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Population genetic analysis of *Oreochromis macrochir* and *O. mweruensis* compared to *O. niloticus* inferred from microsatellite genotyping using next-generation sequencing

Thesis submitted for the award of the title

"Master of Science"

Submitted by

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Supervised by:

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Co-supervised by:

Manuel Antonio Cardoso Curto, Dr (Post Doctorate)

Cyprian Katongo, Dr Associate Univ. Prof. BSc, MSc, PhD

A special project submitted in partial fulfilment for an award of a Masters' of Science degree in Environmental Science with a specialization in Limnology and Wetland Management

Jointly awarded by

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Institute of Integrative Nature Conservation Research (INCR)

Institute of Hydrobiology and Aquatic Ecosystem Management (IHG)

University of Natural Resources and Life Sciences (BOKU), Vienna – Austria

April 2020

Declaration

I hereby declare the sole authority of this work as my own original work that has not been submitted at any other institution/University for the award of a Master's Degree of Science; no assistance apart from what has been used in the form of quotations and concepts taken from published literature from scientific journals over the internet in word or content have been duly acknowledged and the original sources fully accredited. I further declare that websites and other internet sources cited have been duly acknowledged.

Name: **Darlington Besa**

Registration Number: **11836194**

Signature:  Date: 03rd April 2020

Dedication

This work is dedicated to the most important people in my life: my mother (Lear Chipeta), my father (Darlington Maxwell Ngosa Besa), my dearest siblings, Julie (Jelly), Jane (Pamo) and Grace (Graelly) Besa and nephew and niece, David and Joy, I have missed you all so very much.

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I would like the most important people in my life to remember the following wise words on family and friendships:

"*Friendship is one of the sweetest joys of life. Many might have failed beneath the bitterness of their trial had they not found a friend*."

-- Spurgeon

"*It is possible for two people who have wide differences of preference and opinion, of habits, of teaching, of training, of background and belief to enjoy the company of each other in many ways. Indeed, a diversity of friendships is one of life's real enrichments. To learn of the goodness of those who are unlike-their worth, their sincerity, their good hearts, their good minds, their good company-is rich and rewarding. It is wonderful to have a wide range of choice friends who can be counted on, friends who can be enjoyed and loved and trusted. Such is the meaning of friendship*."

– Evans

I would like all of them to remember these closing wise words:

"I am responsible for my own well-being, my own happiness. The choices and decisions I make regarding my life directly influence the quality of my days."

-- Kathleen Androus

"***You must go after your wish. As soon as you start to pursue a dream, your life wakes up and everything has meaning***."

-- Barbara Sher

With an immeasurable love,

Darlington 😊

To my fellow scientists and researchers, I would like them to remember the following words:

"Science is moving forward at an unprecedented speed… Sharing of results early allows other researchers to build upon existing research faster."

-- Raja Mazumder, George Washington University Medical Centre, Washington, DC, United States of America

"There are no solutions, only trade-offs"

– Thomas Somwell

"Life is uncertain and will always remain uncertain and the consensus of a scientific community may not always be right but the potency that comes through the discovery of certainty and through what is right in science adds a degree of certainty to life making it more certain as we ponder on to relativism or absolution of what is right. Such a journey in life is worth exploring meaningfully"

– Darlington Besa,

Personal chronicles at Haus Panorama 1200, Vienna – Austria

Yours in Science and the unwavering service to mankind,

Darlington Besa 😊

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List of Acronyms

DoF – Department of Fisheries

ZAEDP – Zambia Aquaculture Enterprise Development Project

IUCN – International Union for Conservation of Nature

DNA – Deoxyribonucleic acid

SSR – Single Sequence Repeats

mtDNA – mitochondrial DNA

NIST – National Institute of Standards and Technology

PCoA – Principle coordinate analysis

SSR – simple sequence repeats

NARDC – National Aquaculture Research Development Centre

AMOVA – Analysis of Molecular Variance

HWE – Hardy-Weinberg Equilibrium

Abstract

Oreochromis macrochir and *O. mweruensis* are two important cichlid fish for capture fisheries and aquaculture development in Zambia. However, in Zambian freshwater systems, the biomass yield that comes from mainly capture fisheries for the species *O. macrochir* has greatly declined and the current population of *O. mweruensis* remains unknown. In the study, 33 microsatellite DNA markers developed from *O. niloticus* were used for cross-amplification to evaluate population genetic structure, allelic diversity and biogeography of *O. macrochir* and *O. mweruensis* in the entire natural distribution range in Zambian freshwater systems. The study involved 276 fish sampled from four Lakes and three government fish farms. Analysis of molecular variance (AMOVA), pairwise F_{ST} values, model-based clustering and principle coordinate analysis (PCoA) and STRUCTURE analysis was conducted to genetically characterise the populations of *O. macrochir* and *O. mweruensis* based on their differentiation, structure and diversity. Results showed that the species from the wild populations had an excess in heterozygotes and that both species maintained a high level of genetic diversity and formed four cluster groups. Deviations from Hardy Weinberg Equilibrium (HWE) were observed across the loci from almost all the populations. Aquaculture populations clustered closely with the populations from Lake Mweru suggesting the natural populations to be the source. The highest genetic variation was observed within individual samples (57%) for *O. macrochir* and 65% within individuals for *O. mweruensis*. The population structure of the species O. macrochir revealed a distinct population for Lake Bangweulu and Lake Kariba. For the implementation of aquaculture programmes, much care should be taken when recruiting fish genetic stocks from the wild for up-gradation programs in selective breeding to avoid mixing genetic material from different populations without clearly understand the genetic make-up of the fish species.

Keywords: **genetic characterisation, genetic diversity, population differentiation, aquaculture, germplasm**

Abstrakt (in German)

Oreochromis macrochir und *O. mweruensis* sind zwei wichtige Buntbarsche für die Fangfischerei und die Entwicklung der Aquakultur in Sambia. In sambischen Süßwassersystemen ist der Biomasseertrag, der hauptsächlich aus der Fangfischerei für die Art *O. macrochir* stammt, jedoch stark zurückgegangen, und die derzeitige Population von *O. mweruensis* ist unbekannt. In der Studie wurden 33 aus *O. niloticus* entwickelte Mikrosatelliten-DNA-Marker zur Kreuzamplifikation verwendet, um die populationsgenetische Struktur, die allelische Diversität und die Biogeographie von *O. macrochir* und *O. mweruensis* im gesamten natürlichen Verbreitungsbereich in sambischen Süßwassersystemen zu bewerten. Die Studie umfasste 276 Fische aus vier Seen und drei staatlichen Fischfarmen. Die Analyse der molekularen Varianz (AMOVA), der paarweisen FST-Werte, der modellbasierten Clusterbildung und der Hauptkoordinatenanalyse (PCoA) sowie der STRUKTUR-Analyse wurde durchgeführt, um die Populationen von *O. macrochir* und *O. mweruensis* anhand ihrer Differenzierung, Struktur und Diversität genetisch zu charakterisieren. Die Ergebnisse zeigten, dass die Arten aus den Wildpopulationen einen Überschuss an Heterozygoten aufwiesen und dass beide Arten ein hohes Maß an genetischer Vielfalt beibehielten und vier Clustergruppen bildeten. Abweichungen vom Hardy-Weinberg-Gleichgewicht (HWE) wurden über die Loci von fast allen Populationen beobachtet. Die Aquakulturpopulationen gruppierten sich eng mit den Populationen aus dem Mweru-See, was darauf hindeutet, dass die natürlichen Populationen die Quelle sind. Die höchste genetische Variation wurde in einzelnen Proben (57%) für *O. macrochir* und 65% in einzelnen Proben für *O. mweruensis* beobachtet. Die Populationsstruktur der Arten *O. macrochir* ergab eine unterschiedliche Population für den Bangweulu-See und den Kariba-See. Bei der Verbesserung der Aquakulturprogramme sollte bei der Rekrutierung von Fischgenetikbeständen aus der Wildnis in der selektiven Züchtung große Sorgfalt angewendet werden, um zu vermeiden, dass genetisches Material aus verschiedenen Populationen gemischt wird, ohne die genetische Zusammensetzung der Fischarten klar zu verstehen.

Schlüsselwörter: **genetische Charakterisierung, genetische Vielfalt, Populationsdifferenzierung, Aquakultur, Keimplasma**

Chapter 1

1. Introduction

Oreochromines are a subfamily of fish native to Africa belonging to the Cichlidae family that have been largely commercialised due to their economic importance in both capture fisheries and aquaculture development (Trewavas, 1983). The Longfin tilapia (also known as the Green-headed bream) (*Oreochromis macrochir*, Boulenger, 1912) and the Mweru tilapia (*Oreochromis mweruensis*, Trewavas 1983) are two native cichlid species that are mainly distributed in the tropical region, particularly found in Southern Africa belonging to a family of more than 3,000 species (Nagl et al, 2001; Skelton 2001). Cichlids in Southern Africa are further sub-divided into the haplochromines and the tilapiines constituting the three main genera *Tilapia*, *Sarotherodon* and *Oreochromis* based on their feeding behaviour, reproduction, and biogeographical location (Trewavas, 1983; Skelton, 2001). In Zambia, cichlids constitute of 186 described fish species of the 433 identified fish species of Southern Africa (Fish base, 2016).

These cichlids in Southern Africa are a major source of proteins in most developing countries such as Zambia natively distributed to the Chambeshi and Luapula watersheds particularly found in the Bangweulu complex, the Mweru-Luapula fishery of Luapula and part of Northern Provinces and the Zambezi watersheds on in the mid-Zambezi River basins (Marshall and Tweddle, 2007). Due to their economic importance, the species have been extensively used for fish restocking activities in ponds, dams in the Kafue, upper Zambezi, and Congo River system. They have also been reported to be introduced in other parts of Africa and in the Hawaiian Islands, Okavango and Ngami region, Cunene basin, Chambeshi basin, Bangweulu region and other parts of Southern Africa (Trewavas, 1983).

In Zambia, the steady growth of the aquaculture industry has been growing exponentially over the last decade. In 2004, the total aquaculture production was estimated at approximately 4,500 MT and has increased by 87.54% over the past 12 years to a production total of 36,105 MT in 2018 with over three times coming from the commercial sector comprising cage culture and pond rearing systems (DoF, 2019). And being one of the largest producer of tilapia in the Southern African Development Community (SADC), the aquaculture sector has received a lot of recognition that has opened avenues to seed and feed producers in an effort to improve their food and nutritional security at all sector levels

(Genschick et al, 2017), that often constrains aquaculture production (Chirwa, 2008). Of the cichlid family, tilapia species have been widely dispersed because of their quick adaptability to different environmental conditions outside their native regions, particularly the species being used for aquaculture. Studies indicate that the introduction of non-native species often results in establishments of feral populations that threaten the biodiversity by causing contention over habitats and food with similar congeneric species (Canonico et al, 2005).

The cichlids have also found their use in studies of evolution and sympatric speciation in Zambian freshwater systems (Katongo et al, 2005). In a more recent study, rapid speciation processes have been hosted in some Lakes with similar depth as Lake Mweru and Lake Bangweulu while showing no *in situ* speciation having taken place in others as was the case in Lake Bangweulu with no radiation having occurred (Meier et al, 2019). In the Great East African Lakes, allopatric speciation has attracted a lot of attention with over 2,000 cichlids having evolved in the recent epoch occurring in similar or overlapping geographic regions (Kocher, 2004). And like other cichlids, most tilapiines have experienced evolutionary radiation leading to recent or incomplete speciation processes causing them to easily hybridise with other species and exploited quality by aquaculturists (Wohlfarth and Hulata, 1981; Trewavas, 1983; D'Amato et al, 2006). Evidence of hybridization genetically has been quantified using mitochondrial and traditional microsatellite (SSR) DNA genotyping between the exotic *O. niloticus* and other native and endemic *Oreochromis* species of Southern Africa (D'Amato et al, 2006; Deines et al, 2014; Bbole et al, 2014), Western Africa (Lind et al, 2019) and Eastern Africa (Tibihika et al, 2018).

The culturing of tilapia species in Sub-Saharan Africa is braced for rapid growth in the coming years increasing the need to better understand the distribution of wild genetic resources and population structural patterns in Southern African freshwater systems (Eknath and Hulata, 2009; Lind et al, 2012; 2019). Understanding the population genetics of a particular fish species is crucial to the successful implementation of conservational strategies, sustainable management and utilisation of genetic resources and planning of up-gradation programmes fostering capture fisheries and aquaculture development (Sanudi et al, 2020). Geographical locations inhabiting different population structures or sub-populations that are under different ecological and anthropogenic pressures may require dissimilar levels of conservation and management (Bbole et al, 2019; Sveegaard et al,

2015), due to the limited information on the genetic diversity of the native species (Abdul-Muneer, 2014; Bentsen et al, 2017;). In aquaculture production systems, seed quality and growth performance of particular fish can be enhanced through performing selective breeding programmes by applying molecular tools such as microsatellite markers, mitochondrial markers or single nucleotide polymorphisms (SNPs) (Xu et al, 2015; Lind et al, 2019).

Molecular studies have also found their application in determining the genetic diversity and clear population differentiation of *O. niloticus* between East and Western Africa (Bezault et al, 2011; Tibihika et al, 2018). In Southern Africa, particularly in Malawi, studies on *O. shiranus* focused on the genetic variation in the entire distribution range as a basis to improve aquaculture development (Sanudi et al, 2020). In Zambia, studies by Bbole et al, (2018; 2019) focused on genetically characterising the species *O. andersonii* and *O. macrochir* from part of Lake Bangweulu, the upper Zambezi River and the Kafue River using mitochondrial and microsatellite DNA markers. The authors in these studies endeavoured to characterise two species of populations of tilapia species and recommended a more robust study of the genetic diversity in river systems by identifying and protecting conservational sites of the native species' gene pools. Although attention is slowly been given to the characterisation and population genetic structure of *O. andersonii* and *O. macrochir*, these relative few studies have not focused on the entire distribution of *O. macrochir* in the major Zambian Lakes (Lake Bangweulu, Lake Mweru, Lake Mweru-Wantipa and Lake Kariba), that provide genetic resources for aquaculture development, and the genetic population structure patterns remains uncertain (Bbole et al, 2018). The species *O. mweruensis* native to Lakes Mweru and Mweru-Wantipa is often neglected due to limited knowledge about its distribution in Zambian freshwater systems and possible misidentification with *O. macrochir* (Schwanck, 1994; De Vos et al, 2001; Van Steenberge et al, 2014).

This study endeavoured to better understand the population genetic structural patterns of *O. macrochir* and *O. mweruensis* in Southern African freshwater systems in the wild and cultured populations influenced by ecological and anthropogenic factors by building on existing research. The main objective of the study was to genetically characterise and assess the genetic diversity of wild and cultured fish populations of the Longfin tilapia, the Mweru tilapia and the Nile tilapia in four major fisheries (Bangweulu, Mweru-Luapula, Mweru-Wantipa and Kariba) and comparing them to their farmed counterparts in three government

stations (NARDC, Misamfu and Fiyongoli) using 47 microsatellite markers and 33 mitochondrial markers across two river basins. The specific objectives of the study were: 1) to genetically characterize and compare the wild and cultured populations of the Longfin tilapia, Mweru tilapia and Nile tilapia in Southern African populations and compare them with the Eastern/Western African populations; 2) to quantify comparatively the differences in population genetic structure in the fish populations of the Longfin tilapia, Mweru tilapia and Nile tilapia in wild populations; 3) to evaluate the major factors influencing the genetic diversity in the natural distribution of the Longfin tilapia, Mweru tilapia and Nile tilapia in four major fisheries (Lake Bangweulu, Lake Mweru, Lake Mweru-Wantipa and Lake Kariba); 4) to determine the genetic population structure and genetic diversity of cultured populations of the Longfin tilapia from three farms/stations (Misamfu, Fiyongoli and NARDC); 5) to propose an optimal strategy for effective management of fish genetic resources for Southern African wild and farmed cichlid species.

The main output of this project was to provide a clear insight into the natural populations of conservation value and create an evaluated baseline survey based on molecular genetic characteristics of the Longfin tilapia for use in the on-going aquaculture developmental projects in Zambia by 1) providing an in-depth understanding of the population structure in the wild to better manage fish genetic resources (germplasm) in Zambian freshwater systems that may be impacted by escapees coming from the farmed populations; and 2) to provide a clear differentiation genetically of wild populations and their potential for selective and non-selective breeding programs in the aquaculture sector, that expects a wide genetic diversity in their founding populations to accomplish aquaculture productivity goals and avoid problems of inbreeding over time and conservation measures.

1.1. The *Oreochromis* genera in Southern Africa of Zambia

Cichlids constitute a large group of fishes that are diverse in morphology, ecology and behaviour patterns (Bbole, 2019). The family Cichlidae under the order Perciformes is comprised with more than 3,000 fish species that are dispersed across almost all continents, namely: Africa, Central and Southern America, Madagascar, Syria, Israel, Sri Lanka, West Indies and the coastal area of India (Fryer and Iles, 1972; Kocher, 2004). Of the 3,000 species described, about 2,000 species constituting the fresh and brackish water fishes were assumed to be concentrated among the three great lakes of Africa i.e. Victoria, Tanganyika and Malawi (Skelton, 2001; Kocher, 2004; Salzburger and Meyer, 2004). The East African

cichlids were divided primarily into haplochromines and tilapiines, lineages that are distinguished by a bony appendage or apophysis found on the skull used for joining the upper bones (pharyngeal) (Regan, 1920; Nagl et al, 2001). These major lineages within the cichlid family have been divided in Southern Africa into tilapiines and haplochromines, with the tilapiines grouped based on their adaptability (to extreme environmental conditions such as water temperature, salinity and alkalinity in tropical and temperate regions), feeding habits, morphological characteristics and biogeographical location into three main genera: *Oreochromis, Sarotherodon* and *Tilapia* (or *Coptodon*) (Beveridge and McAndrew 2000; Skelton, 2001). Skelton (2001) divided the two major lineages in the cichlid family in Southern Africa into tilapiines and haplochromines with the tilapiines grouped based on their adaptability, reproductive behaviour, feeding habits, morphological characteristics and biogeographical location into three main genera: *Oreochromis, Sarotherodon* and *Tilapia* (or *Coptodon*).

Tilapiines, on the other hand, have been divided into three major categorical genera based on their reproductive behaviour or breeding patterns: 1) the maternal mouthbrooders of the genera *Oreochromis* comprising 31 fish species; 2) the paternal and bi-parental mouthbrooding species of the genera *Sarotherodon* composed of 3 fish species, and 3) the substrate spawners of the genera *Tilapia* composed of 8 fish species (Trewavas, 1981, 1982, 1983; Nagl et al, 2001). All the species under these three genera according to Trewavas (1966a, b; Thys Van den Audenaerde, 1968) are usually referred to as "tilapias". Trewavas (1989) further divided the species under the genera *Oreochromis* into five sub-genera namely, *Oreochromis, Vallicolla, Neotilapia, Nyasalapia* and *Alcolapia* though Peters and Berns (1982) could not find any scientific consideration as to why the five sub-genera ought to be and proposed putting all the main tilapiine fish species as the genus "Tilapia".

In Southern Africa, about 8 genera constituting 42 fish species of cichlids have been documented and divided into the tilapiine and haplochromine lineages with the tilapiines constituting two main genera: 1) *Oreochromis* and 2) *Tilapia* (*Coptodon*). The genus *Oreochromis* comprises 33 species with about 6 being endemic to Southern Africa (i.e. *O. shiranus, O. mossambicus, O. andersonii, O. mortimeri, O. macrochir* and *O. placidus*) and about 2 alien species (*O. aureus* and *O. niloticus*) having been introduced (Skelton, 2001). The genus *Coptodon* consists of 31 species (Froese and Pauly, 2019; Dunz and Schliewen, 2013) with about 4 species being found in Southern Africa (Skelton, 2001). These two

groups form an important resource for aquaculture development (Skelton, 2001; Cononico et al, 2005).

Zambian cichlids are one of the largest groups of fish fauna with about 431 species of fish with about 183 belonging to the order *Perciforms* and subfamily *Pseudocrenilabrinae* constituting 1 fish species under the genus *Coptodon* (*Coptodon rendalli*) and 9 species under the *Oreochromis* genus consisting of the species *O. andersonii* (three spotted tilapia) growing to a maximum total length of 61 cm, *O. aureus* (Blue tilapia) growing to a maximum length of 45.7 cm, *O. karomo* (Karomo tilapia) growing to a maximum of 28 standard length, *O. macrochir* (Longfin tilapia) growing to a total length of 43 cm, *O. mortimeri* (Kariba tilapia) growing to a total length of 48 cm, *O. mweruensis* (Mweru tilapia) growing to a total length of 23.8 cm, *O. niloticus* (Nile tilapia) growing to a total standard length of 60 cm, *O. spilurus* (Sabaki tilapia) growing to a standard length of 19.2 cm and *O. tanganyicae* (Tanganyika tilapia) growing to a maximum total length of 42 cm (Froese and Pauly, 2019). Of these 9 species, about 4 fish species particularly *O. andersonii*, *O. niloticus*, *O. macrochir* and *C. rendalli* are the most common cultured fish species of commercial importance in Zambia.

1.2. Description of the investigated Southern Africa cichlids

1.2.1. *Oreochromis macrochir* (Boulenger, 1912)

O. macrochir (Longfin tilapia) is a fish species native to Africa particularly found in the Kafue, the Upper Zambezi, the Congo River systems and the Bangweulu region (Trewavas, 1983). The species feeds on blue-green algae, diatoms and mostly detritus though in their juvenile stage they may also feed on small invertebrates and zooplankton a feeding habit they lose as they grow older into adulthood (Jackson, 1961; Bell-Cross, 1976; Skelton, 1993; Lamboj, 2004). The species has an active reproductive period beginning from August to March in the tropical regions of Western and Southern Africa (Lamboj, 2004). The figure below shows the distribution of Longfin tilapia in Africa and around the world.

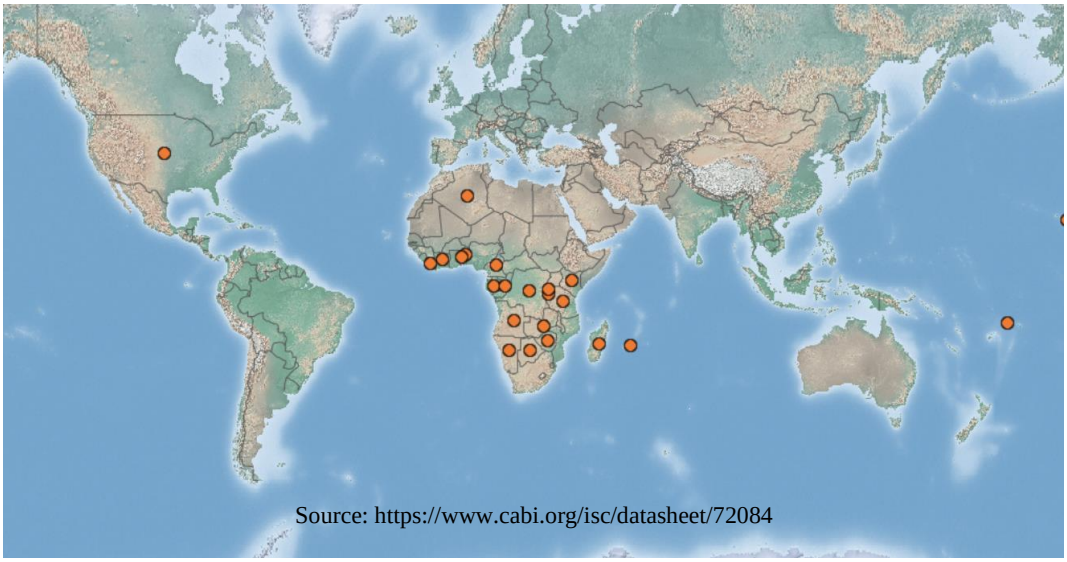

Figure 1 Oreochromis macrochir (Longfin tilapia) in Africa and other parts of the world

The males construct a nest usually in shallow waters and attract female fish to the already prepared nest for mating (Bruton *et al.*, 1982). The males usually mate with several females in succession to one another while the females may be able to mate with more than once in summer after which they take time to rest which ensures a very large population of their young (Bruton *et al.*, 1982; Lamboj, 2004). Trewavas (1983) observed that when males successfully mate with a female, the female is able to carry a large number of eggs usually between 400 – 1300 eggs due to the expanse of the buccal cavity during reproduction. See figure below.

Figure 2 Oreochromis macrochir (Longfin tilapia)

When eggs hatch into small hatchlings they start to slowly swim out of the mouth of the female and back when frightened. They continue to move in and out of the mouth of the female until after about 21 days when they are usually larger and start fending for themselves (Norris and Skelton, 1996).

According to Marshall and Tweddle (2007), the species is threatened by the introduction of alien species such as *O. niloticus* (Nile tilapia) that is now widely distributed in the Kafue, Zambezi and Limpopo freshwater systems and has been listed as a vulnerable species under the IUCN Red List. The impacts of hybridization by the Nile tilapia with the native species where they have been introduced have been observed to have dangerous effects on the natural ecosystems and has become a growing concern on biodiversity and conservation of native species (Canonico *et al*., 2005; Deines, *et al*., 2014; Bbole *et al*., 2015). Lind *et al*., (2012a) observed that the species *O. macrochir* (Longfin tilapia or green-headed bream) is significant in both capture fisheries and aquaculture sectors in many parts of Southern Africa and indicated a dilemma faced by the two sectors in development and conservation of the native species. The native species has proven potential for aquaculture development programmes in Southern Africa and Western parts of the continent and possesses phenotypic culturable traits for aquaculture growth (Kefi *et al*., 2011; Lind *et al*., 2012a).

1.2.2. *Oreochromis mweruensis* (Trewavas, 1983)

O. mweruensis (Mweru tilapia) is an endemic fish species to Africa that is particularly found in Lake Mweru, the nearby swamps, the lower and middle Luapula River systems and Lake Mweru-Wantipa in the upper and middle region of the upper Congo River Basin in the Democratic Republic of Congo (Trewavas, 1983; Van Steenberge *et al*., 2014), and was then later translocated to Lake Kivu of Rwanda where it was introduced as reported by De Vos *et al.*, (2001).

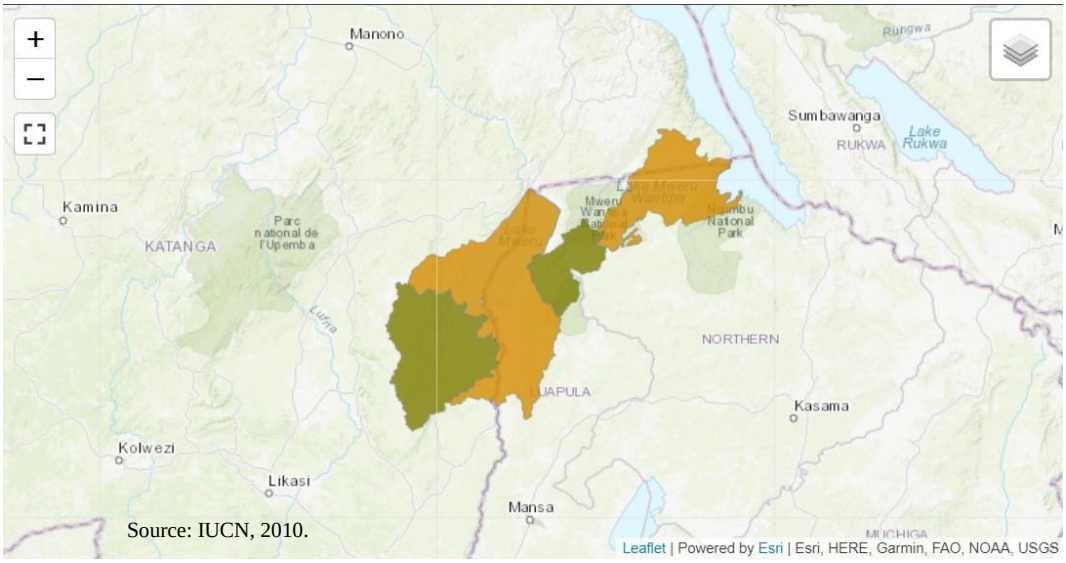

Figure 3 Distribution of Oreochromis mweruensis (Mweru tilapia)

The fry (newly hatched fish) of *O. mweruensis* feed on algae in swampy areas that are threadlike and also feed on small macroinvertebrates and zooplankton like the Longfin tilapia, a feature which they too lose as they mature (Trewavas, 1983; Lamboj, 2004). Further observations by Trewavas (1983) showed that the juveniles feed on threadlike algae and plant detritus but start to exclusively feed on plankton in their adult stages. Their reproductive patterns are very similar to the Longfin tilapia as they build nests, attract females to the nests and are able to mate with more than one female recurrently (Trewavas, 1983). The figure below shows an image of the Mweru tilapia.

Figure 4 Oreochromis mweruensis (Mweru tilapia)

According to Moelants (2010), the species *O. mweruensis* is considered to be the 'least concerned' fish species despite its population steadily declining and in many

cases, it has been reported to occur in a combination of *O. macrochir*. Despite its importance for aquaculture in Southern and Central Africa, the Mweru tilapia is reported to be threatened mostly by the loss of wetland habitats along rivers, swamps, floodplains and the shallow areas around lakes suitable for breeding (Moelants, 2010).

Furthermore, the distribution region of *O. macrochir* that may have been caused by introduction are believed to have been misidentifications of the species *O. mweruensis* considered distinct subspecies of *O. macrochir* then but now viewed distinguishably at specific levels of identification (De Vos *et al*., 2001; Schwanck, 1994). Other reports by Van Steenberge *et al*., (2014) have shown that the distribution of *O. mweruensis* may actually be larger, indicative of being native to the Mweru-Luapula subregion and other parts of the Bangweulu-Chambeshi region and not endemic to the Mweru-Luapula region. The introduction of the invasive species *O. niloticus* have been reported in the Luongo, a tributary of the middle Luapula river (DoF, 2017 Provincial Report). The upper Luongo is rich with fauna generally found in the Bangweulu-Chambeshi subregion while the lower part is rich in fauna from the Mweru-Luapula subregion (Balon and Stewart, 1983).

1.2.3. *Oreochromis niloticus* (Linnaeus, 1758)

The Nile tilapia (*O. niloticus*) is one of the most important food resources in the world; a freshwater cichlid native to various Lakes in Africa in the rivers of the Niger, Volta, Benue, Senegal, and Israel; the Nile basin (constituting Lakes Tana, Edward and Albert), Lakes Kivu (Rwanda), Tanganyika (shared by 1) Zambia, 2) Burundi and the majority by 3) Tanzania and 4) the Democratic Republic of Congo DRC), Lakes Turkana (Kenya and Ethiopia) and Baringo (Kenya), Rivers Awash (Ethiopia) and Suguta (Kenya), and the Omo river system (Ethiopia) that is located outside the Nile basin (Trewavas and Teugels, 1991; Trewavas, 1983; Teugels and Thys van den Audenaerde, 2003; Greenwood et al, 1987). Due to its hardy quality, the Nile tilapia has been widely distributed and augmented in capture fisheries and aquaculture in most tropical regions (Welcomme, 1988). The figure below shows the distribution of the species globally.

