shows the population of *O. andersonii* grew for a particular time and stabilized afterwards. BSP also indicates that the *O. mossambicus* population has declined early and never recovered. Both populations of *T. rendalli* experience an onset decline, however, the population stabilized over time but never recovered the initial loss.

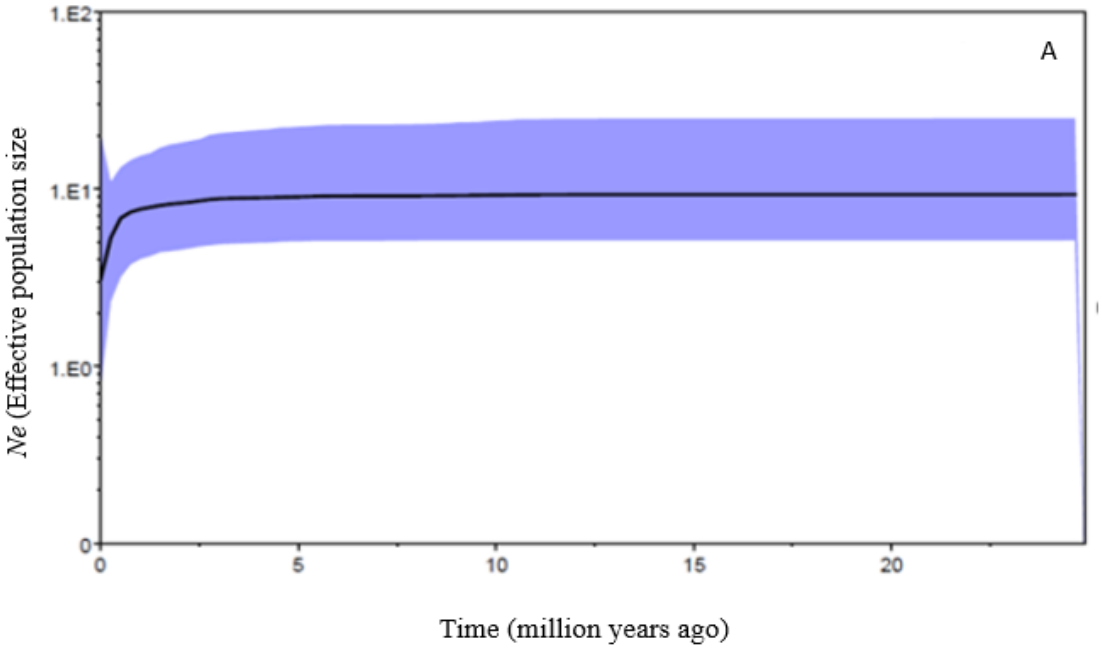
Table 6: Location of the samples and the summary statistics of demography.

Species	Locality	FLD	FLF	Fs	T's D
O. niloticus	Nandoni	-2.01	-2.20	-7.60	-1.63
O. niloticus	Downloaded	-1.42	-1.91	-11.4	-1.80
O. andersonii	Nandoni	-7.32	-6.56	-3.42	-2.79
O. andersonii	Albasini	-3.97	-4.18	1.52	-2.45
O. andersonii	Downloaded	1.50	2.19	2.19	3.12
O. mossambicus	Downloaded	-0.85	-0.99	-15.3	-0.78
O. aureus	Downloaded	0.14	0.24	-0.60	0.31
O. spilurus	Downloaded	-1.11	-1.18	-1.02	-0.92
O. esculentus	Downloaded	-1.33	-1.45	-4.66	-1.32
T. rendalli	Albasini	-1.03	-1.44	0.12	-1.27

Note: **FLD**: Fu and Li's D* test statistics, **FLF**: Fu and Li's F* test statistics**, Fs**: Fu's fs statistics, **T's D,** Tajima's D, Downloaded: from GenBank. This analysis was done for species with more than 4 samples.

3.6. Bayesian Skyline Plots.

Bayesian Skyline Plot shows populations of *O. andersonii* growing, in figure 11 (samples from Nandoni, Albasini and Downloaded samples). Figure 12 shows a population of *O. (mossambicus* (downloaded) that started by dropping and then stabilizers in time. Figure 13 shows a growing population of *O. niloticus* From the Nandoni Dam and the downloaded samples. While Figure 14 shows a growing population of *T. rendalli* from Albasini and the downloaded samples.