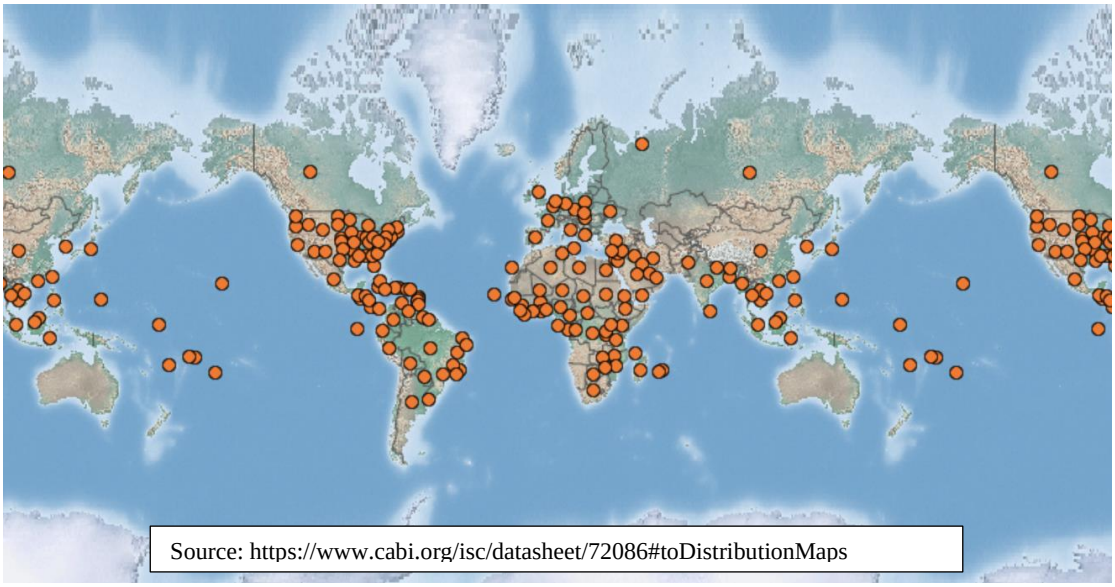

Figure 5 Oreochromis niloticus (Nile tilapia) distribution in Africa and around the world

It is often described to have a relatively small head, with genital papilla of the males not tasselled (Trewavas, 1983; Genner et al, 2018). The Nile tilapia is mostly distinguished by the presence of upright bands or perpendicular strips located at the caudal fin found in all life stages (Eccles, 1992; Teugels and van den Audenaerde, 2003). In juvenile fish, the vertical bands form an arc at the base of the tailfin with dark colours in males (blue-pink; having a dark throat, belly, pelvic and anal fins) and females with a brown colour (having a white/yellow colour on their belly) (Genner et al, 2018).

Figure 6 Oreochromis niloticus (Nile tilapia)

Due to its high adaptability, the Nile tilapia often occurs in different freshwater habitat systems ranging from streams, sewage canals, rivers, lakes, irrigation channels and brackish waters but does not seem to thrive well in saltwater (Bailey, 1994; Lamboj, 2004). The fry of the Nile tilapia feed mainly on benthic algae and phytoplankton in addition to detritus while the juveniles incline to be more all-devouring (omnivorous) in feeding habits than adults (Lamboj, 2004). The females are mouth-brooders with the males building simple conical nests in shallow waters (Trewavas, 1983). It has received a lot of attention in aquaculture and has supported captured fisheries in areas where the species has been established (Genner et al, 2018). The Nile tilapia is regarded as a highly invasive species as it has been documented to hybridize with many of the local indigenous *Oreochromis* species and thus prohibited in a number of countries such as Zambia, Malawi and South Africa (Bbole et al, 2014; Deines et al, 2014; Genner et al, 2018) with an exception of areas where it has already been established such as the Kafue fishery for Zambia.

1.3. Problems associated with translocations and introductions

Some of the major problems associated with the introduction of fish can be perceived as either ecological, economical or biological. Species' introduction or translocation beyond their native range, whether directly or indirectly, or whether intentionally or accidentally, as a result of anthropogenic activities often cause changes in the freshwater ecosystem when introduced and these can be a bane and a boon to society (Ewel et al, 1999; Gozlan, 2008; Jeschke et al, 2014). There are also many cases in which ecological factors may be the driving forces for change in natural aquatic ecosystems (Eissa and Zaki, 2011; Stendera et al, 2012; Trolle et al, 2019).

Fish translocations or introductions provide a number of ecosystem services such as the provision of food to fill up an ecological niche, sport fishing or angling, capture fisheries foraging and using them as biological controls (Gozlan, 2008). But these introductions that are conducted with the intention to improve fish production in most water bodies whose fish stocks are dwindling may often result in deleterious effects on the native fish populations (Madeira et al, 2005). Their introduction has been reported to cause diseases, competition for food and habitat, and a loss of these by the native species might lead to their displacement or extinction of the local species (in some extreme cases) (Lynch and O'Hely, 2001).

In some cases, they may co-exist together, but such cases are very few that have been reported. Due to their high adaptability to different environmental conditions, most tilapiines have been widely distributed globally particularly for aquaculture purposes and this has raised concern and much debate as to whether their introductions or translocations into other freshwater systems are beneficial or destructive (Canonico et al, 2005; Gozlan, 2008; Jeschke et al, 2014).

The tilapia species, Nile tilapia (*O. niloticus*) and Mozambique tilapia (*O. mossambicus*) are one of the widely distributed species contributing to the total global aquaculture with the Nile tilapia contributing over 75% globally (Prabu et al, 2019). The Nile tilapia has been introduced in many countries due to its wide tolerance to environmental conditions such as high temperatures, changes in salinity, low oxygen levels, and its fast growth standing out as its distinguishing characteristic feature (Canonico et al, 2005).

In Zambia, the introduction and translocation of native and exotic species date back as far as the 1940s and 1950s when the first initiatives to improve the food security through aquaculture production was necessitated by the Zambian Government through the Department of Fisheries (Haight, 1987; Utsugi and Mazingaliwa, 2002). Tilapia introductions were first reported during the period 1945 – 1950s with other introductions into Lakes Kariba and Lake Itezhi-tezhi (Haight, 1987). Large companies and commercial farmers such as Nakambala Estate (fish farm), Mubuyu (fish farm), Kafue Fisheries Estate (fish farm), and Kariba Bream farm imported the *O. niloticus* and *O. aureus* from countries such as Kenya, Germany, Israel and the University of Stirling in Scotland (Haight, 1987; Schwanck, 1995).

A study conducted towards the end of the 1980s indicated that the Kafue River was free of exotic species and only contained the native species *O. andersonii, O. macrochir, T. rendalli* and *T. sparmanii* (Welcomme, 1988). Towards the end of the 1980s, the Kafue Fisheries Estate experienced a flood that caused the Nile tilapia (an exotic fish species to Zambia) to escape into the Kafue River (Utsugi and Mazingaliwa, 2002). A study based on morphometric analysis indicated the distribution of the Nile tilapia that had escaped through the flood having established itself in the Kafue River (Schwanck, 1995). In 2014, two studies conducted affirmed the presence of hybridisation of non-native species such as the Nile tilapia (*O. niloticus*) with the native

species (*O. andersonii* and *O. macrochir*) using molecular tools such as microsatellite markers on the presence of hybrids in the Kafue River floodplains and rapid growth upstream and downstream from its introduced area (Deines et al, 2014; Bbole et al, 2014). There has not been any reported case on the distribution of the Nile tilapia on Lakes Mweru, Lake Mweru-Wantipa and Lake Bangweulu based on either morphology or molecular analyses to determine the current distribution of exotic species in Zambian freshwater systems.

In both aquaculture and capture fisheries, the introduction or translocation of the same species from a different geographical origin may introduce alterations to the genetic structure in the native populations due to allochthonous gene introgression with the wild fish populations (Huxel, 1999). This unavoidable need to sustain the natural fish stocks and develop aquaculture has necessitated the need to assess the genetic variation to commercial species of importance to determine if the native populations are a single unit or consist subunits by genetically characterizing the cichlids of Southern Africa into strains based on their hailing populations (Ferguson et al, 1995; Bbole et al, 2018). The geographic distance and physical barriers are reported to enhance reproductive isolation by limiting migration and while increasing genetic differentiation between populations (Ryman, 2002). The effect of migration and/or gene flow on genetic differentiation has been reported to depend on the size of the recipient populations (Chauhan and Rajiv, 2010). In aquaculture, the major concern that has been reported with farmed fish is their ability to escape into the natural environment causing introgression with the wild genomes (Chauhan and Rajiv, 2010). A lack of genetically characterizing wild and cultured fish populations and their conservation remains a challenge as genetic purity is lost when broodstock strains, recruited from the wild, interbreed and hybridize with closely related genera of fish from different biogeographical areas (Changadeya et al., 2003; Bbole et al., 2014)

1.4. Importance of capture fisheries and aquaculture in Zambia

Fish is an important product in Zambia in both capture fisheries and aquaculture sectors as they contribute to the national economic development of the country. The two sectors provide employment and income to both fisherfolk and fish farmers particularly those living in rural areas of the country for whom about 20% of animal protein comes from fish products (Musumali et al, 2009). In 2014, Zambia became the sixth largest producer of farmed fish (mainly through the culturing of breams or

tilapiines belonging to the *Oreochromis* genera) in Africa and the largest in the SADC region (Tran et al, 2019).

Zambia constitutes about 40% of Southern Africa's freshwater and is seasonally nearly 20% of the country with 12 million (ha) of water in the form of lakes, rivers, and wetlands supporting extensive production in fisheries, livestock and agriculture contributing to almost 25 per cent of the population in the country (ADP, 2009; CGIAR, 2011). Fish and fish products in the country play a very important role in the nations' food and nutritional security of the Zambian people particularly those living with HIV and AIDS (Musumali et al, 2009).

The endowment of 12 million hectares represents a large fishery and aquaculture resource segmented into three major water basins: 1) the Congo basin represented by Lake Tanganyika, 2) Luapula basin represented by the Chambeshi River, Lake Bangweulu, Luapula River, Lake Mweru and Lake Mweru-Wantipa, and 3) the Zambezi basin considered the largest represented by the Luangwa River, Kafue River, Lukanga swamps, and the Zambezi and the upper and lower Zambezi (Kefi and Mukuka, 2015). As of 2014, the fisheries sector in Zambia was projected to have contributed 0.4 per cent to the Agricultural sector supporting people directly and indirectly (CSO, 2014; DoF, 2014). According to the FAO, fish contributed about 53.4% of animal protein and even though the global consumption increased to 19.2 kg in 2012 (FAO, 2012a,b), Zambia's per capita consumption for the year 2014 was estimated at 11 kg when compared to a per capita of 6.8 kg recorded in 2011 (Tran et al, 2019).

The annual production from capture fisheries increased from the estimated 40,000 tons produced in a year in the late 1960s to 89,195 MT in 2018 and with the aquaculture sector growing to 36,105 MT in 2018 from the 4,500 tons reported in 2004 (DoF, 2017; 2018). Since the total human population in Zambia has grown from 9,885,591 captured in the year 2000, the population as captured by the 2010 census has increased to 13,092,666 (CSO, 2012). According to the Worldometer (www.worldometer.info/) of providing the latest data from the United Nations, the human population is expected to have grown in Zambia from 13,000,000 to 18,228,190 as of March 17, 2020, constituting 0.24 per cent of the world population. This increase in human population

and an increase in fishers has been one of the reasons as to why most capture fisheries production statistics have stagnated in Zambia and around the world (DoF, 2018).

In an effort to meet the high demand for fish products in Zambia, the government through the Department of Fisheries has embarked on an aquaculture development project to grow the aquaculture industry in the country (AfDB, 2016). In an effort to improve the local fish populations in terms of growth, the fish species *O. andersonii* was adopted as a species for genetic improvement through selective breeding programs under Zambian WorldFish Centre to improve the quality of fish stocks being cultured (AfDB, 2016; DoF 2017). With these initiatives, the aquaculture industry is expected to grow at commercial and artisanal levels in the culturing of cichlids, namely: *O. macrochir, O. andersonii, O. tanganyicae, C. rendalli*, *O. niloticus* and other species such as *C. carpio* and *C. gariepinus* (Nsonga and Mwiya, 2013), to meet the huge demand for fingerlings for aquaculture development (DoF, 2018). The aquaculture sector is expected to expand in the culture of Oreochromiine tilapia species in the face of stagnant and declines coming from capture fisheries (Brummett, 2008).

1.5. Molecular methods and techniques in fisheries and aquaculture

Fish species like any other organisms undergo mutations due to the normal cell operations and interactions with the natural environment causing polymorphisms or genetic variations within a population (Chauhan and Kumar, 2010). Avise (1994) observed that molecular markers are important powerful tools that can be applied to understand the evolutionary forces such as natural selection and genetic drift, some of the factors often to genetic differentiation. The application of molecular markers has found considerable to determine genetic diversity in fields of research, management and conservation of natural resources as well as genetic improvement programmes in the two sectors of capture fisheries and aquaculture (Ferguson et al, 1995; Wasko et al., 2003; Liu and Cordes, 2004). According to Chauhan and Kumar (2010), molecular tools such as DNA markers have found wide acceptance in population genetics studies by exploring genetic variation in the whole genome of a particular species through the application of mitochondrial DNA and genomic DNA.

Some of the commonly applied DNA markers include allozymes markers, restricted fragment length polymorphism (RLFP) markers, DNA barcoding as a species identification method, mitochondrial DNA markers, random amplified polymorphic

DNA (RAPD) markers, amplified fragment length polymorphism (AFLP) markers, microsatellite markers, single nucleotide polymorphism (SNP) that have being used more due to them being more common, expressed sequence tag (EST) markers and restricted site-associated DNA sequencing (RAD-seq) markers (Botstein et al., 1980; Welsh and McClelland, 1990; Hebert et al., 2003; Daw et al, 2005; Lui, 2007a; Chauhan and Kumar, 2010). Microsatellites markers or single sequence repeats (SSRs) or short tandem repeats (STRs) have proved to be the ideal markers of choice in population genetic studies although their developing and genotyping remains a technical challenge (Selkoe and Toonen, 2006; Tibihika et al, 2018). The usage of SSRs and more recently the use of SNPs, have been used to delimit genetic stocks, the phylogeography of species, identification of hybridization, determining of translocation routes and identifying genetic traits of interest among other things (Wang et al, 2016; Stobie et al, 2017; Sundaray et al, 2016). According to O'Brien (1991), these molecular markers have been classified into types I and II markers where type I is related with genes of known function and type II markers being related to unknown regions in the genome of a species. These markers have been observed to find far-flung use in population genetic fields to characterize genetic deviation or divergence existing between and amongst different populations and/or species (Brown and Epifanio, 2003).

In Zambia, molecular tools such as mitochondrial DNA markers (mtDNA) have been used to phylogenetically investigate the position of the species *O. macrochir* amongst the other cichlids of Southern Africa based on the complete mitochondrial genome sequences (Bbole, *et al*., 2018). This is an attempt made to establish areas from which invasive species such as the Nile tilapia can be excluded and foster the culture of native stocks in areas where the species has started to or almost disappeared (Marshall and Tweddle, 2007). Other studies in this regard have also focused on the phylogeographic structuring of the *Pseudocrenilabrus philander* in river systems in Zambia (Katongo *et al.,* 2005). Recent molecular studies on *O. mweruensis* have focused on the phylogeny of the genus using mito-nuclear multiple markers and adaptation to soda conditions, tolerance to high temperatures and high salinity levels, and identifying traits of interest to evolutionary biological research and aquaculture research (Ford *et al.*, 2019).

1.6. Phylogeographical distribution of the *Oreochromis* genera in Zambia

Geographical barriers have often been a deciding factor in species identification and over the past 40 to 60 years, as species identification has become more bewildered by the introduction of none-indigenous species extensively that have been taken outside their native environments with examples of Lake Victoria (Agnese et al., 1999) and the man-made Lake Kariba (Bbole et al., 2014). These hybridizations between the native species and their introduced counterparts form hybrids that make it exceedingly difficult to identify morphologically (Gregg et al., 1998; Nagl et al., 2001). The applicability of biochemical tools of allozyme typing (B-Rao and Majumdar, 1998) and molecular tools that are founded on different types of satellite DNA markers have greatly facilitated in understanding some of the hybridization problems that may be caused by translocation of non-native species into a similar population (Franck et al., 1992; McAndrew, 2000). Moritz et al., (1987) postulated that because mutations occur more rapidly than other sections, the applicability of mitochondrial DNA (mtDNA), would assist in the discrimination of comparative phylogeny in tilapiines species.

Chapter 2

2. Methodology

2.1. Sample locations and collection scheme

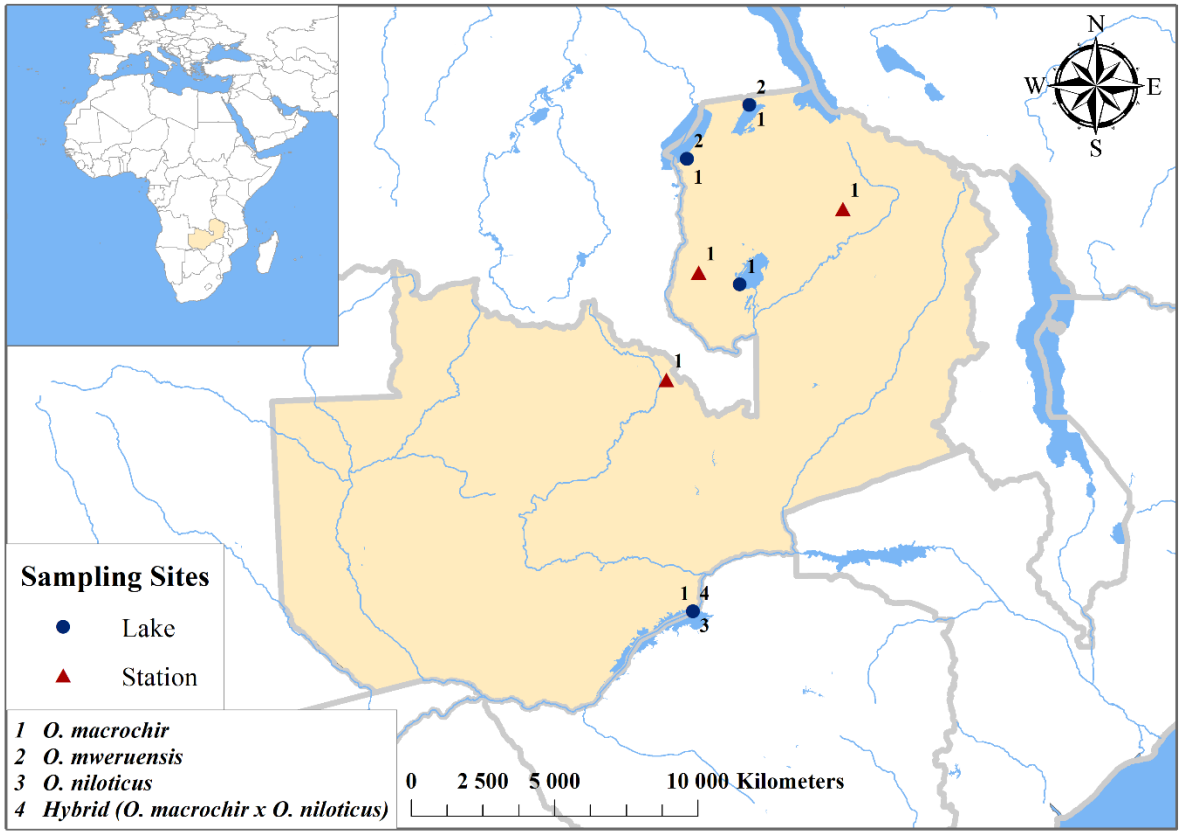

Figure 7 Map of Zambia showing sampling locations for the three species: O. macrochir (Mwela landing site in Samfya District of Lake Bangweulu; Nchelenge Landing site on Lake Mweru in Nchelenge District; and Siavonga market from Lake Kariba in Siavonga District), O. mweruensis (Nchelenge Landing site on Lake Mweru in Nchelenge District and Mwenda Na Nsapato Landing site on Lake Mweru-Wantipa in Kaputa District) and O. niloticus (Siavonga market on Lake Kariba in Siavonga District)

A total of 557 fish samples were collected from the four important wild fish populations from various Lakes in Zambia (see Figure 1 above, and Table 1 below), namely; Lake Bangweulu at Mwela landing site in Samfya District of Luapula Province, Nchelenge landing site and Kashikishi market on Lake Mweru of Nchelenge District of Luapula Province, Mwenda Na Nsapato landing site (translated into English meaning "walking with shoes") on Lake Mweru-Wantipa in Kaputa District of Northern Province and Siavonga market on Lake Kariba in Southern Province where the fish species *O. macrochir*, *O. mweruensis* are native/endemically located. Samples of *O. niloticus* that was introduced into the Zambezi River basin accidentally from a fish farm in the 1980s (Schwanck 1995) was also collected from Lake

Kariba for comparative purposes with the different populations of *O. niloticus* previously collected from Eastern and Western Africa by Papius Dias Tibihika during his doctoral degree studies (PhD) at the University of natural resources and life sciences (BOKU), Vienna – Austria. Additionally, because of the steady growth in the aquaculture sector by both public and private sectors in the country, fish samples of *O. macrochir* commonly being cultured from Government research stations namely, Misamfu Aquaculture Research Station, Fiyongoli Aquaculture Research Station and the National Aquaculture Research Development Centre (NARDC) were collected for comparative analyses amongst the different populations studied.

Table 1 Fish sampling sites, location, date and number (N) of fish samples collected for DNA analysis

Species	**Site name**	**Farm/Lake**	**GPS Location**		**Date sampled**	**N individuals**
			Longitude (E)	**Latitude (S)**		
O. macrochir	Mwela Landing site (market), Samfya district	Lake Bangweulu	29°33'43.873"E	11°21' 20.964"S	13/10/2019 & 14/10/2019	108
O. macrochir	Nchelenge Landing site - Kashikishi market, Nchelenge District	Lake Mweru	28°44'24.746"E	09°21'12.337"S	15/10/2019 & 16/10/2019	184
O. mweruensis	Nchelenge Landing site - Kashikishi market, Nchelenge District	Lake Mweru	28°44'24.746"E	09°21'12.337"S	16/10/2019	20
O. macrochir	Mwenda Nsapato, Kaputa District	Lake Mweru-Wantipa	29°44'44.016"E	08°30'20.208"S	17/10/2019 & 18/10/2019	58
O. mweruensis	Mwenda Nsapato, Kaputa District	Lake Mweru-Wantipa	29°44'44.016"E	08°30'20.208"S	18/10/2019	47
O. niloticus	Siavonga market, Siavonga District	Lake Kariba	28°42'48.312"E	16°32'05.352"S	21/10/2019 & 22/10/2019	14
O. macrochir	Siavonga market, Siavonga District	Lake Kariba	28°42'48.312"E	16°32'05.352"S	21/10/2019 & 22/10/2019	16
O. macrochir	MARS [1], Kasama District	Government Station/farm	31°14'02.478"E	10°10'24.774"S	19/10/2019	39
O. macrochir	FARS [2], Mansa District	Government Station/farm	28°54'17.940"E	11°09'47.022"S	20/10/2019	33
O. macrochir	NARDC [3], Mwekera - Kitwe District	Government Station/farm	28°21'23.118"E	12°51'18.458"S	20/10/2019	36
Hybrid *(O. macrochir x O. niloticus)* ***	Siavonga market, Siavonga District	Lake Kariba	28°42'48.312"E	16°32'05.352"S	22/10/2019	2

In consultation with the responsible fisheries officers in the targeted sampling sites and commercial trading points in the different localities, fish samples were collected from the local fresh catches and identified using the "Field Guide to Zambian Fishes" developed by Utsugi

and Mazingaliwa (2002), and "A complete guide to the freshwater fishes of Southern Africa" by Paul Skelton (2001) based on their morphological characteristics from the local fish catches carefully. The species *O. macrochir* was identified by having a round humped or bulging forehead, a greenish head colour with dark spots and a long pectoral fin and the genital tassel bearing a legion of orange or yellow blots (see Figure 2). *O. mweruensis* was identified by having a steep head profile like in other tilapia species with a white and less branched genital tassel shooting a few extended strands or filaments (see Figure 3).

Figure 8 Oreochromis macrochir (Green-headed bream or Longfin tilapia) identified from Lakes Bangweulu, Lake Mweru, Lake Mweru-Wantipa, Lake Kariba and the government fish stations

Figure 9 Oreochromis mweruensis (Mweru tilapia) identified from Lake Mweru and Lake Mweru-Wantipa

Additionally, *O. niloticus* was identified by having a large deep body with a small head, a genital papilla usually not tessellated like in *O. macrochir* and *O. mweruensis* and mostly differentiated by bearing regular vertical bands located at the caudal fin (see Figure 4).

Figure 10 Oreochromis niloticus (Nile tilapia) identified from Lake Kariba

Fin clips (caudal fin) approximately 2-3 g were collected immediately on landing and preserved in 2 ml microtubules with 96 % ethanol and kept frozen for DNA extraction.

Permission was obtained from the Department of Fisheries in collecting the fish samples from the Lakes and the government stations. After collection, samples were transported to the Institute of Integrative Nature Conservation Research at the University of Natural Resources and Applied Life Sciences (BOKU) in Vienna for DNA extraction. All animal rights were adhered to during the collection processes and a permit for exporting of fish samples for study purposes granted.

2.2. Methodological procedure

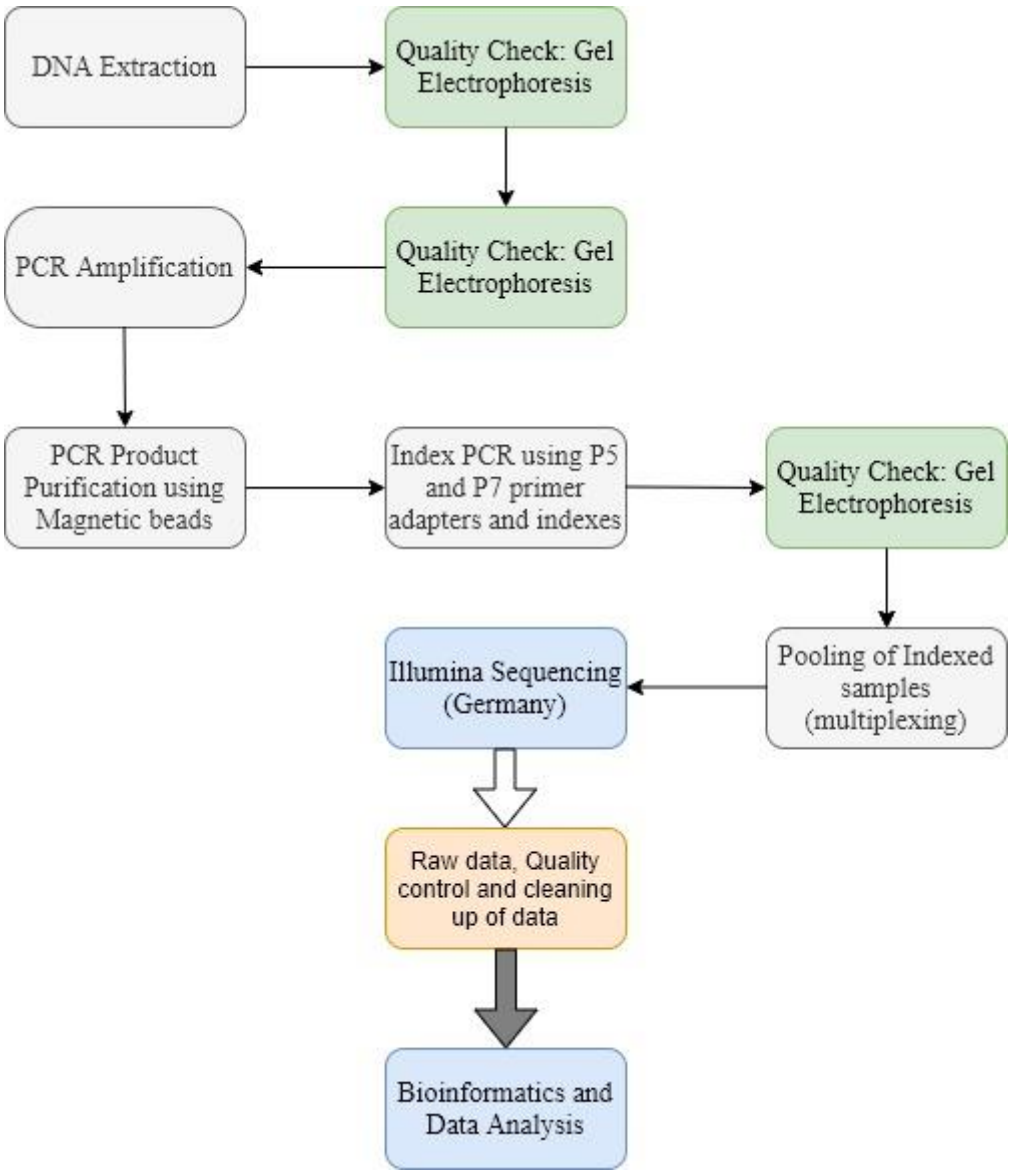

Figure 11 Shows a Flow Chart summarising the steps undertaken from DNA isolation/extraction to bioinformatics/data analysis

2.2.1. DNA Extraction

DNA from the preserved samples in 96% ethanol was extracted using 96 well Econospin plates. Firstly, a fish sample of the collected species was prepared by drying the ethanol from the fin clips using the Econospin Plate protocol to remove the ethanol and then transferred to a 2 ml vial tube or 1.5 ml tube in a case where the former was lacking. After drying the samples, 300 ul of lysis buffer (2% SDS, 2% PVP, 250m MNaCl, 200 M Tris-HCl, 5mM EDTA, pH 8) and proteinase K enzyme [10mg/ml] was added to the tube and then vortexed shortly. Samples

were incubated at a temperature of 56ºC at 400 rpm for 3h-overnight using a PEQLAB mixing block (see Figure 6 below).