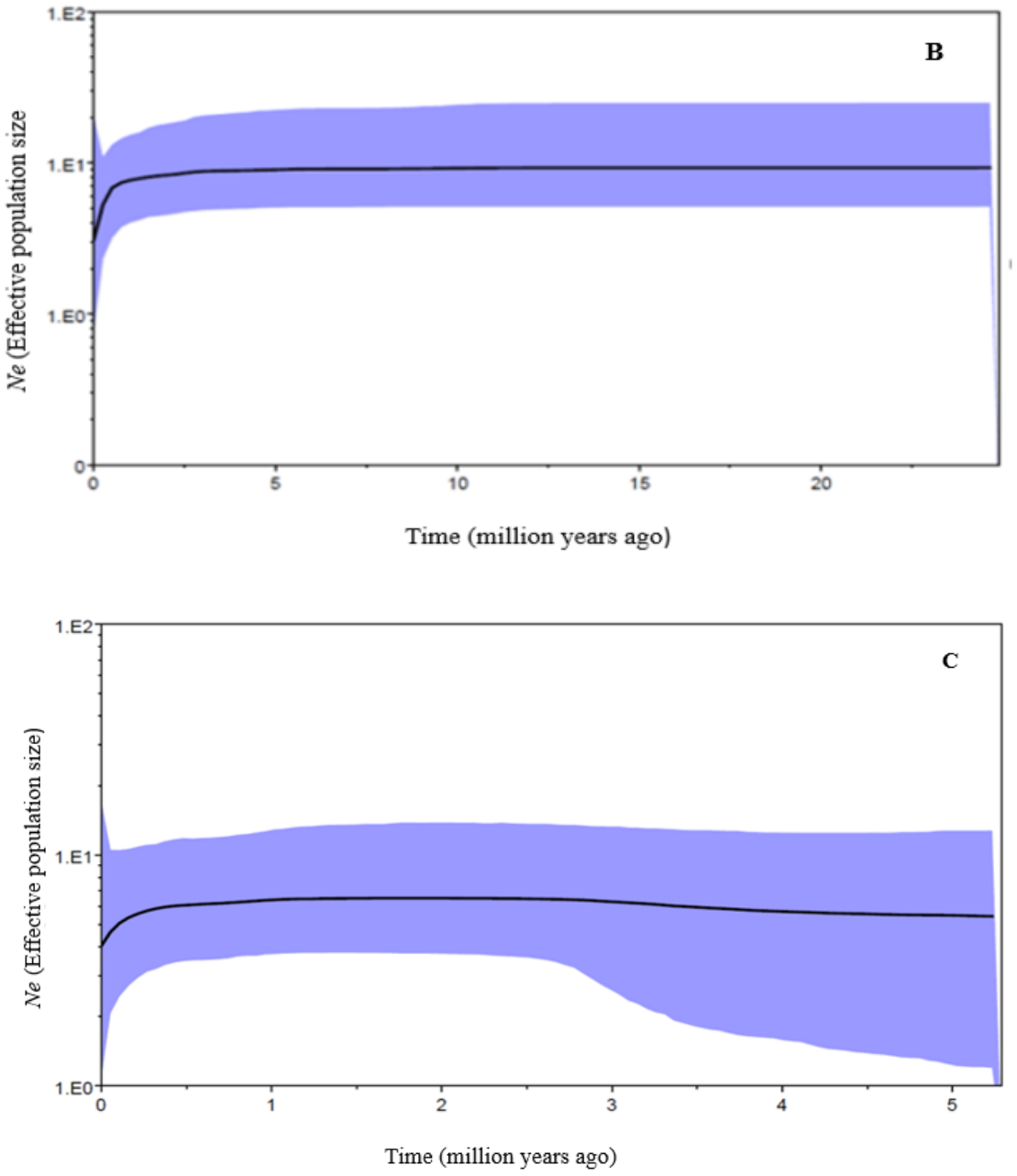


Figure 6: Showing the Bayesian skyline plots of (A) *O. andersonii* from Genbenk and our samples, (B) *O. andersonii* from Albasini Dam and (C) *O. andersonii* from Nandoni Dam. The black solid line is the estimated median and the shaded blue area shows the 95% HPD limits.

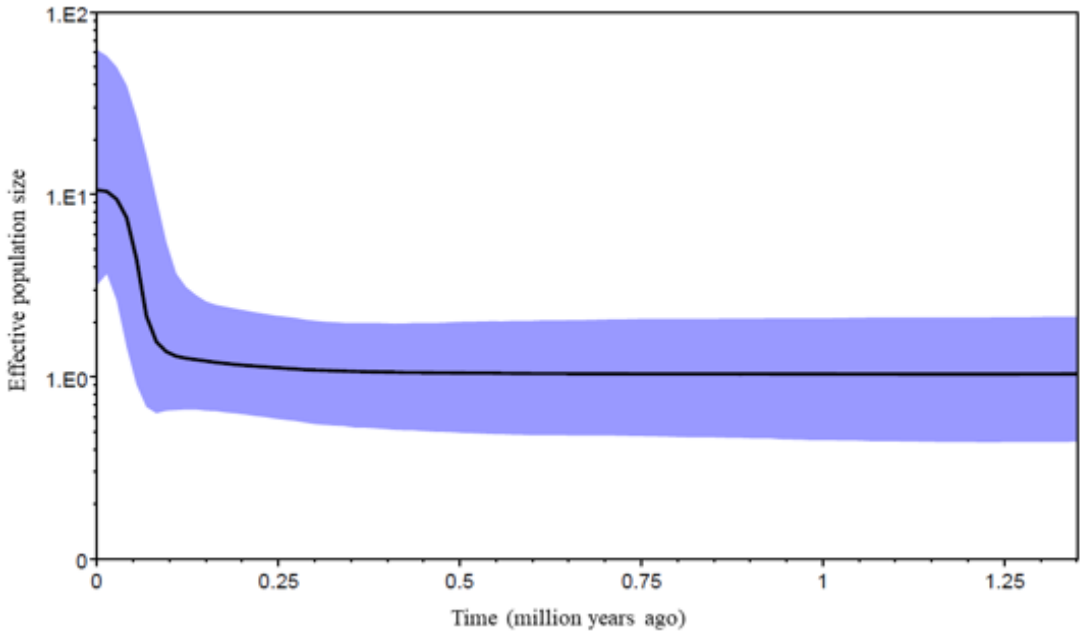
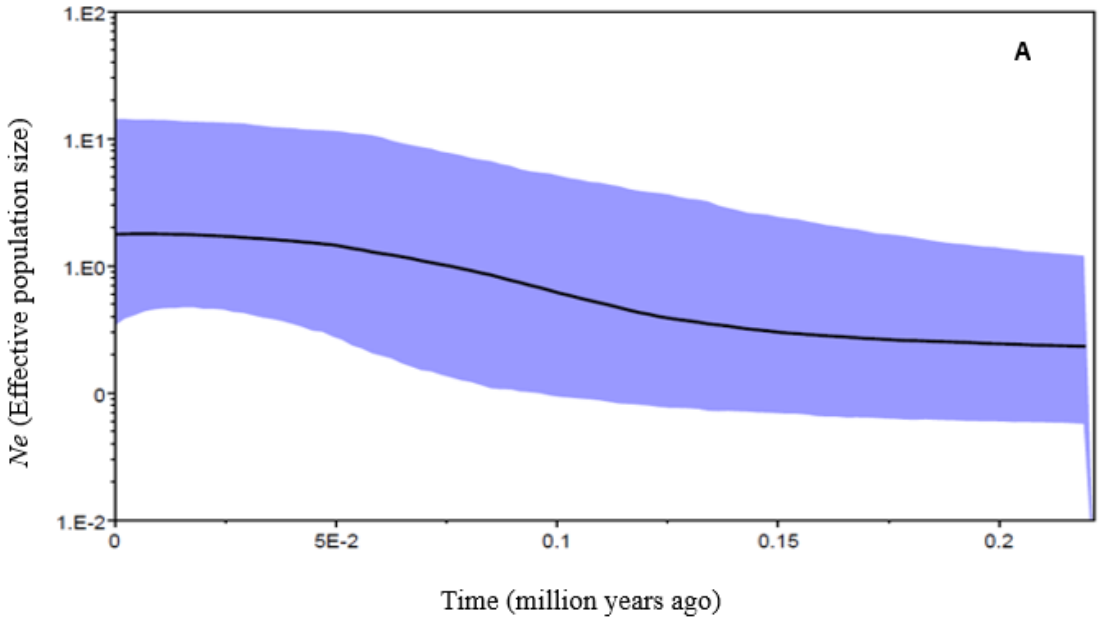