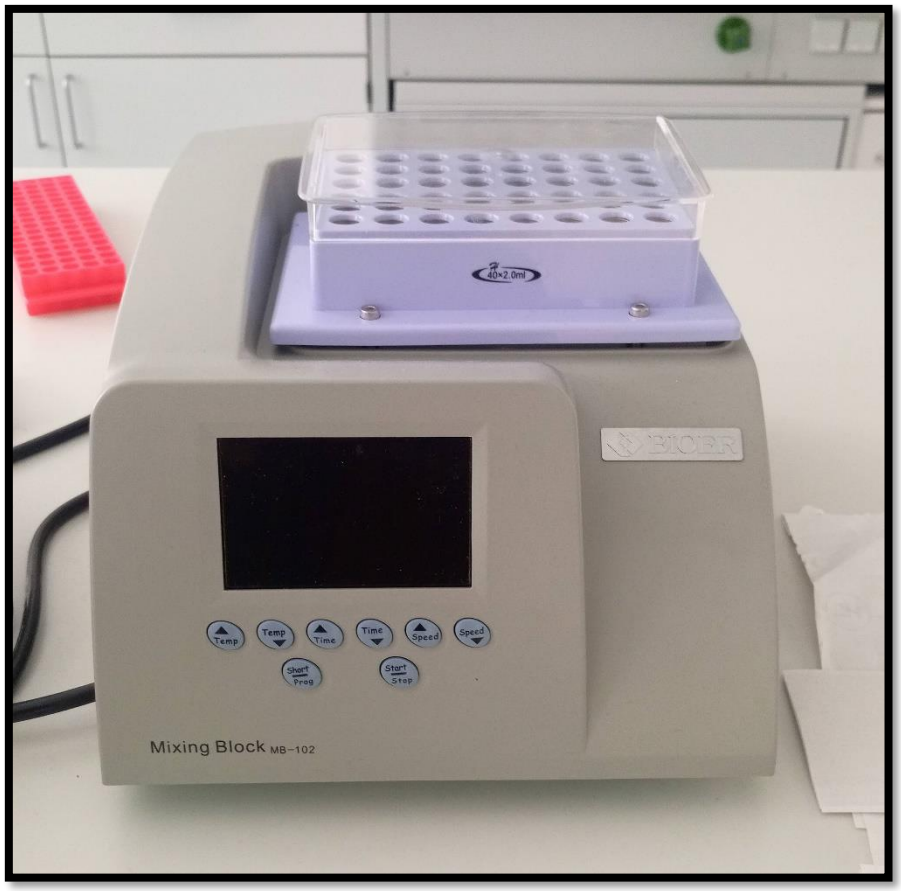

Figure 12 PEQLAB Mixing block used to incubate the samples overnight for genomic DNA extraction of O. macrochir, O. mweruensis and O. niloticus

After digesting the sample for 3hrs-overnight, RNase (10 mg/ml) with a volume of 10 ul and 75 ul of potassium acetate (3M) was added and then incubated while mixing again with a PEQLAB mixing block for 15 minutes. After the samples were put on ice for 20 minutes or stored overnight at -20ºC. When ready to be worked on, the samples were removed from the freezer at allowed to thaw at room temperature. The samples were then centrifuged using two centrifuge machines: an Eppendorf Centrifuge 5430R and Multifuge X3R centrifuge (see Figure 7 below) for 1 minute at 1,000 rpm, 1 minute at 2,000 rpm, 1 minute for 4,000 rpm, 1 minute at 8,000 rpm and 7 minutes at 11,000 rpm. After centrifugation, the 1.5 ml or 2 ml tubes with the samples were removed and the supernatant or lysat of 370 ul transferred into a deep well plate using a single pipetting procedure. A volume of 545 ul binding buffer (2 M GuHCl in 95% ethanol) was added to each sample and mixed by pipetting 10 times (10X). After thoroughly mixing, 545 ul of the sample solution was added to a new Econospin plate and

centrifuged for 15 seconds at 2,000 rpms and then adjusted to 1 minute at 4,000 rpm. The remaining 370 ul supernatant of the sample was transferred to the same Econospin plate and centrifuged at the same respective times as above.

Figure 13 Centrifuges used in the DNA extraction process: Eppendorf Centrifuge 5430R (left) and Thermal Heraeus Multifuge X3R Centrifuge (right)

After, the samples were washed 3 times with 80% ethanol (EtOH) by adding 600 ul of 80% EtOH to each sample and then centrifuged for 1 minute at 4,000 rpm as step 1. Step 2 required the washing with 600 ul of 80% EtOH and centrifuged for 1 minute at 4,000 rpm. In Step 3, the same procedure was repeated for 1 minute at 6,000 rpm. The plates containing the sample DNA was allowed to dry in a Hood Chamber for 15 minutes at room temperature.

After 15 minutes, the sample plate containing the DNA samples was eluted three (3) times using the Elution Buffer 10 mM Tris, pH 8. The elutions were prepared in the following volumes:

1st Elution of Sample Plate → 50 ul of Elution Buffer

2nd Elution of Sample Plate → 50 ul of Elution Buffer

3rd Elution of Sample Plate → 80 ul of Elution Buffer

The elution buffer was prepared and mixed using a PEQLAB mixing block at 65ºC for 5-10 minutes. To prepare the 1st Elution, 50 µl of the Elution Buffer was added to the Econospin

plate having the DNA samples and incubated at room temperature for 3 minutes and then after centrifuged for 5 minutes at 12,000 rpm. For the 2nd Elution, the same volume of 50 µl of Elution Buffer was added and allowed to incubate at room temperature for 3 minutes and centrifuged for 1 minute at 12,000 rpm. To prepare the 3rd Elution, 80 µl of Elution Buffer was added and incubated at the same time for 3 minutes as the previous elutions prepared and centrifuged at 12,000 rpm for 1 minute.

After these steps were completed, the DNA extraction process was completed and the quality check of the DNA using the Gel Electrophoresis.

2.2.2. DNA Quality Check using the Gel-Electrophoresis chamber

The DNA was checked using the Biozym LE Agarose for gel electrophoresis under a 0.8% agarose gel using a MIDI medium gel 5 mm chamber. To prepare the gel, 75 ml of 1xTAE was added to 0.60 g of agarose gel and mixed while heating under a laboratory microwave machine and regularly stirring until the agarose completely dissolves into the solution. After mixing the solution thoroughly and allowed to cool while still warm, 1 µl of dye (HDgreen+) was added to 1xTAE of 75 ml of gel while warm and allowed to cool and solidify. After the Gel was ready, 6 µl of DNA sample and Loading Buffer (LB) was loaded to each well in the following order:

"5 µl DNA + 1 µl 6x LB"

The samples were loaded in the gel wells and an empty well used as a reference in which 6 µl of the ladder (=250 ng) per lane by using λ ladder mix for DNA. After finishing loading the DNA samples and λ ladder in the gel electrophoresis chamber, allow the electric current to run to have the DNA move from the negative (-) side to the positive (+) side at 80 V. The gel was examined for quality check under white light and UV light for the 1st, 2nd and 3rd Elutions.

2.2.3. PCR Amplification and Genotyping of SSR and mtDNA markers

PCR amplification was conducted using microsatellite (SSR) markers and mitochondrial (mtDNA) markers using a 96 Cel Z PCR plate and a 384 PCR plate for all the samples.

A total of 47 microsatellite loci (see Table 2) were employed to genotype the DNA of the fish samples. This was done using a 96 PCR plate in which an individual well contained 5.0 µl of Master mix mixed with 1.0 µl of the Primer mix done respectively for the five mixes PM_Ti2a, PM_Ti2b, PM_Ti3, PM_Ti4 and PM_Ti5), 1 µl of sample DNA and 3.0 µl of distilled H_2O and then centrifuged the sample plate for 30 seconds at 2,000 rpm. For a 384 PCR plate per

well, 2.5 µl of Master Mix was mixed with 0.5 µl of Primer Mix, 1.5 µl H_2O and 0.5 µl of DNA added using a single pipetting procedure with a multi-channel pipette and replenishing the pipette tips to avoid cross-contamination (See Figure 8 below).

Figure 14 RAININ Multi-Channel Pipette

The samples were then inserted into a BIO-RAD C1000 Touch Thermal Cycler PCR machine (see Figure 9 below) using the program LT PCR 30 (see Figure 10 below) and initialized at 95^0C for 15 minutes, followed by denaturation for 30 cycles at 95^0C for 30 seconds, annealing at 55^0C for 1 minute and elongation for 1 minute at 72^0C and the last extension steps at 72^0C and incubation at 10^0C for 10 minutes. For mitochondrial genotyping, a total of 33 mitochondrial markers (see Table 3 below) were used to genotype the DNA samples with two respective mixes i.e. PM_mt_Ti2 and PM_mt_Ti3 and the same steps as the microsatellite procedure repeated at the same times and temperatures for the same number of cycles. The quality of the PCR product was checked using the Biozym LE Agarose used for gel electrophoresis.

Table 2 Microsatellite loci used to screen the samples of *O. macrochir*, *O. mweruensis* and *O. niloticus*

Marker Name	Forward Sequence	Reverse Sequence	Primer Mix
TI1_TG	TTATCACTGCTGAACGTCTT	GTTTTGGCTGCTACACATTC	PM TI 2a
TI2_CA	TTCTGGGCTAACACACAAG	AAGGTGTCACACAGTTTAGG	PM TI 2a
TI4_GT	TGTGCAGAATAGAATAGCCC	GAAAGGAAAAATGTTGGTGGT	PM TI 2a
TI5_CA	AAGGAGGATGATCAGGACAC	AGACCTCCACTGTGATCTTA	PM TI 2b
TI6_GA	CAGCTCTCATGAACACTTGA	ACCCATAAATCACACCAGTC	PM TI 2b
TI7_AC	TCTTTGTGTCAGAACTGTGT	ACTCTGCTTTTAGCCAATCA	PM TI 2b
TI8_AC	CTGAAGTCCTGCTGAGATTT	CATTGTTCTTGGCACCTCTA	PM TI 2b
TI9_AC	CTCAGTGACGAAGCCAAA	CCTGGCAATCAAAAGAACAA	PM TI 2a
TI12_TAC	GCCACCAAAATATTCGTGTT	CCATGTTCTGTCTCCTTGAA	PM TI 2a / PM TI 3
TI13_ATG	AATCCGTTAGCTGCAGATAG	GCTGATTAAACACAAAGTTGG	PM TI 2a
TI14_TAA	TCCCTAAAATATGCCACCAA	TAGTGCTTTAATGGCTCTGG	PM TI 2a
TI15_TGC	GCTGTGATCATCTGGAGAAA	AGGATCTAGAACCTCCAACC	PM TI 2b
TI16_AAC	CAGACGTAGGCGATAAATCT	GAACACATCCATTTCCACAC	PM TI 2b
TI17_GAA	AACTGAAGAAGAAGCCTTGG	ATCATCTTCCTCTACTGCCT	PM TI 2a / PM TI 3
TI18_ATCT	AGCAAGTGAGATAAGCACTG	TACATAGCAGTGCAGTTTGC	PM TI 2a
TI22_CTAT	ACTGACCAAGTGCTTTGTAT	AACTGCTGTGTTGAACTTTG	PM TI 2a / PM TI 3
TI24_TTAC	ACTGACAACATAAAGACATATGC	CACAGTTTGAATCCACCATC	PM TI 2b
TI26_ACAA	ATTGCTTCATCCCTTGAGTT	ACACGGAAAACCTAATGACA	PM TI 2a
TI27_TTTG	CTGTCTTTCTTGATGTGGGA	ATGCACAAATTTTAAGGGCC	PM TI 2a
TI28_ATTCA	TGTCTTGGGATTTGAGATCA	CGGAGGTTTCTTCCTGTTAA	PM TI 2a / PM TI 3
TI29_TAAAA	AGGTCAAGATCAAGCAGTTT	CATCAACATAATTCAGTGTGGA	PM TI 2a / PM TI 3
TI31_CTAAT	GAAACTATCCACAGAAGCCA	AGGCTTCTTACAGTTGGATG	PM TI 2a / PM TI 3
TI32_AAAAT	CAGGAAATGGCTCCAAAATG	TTGTAGCTAGGAATCAGTGC	PM TI 2b
TI33_TTCAA	GCTTATGGCTGTATGGAGTT	CGACTTCTGTTGTGATTTGG	PM TI 2b
TI34_TCTCT	GCTTACAGTACATTGTGTGC	CTGATGAGAAAAACAGACGC	PM TI 2a
TI35_AAAAG	TCAACCACAAACTCCTCTTT	AAACTAAGTGCAGCTCATGA	PM TI 2a
TI36_ATT	GCCGTAATGGAGCTGACAGA	CCAAGATGTCGGCAAACTGC	PM TI 4
TI37_TTA	GCATGCACTAAACCACGCAT	CGAGACTGTGGCGGATTAGG	PM TI 4
TI38_TAT	ACTCCACACAGTGAACTACTCT	TGAGACTCTCACGTAGGCCA	PM TI 4
TI39_ATGG	TACCTGCCAGTCATGTGCTG	TGCTCAGACTGGTCCCTTCT	PM TI 4
TI41_AAAC	TCGCAGCTGCTCCTGTTTAA	TTGTGCACGTGGACATGTTG	PM TI 4
TI43_GAATA	ATTGCCATCACCAGGAACCA	TGCTAGCCCAGAGCATTTGA	PM TI 4
TI44_GAAAA	TGCTCCTGACTCAGCATCAC	GCAGCACTCTGACATGAAGC	PM TI 5
TI45_ATATA	CCTGCTGAAGCTAAACCTGC	TCAAAGGACATTATGGTCTGACT	PM TI 4
TI46_TAT	ACTCCACACAGTGAACTACTCT	TGAGACTCTCACGTAGGCCA	PM TI 5
TI49_TGT	TCGAAGTAGCGTGGAAAACCT	ACAACAACAACAGGTCGGGA	PM TI 4
TI50_ATGG	CCTGTGACAGACTGGTGACC	ACACTGATGCGGTTTACGGT	PM TI 5
TI51_TGT	TGCTAAACGCCAGCTGATGA	TTACCACACGATGTCGCAGG	PM TI 5
TI52_TAT	GAGAAACGTCCAGTGGCAGA	TTTCGATCTGCTGCCCCTTT	PM TI 5
TI53_ATAG	ATGAGCCAGCGTTGAGTCAA	TTCCGAACACCTTGGTGTCC	PM TI 5
TI54_GGAT	TTTCTTGCCAGCAAAAACAGT	CAGATTCTTCCAGTGCTTGTGC	PM TI 5
TI55_TCTA	GAGCCCAGACAGCAGACAAT	AGGACCTTCTATGGCCCTGT	PM TI 4
TI56_TGTT	TGCAGTGAATTTGGCACCTG	AGCCTGAGATACCTGTGCCT	PM TI 4
TI57_TCCA	CAGTGGGAGGAAGCTCCAAA	GCTGCATGGATCCAATAGGC	PM TI 4
TI59_AGGA	ATGGACTTAAGCTGCACCCC	TGAGCATTTGACCCCAGCAT	PM TI 5
TI60_ATCC	GAGCCGCCATAGTGTCACTT	CCTGCTCTCACTCAAAGAGGG	PM TI 5
TI61_TGGA	GCTACACAGGAAAGCAGAGC	ACTCAATGCTGGACGTGACC	PM TI 5

Table 3 Mitochondrial DNA loci that were used to screen the species *O. macrochir*, *O. mweruensis* and *O. niloticus*

Marker Name	Forward Sequence	Reverse Sequence	Primer Mix
1-110-511	TTACACATGCAAGTCTCCGC	GGGGTTATCGGTTCTAGAACA	PM mtTI 1a
2-497-999	ACAAACTGGGATTAGATACCC	ATGTTACGACTTGCCTCCC	PM mtTI 2
3-920-1459	ACACACCGCCCGTCACTC	ACTCTTTTGCCACAGAGAC	PM mtTI 3
4-1441-2071	GTCTCTGTGGCAAAAGAGT	CTTGTGTTTGCCGAGTTCCT	PM mtTI 1a
5-2082-2664	AAGGAACTCGGCAAACACAA	TAATAGCGGCTGCACCATTA	PM mtTI 2
6-2631-3124	GGATCAGGACATCCTAATGG	AGTCCGTCTGCAATTGGTTG	PM mtTI 3
7-2876-3398	CTTGTTAAAGTGGCAGAGC	ACTTCGTAGGAAATAGTCTGTG	PM mtTI 1b
8-3384-4005	GCACAGACTATTTCCTACGAAG	CACTCTATCAAAGTGGCCCTT	PM mtTI 2
9-3987-4538	AAGGGCCACTTTGATAGAGT	TCCTTGYAGAACTTCGGGG	PM mtTI 3
10-4521-5099	TTGRCTCCCCGAAGTTCT	TTATTGCGTAGGAGAGGCG	PM mtTI 1b
11-5077-5649	CGCCTCTCCTACGCAATA	CGTGTGATTGCCACAGGTA	PM mtTI 2
12-5403-5840	AAGGCCTCGATCCTACAAAC	GYATTACTATAAAGAAAATTATTAC	PM mtTI 3
13-5803-6342	TGTAATTGTTACAGCACATGC	AAAGAATCAGAATAAGTGTTGG	PM mtTI 1a
14-6330-6831	ACCAACACTTATTCTGATTCT	GAATCAGTGGACGAAGCC	PM mtTI 2
15-6818-7260	GCTTCGTCCACTGATTCC	GGGTTCAATTCCTCCCTTTC	PM mtTI 3
16-7241-7800	CGAGAAAGGGAGGAATTGAA	AACYACTATTCGATGGTCTG	PM mtTI 1b
17-7832-8342	GARACAGACCATCGAATAGT	GTATGTGGGGCTCATAAATTG	PM mtTI 2
18-8352-8811	CAATTTATGAGCCCCACAT	TGCGGTTAGTCGAACTCC	PM mtTI 3
19-8861-9320	GAGTTCGACTAACCGCAAA	GAGTGGTARAAGGCTCAGAA	PM mtTI 1b
20-9307-9795	TTCTGAGCCTTYTACCACT	TATGAGCCTCATCAGTAAAT	PM mtTI 2
21-9778-10255	ATTTACTGATGAGGCTCAT	TGAGCCGAAATCAAGGGT	PM mtTI 3
22-10247-10872	ACCCTTGATTTCGGCTCA	GATGTAGGYTCGTTGGCG	PM mtTI 1a
23-10857-11356	GCCAACGARCCTACATCAC	GGRTAACTGAGTTCTTTGGT	PM mtTI 2
24-11411-11891	CCTYACCAAAGAACTCAGTTA	GTGTTCTCGGGAGTGTGT	PM mtTI 3
25-11885-12421	AACACACTCCCGAGAACA	AGGTGTGGGTRTTTATTCAAGT	PM mtTI 1a
26-12401-12925	CTTGAATAAAYACCCACACCT	AGTAGGGCAGAGACCGGT	PM mtTI 2
27-12932-13426	ACCGGTCTCTGCCCTACT	CTCAGGCGTTTAGGTAAGAT	PM mtTI 3
28-13406-13958	CATCTTACCTAAACGCCTGA	GATTATACCTTGTTGAATATTGTT	PM mtTI 1b
29-13952-14495	ACAAGGTATAATCAAAACCTACC	TCCTGGCAGAAACTATGGT	PM mtTI 2
30-14548-15013	GCCAGGACTTTAACCAGGA	ATTTGTCCTCATGGGAGGAC	PM mtTI 3
31-15021-15460	CTCCCATGAGGACAAATRTCA	GCGTAGGCAAATARGAAGTA	PM mtTI 1a
32-15441-15889	TACTTCYTATTTGCCTACGC	GAATCCTAGCTTTGGGAGTT	PM mtTI 2
33-15870-16382	AACTCCCAAAGCTAGGATTC	GAACCAGATGCCAGGAATA	PM mtTI 3

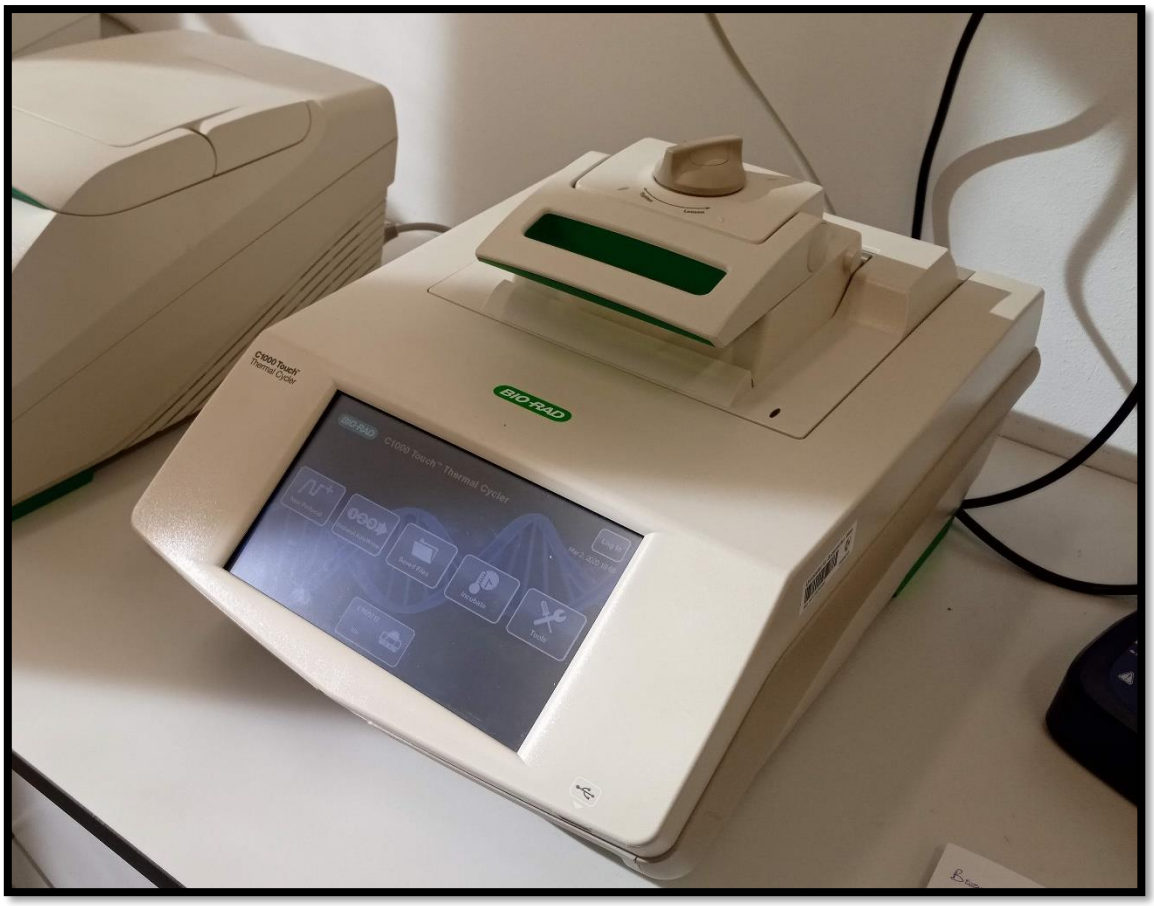

Figure 15 Bio-Rad PCR Thermal Cycler used to amplify samples of O. macrochir, O. mweruensis and O. niloticus

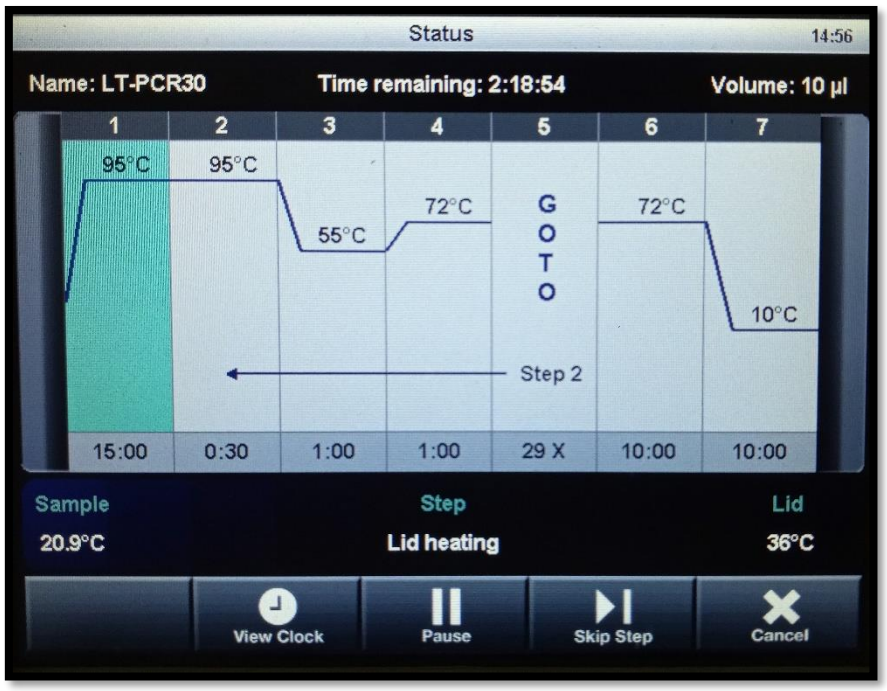

Figure 16 PCR machine running using the program LT-PCR30 for microsatellites and mitochondrial markers

The samples upon completion of the PCR amplification run were checked for DNA quality using 1.5% Agarose Gel following the volumes as previously indicated for the MIDI Medium size chamber on the Agarose gel prepared as previously indicated. After this, primer pairs were used for multiplexing the PCR products.

2.2.4. Amplicon library preparation, PCR clean-up and multiplexing

In this process, two PCR steps were conducted as part of the library preparation process in readiness for Illumina sequencing. Two primer adapters P5 (TCT TTC CCT ACA CGA CGC TCT TCC GAT CT) and P7 (CTG GAG TTC AGA CGT GTG CTC TTC CGA TCT) were attached at the 5' end of the forward and reverse primer in the first PCR (see Figure 11 below).

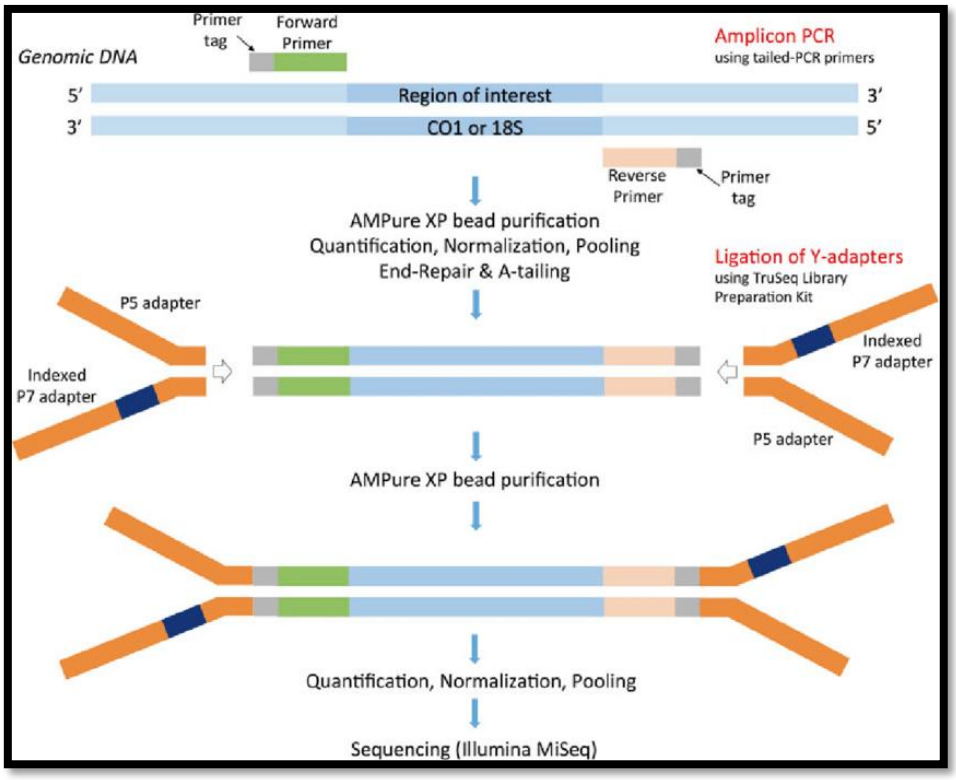

Figure 17 Library preparation scheme for Illumina MiSeq sequencing using the P5 and P7 tailed PCR primers (1st PCR) and single indexing using the P5 and P7 or Y-adapters (2nd PCR)

(Picture by Matthieu Leray from https://www.researchgate.net/figure/Scheme-for-Illumina-MiSeq-multiplex-library-preparation-using-the-tailed-PCR-primers-and_fig1_305673701)

In the second PCR, two indexes with 8 base pairs each (bp) were added to each sample using a single approach with the P5 (AAT GAT ACG GCG ACC ACC GAG ATC TAC AC[index]ACA CTC TTT CCC TAC ACG ACG) and P7 (CAA GCA GAA GAC GGC ATA CGA GAT[index]GTG ACT GGA GTT CAG ACG TGT) as the ligation step to allow for the

pooling of large samples in preparation for other steps to follow. A combination of the primers analysed under the gel electrophoresis were multiplexed and run in the first PCR reaction in a total volume of 10 µl consisting of 5 µl Master Mix, 1.0 µl Primer mix, 3.0 µl H_2O for a 96 PCR plate and for a 384 PCR plate a total volume of 5.0 µl consisting of 2.5 µl Master mix, 0.5 µl Primer mix, 1.0 µl of H_2O and 1.0 µl of sample DNA. All this was conducted using a Liquid Handling Station robot with a data pool version 2.1.14 (see Figure 12).

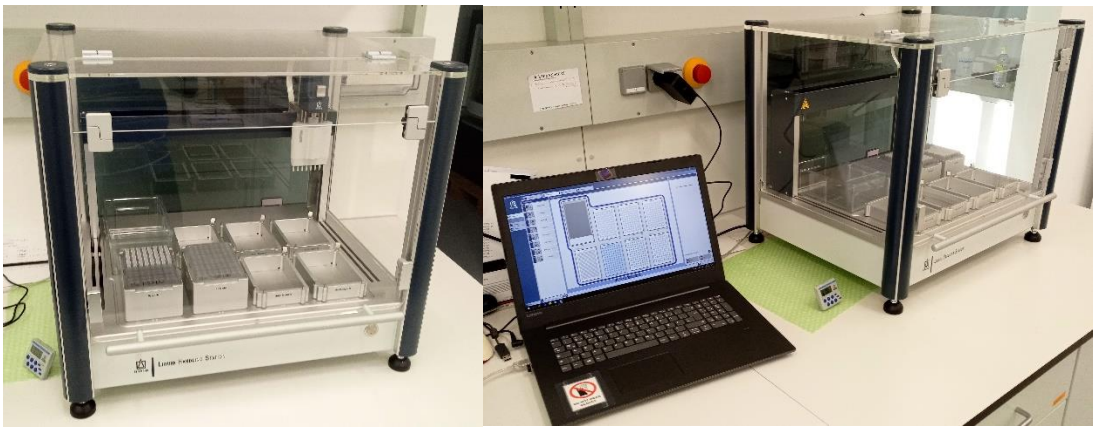

Figure 18 BRAND Liquid Handling Station used in the running of PCR products and indexing of Tilapia fish samples (left – Liquid Handling Station; Right – laboratory laptop used to run the Liquid Handling Station robot)

The samples were then put in a Thermal cycler PCR machine and run for 30 cycles as previously described above (see Figure 9 and 10 above). To clean up the PCR products, AMPure magnetic beads were used according to the Agencourt AMPure XP PCR purification protocol. An aliquot of 7.5 µl of pooled PCR product from each of the five sample trays was mixed with 5.4 µl magnetic AMPure XP beads and incubated at room temperature for five minutes. A magnetic inverted device (see Figure 13) was used to collect the beads, and these were washed in 200 µl of ethanol (80% EtOH) for 45 seconds and then washed again in fresh ethanol for the same seconds as before.

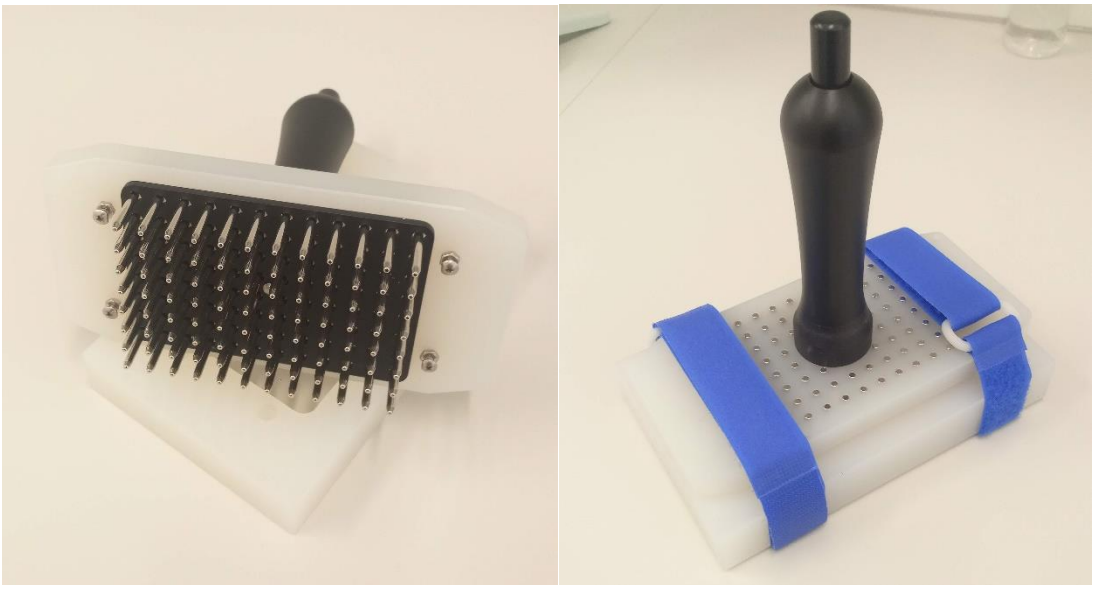

Figure 19 Inverted magnetic bead extraction device VP 407-AM-N (V & P Scientific, Inc.)

These were later allowed to dry for a period of five minutes at room temperature before eluting the samples with a warm Elution Buffer (10mM Tris-HCL, pH 8) of 17 µl.

The second index PCR step was undertaken using a total volume of 5 µl containing 2.5 µl of Master mix, 0.25 µl of P5 (with a concentration ratio of 1:25), 1.0 µl of P7 (with a concentration ratio of 1:100), 0.25 µl of H_2O and 1.0 µl of purified PCR product DNA sample per well in a 384 PCR plate. The indexed PCR was initialized at 95^0C for 15 minutes that was followed by denaturation at 95^0C for 30 seconds, annealing at 58^0C for 1 minute, and elongation at 72^0C for 10 cycles. The final extension was run at 72^0C for five minutes (see Figure 14 below).

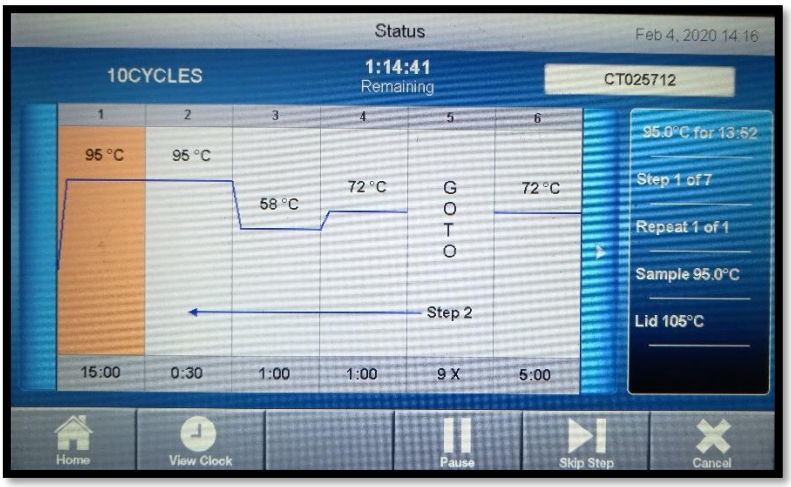

Figure 20 Index PCR run for microsatellite (SSRs) and mitochondrial (mtDNA) markers respectively run for O. macrochir, O. mweruensis and O. niloticus

After indexing the PCR products, all the samples that were marked using 47 microsatellite markers were pooled into a single 1.5 ml tube. Additionally, all the samples that were marked with the 33 mitochondrial markers were also pooled into a separate 1.5 ml tube using the multiplex PCR approach over the traditional PCR method (see Figure 15 below). The indexed PCR product samples in the two tubes were then sent for Illumina MiSeq (PE300) sequencing at the Genomics Service Unit at the Ludwig-Maximilians-Universität München (LMU) Biocenter in Germany.

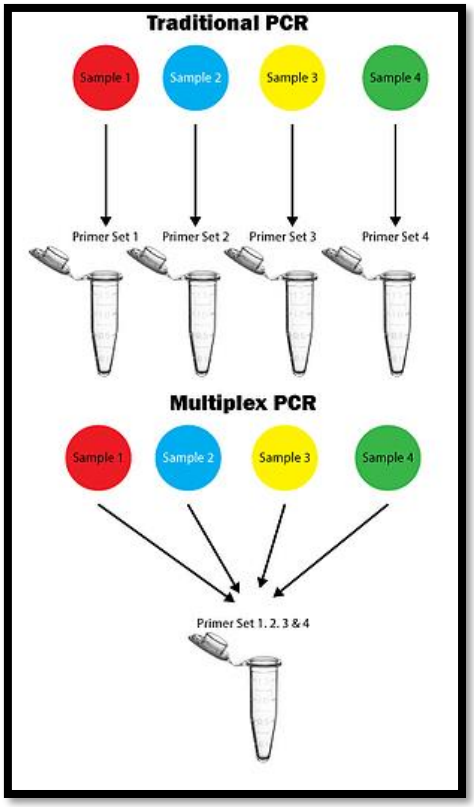

Figure 21 Advantages of multiplex PCR over traditional PCR of Indexed PCR product samples (Source: https://info.gbiosciences.com/hs-fs/hubfs/Multiplex_PCR.png?width=300&name=Multiplex_PCR.png)

2.2.5. Bioinformatics: Sequence analysis, genotyping and allele calling of microsatellites (SSRs) and mitochondria (mtDNA)

After receiving the reads from the Genomics Service Unit, the raw data of the reads from Illumina MiSeq platform when received were subjected to quality control using FastQC version 0.11.9 (Andrews, 2010) and trimmed to remove artefact adapters and poor quality regions (having a Phred less than 20) using Trimmomatic version 0.39 (Bolger et al, 2014) available from http://www.usadellab.org/cms/index.php?page=trimmomatic. From this quality

control, two sets of sequences were obtained having the forward and reverse sequences after de-multiplexing the multiplexed sequences into an individual sample per loci using an in-house python script. The python script searched for mismatches between the motif adapters in the forward and reverse primer sequences at the beginning and end of the sequences, respectively and considered only reads having primer pairs below two.

After the identification of the mismatches, allele calling was employed using the SSR-GBS-Pipeline python script program available on GitHub (https://github.com/mcurto/SSR-GBS-pipeline) with a few modifications written by Manuel Curto used in the publications by Curto et al, (2019) in investigating European Hedgehogs and Tibihika et al, (2018) in investigating anthropogenic factors influencing the distribution of the Nile tilapia showing advantages over the traditional SSR method.

In allele calling, two steps were undertaken specifically 'genotype calling' to determine the genotype of an individual at a given amplicon length and 'SNP calling' to identify sites that varied within the alleles having the same length. After running the python script for the first allele call using the program "SSR-GBS-pipeline_Allele-Length-Call_win_0.12.py", a codominant matrix was produced as one of the output files showing that incorporated length variation at each repeated motif and flanking regions, and histograms that were based the length of the sequence. Genotypes with 10 reads were considered as the minimum depth for homozygous genotypes having a frequency length equal to or above 90 per cent of the aggregate total number of reads. Heterozygous genotypes were accepted if two frequency lengths were above 90 per cent and were not different by a margin of 20 per cent. Using the 'marker plots' showing the histograms produced as one of the output files, if the genotypes did not meet the above-mentioned criterion automatically these were subjected to manual quality control.

A consensus sequence with about 70 per cent was produced from the allele calling of the genotype and later used for SNP calling using the python script "SSR-GBS-pipeline_SNP-call_win_0.122.py". For SNP calling, an ambiguous base 'N' was assigned to positions having a common nucleotide frequency falling below the 70 per cent threshold value and coded thus as possible heterozygous SNPs having different alleles at matching loci in the chromosome. In cases where more than one SNP position was discovered, these positions were counted to be connected and the commonest nucleotide combinations retrieved. The second run outputted the files in a similar manner as the genotype calling into a codominant matrix resulting in

sequences for the samples that would be subjected to microsatellite and mitochondrial statistical analyses.

2.3. Statistical Analyses

To address the main objective of genetically characterize the populations of the three species under investigation, descriptive statistics were conducted using various software programs for the analysis of microsatellite DNA markers and mitochondrial DNA markers on *O. macrochir, O. mweruensis* and *O. niloticus.*

2.3.1. Microsatellite DNA analysis

To assess the informativeness of the genetic markers used in the study in investigating the species *O. macrochir, O. mweruensis* and *O. niloticus* from wild and cultured populations, principal coordinate analysis (PCoA) was conducted from pairwise genetic distances using GenAlEx version 6.51 (Peakall and Smouse 2006, 2012, 2017; Smouse *et al.*, 2017). To analyse the population structure per population and compare the four wild populations and farmed populations for the complete sample datasets, the program STRUCTURE version 2.3.4 (Pritchard et al., 2000; Hubisz et al., 2009; Porras-Hurtado et al, 2013) was used to visualize the genetic divergence between samples and their locations. The method required the use of Markov Chain Monte Carlo (MCMC) to provide an estimation of the allele frequencies by also identifying the optimal count of population (K) groupings (groups and subgroups) from the total samples. The program was set at 20,000 generations of burn-in period followed by 20,000 Markov Chain Monte Carlo (MCMC) runs at 6 replications with cluster (K) values ranging from 2 – 12. Implementation of STRUCTURE for the admixture model and correlation of allele frequencies was run using the default settings. The detection and classification of the best fitting genetic cohorts that exhibited similar genetic variation patterns and also useful in the identification of subpopulations structures were assessed using the program STRUCTURE HARVESTER version 0.6.94 (Earl and Von Holdt, 2011) to compare the results obtained from the STRUCTURE analysis following Evanno et al, (2005). The maximum average K value related to the Delta K value was analysed to distinguish the cluster groups best describing the data. To present informative population structure inferences made across K, the method CLUMPAK (Cluster Markov Package Across K) (Kopelman et al, 2015) was run to process the model-based STRUCTURE results graphically.

Genetic diversity statistics and genetic differentiation between and within populations was inferred using the software ALEQUIN version 3.5.2.2 (Excoffier and Lisher, 2010) and

the test for the presence of recent gene flow between wild and farmed populations was estimated using the Mantel test in the Isolation by Distance (IBD) software program (Bahonak 2002). GenAlEx. To determine the genetic variation between and within populations, Wright's F-statistic (Wright, 1969, 1978) and F_{ST} values at 95% confidence level were calculated for allelomorphic richness and the number of allelomorphs per locus using the software program FSTAT version 2.9.4 (Goudet, 2002). Micro-Checker version 2.2.3 (Van Oosterhout *et al.*, 2004) was used to screen for genotyping errors and estimate the presence of null allele contribution to avoid incorrect assignment of microsatellite genotypes inferred from predetermined population genetic analyses by detecting null alleles induced by a lack of PCR amplification, grading of shutter peaks (removal of artefacts), and as well identifying of typographical errors.

To test if there were any departures from Hardy-Weinberg Equilibrium (HWE) per loci the program FSTAT version 2.9.4 (Goudet, 2002) was used and the fixation index (F_{IS}) determined by applying the Markov chains method run at different parameters for all trials run for batches and iterations using the software program Genepop'007 version 4.7.2 (Rousset, 2008). According to De Meeûs (2017); Stoceckel and Masson (2014), positive F_{IS} values indicate a high level of homozygosity and negative F_{IS} values indicate a high level of heterozygosity (outbreeding coefficient). To determine the loci polymorphic information content (PIC), expected heterozygosity (H_e) and the observed heterozygosity (H_o) using the software Cervus version 3.0.7 (Kalinowski et al, 2007). When the PIC value of the PIC > 0.5, the locus was considered to display high polymorphic diversity locus (Kalinowski et al, 2007). To determine statistical power, the program POWSIM version 5.0 (Ryman and Palm, 2006) was used to observe if there was any genetic divergence in all the samples analysed from the primer markers used. A genetic distance matrix containing pairwise FST estimates was used to run a hierarchal analysis of molecular variance (AMOVA) in GenAlEx. The mean values obtained for each of the analyses per populations were calculated using the software R version 3.6.1 (Team, 2019) for each locus.

Chapter 3

3. Results

3.1. DNA Isolation of samples

A total of 557 samples collected from four different lakes namely Lake Bangweulu, Lake Mweru, Lake Mweru-Wantipa and Lake Kariba. Additionally, populations from three aquaculture government fish farms namely, Fiyongoli Aquaculture Research Station, Misamfu Aquaculture Research Station and the National Aquaculture Research Development Centre (NARDC) were collected for comparative purposes. Samples that were previously collected by Papius Dias Tibihika (during his doctoral studies - published), Genanaw (unpublished), Esayas (published) and John (unpublished) from Western and Eastern Africa whose DNA was successfully isolated were included in the dataset to compare Southern, Western and Eastern African populations of the Nile tilapia (*O. niloticus*).

3.2. Marker quality control and compilation of dataset

A total of 47 microsatellite markers were genotyped for all the 557 samples collected from Zambia constituting the Longfin tilapia (*O. macrochir*), Mweru tilapia (*O. mweruensis*) and the Nile tilapia (*O. niloticus*). Additionally, 33 mitochondrial markers were genotyped for all the samples as well. Both SSRs and mtDNA markers used were previously developed for the Nile tilapia (O. niloticus) and used by Tibihika et al, (2018; 2020). From a total number of samples, 289 samples were used in the final dataset for all the samples collected from Zambia due to failure in PCR amplification. Eight (8) microsatellite loci showing more than 40% missing genotypes were removed from the dataset leaving a total of 33 single sequence repeats (SSR) markers used in the final dataset showing successful genotyping. Furthermore, fourteen (14) SSR markers that showed 40% missing genotypes after removing the Nile tilapia were successfully used in the final dataset constituting a total of 276 samples with 33 microsatellite loci after removing the markers that failed genotyping. Because the study required inferring using SSRs and mtDNA markers, there was not enough time to process the mtDNA markers to further answer some research questions under investigation on the tilapia species and this part was left out of the thesis.

3.3. Population genetic differentiation

3.3.1. Population genetic differentiation by regional clustering

To assess the placement position in a large dataset of tilapia samples collected from different regions of Africa, 39 microsatellite loci used from the previously developed microsatellites by Tibihika et al, 2018 were used to envision the genetic distances of the populations from Burkina Faso, Ethiopia, Uganda, Kenya and Zambia, constituting samples from Eastern, Western and Southern Africa was conducted in a principal coordinate analysis (PCoA) (see Figure 22).

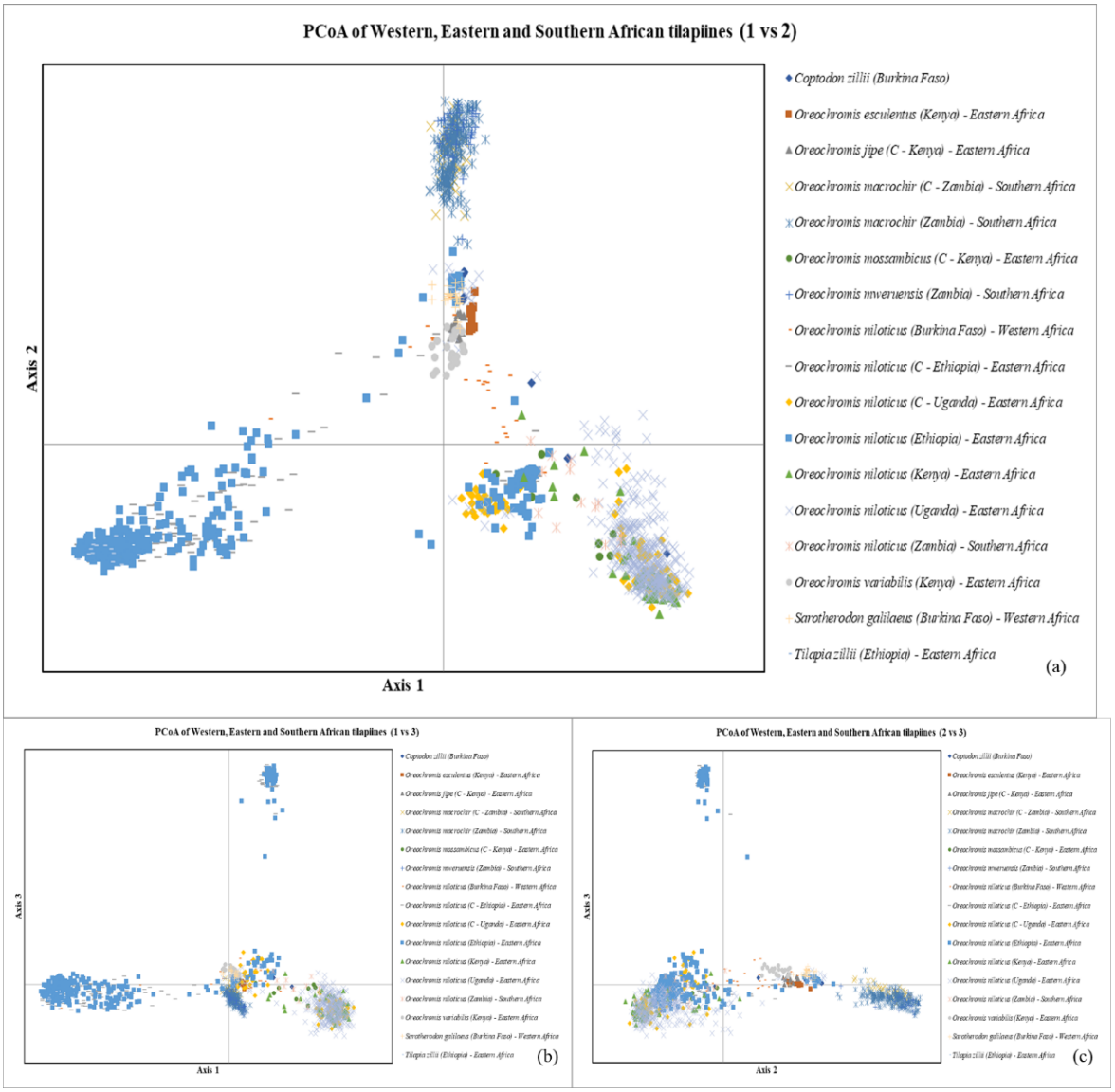

Figure 22 Principle coordinate analysis (PCoA) showing the four major cluster groups differentiating the populations from Uganda, Kenya, Burkina Faso, Ethiopia and Zambia constituting Western, Eastern and Southern Africa populations explained by the vertical and horizontal axes represented by (a) variation of 17.64%(1) and 30.71%(2), (b) variation of 17.64%(1) and 35.92%(3), and variation of 30.71%(2) and 35.92%(3) using the pairwise genetic distances

The principal coordinate analysis (PCoA) above in Figure 22 showed a clear differentiation between the regional populations under investigation from the Zambian populations in Southern Africa and how differentiated from other tilapia species from Ethiopian, Burkina Faso, Ugandan and Kenyan regions. From a total sample size of 1,466 constituting 17 populations from Lakes and farms, 17.65% explained the variation by the first axes, 30.71% by the second axes, and 35.92% by the third axes. To further investigate the differentiation and structure of the population within the Zambian freshwater systems, a PCoA in GenAlEx version 6.51 (Peakall and Smouse 2006, 2012, 2017; Smouse *et al*., 2017) was conducted and the file exported for a structure analysis using the program STRUCTURE version 2.3.4 (Pritchard et al., 2000; Hubisz et al., 2009; Porras-Hurtado et al, 2013) reported in the section below.

3.3.2. Population genetic differentiation of freshwater cichlids of Zambia

The principal coordinate analysis for all the eleven (11) samples collected from four different lakes geographically and three (3) government farms culturing the Longfin tilapia were conducted as shown in the Figure 23 below. The dataset analysed comprised 289 samples including the Nile tilapia and 39 microsatellite loci. These fish populations formed four major groups from Lake Bangweulu, Lake Mweru, Lake Mweru-Wantipa, Lake Kariba and the government fish farms differentiated by three axes vertically and horizontally explaining the variation with 13.65% at the first axes, 20.50% at the second axes and 26.83% at the third axes. The cultured populations formed one large cluster with the fish populations from Lake Mweru. The Nile tilapia population from Lake Kariba was removed from the dataset due to its clear distinction from the Longfin and Mweru tilapia. After the removal of the Nile tilapia, the six (6) microsatellite loci were removed due to the amount of missing data leaving only 33 informative microsatellite loci and a total population of 276 samples comprising only the two species: *O. macrochir* and *O. mweruensis*.

The principle coordinate analysis (PCoA) was conducted to differentiate the populations of *O. macrochir* from Lakes 1) Mweru, 2) Bangweulu, 3) Mweru-Wantipa and 4) Kariba using the first three axes. The percentage variation within the four lakes was explained and represented by 18.38% at the first axes, 25.43% at the second axes, and 29.22% at the third axes. The population according to the PCoA showed that the Longfin tilapia from Lake Kariba was closer to the Longfin tilapia from Lake Bangweulu. The population of *O. macrochir* from Lake Mweru-Wantipa formed a strong cluster with the population from Lake Mweru. The

PCoA also showed the differentiation of the same species between the two major Zambian fisheries, Lake Mweru and Lake Bangweulu (see Figure 25 below).

To investigate whether there was genetic differentiation between the two species *O. macrochir* and *O. mweruensis* in Lake Mweru-Wantipa, a PCoA was conducted to detect genetic differences between populations. The two species from the lake showed no clear differentiation into clusters with a percentage of variation being explained by the first (3) axes with the vertical and horizontal axes represented by 8.73% (1), 13.83% (2) and 17.85% (3) using the genetic distances (see Figure 26).

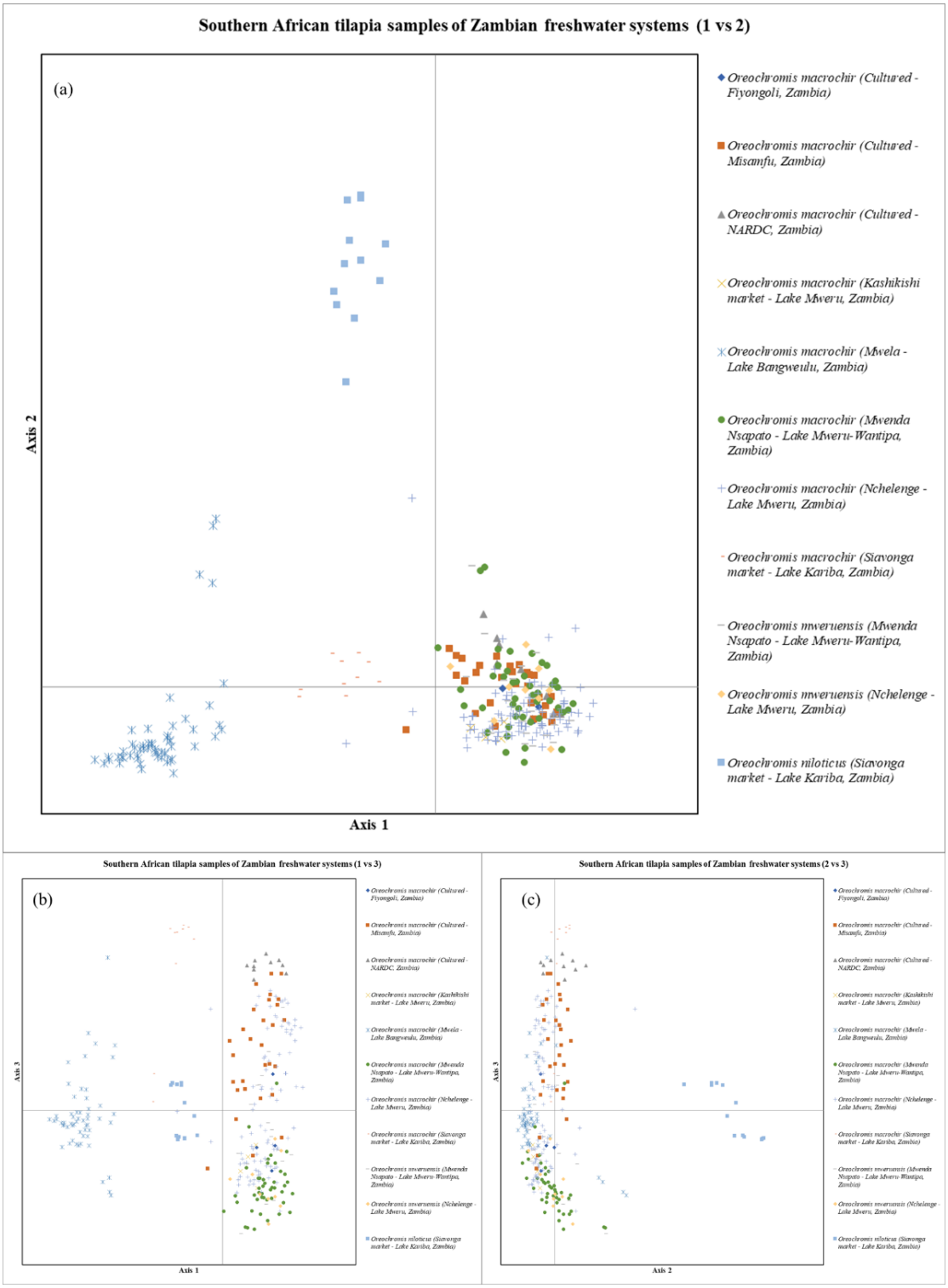

Figure 23 Population genetic differentiation of eleven (11) populations of the Longfin tilapia, Mweru tilapia and Nile tilapia forming four major cluster groups explained by the vertical and horizontal axes with a percentage variation for (a) 13.65%(1) and 20.50%(2); (b) 13.65%(1) and 26.83%(3); and 20.50%(2) and 26.83%(3) using the genetic pairwise distance

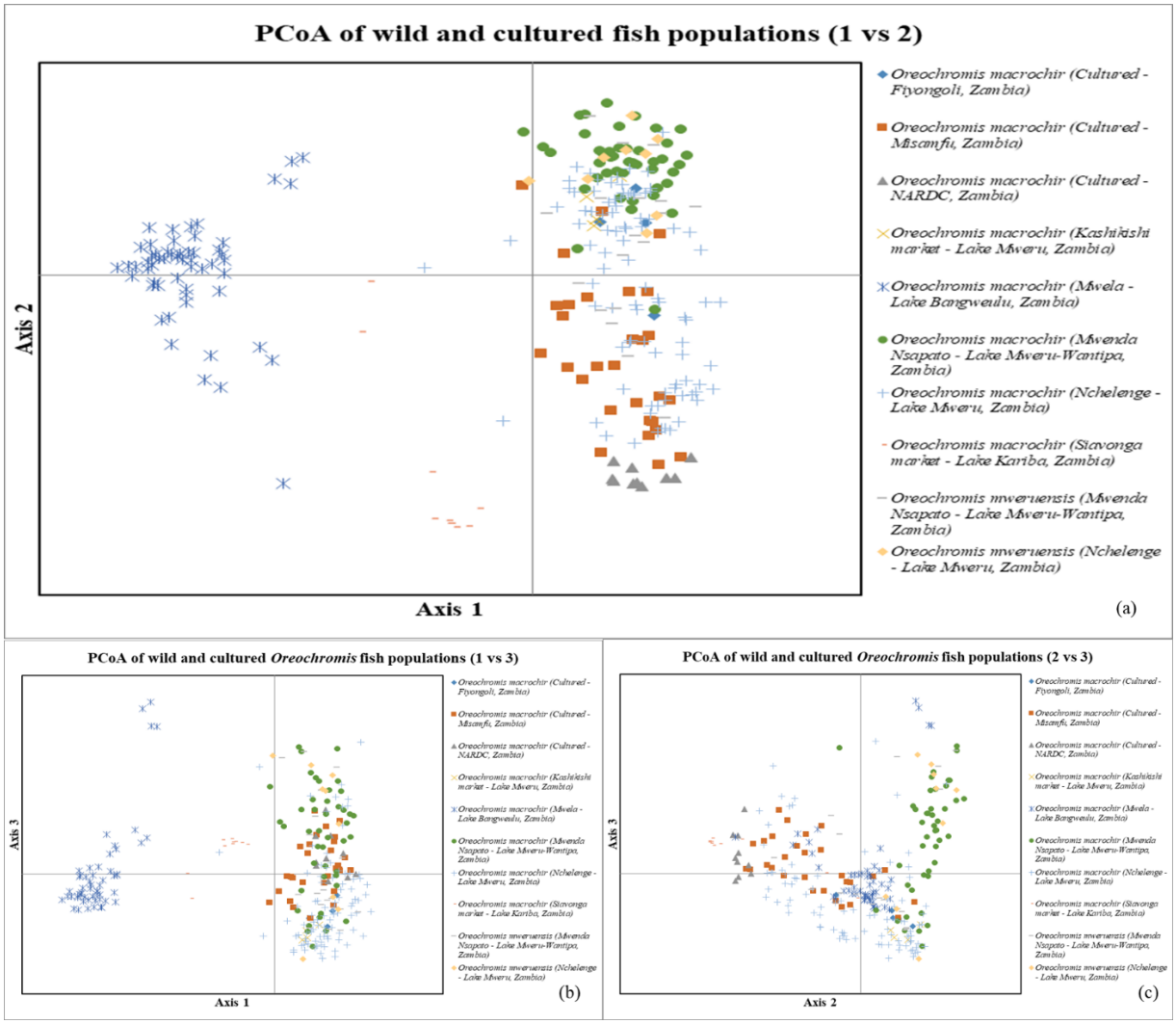

Figure 24 Principle coordinate analysis (PCoA) showing all the ten (10) different fish populations of O. macrochir and O. mweruensis forming cluster groups distinguished by the percentage variation explained by the vertical and horizontal axes with (a) represented by 14.90%(1) and 22.35%(2); (b) represented by 14.90%(1) and 25.81%(3); and (c) represented by 22.35%(2) and 25.81%(3) using pairwise genetic distances