Figure 7: Showing a Bayesian skyline plot of the downloaded *O. mossambicus*. The black solid line is the estimated median and the shaded blue area shows the 95% HPD limits.



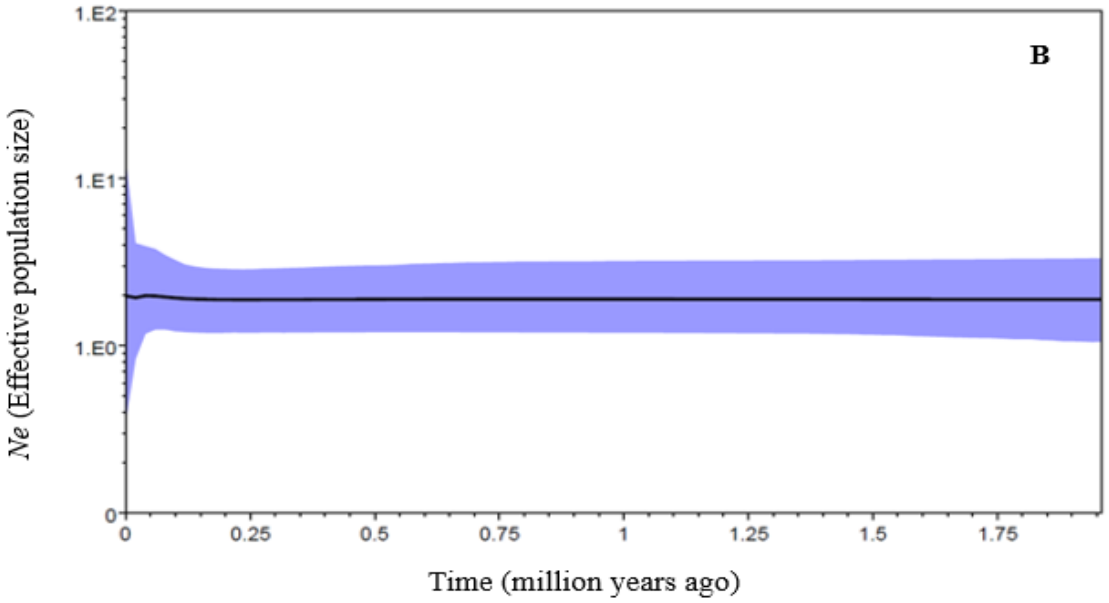
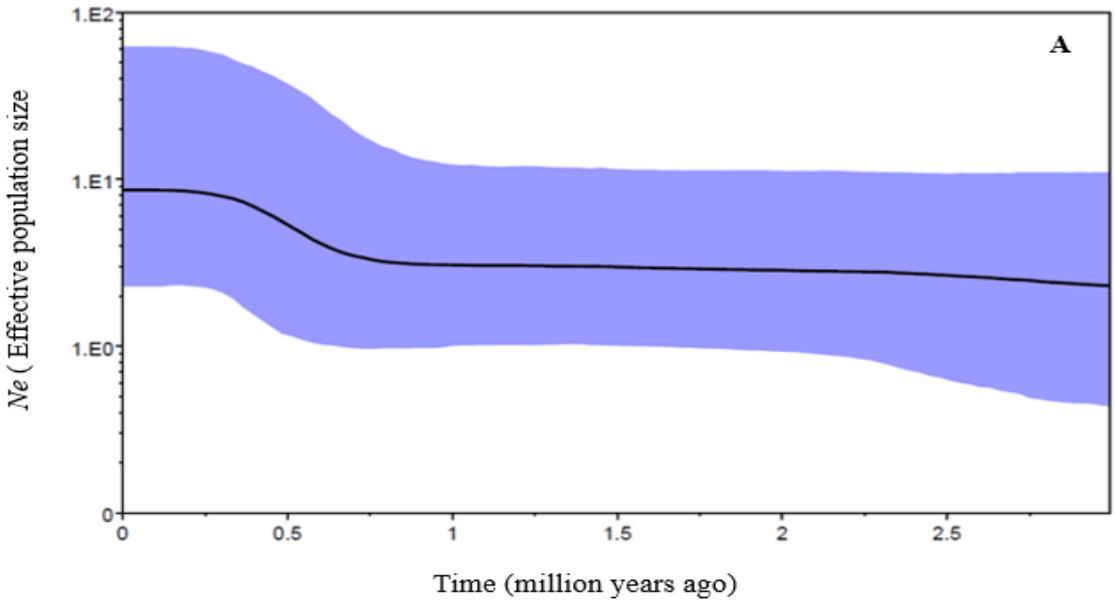


Figure 8: Showing the Bayesian skyline plots of *O. niloticus* that have been downloaded in (A) and the *O. niloticus* samples from Nandoni Dam in (B). The black solid line is the estimated median and the shaded blue area shows the 95% HPD limits.