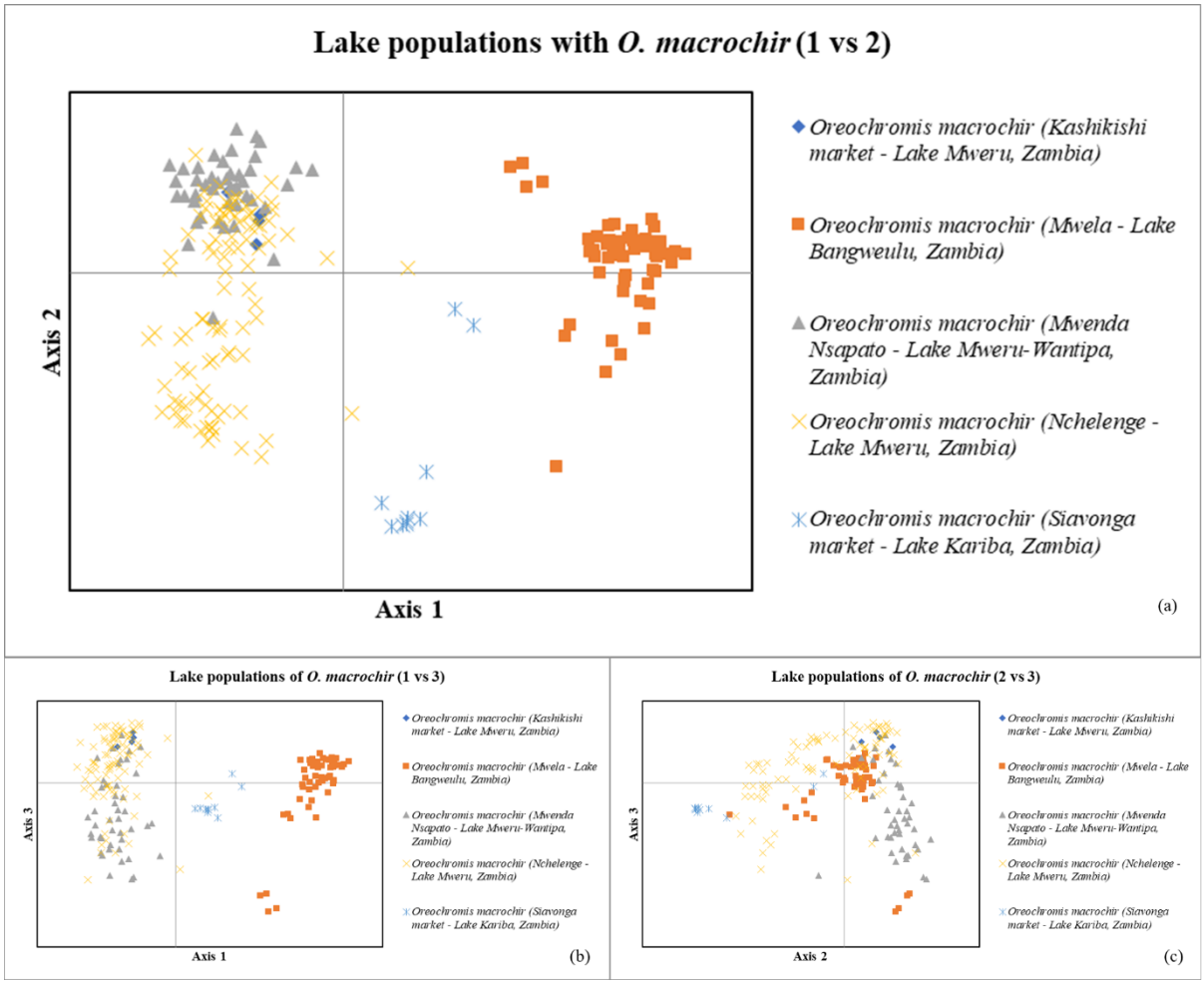

Figure 25 PCoA of five (5) fish populations of O. macrochir from Lakes Mweru, Bangweulu, Mweru-Wantipa and Kariba forming three cluster groups explained by the vertical and horizontal axes with a percentage variation at the first three axes of 18.38%(1), 25.43%(2) and 29.22%(3) using the genetic pairwise distances

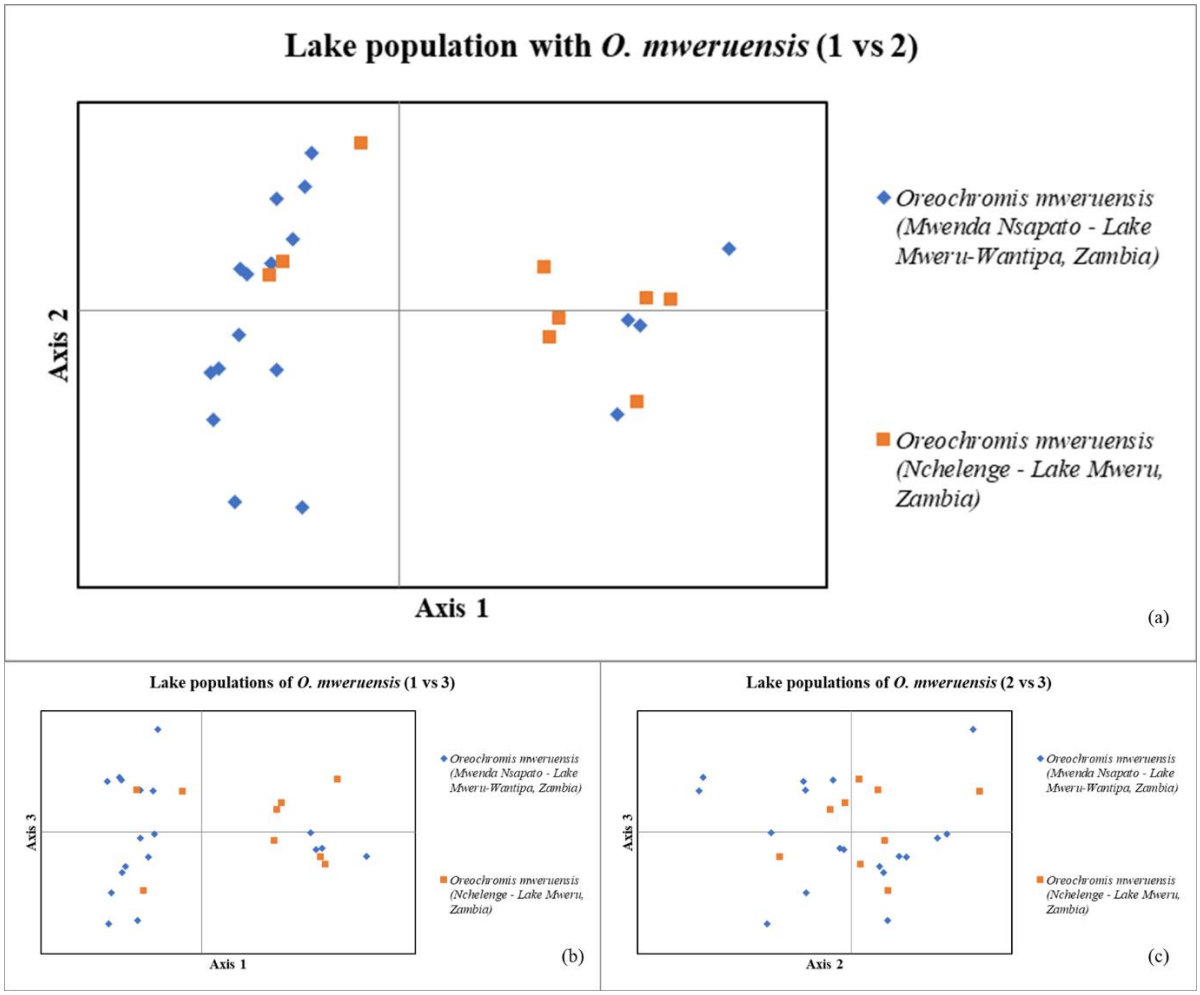

Figure 26 PCoA of two fish populations of O. mweruensis from Lake Mweru and Lake Mweru-Wantipa not forming a distinct cluster group explained by the vertical and horizontal axes with a percentage variation at the first three axes represented by (a) 12.79%(1) and 20.69%(2), (b) 12.79%(1) and 26.95%(3), and (c) 20.69%(2) and 26.95%(3) using pairwise genetic distances

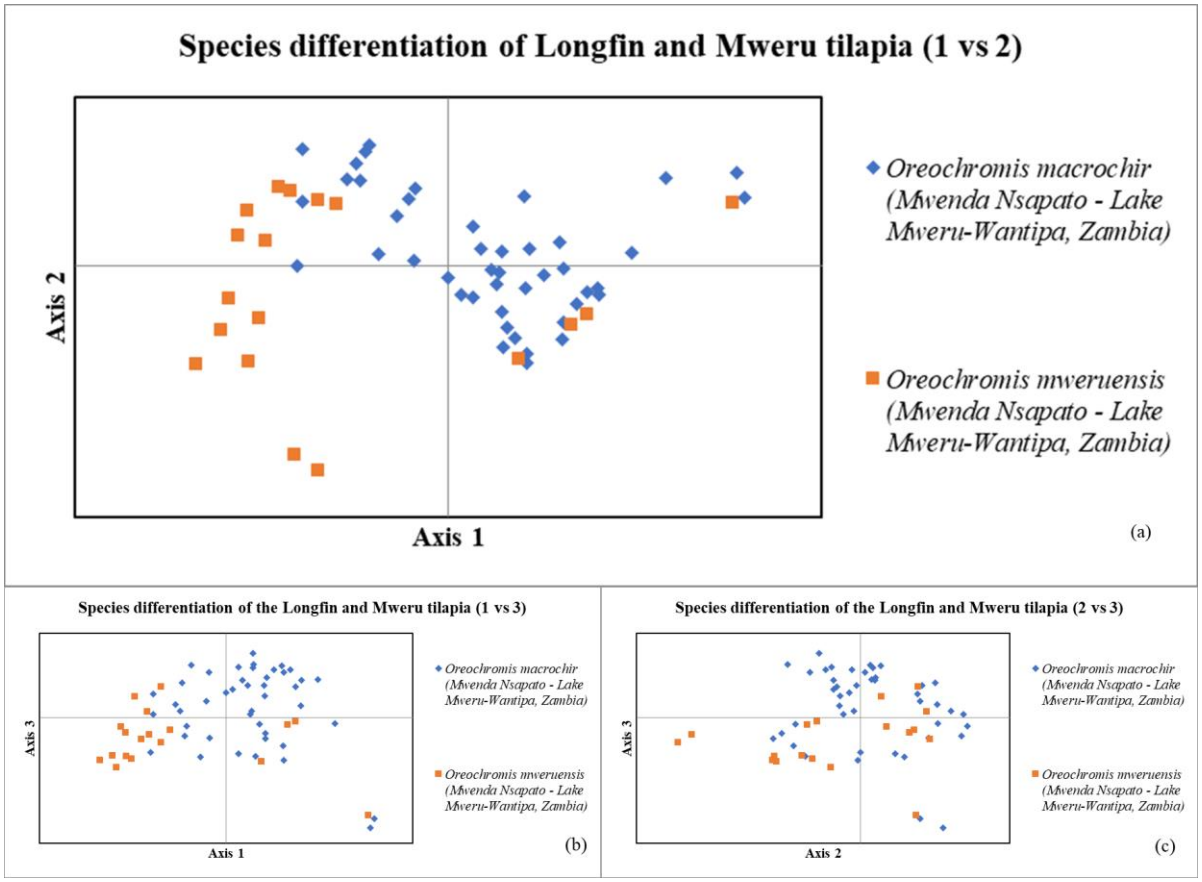

Figure 26 PCoA of species differentiating O. macrochir and O. mweruensis from Lake Mweru-Wantipa with no distinct cluster group explained by the variation percentage represented by three axes of 8.73% (1), 13.83%(2) and 17.85%(3) for (a), (b) and (c) using genetic pairwise distances

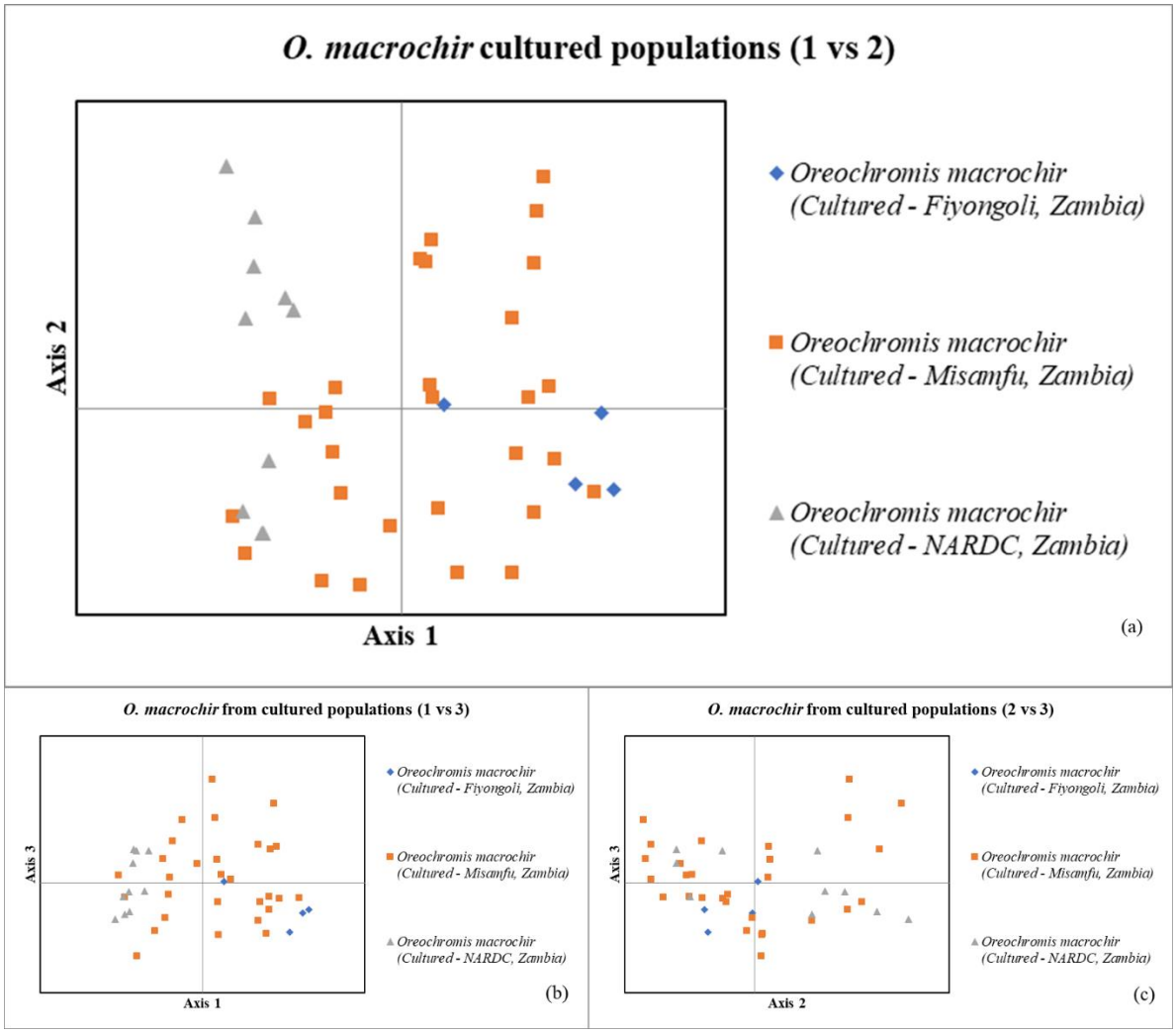

Figure 27 PCoA differentiating the cultured species of O. macrochir forming two distinct cluster groups between the populations from Fiyongoli and NARDC explained by the vertical and horizontal axes with a percentage variation at the first three axes represented by (a) 12.47%(1) and 19.72%(2), (b) 12.47%(1) and 25.56%(3), and (c) 19.72%(2) and 25.56%(3) using genetic pairwise distances

Structure bar plot key for population structure patterns:

1) Fiyongoli – *O. macrochir*

2) Misamfu – *O. macrochir*

3) NARDC – *O. macrochir*

4) Lake Mweru (Kashikishi market) – *O. macrochir*

5) Lake Bangweulu (Mwela landing site) – *O. macrochir*

6) Lake Mweru-Wantipa (Mwenda Nsapato landing site – *O. macrochir*

7) Lake Mweru – *O. macrochir*

8) Lake Kariba – *O. macrochir*

9) Lake Mweru-Wantipa – *O. mweruensis*

10) Lake Mweru – *O. mweruensis*

11) Lake Kariba – *O. niloticus*

For the STRUCTURE analysis nine barplots diagrams were constructed during which all the 289 samples constituting eleven (11) populations were included in the analysis (see Figure 30). Two optimal values of K were revealed with the first Delta K being optimal according to the Evanno method (Evanno et al, 2005) at K = 4 with 60.367 (see Table 5 and Figure 31 and Figure 32) and the probability by K graph using the median values of Ln(Pr Data) the k for which Pr(K=k) being highest at K = 8 with 25.251. The Delta K values were calculated using STRUCTURE Harvester (Earl and vonHoldt, 2012) free available at http://taylor0.biology.ucla.edu/structureHarvester/# and the Delta K graphs obtained using CLUMPAK (Kopelman et al, 2015) web-version freely available at http://clumpak.tau.ac.il/index.html that uses CLUMPP and DISTRUCT to visualise population structure patterns. At an optimal Delta K= 4, the structure analysis revealed that the Longfin tilapia and Mweru tilapia from Lake Mweru obtained at Kashikishi market, Nchelenge landing site and Mwenda Nsapato landing site on Lake Mweru-Wantipa did not show any distinct structure patterns as these were all clustered together. According to the structure analysis in Figure 30, the optimal DeltaK value (K = 4), assigned populations 1 to 3 (constituting *O. macrochir* farmed from Fiyongoli, Misamfu and NARDC government fish farms) into the same cluster group with population 6 (of *O. macrochir* from Lake Mweru-Wantipa), population 7 (of *O. macrochir* from Lake Mweru), population 9 (of *O. mweruensis* from Lake Mweru-Wantipa) and population 10 (of *O. mweruensis* from Lake Mweru) forming a second single cluster group with populations 5 (from Lake Bangweulu of the Longfin tilapia) and 8 (from Lake Kariba) forming the other two distinct cluster groups at an optimal Delta K = 4 (see Figure 30). These results supported the principle coordinate analysis (PCoA) in Figure 23 showing four major cluster groups.

A structure analysis showing nine five (5) bar plot diagrams with only a total of 276 samples constituting ten (10) populations (see Figure 33) excluding one (1) population of the Nile tilapia from the dataset investigating the Longfin tilapia (*O. macrochir*) and Mweru tilapia (*O. mweruensis*) from the four Lakes (Bangweulu, Mweru, Mweru-Wantipa and Kariba) and the three farms (Fiyongoli, Misamfu and NARDC) revealed an optimal Delta K by Evanno at K = 3 with a Delta value of 53.435, and the probability by K graph using median values of Ln(Pr Data) of k for which Pr(K=k) was highest at 9 with a value of 27.061 (see Table 4 and Figure 29). Populations 1 to 3 (of *O. macrochir* from Fiyongoli, Misamfu and NARDC) formed a cluster group forming a close cluster group with populations 6 (of *O. macrochir* from Lake Mweru-Wantipa), population 7 (of *O. macrochir* from Lake Mweru), population 9 (of O.

mweruensis from Lake Mweru-Wantipa), and population 10 (of *O. mweruensis* from Lake Mweru). Population 5 (of *O. macrochir* from Lake Bangweulu) formed a single cluster group and population 8 (of *O. macrochir* from Lake Kariba) formed two distinct groups. These results from the structure analysis supported the principle coordinate analysis (PCoA) in Figure 24 showing three major cluster groups at an optimal Delta K value of 3. →

A genetic differentiated pattern was observed using a pairwise F_{ST} values conducted in GenAlEx for all the sampling points (Table 4). A locus-by-locus Analysis of Molecular Variance (AMOVA) was also conducted on the ten populations in GenAlEx that supported the PCoA analysis by clustering the fish species into three major cluster groups based on water basins; Luapula-Congo basin (Lake Mweru and Lake Mweru Wantipa), the Zambezi river basin (Lake Kariba) and the Chambeshi river basin (Lake Bangweulu). For *O. macrochir*, genetic variation among populations from the different basins of the Zambian freshwater Lakes was described by 18.37% variation, as 25.11% was accounted for by variation among individual samples, and 57.52% of variation within individual samples of the total genetic variation (Figure 28) and vice versa for *O. mweruensis* below.

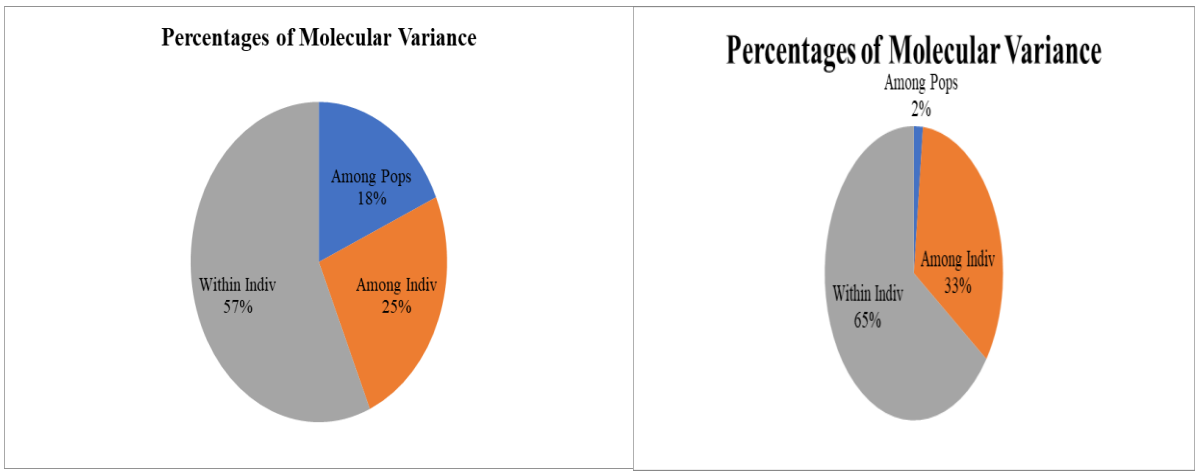

Figure 28 Proportion of molecular variance of O. macrochir (left) and O. mweruensis (right)

These results further corresponded with the F-statistic values that showed significant values at a P(rand >= data) value of 0.001 over all the loci of F_{ST} (0.184), F_{IS} (0.308) and F_{IT} (0.435) based on the standard permutations across the complete dataset.

Table 4 Pairwise FST values for showing the differentiation of the Longfin tilapia (LT) and Mweru tilapia (MT) among the from different sampling locations from four wild populations and three government farms in Zambia

				Lake Mweru (KM) - LT	Lake Bangweulu (M) - LT	Lake Mweru-Wantipa (MN) - LT	Lake Mweru (N) - LT	Lake Kariba (SM) - LT	Lake Mweru-Wantipa (MN) - MT	Lake Mweru (N) - MT
	Fiyongoli - LT	Misamfu - LT	NARDC - LT							
Fiyongoli - LT	0.000									
Misamfu - LT	0.103	0.000								
NARDC - LT	0.247	0.073	0.000							
Lake Mweru (KM) - LT	0.098	0.084	0.210	0.000						
Lake Bangweulu (M) - LT	0.361	0.300	0.393	0.300	0.000					
Lake Mweru-Wantipa (MN) - LT	0.119	0.124	0.245	0.046	0.305	0.000				
Lake Mweru (N) - LT	0.083	0.064	0.129	0.017	0.273	0.058	0.000			
Lake Kariba (SM) - LT	0.346	0.213	0.238	0.286	0.313	0.318	0.222	0.000		
Lake Mweru-Wantipa (MN) - MT	0.060	0.065	0.175	0.029	0.295	0.033	0.025	0.261	0.000	
Lake Mweru (N) - MT	0.102	0.106	0.244	0.053	0.313	0.000	0.053	0.325	0.016	0.000

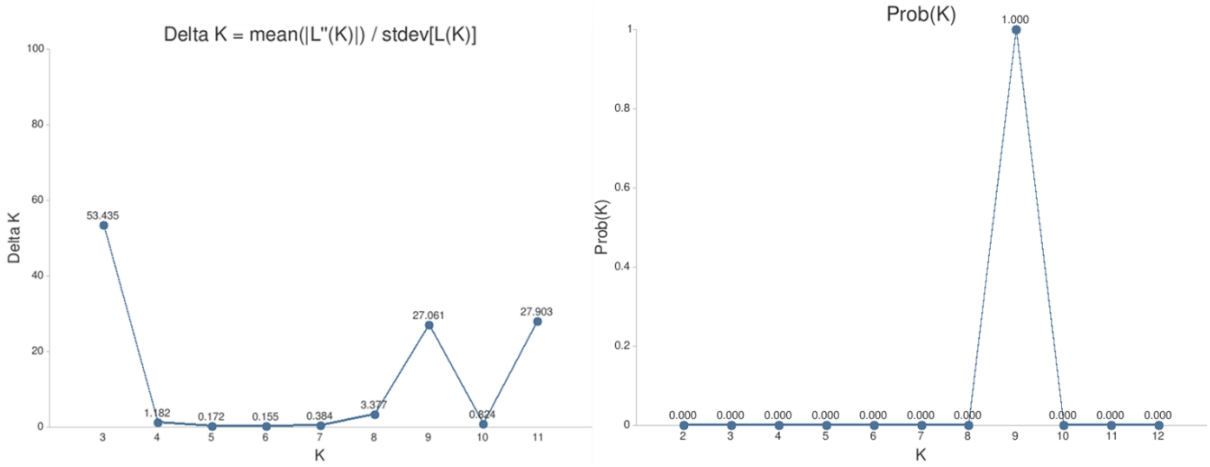

Figure 29 DeltaK graph (left) showing the optimal K by Evanno of 3 and the probability by K graph (right) using the median values highest at 9

Table 5 Evanno output table showing the optimal Delta K value

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
2	6	-26374.300000	51.929183	—	—	—
3	6	-25045.866667	15.668014	1328.433333	837.216667	53.434766
4	6	-24554.650000	233.181884	491.216667	275.666667	1.182196
5	6	-24339.100000	226.396599	215.550000	38.916667	0.171896
6	6	-24162.466667	235.140321	176.633333	36.400000	0.154801
7	6	-23949.433333	199.139596	213.033333	76.533333	0.384320
8	6	-23659.866667	79.518769	289.566667	268.533333	3.376981
9	6	-23638.833333	131.866746	21.033333	3568.383333	27.060525
10	6	-27186.183333	8640.834005	-3547.350000	7123.533333	0.824403
11	6	-23610.000000	132.975712	3576.183333	3710.466667	27.903341
12	6	-23744.283333	293.012985	-134.283333	—	—

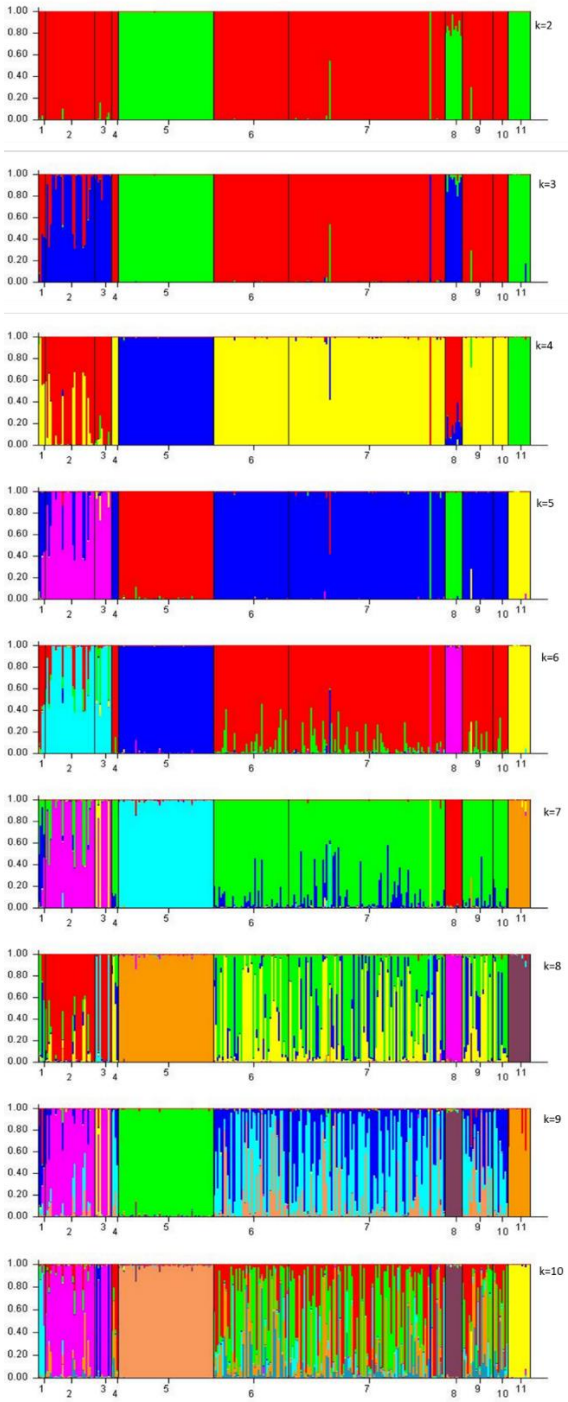

Figure 30 STRUCTURE analysis with all the eleven (11) fish populations of Oreochromis species 1) O. macrochir (Fiyongoli Government fish farm, Mansa), 2) O. macrochir (Misamfu Government fish farm, Kasama), 3) O. macrochir (National Aquaculture Research Development Centre (NARDC), Mwekera – Kitwe), 4) O. macrochir (Kashikishi market – Lake Bangweulu, Nchelenge), 5) O. macrochir (Mwela landing site – Lake Bangweulu, Samfya), 6) O. macrochir (Mwenda Nsapato landing site – Kaputa), 7) O. macrochir (Nchelenge landing site – Lake Mweru, Nchelenge), 8) O. macrochir (Siavonga market – Lake Kariba, Siavonga), 9) O. mweruensis (Mwenda Nsapato landing site – Lake Mweru-Wantipa, Kaputa), 10) O. mweruensis (Nchelenge landing site – Lake Mweru, Nchelenge), and 11) O. niloticus (Siavonga market – Lake Kariba, Siavonga): K=4 (optimal Evanno) and K=8 (using median values of Ln(Pr Data) for which the Pr(K=k) was highest at a burning length period of 20,000 and an MCMC after burnin at 20,000 for 4 iterations (K: 2-10)