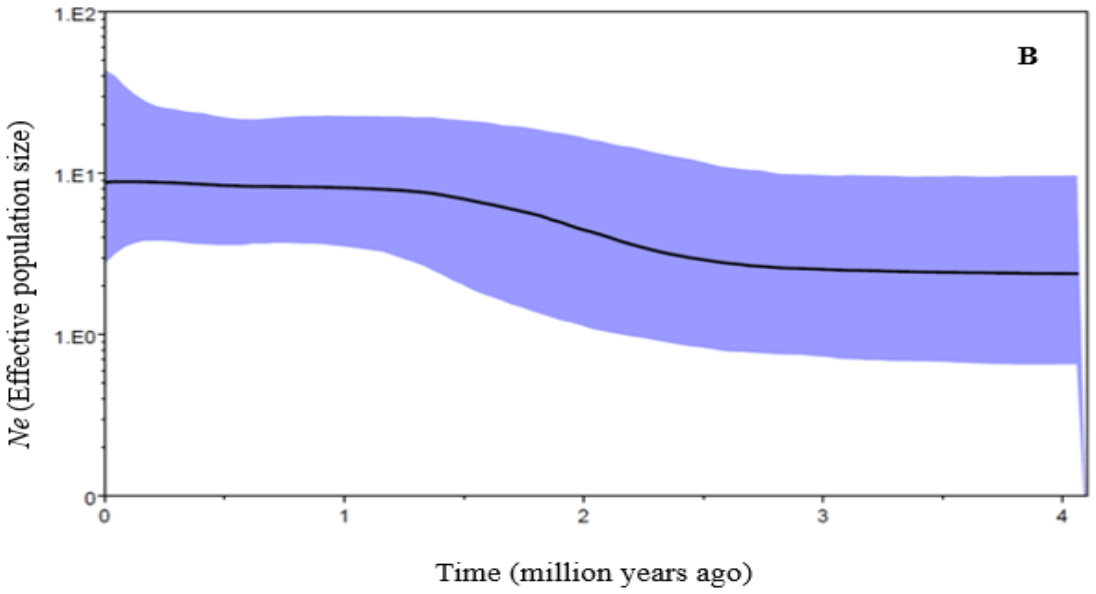
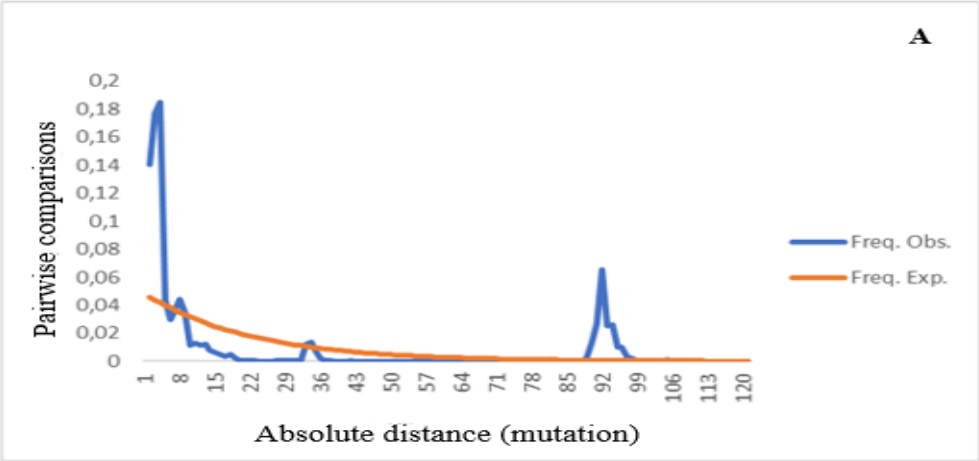
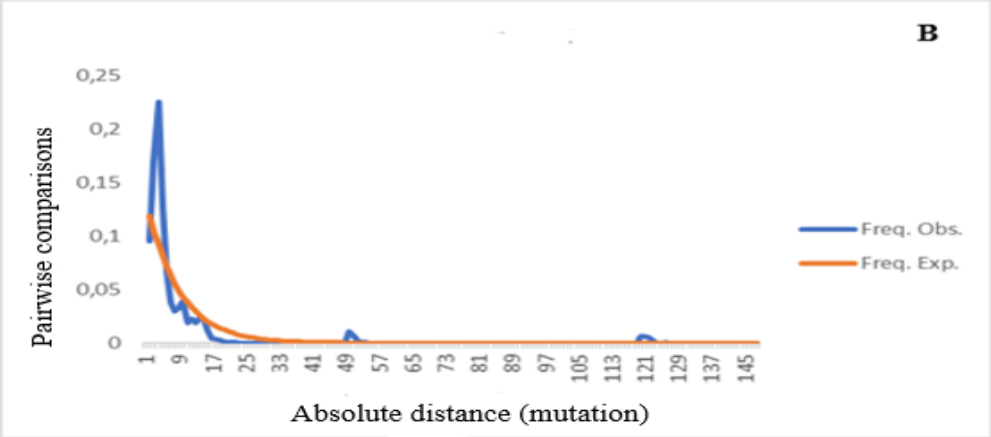


Figure 9: Shows the Bayesian Bayesian Skyline Plots of (A) downloaded *T. rendalli* and *T. rendalli* from the Nandoni Dam. (B) *T. rendalli* from Nandoni Dam. The black solid line is the estimated median and the shaded blue area shows the 95% HPD limits.

3.7.Mismatch distributions.

Figure 11 shows a population expansion of *O. andersonii* population in the Nandoni Dam, but not in download samples and Albasini Dam. Figure 12 shows a population of *O. mossambicus* (downloaded samples) that is not experiencing growth or expansion. Figure 13 shows an expanding population of *O. niloticus*, in Nandoni Dam but there is no expansion in figure 13 (I) all *O. niloticus.* While Figure 14 shows no expansion for the population of *T. rendalli.*