Table 6 Evanno output table showing the optimal Delta K value

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
2	4	-29902.425000	373.909565	—	—	—
3	4	-27902.600000	303.719201	1999.825000	512.025000	1.685850
4	4	-26414.800000	19.016309	1487.800000	1147.950000	60.366605
5	4	-26074.950000	353.286178	339.850000	98.975000	0.280155
6	4	-25834.075000	368.962007	240.875000	4158.100000	11.269724
7	4	-29751.300000	5066.737308	-3917.225000	8320.325000	1.642147
8	4	-25348.200000	190.316421	4403.100000	4805.775000	25.251499
9	4	-25750.875000	822.185358	-402.675000	685.950000	0.834301
10	4	-25467.600000	187.125466	283.275000	—	—

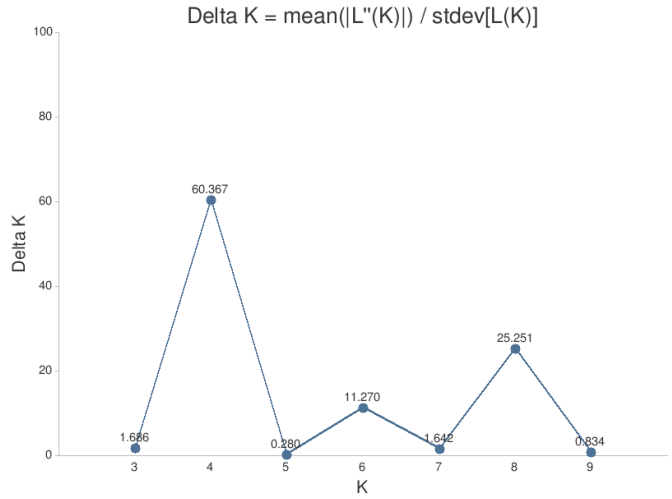

Figure 31 DeltaK Graph showing the optimal K value by the Evanno method on the population structure

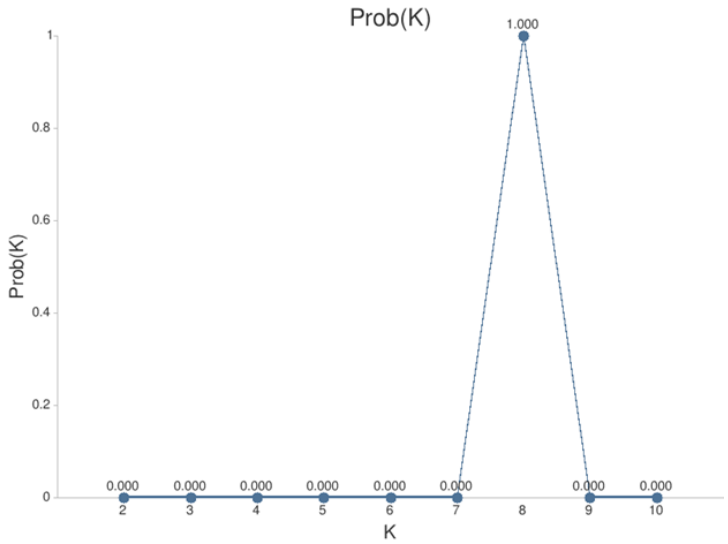

Figure 32 Probability by L graph using the median values of Ln(Pr Data) the k for which Pr(K=k) is highest: 8

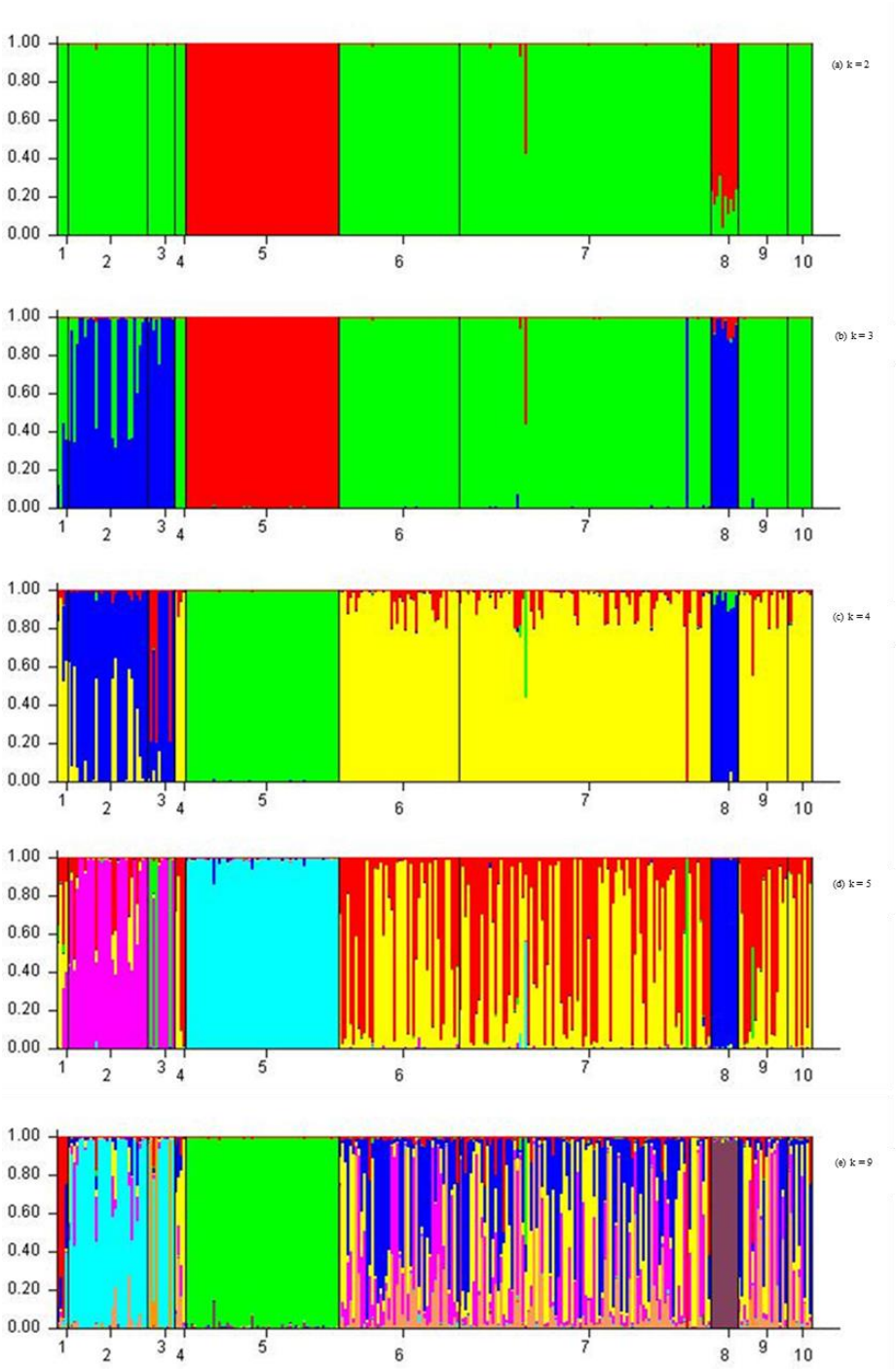

Figure 33 STRUCTURE analysis with all the Longfin and Mweru tilapia samples: 1) O. macrochir from Fiyongoli (government fish farm (Mansa), 2) O. macrochir from Misamfu (government fish farm) (Kasama), 3) O. macrochir from the National Aquaculture Research Development Centre (NARDC) (government fish farm) (Mwekera – Kitwe), 4) O. macrochir from Kashikishi market (Nchelenge), Lake Mweru, 5) O. macrochir from Lake Bangweulu (Mwela Landing site – Samfya), 6) O. macrochir from Lake Mweru-Wantipa (Mwenda Nsapato Landing site – Kaputa), 7) O. macrochir from Lake Mweru (Nchelenge landing site – Nchelenge), 8) O. macrochir from Lake Kariba (Siavonga market – Siavonga), 9) O. mweruensis from Lake Mweru-Wantipa (Mwenda Nsapato landing site – Kaputa), & 10) O. mweruensis from Lake Mweru (Nchelenge landing site – Nchelenge): K=3 (optimal K by Evanno) and K = 9 (showing the median values of In(Pr Data) the value of k for which Pr(K=k) is highest); Length of burnin period: 20,000 and the MCMC after burning repeats of 20,000, represented by (a) K = 2, (b) K = 3, (c) K = 4, (d) K = 5 and (e) K = 9

3.4. Genetic diversity of *O. macrochir* and *O. mweruensis* populations

A total of 33 microsatellite loci were used for *O. macrochir* and *O. mweruensis*. In O. macrochir, there were significant departures from Hardy-Weinberg Equilibrium (HWE) ($P < 0.05$). Deviations observed for *O. macrochir* were three loci (Ti31_AAAAT, Ti34_TCTCT and TI54_GGAT) of the farmed population at Fiyongoli, three loci (Ti5_CA, Ti15_TGC, and Ti54_GGAT) for the cultured populations at Misamfu, one loci (Ti26_ACAA) for the cultured populations at NARDC, one loci (Ti27_TTTG) from Kashikishi market on Lake Mweru, two loci (Ti1_TG and Ti31_CTAAT) from Mwela landing site on Lake Bangweulu, one loci (Ti16_AAC) from Nchelenge landing site on Lake Mweru, four loci (Ti7_AC, Ti22_CTAT, Ti32_AAAAT, Ti49_TGT), and *O. mweruensis* were two loci (Ti32_AAAAT and Ti49_TGT). No linkage equilibrium was observed among the loci in the populations constituting both species showing unique variation at all the informative loci used during the study.

To analyse the genetic diversity of the different major cluster group of the major fish species under investigation, the heterozygosity, a pairwise comparison using Fst/NeiP distance, and the number of polymorphic loci were evaluated. The average count of alleles per locus throughout the dissimilar populations was 6.779±0.741. The Longfin tilapia (*O. macrochir*) from Nchelenge landing site of Lake Mweru had the highest observed number of alleles (Na) = 16.333±2.109 while *O. macrochir* from the government farm at Fiyongoli had the lowest observed number of alleles (Na) = 2.939±0.292. The average number of different allele frequencies >5% was 4.070±0.395.

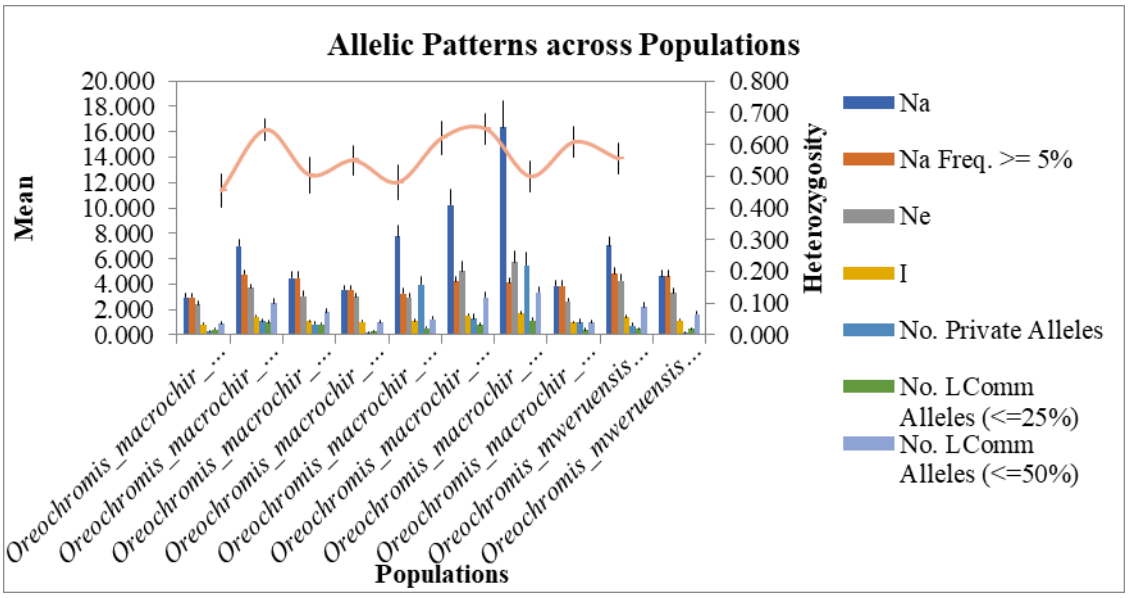

Figure 34 Allele frequency patterns across ten (10) different populations of the Longfin tilapia and the Mweru tilapia in Zambia

The number of different allele frequencies >5% (Na) was highest for the Mweru tilapia (*O. mweruensis*) from Lake Mweru-Wantipa with an observed number of alleles (Na) = 4.818±0.474 of alleles and seconded by *O. macrochir* from the government fish farm at NARDC with an observed number of alleles (Na) = 4.788±0.310 while the lowest number of allele (Na) was 2.939±0.292. The average number of effective alleles (Ne) was observed at 3.608±0.443 across all the populations. The Longfin tilapia from Lake Mweru at Nchelenge landing site showed the highest observed number of effective alleles (Ne) = 5.742±0.889 while the lowest was the Longfin tilapia from the government fish farm at Fiyongoli with 2.384±0.249.

The average Shannon Information Index (I) across the ten populations was 1.216±0.123 outside the recommended index ranges between 0 and 1 (NIST, n.d.). The highest index across the populations was observed in the Longfin tilapia from Nchelenge landing site on Lake Mweru with 1.707±0.166 while the lowest was observed on the cultured Longfin tilapia from Fiyongoli government fish farm with 0.828±0.100. The mean allele richness across the different populations or sampling sites was 28.79±3.36. The microsatellite locus TI43_GAATA had the highest number of alleles with 83 and the locus TI12_TAC with the least alleles (4) for the Longfin tilapia from Fiyongoli government farm and Kashikishi market on Lake Mweru. The average number of alleles across the ten (10) populations was 1.503±0.305. Lake Mweru at Nchelenge landing site had the highest number of private alleles for *O. macrochir* with 5.485±1.014 while the adjacent population no more than 5 km apart at Kashikishi market on Lake Mweru had the lowest private number of alleles with 0.182±0.068. The average observed heterozygosity (Ho) across all the populations per loci was 0.489±0.017. The highest observed heterozygosity (Ho) was 0.563±0.062 for the Longfin tilapia sampled at Kashikishi market of Lake Mweru while the least observed was from Lake Bangweulu at Mwela landing site with a heterozygosity (Ho) with a mean value of 0.412±0.055 though this observation was not significant. The average unbiased heterozygosity (uHe) over the loci and fish populations was 0.594±0.017. The highest unbiased heterozygosity (uHe) was observed for the Longfin tilapia from Misamfu government fish farm with a mean value of 0.663±0.037 and the lowest for the same species observed at Mwela landing with a mean value of 0.487±0.054. The expected heterozygosity (He) over the population and loci was a mean value of 0.557±0.016 with the highest heterozygosity (He) expected of the Longfin tilapia at Nchelenge landing site of Lake Mweru (0.649±0.049) and Misamfu government farm

(0.647±0.036). These estimates values were not found to be significantly different between populations collected at a P value of 0.05.

Slight heterozygote deficiencies were observed throughout all the populations having positive mean values of F_{IS} (0.142±0.047), F_{IT} (0.336±0.041), and F_{ST} (0.227±0.022) though these low values were not significantly different among the sampled populations.

Table 7 Genetic diversity indices according to species and location in Zambia

Species/Population	Na	Na Freq. >= 5%	Ne	I	No. Private Alleles	No. LComm Alleles (<=25%)	No. LComm Alleles (<=50%)	He	uHe
Oreochromis macrochir **(Cultured – Fiyongoli)**	2.939	2.939	2.384	0.828	0.273	0.424	0.879	0.455	0.531
Oreochromis macrochir **(Cultured – Misamfu)**	6.970	4.788	3.689	1.419	1.091	0.970	2.485	0.647	0.663
Oreochromis macrochir **(Cultured – NARDC)**	4.485	4.485	3.042	1.067	0.818	0.818	1.788	0.504	0.535
Oreochromis macrochir **(Kashikishi M – Lake Mweru)**	3.545	3.545	3.023	1.027	0.182	0.273	0.970	0.550	0.640
Oreochromis macrochir **(Mwela – Lake Bangweulu)**	7.758	3.273	2.946	1.094	3.939	0.515	1.212	0.481	0.487
Oreochromis macrochir **(Mwenda N - Lake Mweru-Wantipa**	10.212	4.212	5.020	1.529	1.303	0.788	2.970	0.622	0.633
Oreochromis macrochir **(Nchelenge – Lake Mweru)**	16.333	4.121	5.742	1.707	5.485	1.121	3.364	0.649	0.654
Oreochromis macrochir **(Siavonga M – Lake Kariba)**	3.879	3.879	2.646	0.962	1.000	0.394	0.970	0.500	0.559
Oreochromis_mweruensis_**(Mwenda N_Lake Mweru-Wantipa)**	7.030	4.818	4.262	1.387	0.727	0.455	2.182	0.609	0.632
Oreochromis_mweruensis_**(Nchelenge – Lake Mweru)**	4.636	4.636	3.322	1.139	0.212	0.455	1.636	0.555	0.609
Average	**6.779**	**4.070**	**3.608**	**1.216**	**1.503**	**0.621**	**1.845**	**0.557**	**0.594**

Species/Population	Na	Na Freq. >= 5%	Ne	I	No. Private Alleles	No. LComm Alleles (<=25%)	No. LComm Alleles (<=50%)	He	uHe
Oreochromis macrochir **(Cultured – Fiyongoli)**	0.292	0.292	0.249	0.100	0.090	0.115	0.167	0.051	0.060
Oreochromis macrochir **(Cultured – Misamfu)**	0.518	0.310	0.324	0.094	0.159	0.166	0.348	0.036	0.037
Oreochromis macrochir **(Cultured – NARDC)**	0.563	0.563	0.432	0.131	0.187	0.165	0.281	0.057	0.060
Oreochromis macrochir **(Kashikishi M – Lake Mweru)**	0.311	0.311	0.290	0.100	0.068	0.100	0.182	0.046	0.053
Oreochromis macrochir **(Mwela – Lake Bangweulu)**	0.853	0.397	0.366	0.132	0.664	0.116	0.221	0.054	0.054
Oreochromis macrochir **(Mwenda N - Lake Mweru-Wantipa**	1.233	0.396	0.753	0.153	0.368	0.149	0.412	0.052	0.053
Oreochromis macrochir **(Nchelenge – Lake Mweru)**	2.109	0.366	0.889	0.166	1.014	0.217	0.454	0.049	0.049
Oreochromis macrochir **(Siavonga M – Lake Kariba)**	0.386	0.386	0.233	0.103	0.230	0.115	0.187	0.048	0.055
Oreochromis_mweruensis_**(Mwenda N_Lake Mweru-Wantipa)**	0.696	0.474	0.531	0.130	0.181	0.107	0.349	0.047	0.050
Oreochromis_mweruensis_**(Nchelenge – Lake Mweru)**	0.452	0.452	0.362	0.116	0.084	0.107	0.245	0.049	0.054
Average	**0.741**	**0.395**	**0.443**	**0.123**	**0.305**	**0.136**	**0.285**	**0.049**	**0.053**

Table 8 Genetic diversity indices of eleven fish populations of showing the different number of alleles, effective alleles, Shannon's Information index, observed-, expected-, and unbiased heterozygosity, and the fixation index

Mean and SE over Loci for each Pop									
Pop		**N**	**Na**	**Ne**	**I**	**Ho**	**He**	**uHe**	**F**
***Oreochromis_macrochir*_Cultured_Fiyongoli**	**Mean**	3.455	2.939	2.384	0.828	0.449	0.455	0.531	0.025
	SE	0.185	0.292	0.249	0.100	0.063	0.051	0.060	0.080
***Oreochromis_macrochir*_Cultured_Misamfu**	**Mean**	23.061	6.970	3.689	1.419	0.539	0.647	0.663	0.181
	SE	1.253	0.518	0.324	0.094	0.047	0.036	0.037	0.059
***Oreochromis_macrochir*_Cultured_NARDC**	**Mean**	7.152	4.485	3.042	1.067	0.415	0.504	0.535	0.185
	SE	0.686	0.563	0.432	0.131	0.058	0.057	0.060	0.065
***Oreochromis_macrochir*_KM_LakeMweru**	**Mean**	3.788	3.545	3.023	1.027	0.563	0.550	0.640	-0.018
	SE	0.095	0.311	0.290	0.100	0.062	0.046	0.053	0.074
***Oreochromis_macrochir*_M_LakeBangweulu**	**Mean**	47.818	7.758	2.946	1.094	0.412	0.481	0.487	0.110
	SE	2.274	0.853	0.366	0.132	0.055	0.054	0.054	0.051
***Oreochromis_macrochir*_MN_LakeMweru-Wantipa**	**Mean**	37.576	10.212	5.020	1.529	0.496	0.622	0.633	0.152
	SE	1.780	1.233	0.753	0.153	0.046	0.052	0.053	0.046
***Oreochromis_macrochir*_N_LakeMweru**	**Mean**	80.485	16.333	5.742	1.707	0.540	0.649	0.654	0.177
	SE	2.495	2.109	0.889	0.166	0.048	0.049	0.049	0.050
***Oreochromis_macrochir*_SM_LakeKariba**	**Mean**	7.697	3.879	2.646	0.962	0.452	0.500	0.559	0.063
	SE	0.597	0.386	0.233	0.103	0.056	0.048	0.055	0.068
***Oreochromis_mweruensis*_MN_LakeMweru-Wantipa**	**Mean**	14.758	7.030	4.262	1.387	0.516	0.609	0.632	0.173
	SE	0.698	0.696	0.531	0.130	0.050	0.047	0.050	0.057
***Oreochromis_mweruensis*_N_LakeMweru**	**Mean**	7.273	4.636	3.322	1.139	0.510	0.555	0.609	0.071
	SE	0.395	0.452	0.362	0.116	0.059	0.049	0.054	0.064
Grand Mean and SE over Loci and Pops									
		N	**Na**	**Ne**	**I**	**Ho**	**He**	**uHe**	**F**
Total	**Mean**	23.306	6.779	3.608	1.216	0.489	0.557	0.594	0.115
	SE	1.378	0.354	0.163	0.042	0.017	0.016	0.017	0.019

Where,

Na = Number of different alleles; Ne = number of effective alleles = 1 / (Sum pi^2); I = Shannon's Information Index = -1 * Sum (pi * Ln (pi)); Ho = Observed Heterozygosity = Number of Hets / N; He = Expected Heterozygosity = 1 – Sum pi^2; uHe = Unbaised Expected Heterozygosity = (2N / (2N-1)) * He; F = Fixation Index = (He – Ho) / He = 1 – (Ho / He);

where “pi” is the frequency of the “ith” allele for the population and “Sum pi^2” is the sum of squares of allele frequencies.

Table 9 Summary of the F-Statistics and Estimates of the eleven fish populations per loci

F-Statistics and Estimates of Nm over All Pops for each Locus					
All Pops.	**Locus**	**Fis**	**Fit**	**Fst**	**Nm**
	TI1_TG	-0.080	0.116	0.182	1.124
	TI2_CA	-0.067	0.128	0.183	1.118
	TI4_GT	-0.012	0.172	0.181	1.130
	TI5_CA	-0.059	0.163	0.210	0.941
	TI7_AC	0.100	0.300	0.222	0.876
	TI12_TAC	0.795	0.808	0.061	3.835
	TI13_ATG	0.195	0.334	0.172	1.200
	TI14_TAA	0.097	0.134	0.041	5.851
	TI15_TGC	-0.092	0.029	0.111	2.006
	TI16_AAC	-0.023	0.145	0.164	1.276
	TI17_GAA	0.029	0.323	0.302	0.577
	TI18_ATCT	-0.040	0.104	0.138	1.556
	TI22_CTAT	0.612	0.664	0.134	1.618
	TI26_ACAA	0.170	0.376	0.247	0.761
	TI27_TTTG	0.196	0.412	0.269	0.681
	TI29_TAAAA	0.676	0.739	0.194	1.041
	TI31_CTAAT	0.083	0.270	0.204	0.977
	TI32_AAAAT	0.474	0.566	0.174	1.191
	TI34_TCTCT	0.204	0.289	0.107	2.087
	TI35_AAAAG	-0.029	0.086	0.112	1.991
	TI39_ATGG	-0.128	0.551	0.602	0.166
	TI41_AAAC	0.193	0.471	0.344	0.477
	TI43_GAATA	0.004	0.193	0.190	1.063
	TI49_TGT	-0.125	-0.008	0.104	2.164
	TI50_ATGG	0.576	0.845	0.634	0.144
	TI51_TGT	0.214	0.419	0.261	0.707
	TI52_TAT	0.496	0.683	0.370	0.426
	TI54_GGAT	0.148	0.360	0.249	0.753
	TI55_TCTA	0.204	0.449	0.308	0.561
	TI56_TGTT	0.244	0.372	0.169	1.229
	TI59_AGGA	0.247	0.460	0.283	0.633
	TI60_ATCC	-0.170	0.166	0.287	0.621
	TI61_TGGA	-0.444	-0.044	0.277	0.652
	Mean	0.142	0.336	0.227	1.256
	SE	0.047	0.041	0.022	0.190

Where,

Fis = (Mean He – Mean Ho) / Mean He; Fit = (Ht – Mean Ho) / Ht; Fst = (Ht – Mean He) / Ht; Nm = [(1 / Fst) – 1] / 4; with “Mean He” = Average He across the populations; “Mean Ho” = Average Ho across the populations; “Ht” = Total Expected Heterozygosity = 1 – Sum tpi^2, where “tpi^2” is the frequency of the “ith” allele for the total and “Sum tpi^2” is the sum of the squared total of allele frequencies.

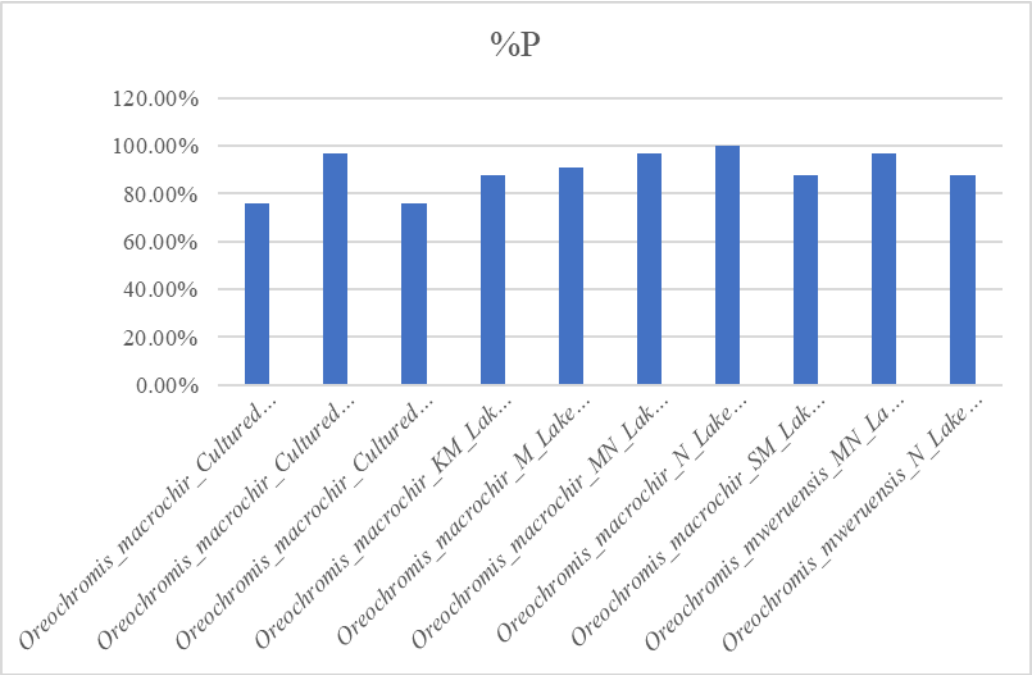

Figure 35 Percentage of polymorphic loci for the ten (10) fish populations

Chapter 4

4. Discussion

4.1. Marker quality and cross amplification of congeneric species

The results of this study revealed a number of important things, most of which had to do with the efficient and successful isolation of DNA during isolation using molecular tools such as microsatellite loci in genotyping congeneric species. The study initially began with the collection of 557 fin clips collected from the caudal fin of a whole sample of fish according to Wu and Yang, (2012). About 78% of the total samples were successfully isolated and proceeded for further analysis leaving about 22% that failed the extraction process completely. This failure could have possibly been due to the DNA isolation protocol used for the Longfin and Mweru tilapia. The protocol used was successfully used by Tibihika et al, 2018 in DNA isolation although their study used Magnet beads whereas this study involved the usage of Econospin plates. The application of DNA extraction methods often tends to yield alternative results. The use of a fish sample collected from the muscle tissue as was used by Tibihika et al, 2018 tends to be the most preferred sample of DNA isolation (Tibihika et al, 2018). A study by Wu and Yang (2012) reported to have used the caudal fin for the extraction of genomic DNA which may be seen by many to be a favourable method of collecting more fish samples during field collection and less costly. Another study by Wasko et al, (2003) described an ameliorated procedure of extraction DNA from fish scales, caudal or anal fins. This study involved the usage of samples collected from fish by-catches from fishers and fish traders to increase the sample size collected, a method that was non-destructive during field sample collection. The study by Wasko et al, (2003) further demonstrated a high efficiency in DNA extraction resulting in high quality DNA. The other challenge that could have led to the failures during DNA extraction from the caudal fins could also be attributed to standardisation of the tissue preservation and extraction protocol (Asahida et al, 1996).

A recent study by Sanudi et al, (2019) reported to have successfully cross-amplified SSR loci in *O. shiranus* with primer pairs developed for congeners with 100% of the loci utilised while retaining about 80% to score in multiplex amplification. Such as a successful amplification is very rare in the scientific community although it is not impossible. The study used 47 microsatellite loci (SSRs) and 33 mitochondrial markers previously developed by Tibihika et al, (2018) in the study to investigate the local adaptation of the Nile tilapia from native and non-native freshwater systems in Eastern and Western Africa to test their

transferability from *O. niloticus* onto *O. macrochir* and *O. mweruensis*. The mitochondrial markers genotyped were not analysed due to time limitations of the study. Using only microsatellite loci, about 70.21% were retained in the study and used to score the microsatellite loci. The two species under investigation were collected based on morphological features in the field from different freshwater systems constituting four Lakes in three river basins and three fish farms. The two species are reported to have been misidentified due to their occurrence and not understanding clearly their distribution (

as the DNA quality check under the Gel electrophoresis was not done for all the samples whose DNA was isolated and cross amplified during the two-polymerase chain reaction (PCR) stages. A study in Malawi during a showed 100% success rate in genomic DNA isolation and amplification of microsatellite loci in a congeneric species such as *O. shiranus* (Sanudi et al, 2019). Such a successful amplification is very rare in the scientific community though it usually leads to repeating of the failed samples. The extraction process is usually difficult to trace during isolation if all the samples have been successfully extracted and if others need to be repeated especially if the sample size is large. All the fish samples collected from the Longfin tilapia (*O. macrochir*), the Mweru tilapia (*O. mweruensis*) and Nile tilapia (*O. niloticus*) were fin clips collected from the caudal fin of the fish (Schwanck, 1994; De Vos et al, 2001; Moelants, (2010). The study set out to test the hypothesis as to whether the two species identified in the field using the field guide by Utsugi and Mazingaliwa, (2002); Skelton (2001) and Schwanck (1994) were different at a molecular level in addition to taxonomic identification and their phylogenetic and biogeographic distribution in genetically characterising the populations from the wild particularly from Lake Mweru and Lake Mweru-Wantipa and their placement position in the Southern African cichlids of commercial importance in Zambia.

After carefully screening the illumina reads using an in-house Genotyping by Sequencing pipeline python script developed by Manuel Curto and successfully employed in the studies on Hedgehogs (Curto et al, 2019) and the Nile tilapia (Tibihika et al, 2018; 2019; 2020), some alleles dropped out due to a lack in not genotyping successfully during the single PCR step or Multiplex PCR steps and the initial DNA isolation protocol used. The mitochondrial markers were incomplete and could not be used to address one of the objectives of the study due to a mix-up with the labelling and sample assignment of the P5 and P7 motif adapters. This fatal anomaly limited the study in distinguishing the two species that have now

been divided as subspecies at a molecular level as most alleles dropped out during the SSR-GBS pipeline run, a problem that only becomes apparent after sequencing of all the samples in a case of large sample sizes (Gagneux et al, 1997; Seiter, A., 2018; Tibihika et al, 2018; Curto et al, 2019). It has been reported in the same study by Gagneux et al, (1997) that alleles that are usually false are less frequent and shown an unusual pattern that has to be manually corrected using maximum likelihood approaches (Wang et al, 2012).

Studies by Bezault et al, (2012) described variable cross amplification among congeneric species with 67% for Tilapia species, 86% for Sarotherodon species, 88% for Oreochromis species among others species although Sanudi et al, (2019) reported 100% cross amplification something that rarely occurs but not impossible. This could have been possibly attributed to the utility of a single species unlike other studies (Bezault et al, 2012; Tibihika et al, 2018). The lower cross amplification shown in these results can be attribute to a high average number of alleles per loci with a mean value of 16.333±2.109 of the Longfin tilapia observed from Lake Mweru at Nchelenge landing site. These results are further supported by Bezault et al, (2012) who obtained a higher average number of alleles (Na) with a mean of 17.8 when compared to the 100% cross amplification success rate obtained by Sanudi et al, (2019) and a wider allelic range from 3 to 56 and also 1.8 to 13.9 respectively (Tibihika et al, 2018).

4.2. Population genetic differentiation and structure

During the study, it was observed that they were a few samples within certain populations that showed a departure from Hardy-Weinberg Equilibrium (HWE) such the *O. macrochir* samples from Kashikishi market on Lake Mweru with P values lesser than 0.05. The PCoA and STRUCTURE analysis clustered the populations of *O. macrochir* and *O. mweruensis* into four major clusters (see Figure 25 above) by providing a historical structural pattern of the Lakes and river basins (Evanno et al, 2005). The Evanno method clustered the species *O. macrochir* populations into four cluster groups. Figure 25 showed that the *O. macrochir* from Lake Mweru-Wantipa clustered together with the same population of the same species from Lake Mweru despite the two Lakes being geographically isolated by a distance of about 40 km (Wikipedia contributors, 2019). The population of *O. macrochir* from Lake Kariba (Africa's largest man-made dam) in the Zambezi river basin seemed to be slightly clustered with the *O. macrochir* from Lake Bangweulu despite the distance between them estimated to be over 700 km apart and no connectivity between them. The distributions of cichlids in Zambian freshwater systems that may be associated with geological events is not clearly understood (Genner et al, 2007; Meier et al, 2019). The hydrogeography of the Zambia allows

for a geographical barrier to gene flow amongst Lake Mweru, Lake Mweru-Wantipa, Lake Bangweulu and Lake Kariba (see Figure 7) corresponding to K = 3 being the likely population structure of *O. macrochir* in Zambia. For *O. mweruensis* the geographical isolation of 40 km west of Lake Mweru acts as a barrier to gene flow between the two Lakes (Mweru and Mweru-Wantipa) both found in the Congo river basin.

Although there is no connectivity between Lake Mweru and Lake Mweru-Wantipa found in the Congo river basin, the PCoA and STRUCTURE analysis did not support the two populations to being genetically differentiated from each other as they were clustered together and this was supported by the structure analysis showing no distinct structure between the two lakes of the same species (see Figures 25, 33). Since Lake Bangweulu is connected to Lake Mweru by the Luapula river we would expect gene flow between the two Lakes though the results of this study indicate the two populations to be genetically differentiated from each other. This was supported by the fact from literature by Thieme et al, (2005) that the fauna between the two lakes were geographically isolated by the Mambatuta (also known as Mumbotuta) falls which prevents upstream gene flow into Lake Bangweulu via the Luapula river and the distance of over 560 km between them. Gene flow downstream from Lake Bangweulu to Lake Mweru is possible however the long distance could be one of the main attributes as to why the populations were distinct. Bbole et al, (2019) investigated the genetic differentiation of *O. macrochir* from Lake Bangweulu using eighteen (18) microsatellite loci that were previously used for SSR loci analysis of the *O. mossambicus* and *O. niloticus* tissue samples according to D'Amato et al, (2007). The findings of this study corroborated with their previous findings although they sampled along the Kafue river that feeds into Lake Kariba and not directly from Lake as was done in this study. The population structure within the four Lakes investigated housing *O. macrochir* indicated gene flow limitation which could have been as a result of the lakes having different geographic distances, biogeographic conditions, and a lack of habitat connectivity (Mills et al, 2007).

4.3. Genetic diversity of *O. macrochir* and *O. mweruensis* in Zambia

The genetic diversity of the two species *O. macrochir* and *O. mweruensis* populations in Zambia in number of alleles ranged from 2.939±0.292 to 16.333±2.109 and expected heterozygosity value ranging between 0.455±0.051 to 0.649±0.049. A similar study by Bbole et al, (2019) on *O. macrochir* reported a high observed heterozygosity of 0.946±0.041 for Lake Bangweulu. The results of this study however showed a very low observed heterozygosity (Ho) of 0.412±0.055 and expected heterozygosity (He) of 0.481±0.054 which were not similar to

the previous findings by Bbole et al, (2019) on the same Lake. Furthermore, the findings from the recent study showed lower heterozygosity values from the studies on O. mossambicus in Mozambique by Simbine et al, (2014) and *O. niloticus* and *O. mossambicus* by D'Amato et al, (2007) that supported the findings from Bbole et al, (2019). The results of this study for *O. macrochir* had an expected heterozygosity of 0.557±0.016 similar to the literature values from DeWoody and Avise, (2000) with an expected heterozygosity of 0.54±0.25. *O. mweruensis* had an expected heterozygosity ranging from 0.555±0.049 for Lake Mweru and 0.609±0.047 for Lake Mweru-Wantipa. These results between the two Lakes were not significant from each other. Therefore, high genetic diversity is necessitated in constantly changing environments to provide genetic baseline for upgradation programs in aquaculture development and promoting the culture of indigenous stocks with novel genetic traits (Takahashi et al, 2016; Kefi and Mwango, 2018; Bbole et al, 2019). Even though the private number of alleles was low with an average of 0.621±0.136, the observed genetic diversity in populations of *O. macrochir* and *O. mweruensis* was relatively high and offered an important baseline for aquaculture development in the country.

Chapter 5

5. Conclusion

The species *O. macrochir* and *O. mweruensis* are two important ecological and commercial cichlids due to the functions and structure they play in the Zambian freshwater ecosystems. With the upgradation programmes in fisheries and aquaculture in Zambia, it calls for critical strategies to conserve genetic resources from the wild populations and ensure the sustainable utilisation of the fish germplasm. The analysis conducted to differentiate the two species using the 33 microsatellite loci using the PCoA and STRUCTURE showed significant differences in the cultured populations from their founding wild populations. The distinction of the four major populations into the three water basins implies that the populations even though they are the same species form different stocks and may thus require different genetic conservation measures and management strategies.

The aquaculture populations showed significant differences in the genetic diversity from the wild populations from the four natural Lakes. The population differentiation and structuring also suggested that the origin of the founding populations in the three government fish farms could have possibly come from Lake Mweru because of their close relationship to the Longfin tilapia on the Congo basin. Great care would have to be taken when undertaking an aquaculture project when recruiting brood stock from the wild populations and avoiding the mixing of similar species that have different a genetic structure. Selective information on the fish population genetic structure and genetic diversity form the baseline on which aquaculture development can continue to foster in ensuring achievement of sustainable development goal number 1 and 2.

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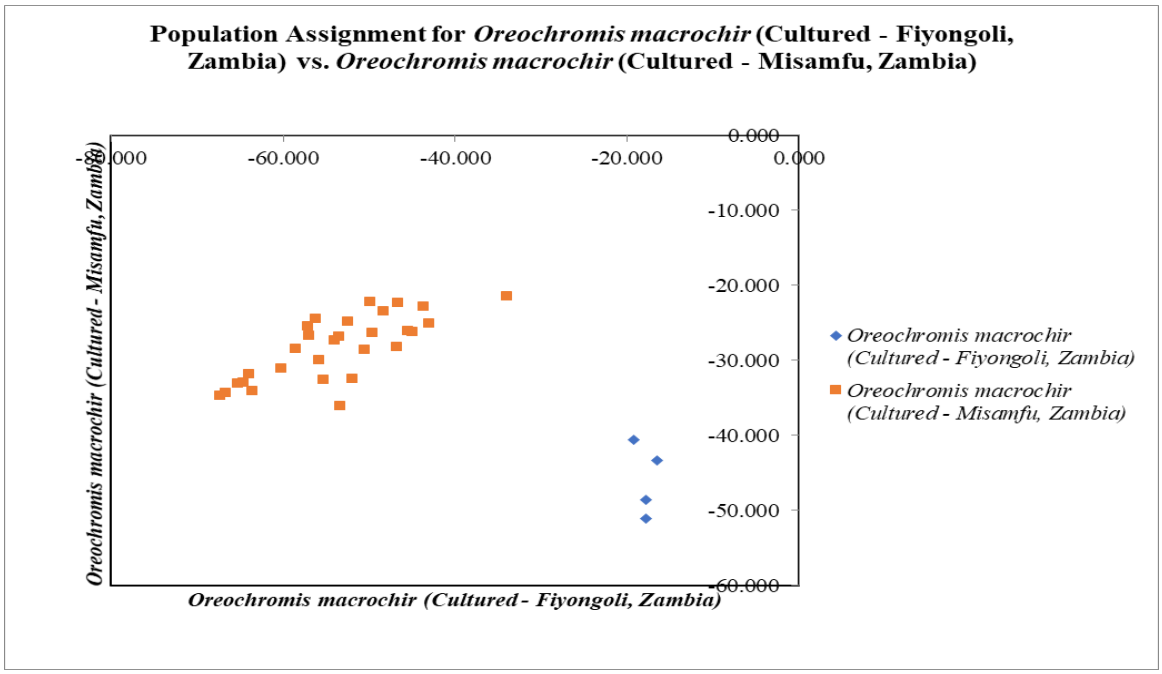
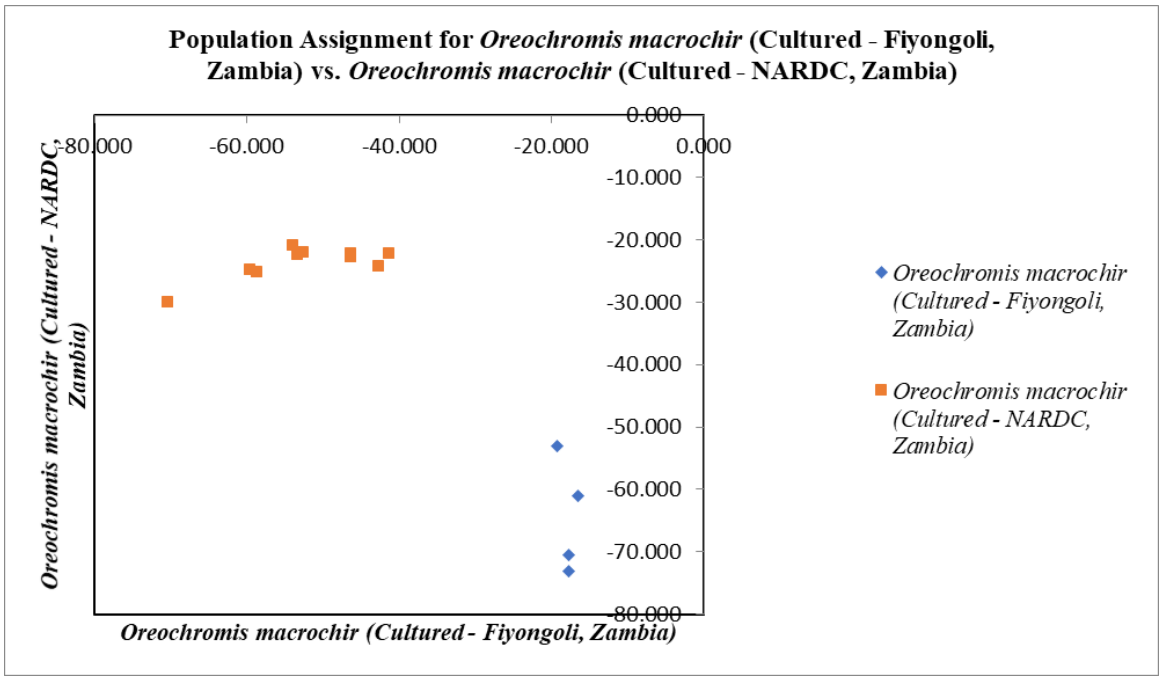
Appendices

Annex 1 Population assignment of Fiyongoli vs Misamfu



Annex 2 Population assignment of Fiyongoli vs NARDC



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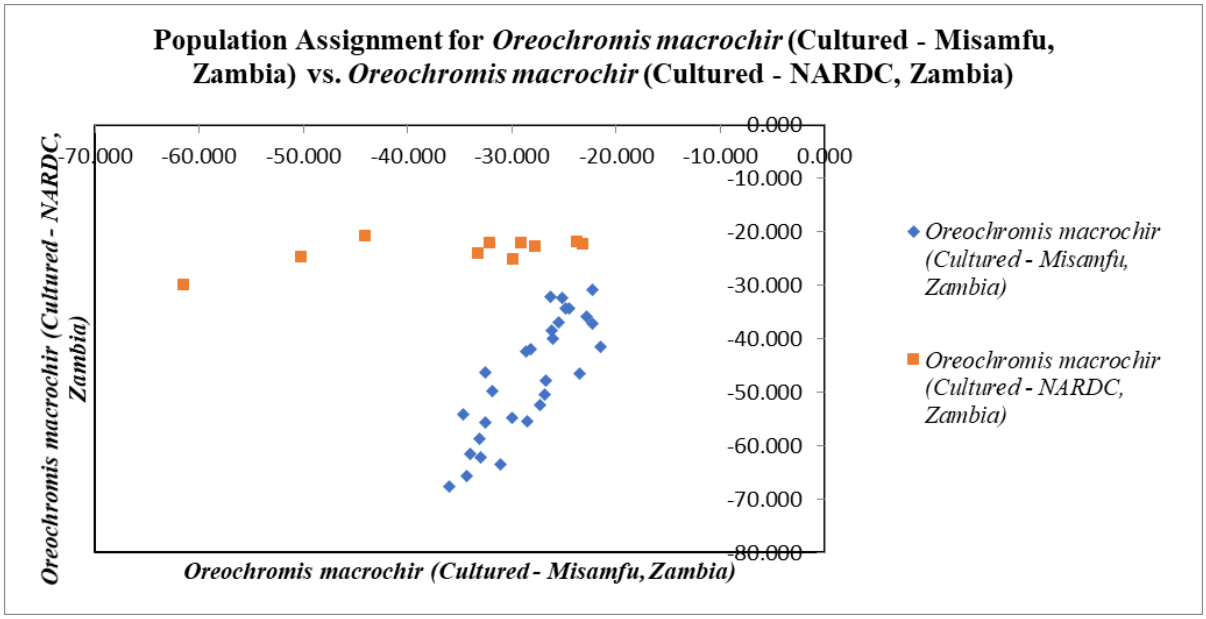
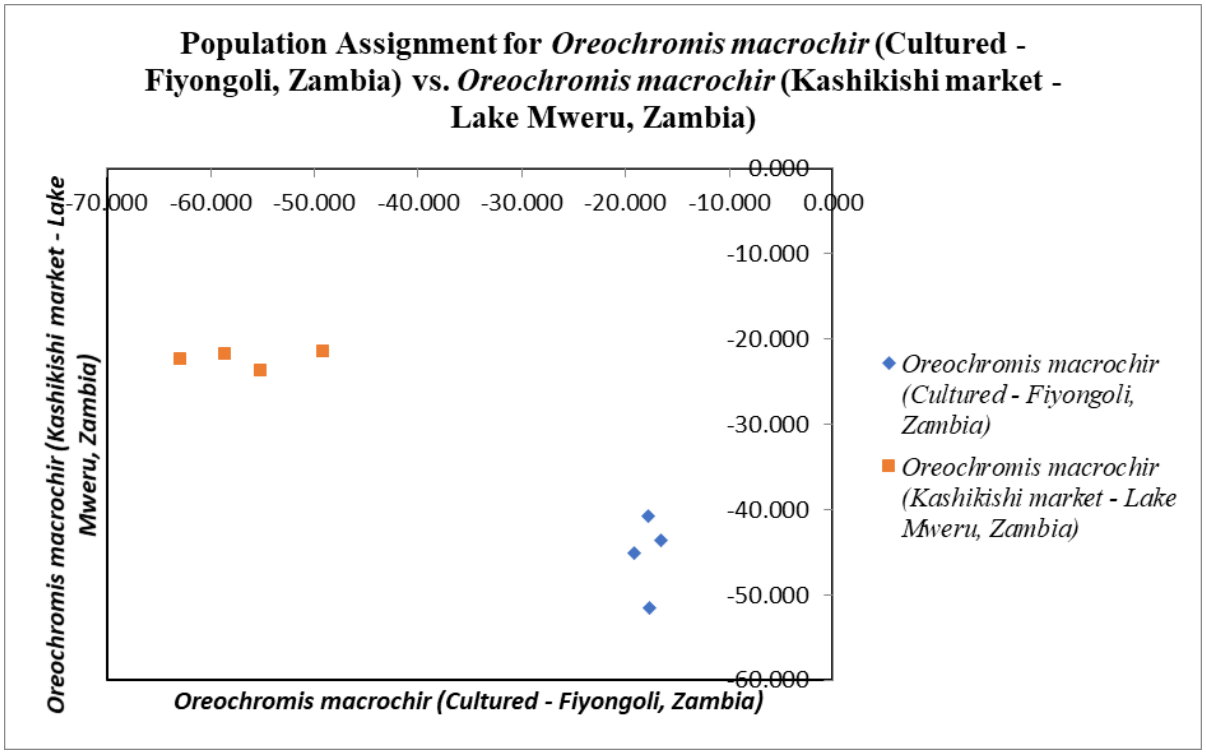
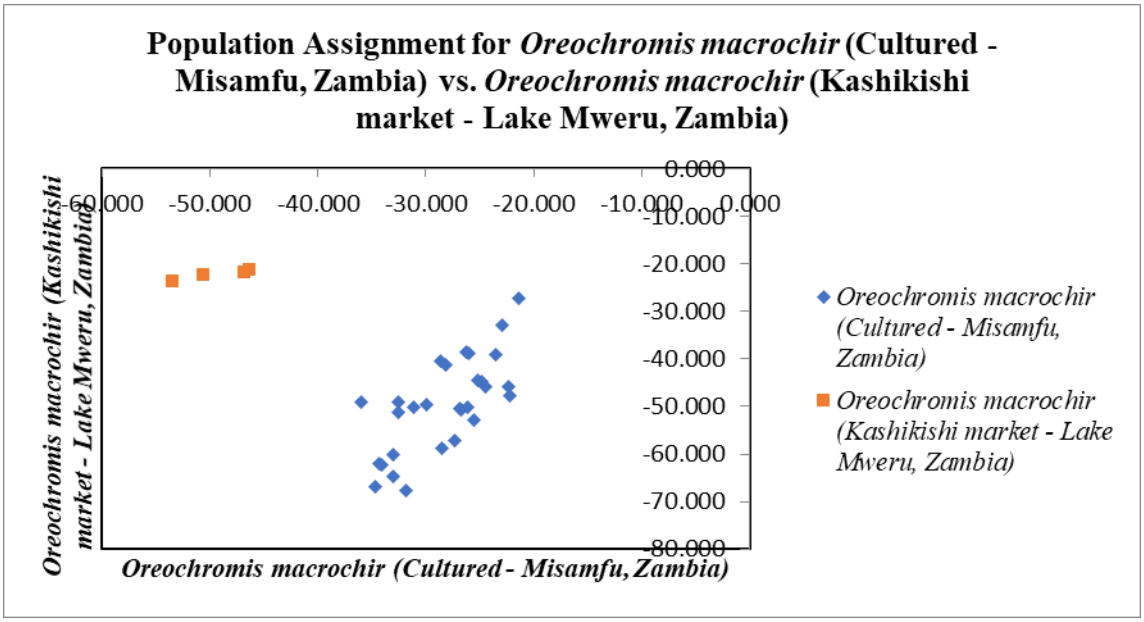


Annex 4 Population assignment of Fiyongoli vs Kashikishi market, Lake Mweru

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Oreochromis macrochir (Kashikishi market - Lake Mweru, Zambia)
Oreochromis macrochir (Cultured - Misamfu, Zambia)
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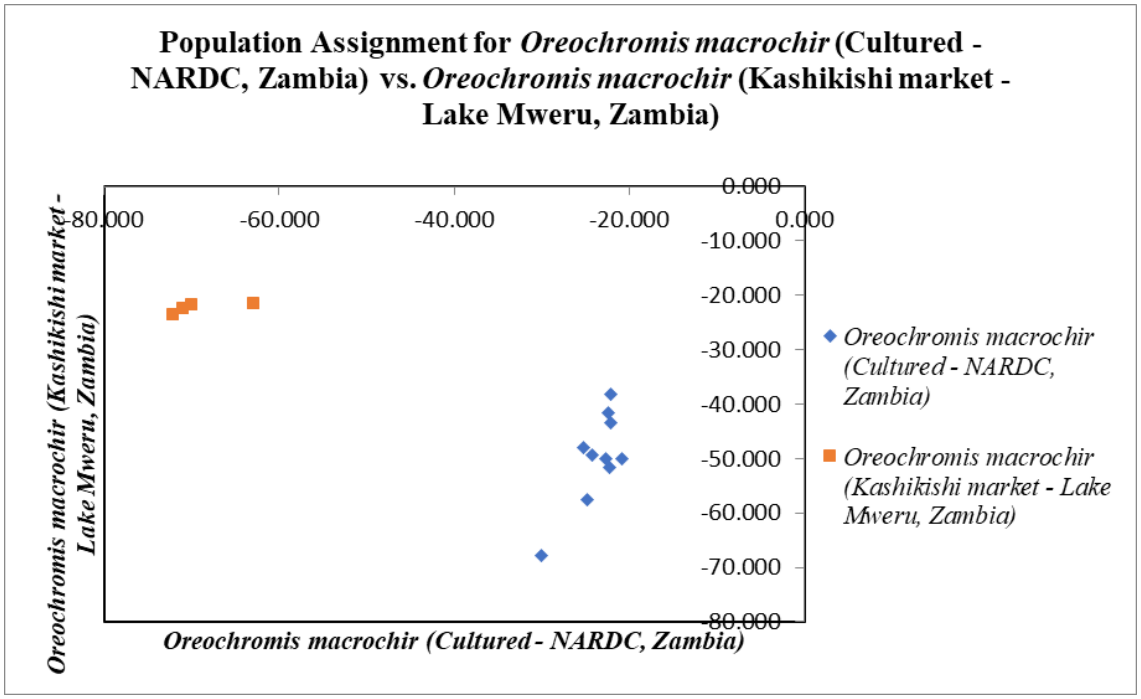
Oreochromis macrochir (Cultured - Misamfu, Zambia)
Oreochromis macrochir (Kashikishi market - Lake Mweru, Zambia)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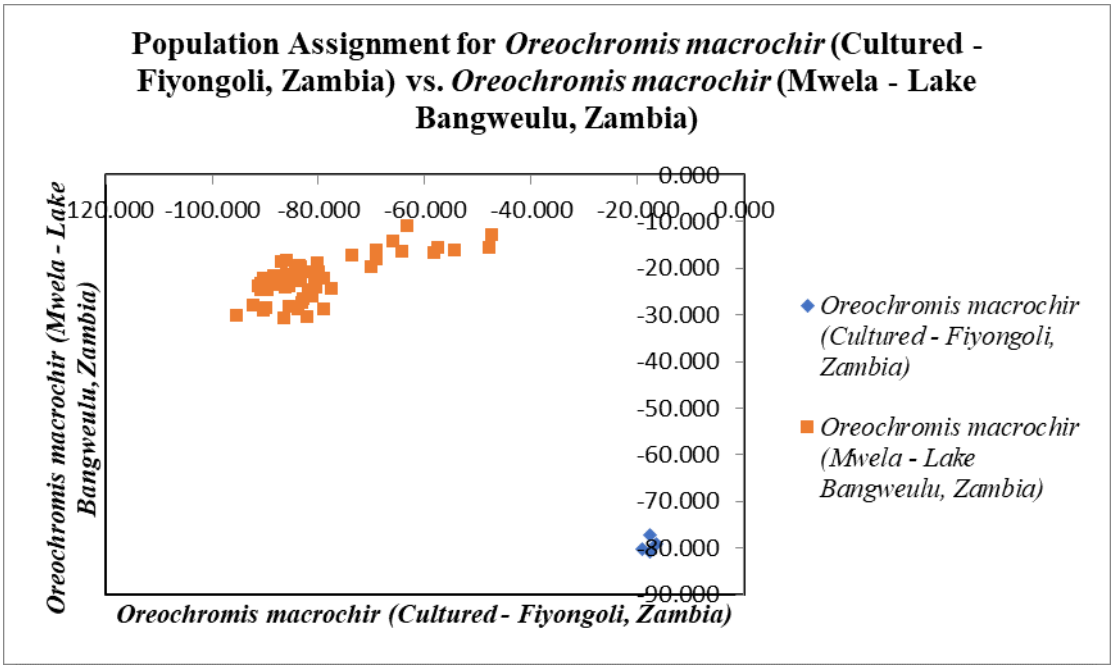
Oreochromis macrochir (Kashikishi market - Lake Mweru, Zambia)