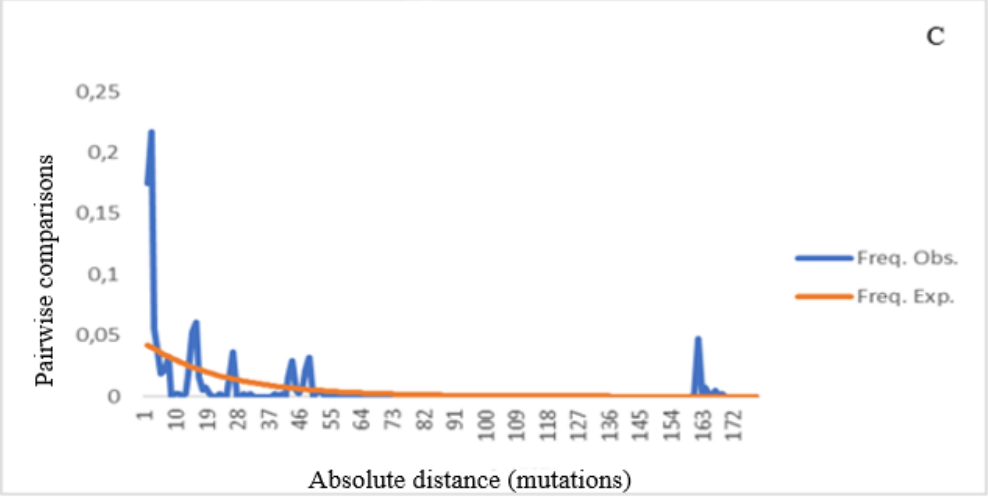


Figure 10: Shows the mismatch distribution of (A) All *O. andersonii*, (B) *O. andersonii* from Nandoni Dam and (C) *O. andersonii* from Albasini Dam.

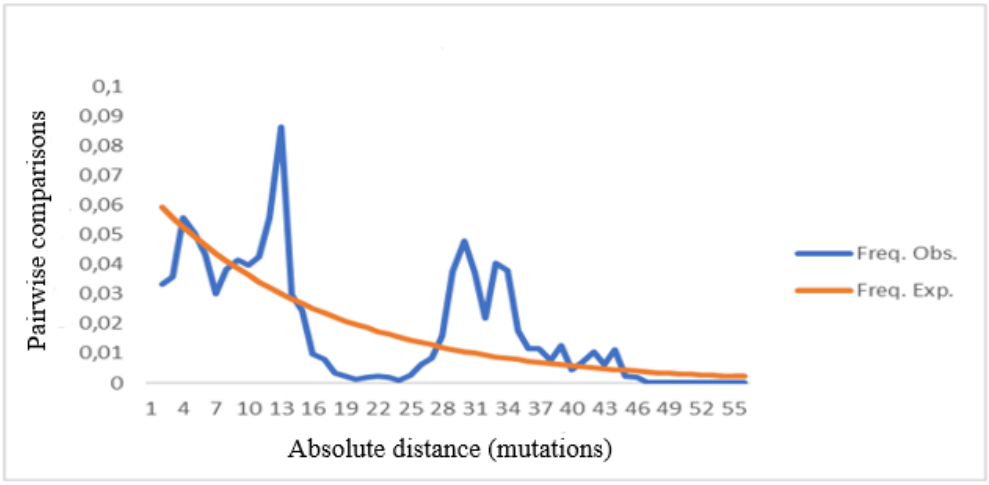
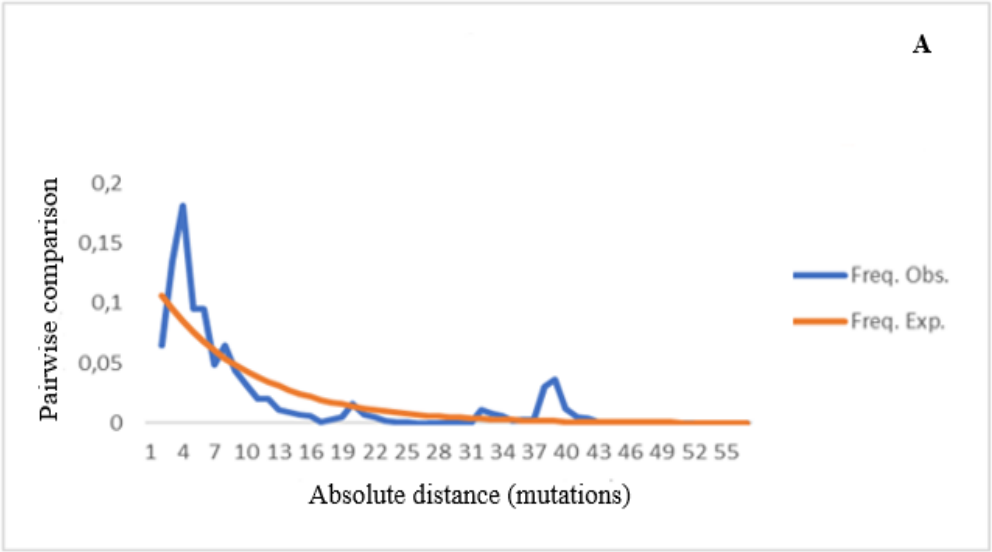


Figure 11: Mismatch distribution of downloaded *O. mossambicus* samples.



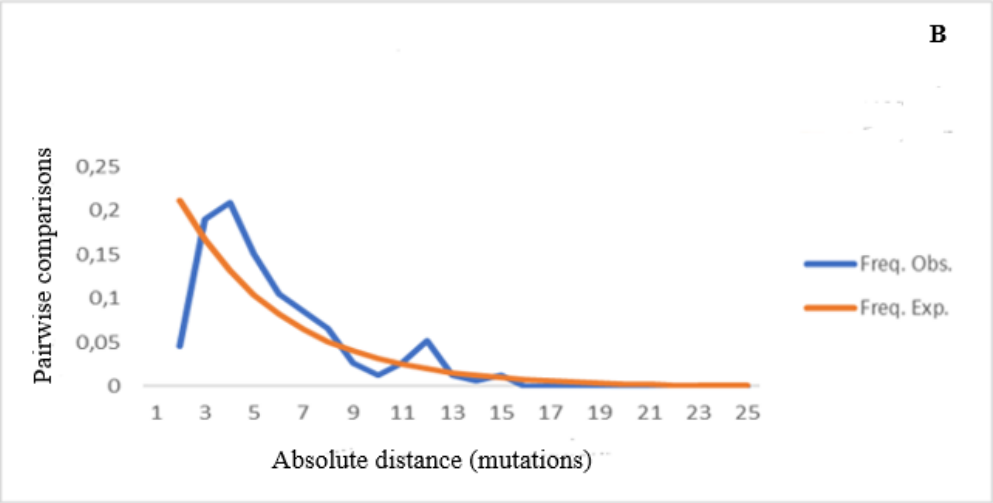
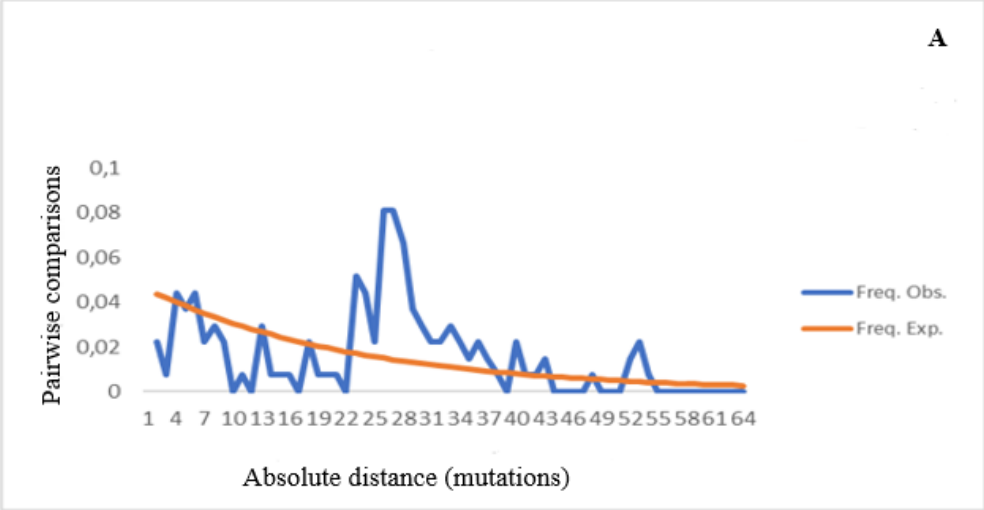


Figure 12: Mismatch distribution showing (A) downloaded *O. niloticus* and (B) *O. niloticus* from Nandoni Dam.



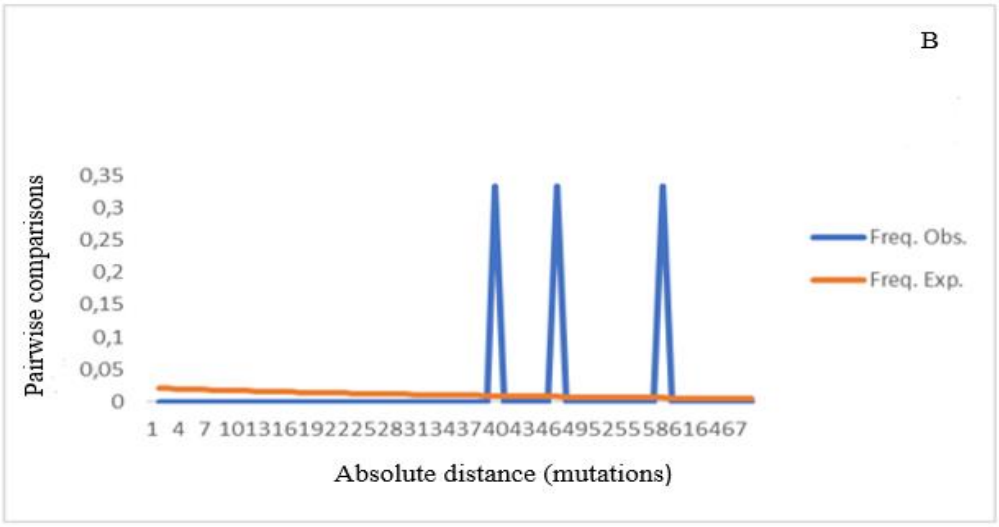


Figure 13: Mismatch distribution showing samples of (A) all *T. rendalli* (B) *T. rendalli* from Albasini Dam

3.8.Hybridization in Nandoni and Albasini.

In a total of 114 samples collected in both Nandoni and Albasini dams, that were morphologically identified as *O. mossambicus*, all turned out to be *O. andersonii* genetically. Only one sample was correctly identified as *O. andersonii* with 3 spots (Ex12 number 23) on the side of the belly. *Oreochromis* hybrids are very challenging to identify morphologically and even impossible when using a mitochondrial study alone. This is because mtDNA will only tell us the maternal species and not the paternal species. Some or most of our samples may be hybrids, but we need to look at the nuclear DNA to be confident in concluding this theory.

4. DISCUSSION.

4.1.Genetic structure and evolutionary history of *Oreochromis.*

The earliest fossil of the genus *Oreochromis* has been dated to be around 6 million years ago (Carnevale *et al*. 2003; Krijgsman *et al*. 1999) (*Oreochromis lorenzoi* +, no genetic data of this extinct species has been generated). This information suggests that this genus is probably older than 6 million years old. The presence of a fossil at that time (6 million years) means that the genus existed before 6 million years ago. The fossil of *O. niloticus* was found to be 5-2 million years old (Trewavas 1983), placing it in the upper Pliocene. According to our tree, *O. niloticus* is much older than *O. mossambicus*.

Oreochromis mossambicus (cluster/ branches 1 and 2 in Figure 3) is not a monophyletic group and evolved at different times based on our Bayesian tree in figure 5. The two branches evolved into the modern-day *O. mossambicus*, which suggest that this species might have originated through two waves of maternal migration. *Oreochromis mossambicus* have evolved to tolerate high salt concentrated environment. Hence today this species can tolerate double the amount of sea salt (Yamaguchi *et al*. 2018; Fiess *et al*. 2007; Trewevas 1983). A similar situation might have happened with *O. andersonii* as it also shows two maternal branches that evolved into the modern-day *O. andersonii*. The two branches of this species seem to have evolved at a completely different time because one branch (cluster 4 in Figure 3) appears to be much older than the other branch (cluster 6 in Figure 3). The genus *Oreochromis* evolved during dramatic environmental changes, which gave it some advantages over other genera, in the sense that this species can tolerate saltwater to some extent and completely for *O. mossambicus*. Hence, they can survive easily in seawater (Trewevas 1983).

Tilapia rendalli was used as an outgroup to root the Bayesian phylogeny (Figure 4) and determine which *Oreochromis* species was most basal. All seven *T. rendalli* haplotypes, therefore, branched much earlier and are the outgroup to the *Oreochromis* genus. Both analyses of structure, the network (Figure 3) and Bayesian tree (Figure 4) show that *O. niloticus* is the most basal of all the *Oreochromis* species.

The branching of *T. rendalli* occurred during the Miocene period (23.03-53.33 million years). During this time a big portion of the continent underwent drying, enhanced seasonality and reorganization of organism's communities (Herbert *et al*. 2016), most water bodies dried out or shrunk in size drastically that the water was very salty, only fish that can tolerate or adapt to high salt content could survive. This was the time when the Sahara Desert was formed (Herbert *et al*. 2016). Perhaps this was the main reason why the genus *Oreochromis* separated into different species since the main branches leading to present-day *Oreochromis* species occurred during this time (Figure 4). Hence some species, such as *O. mossambicus* can tolerate high salt concentration than most of the relatives, and the fact that it can thrive in ocean water (Trewavas 1983; Yamaguchi *et al.* 2018; Fiess *et al.* 2007). This period was followed by a cooling of the temperature toward the late Messinian (5.33 million years ago) (Herbert *et al*. 2016).

4.2. Genetic diversity.

The nucleotide diversity of *O. niloticus* was found to be much lower in Nandoni Dam at 0.01, which could be the result of a small sample number, while the nucleotide diversity of the downloaded sample was 0.03. This suggests that *O. niloticus* may have gone through a

bottleneck after the introduction to Nandoni Dam. Additionally, low nucleotide diversity and the presence of only two very closely related haplotypes (Figure 3) might mean that *O. niloticus* was introduced only once in Nandoni Dam. The fact that *O. andersonii* in Albasini and Nandoni dams appeared to show a high haplotype diversity (0.85 and 0.88) respectively, and the same nucleotide diversity (0.03) as the downloaded sample suggests that there is a possibility that this species might have been existing in the dam for a long time. Although *O. andersonii* has never been reported in the Levubu system, which might have been the result of misidentification and the lack of genetic information in the river and the Dam itself. The fact that some haplotypes of *O. andersonii* were present in the Albasini Dam and Nandoni dams corroborates the idea that *O. andersonii* has been present in the system for some time. This is because Albasini is upstream to the Nandoni Dam, meaning that fish in Albasini could leave the dam during high heavy rains that caused floods and swim downstream. Or there have been several introductions of this species in the dam, hence a high nucleotide diversity.

Alternatively, it is also possible that *O. andersonii* were introduced more than once into the Upper Levubu. This is because some haplotypes found in Nandoni are also absent in Albasini. Where do they come from and why they are more than expected? This gives a bit of a clue on the fact that some fish with different haplotypes could have arrived in the dam at the same time, perhaps as adults. This might mean that floods play an important role in allowing fish to escape the breeding ponds that existed in the Levubu farm settlement area, more especially the flood in the year 2000. Another practical theory is that anglers themselves are responsible for introducing fish into the dams intentionally for sports (Van Schoor 1966). This could also explain the high genetic diversity of this species.

Since the Albasini Dam is upstream of the Nandoni Dam, that means that what happens in Albasini has a greater effect on what happens in the Nandoni Dam. In a way, if *O. andersonii* were already present in the Albasini Dam before the construction of the Nandoni Dam, the floods made it easy for the haplotypes to mix, assuming that some haplotypes were existing in the Levubu river downstream Albasini Dam. The most reasonable explanation is fish could only swim downstream from the Levubu settlement towards the Nandoni Dam but cannot swim upstream into Albasini because of the high wall of the Albasini Dam. The higher haplotype diversity of *O. andersonii* and *O. niloticus* found in the Nandoni Dam could also be boosted by fish escaping breeding pond during heavy rains that coursed floods in Levubu farm settlement.

4.3.Demography.

Oreochromis emerged during a very dramatic Miocene age where the climate was changing frequently, this was the survival of the fittest. The climate perhaps was responsible for separating this group into many different species that remain today. The genus *O. andersonii* and *O. niloticus* however are more derived and seems to have been demographically expanding since they diverged see Figure 4. This could have been also promoted by the reality that they form part of the aquaculture favourite species, where they have been introduced in many rivers around the world.

Oreochromis has been reported as one of the most successful invasive species worldwide. The populations of these genera can easily reproduce and take over a water body in a few years. In our study, we observed a great expansion of *O. andersonii* in Nandini Dam,

supported by Tajima's D statistics of -2.79 and Fu's fs statistics of -656. This was also supported by a mismatch distribution in Figure 10 (B).

Even though the only samples used of *O. mossambicus* were downloaded, meaning they are from different locations, a skyline plot in Figure 8 and mismatch distribution in Figure 11, clearly indicated a decrease in a population in the past. The population is stable at the moment, but it never recovered from the drop. Our results do not show any dramatic expansion.

Similarly, the population of *O. niloticus* in the Nandoni Dam has been expanding for some generations now, this was supported by a mismatch distribution in Figure 12 (B) and the Tajima's D statistics of -1.63 and the Fu's statistics 0f -7.60. It makes sense that the population is expanding because this species has been well distributed all over the world for aquaculture even though it seems to have gone through a bottleneck effect in the Nandoni Dam see the mismatch distribution in Figure 12 (B).

The populations of *T. rendalli* in both dams have been doing well according to the BSP (see Figure 13). Yet the population does not seem to be expanding according to the mismatch distribution in figure 13, however, this could be due to a very low sample number. This was almost the same with downloaded samples. The population seems to be declining. Or simply because of the low sample number which failed to give us enough information.

4.4.Distribution of haplotypes in the Levubu system.

The observed number of haplotypes of *O. niloticus* and *O. andersonii* were higher than expected. Which questions the time of invasion to the Levubu system. The estimated invasion time is about 20 years or more. The highest flood of the year 2000 being the main suspected time

of the invasion. But this alone does not explain why some haplotypes of *O. andersonii* are present in the Albasini Dam but not in the Nandoni Dam. This suggests that *O. andersonii* might have invaded the system much earlier, before either Albasini and Nandoni dams were built. This in a way explains the high haplotypes diversity present to date. This explains why no *O. mossambicus* haplotype was observed (could have been replaced or pushed out). If indeed these species have been in the system longer than expected, then it is most likely that hybridization has been taking place. This probably made it very hard to identify pure species morphologically and using only mitochondrial DNA does not place this study at the advantage of looking into the hybrids.

This means that traces of *O. mossambicus* haplotypes might be present in its paternal genome, and perhaps very few in the maternal genome. Unfortunately, we could not find a specimen that could represent this species in our study. However, this does not mean that there are no haplotypes of *O. mossambicus* in the Nandoni Dam.

4.5. Hybridisation.

There have been researches about *Oreochromis* species in the Levubu river system in the past two decades (Fouché *et al*. 2010), but no one has reported the presence of alien species since then. This could be because there might be hybrids present in the Dams. Hybrids are complicated and not easy to identify morphologically since they resemble any of the parent species (Van der Waal 2000 and Moralee *et al*. 2000). The results by Moralee *et al*. 2000, revealed the case of misidentification of a pure *O. mossambicus* for a hybrid by using morphological data. This is a perfect example of what probably happened to the previous researchers who were not using genetics.

Mitochondrial DNA can only tell us about maternal haplotypes. In this case, a bigger project that looks at the problem more comprehensively (nuclear DNA combined with mitochondrial DNA) needs to be conducted to answer the question of hybridization fully and point out the pure species and hybrids if they are present.

The haplotypes of *O. mossambicus* were not found in this study, which was very concerning because *O. mossambicus* is considered to be a native species. This clearly shows that all the numbers of *O. mossambicus* reported by Fouché *et al* (2010) have been greatly reduced, misidentified or completely whipped out either by hybridization or overfishing. It was completely unexpected that all samples thought to be *O. mossambicus* were specimen carrying a mitochondrional DNA of *O. andersonii*. This reality brought a question of hybridization and near extinction within the Dams. Maybe the nuclear DNA could have told us a different story, it is near impossible to conclude about hybridization with mitochondrial DNA alone.

The 19 specimens of *O. niloticus* from Nandoni Dam produced 14 haplotypes in figure 6, however, once the haplotypes have been merged with the downloaded samples, all the Nandoni haplotypes condensed into two (see figure 3) nested within cluster 3 in blue. *O. niloticus* has never been reported in Nandoni or anywhere within the Levubu river, which means that it invaded the system late or was present but misidentified morphologically as something else.

It has been reported that species of the genus *Oreochromis* are capable of hybridizing and produce fertile offspring (D'Amato *et al.* 2007; Seehausen *et al*. 2003; Agnese *et al*, 1998; Rognon and Guyomond 2003). The cases of hybridization have been well documented in different studies worldwide. One statement provided by (Bezault *et al.* 2012) states that "hybridization event between highly distantly related tilapia species can lead to meiotic classic

process following diploid mendelian segregation and the maintenance of a stable and recombining hybrid gene pool across generations", explains the hybridization situation better.

The fact that most species of *Oreochromis* are omnivorous looks like an advantage but, according to (Agnese *et al*, 1998; Moralee *et al*. 2000) "*O. niloticus* directly compete with our native *O. mossambicus* for breeding space and food". This situation often results in the production of hybrids, which tend to grow bigger than the two parents (pure species) (Moralee *et al*. 2000). However, it is important to mention that hybridization does not always result in the extinction of the native species (Moralee *et al.* 2000).

Oreochromis andersonii, *O. mossambicus* and *O. niloticus* and their hybrids have been reported to be present in the Limpopo river by D'Amato *et al*. 2007; Firmat *et al*. 2013 and others. The presence of invasive species is considered a problem everywhere. However, it is even devastating for the native species if the alien species can hybridize with them. Hybridization is not always bad. If there was no human influence on the introduction of alien species, and it happens naturally in the wild. This could be good for the genus as the hybrid traits that are beneficial will be under positive selection and will thus be inherited by future generations. In this way, hybridization can promote speciation and adaptive processes (Masello et al. 2019; Seehausen 2004).

5. CONCLUSION.

Invasive species have been problematic worldwide, this is especially important in species that can hybridize. *Oreochromis* species is one case that has been a problem for a long time. In our case where we checked for invasive species in Nandoni and Albasini Dams, the outcome was the reality that the Levubu river has been invaded *by O. niloticus* and *O. andersonii*. The populations of these species are expanding, this situation is most likely to encourage hybridization. Even though no genetic study on hybridization in Levubu has been published yet. Relying on our findings, it is most likely that hybridization has taken place and the population of hybrids is expanding. A more detailed study that will deal with mtDNA and nuclear DNA is recommended so that it can reveal the hybrids. The only way we can save *O. mossambicus* is to look at water bodies that are isolated from a large river and try to study and protect them.

It will be a better conservation strategy to conserve *O. mossambicus* that has a great genetic diversity such as the one's found in the headwaters of the Changane drainage and the lakes surrounding the Lower Changane river (Firmat *et al*. 2013). *O. mossambicus* is better adapted to low temperature, high salt concentration, low O_2 and swamps that are prone to experiencing eutrophication, therefore, the swamps could become a refuge for *O. mossambicus* in its home range. Most importantly *O. mossambicus* is well adapted to seawater, this is another refuge space that we can look at. More especially in Mozambique where the Limpopo River release its waters into the ocean. This is where one can find a great genetic diversity of this species as it has been existing and adapting for millions of years here.

We also must acknowledge that the problem of invasion is facilitated by us human beings, confronting the reality head-on might be the only way to solve this problem. Educating

fishermen and professional anglers would be another way to try and control the intentional release of unknown species in Dams.

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