Population Assignment for Oreochromis macrochir (Cultured - Fiyongoli, Zambia) vs. Oreochromis macrochir (Mwela - Lake Bangweulu, Zambia)
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Oreochromis macrochir (Cultured - Fiyongoli, Zambia)
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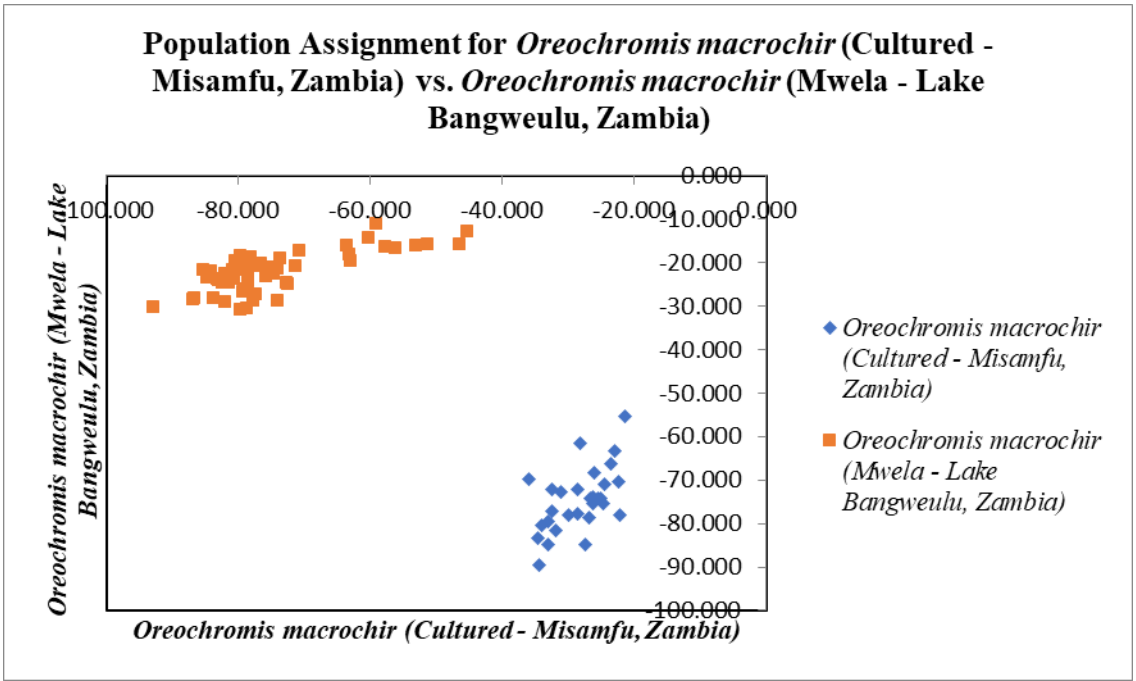
Oreochromis macrochir (Mwela - Lake Bangweulu, Zambia)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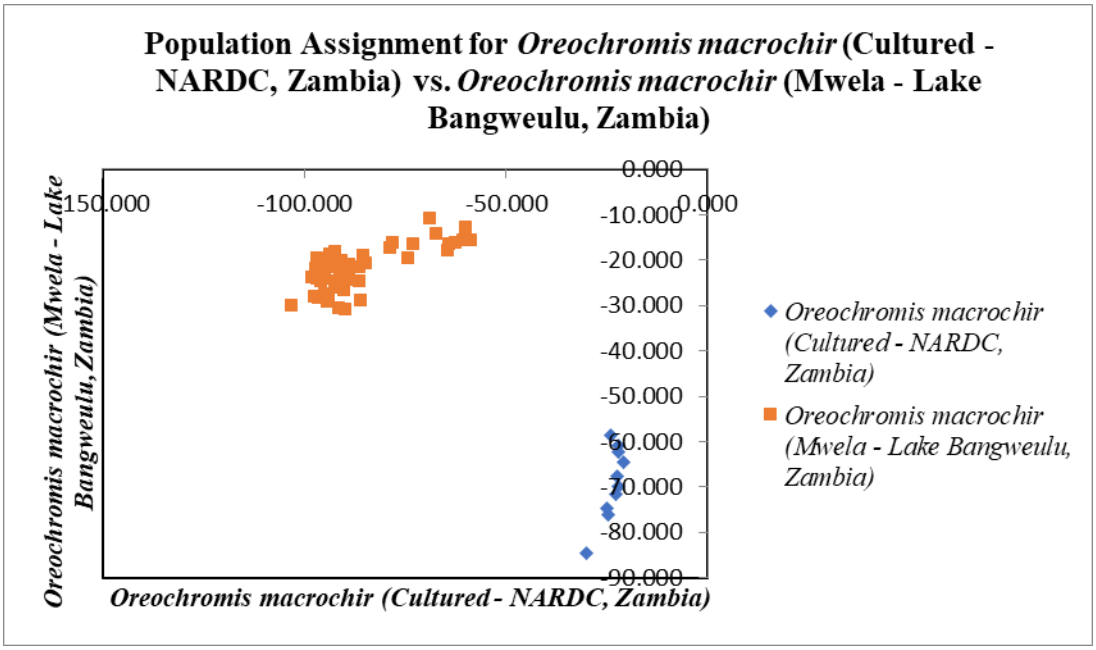
Oreochromis macrochir (Mwela - Lake Bangweulu, Zambia)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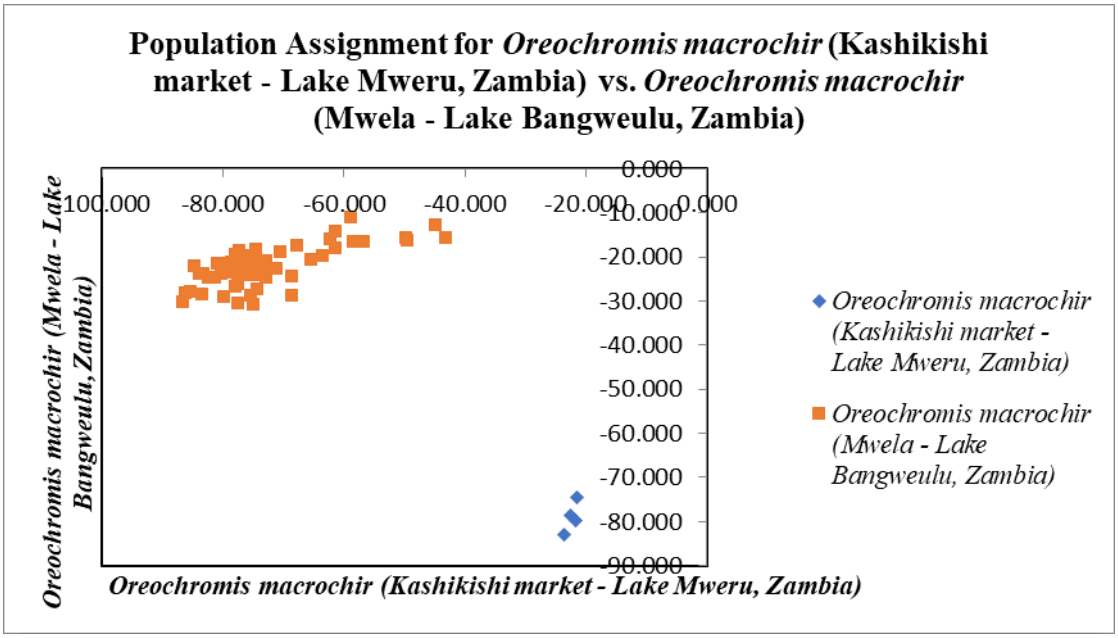
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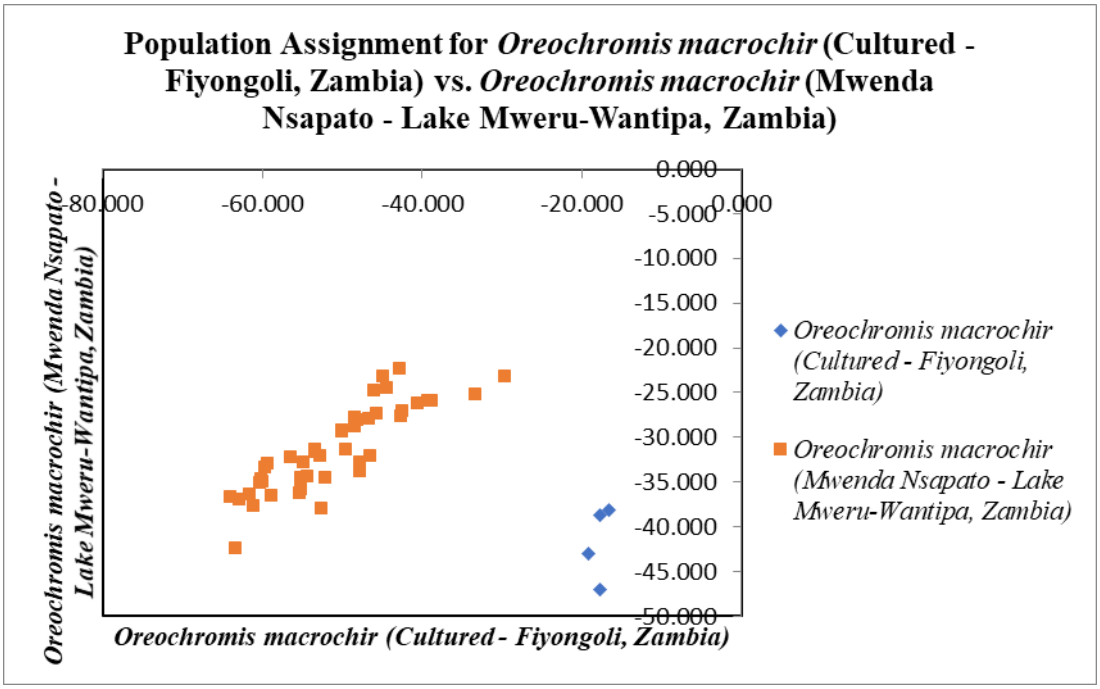
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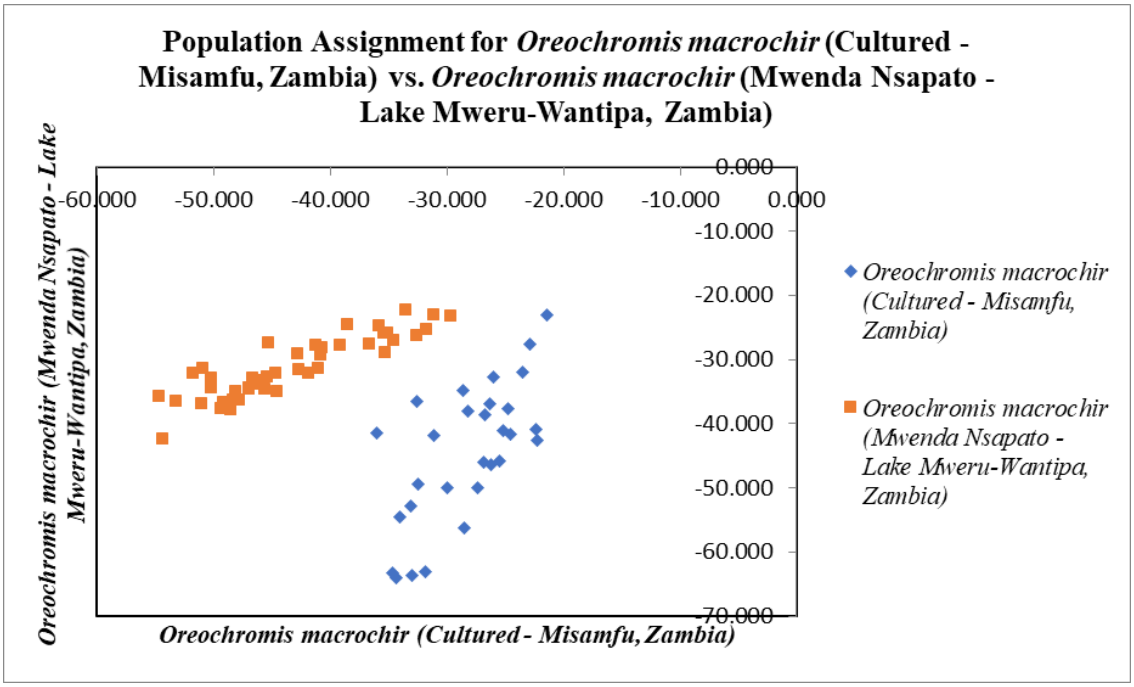
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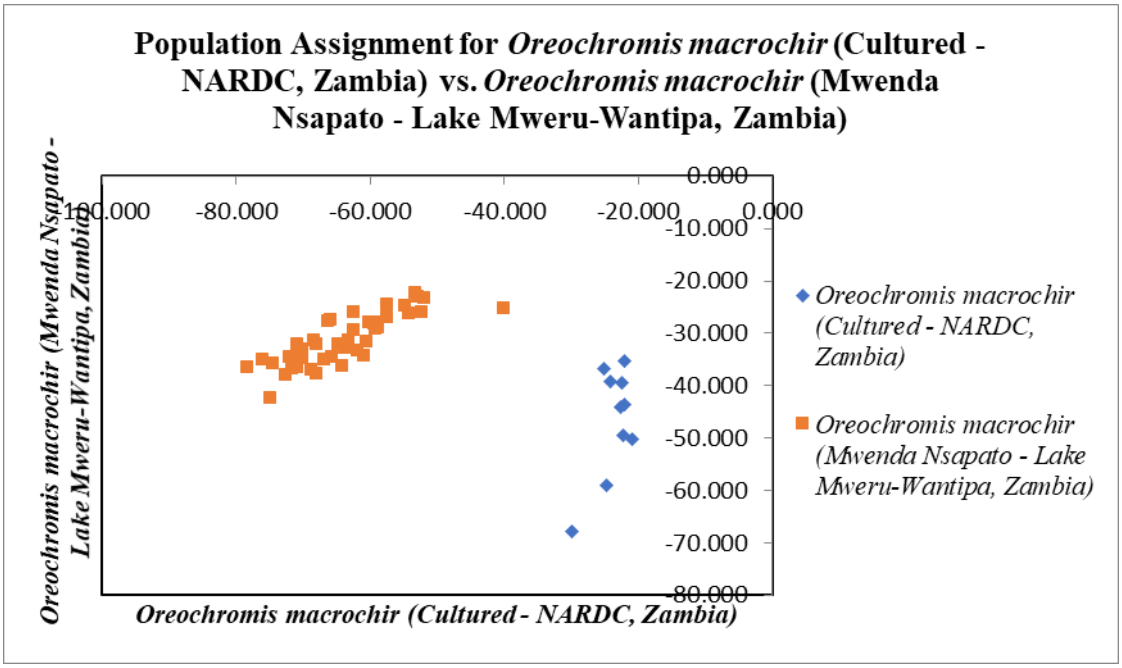
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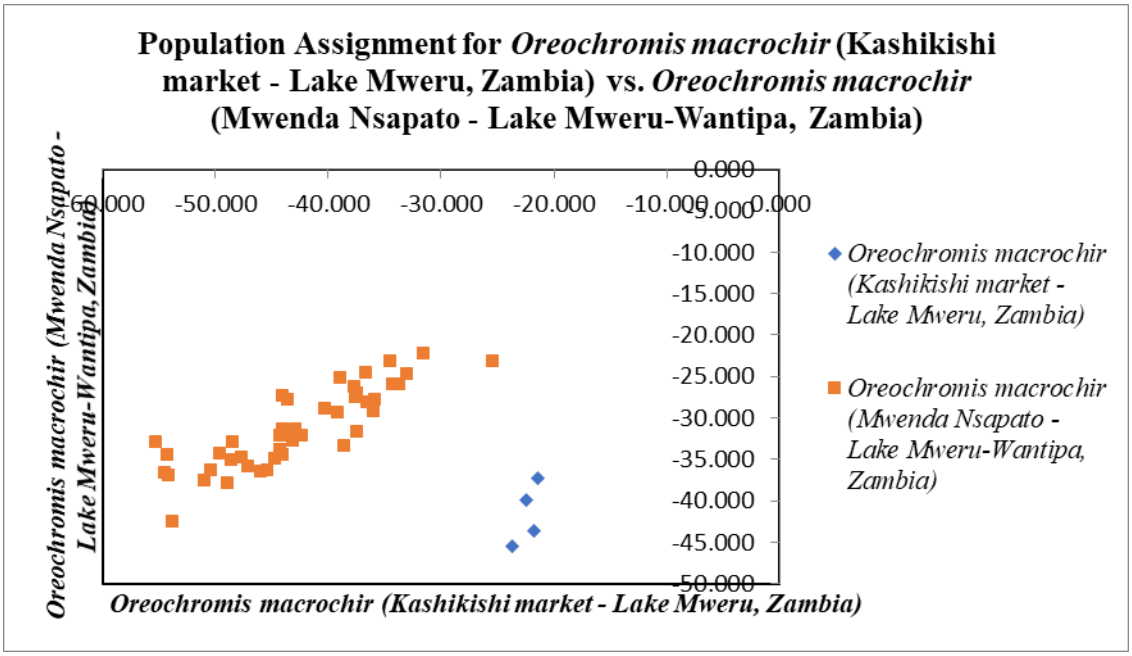
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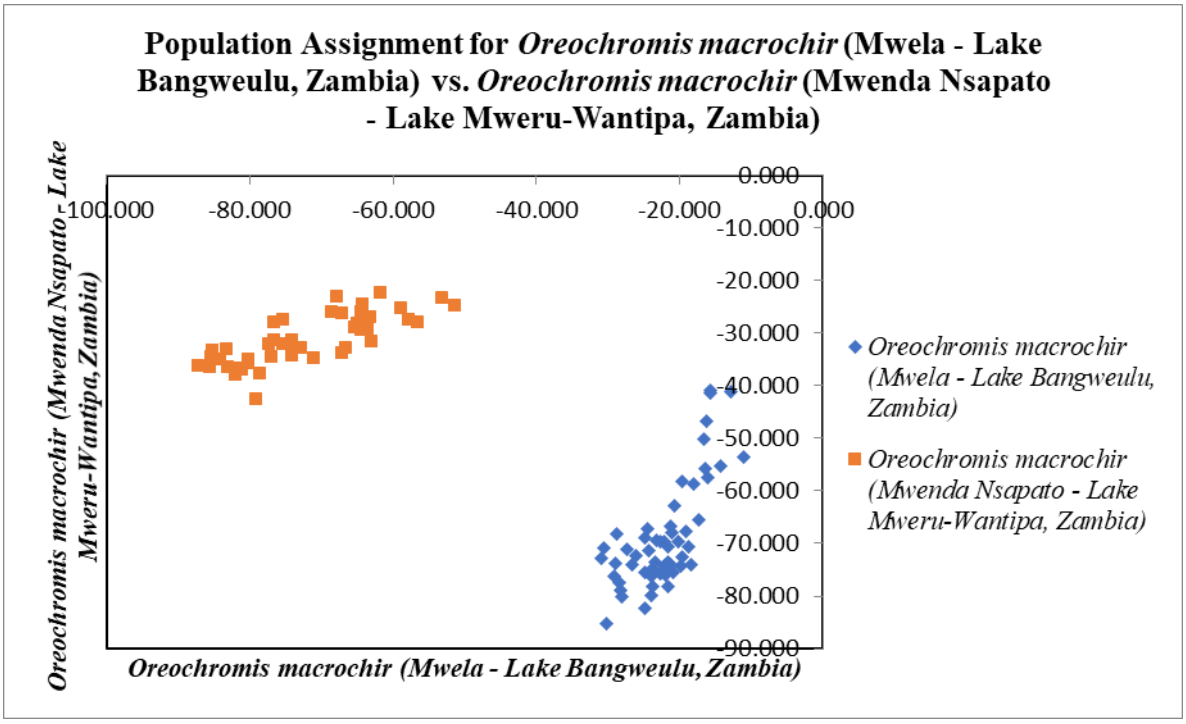
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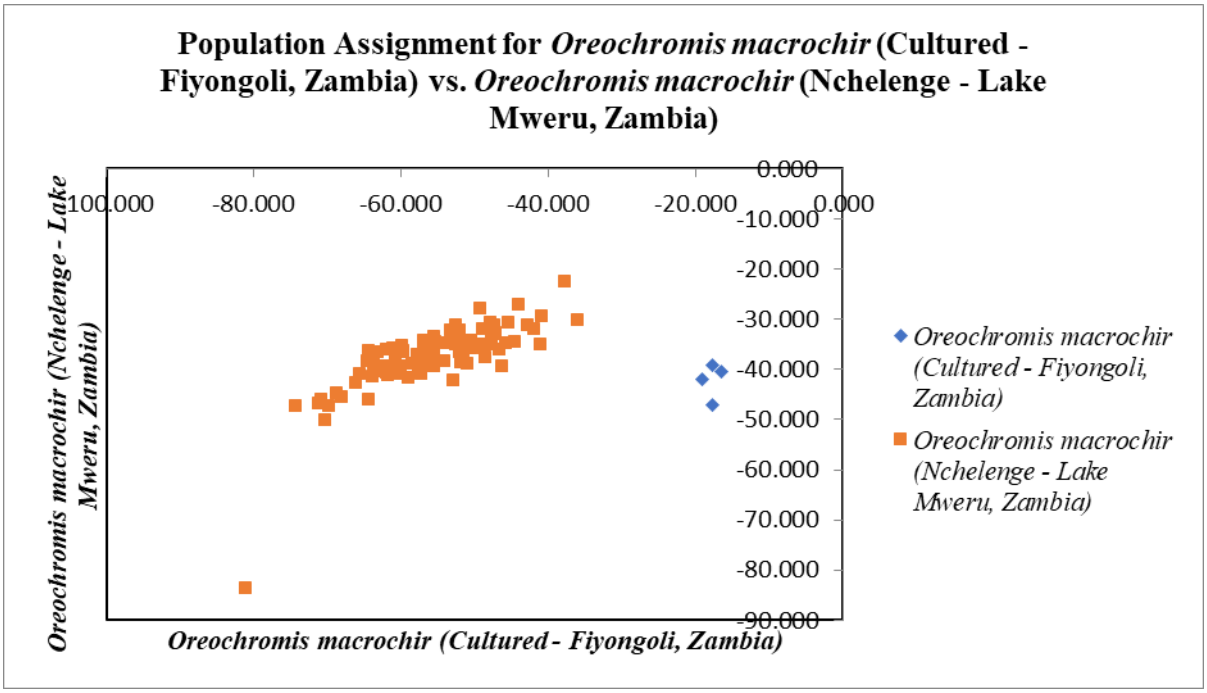
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Oreochromis macrochir (Cultured - Fiyongoli, Zambia)
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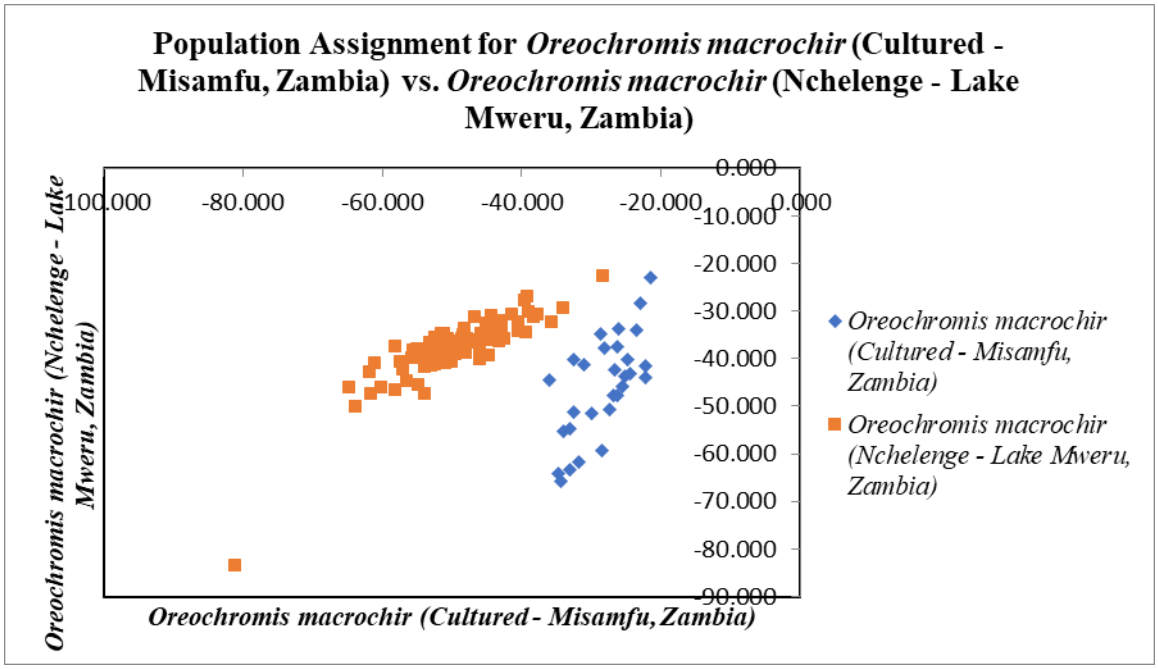
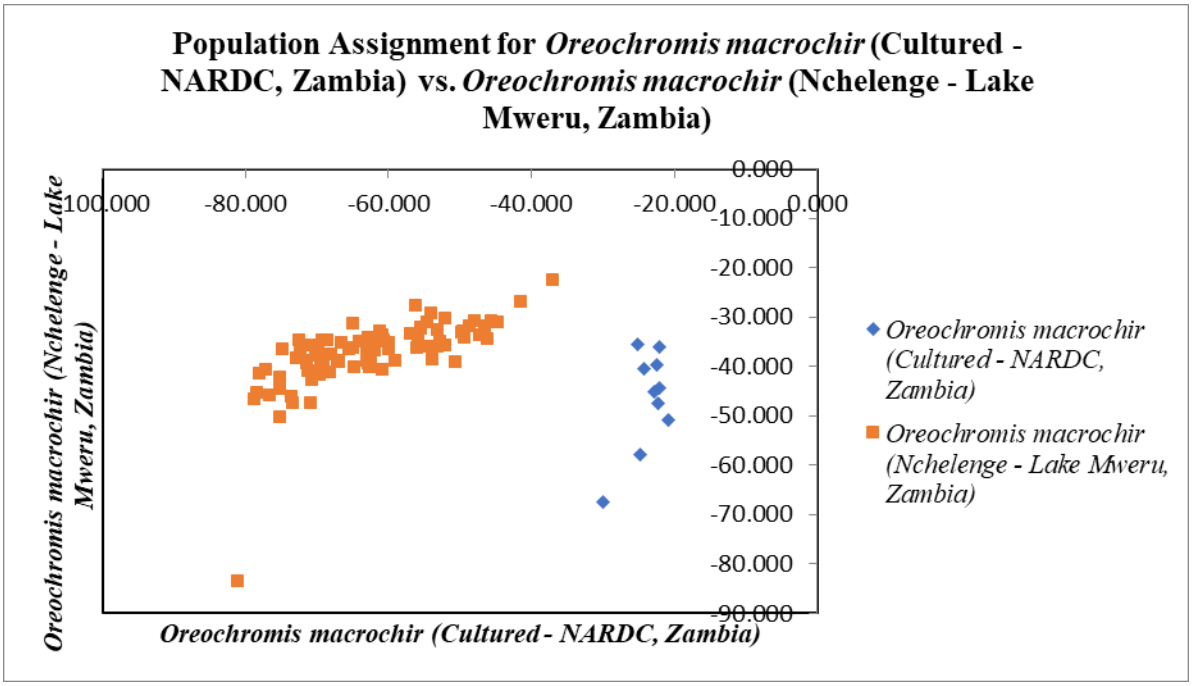
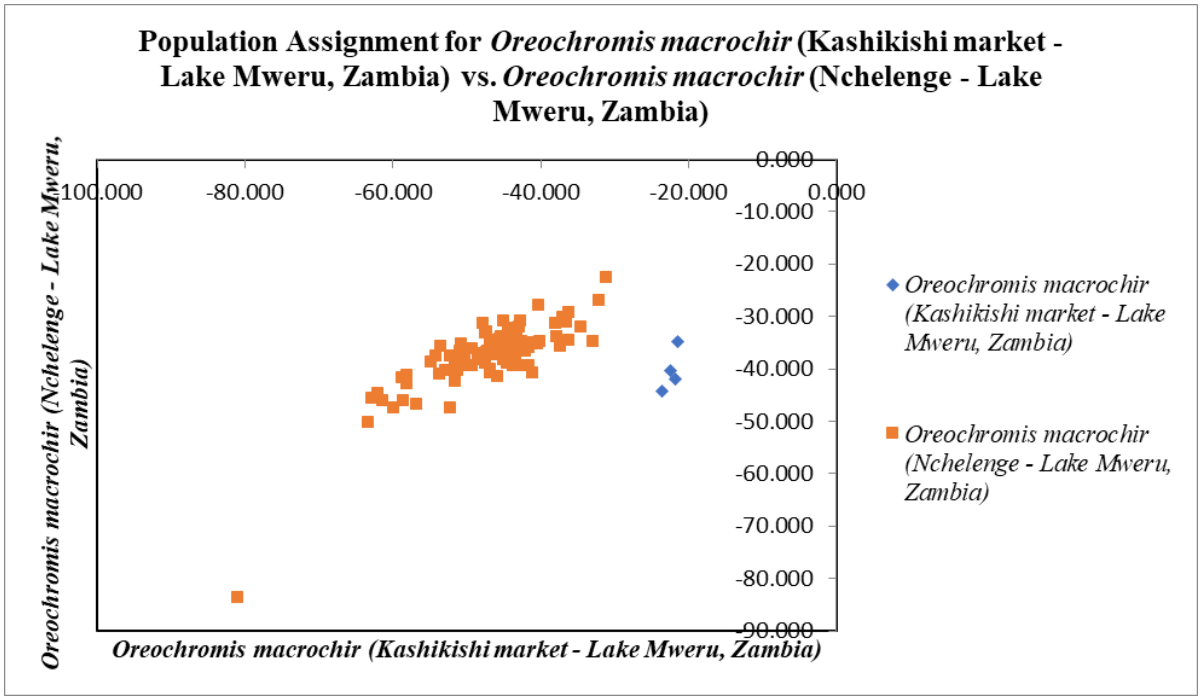
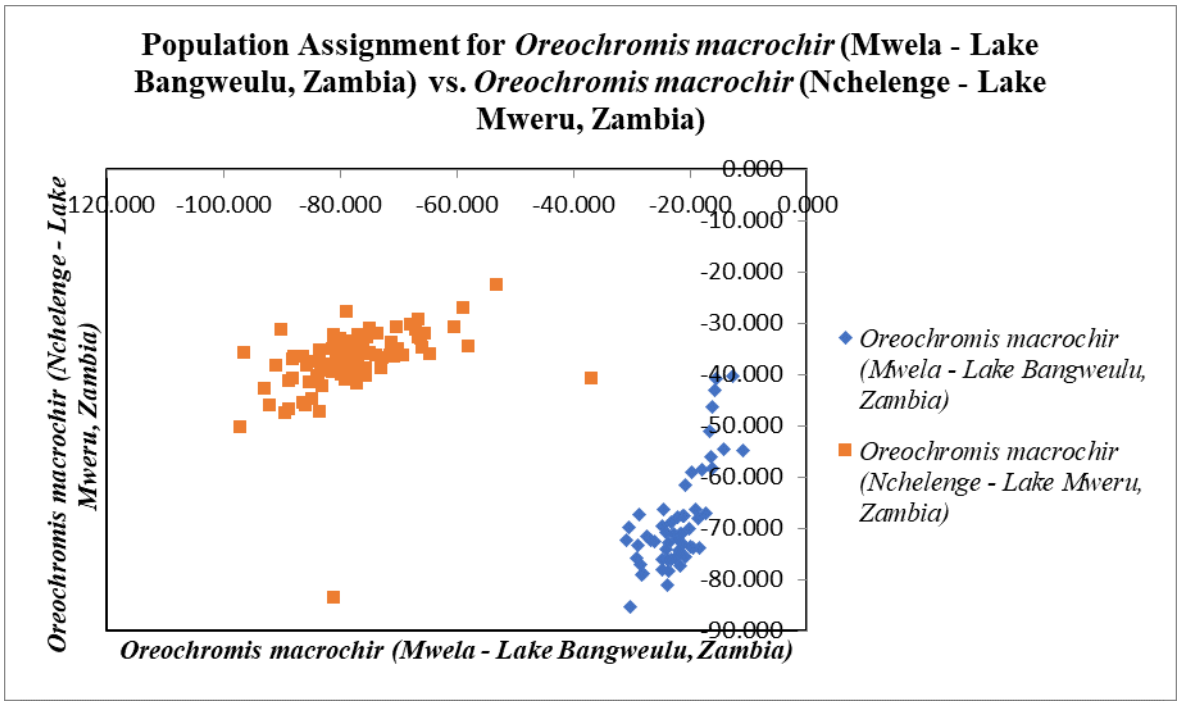
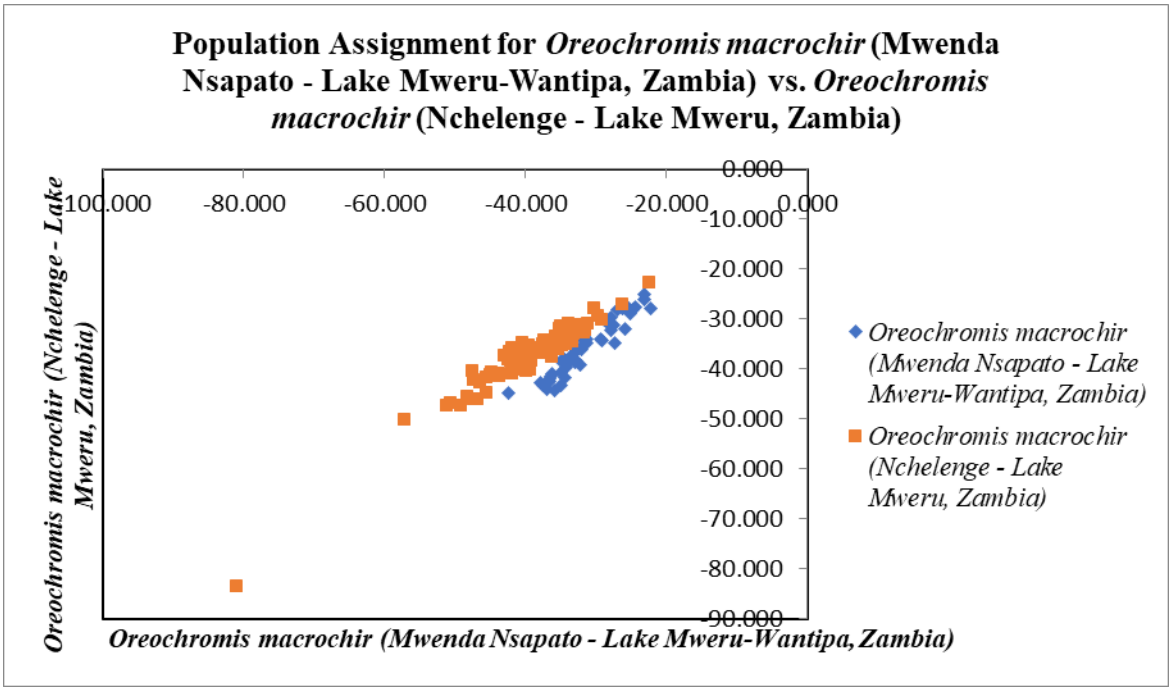
-80.000
-60.000
-40.000
-20.000
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-90.000
Oreochromis macrochir (Cultured - Fiyongoli, Zambia)
Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)

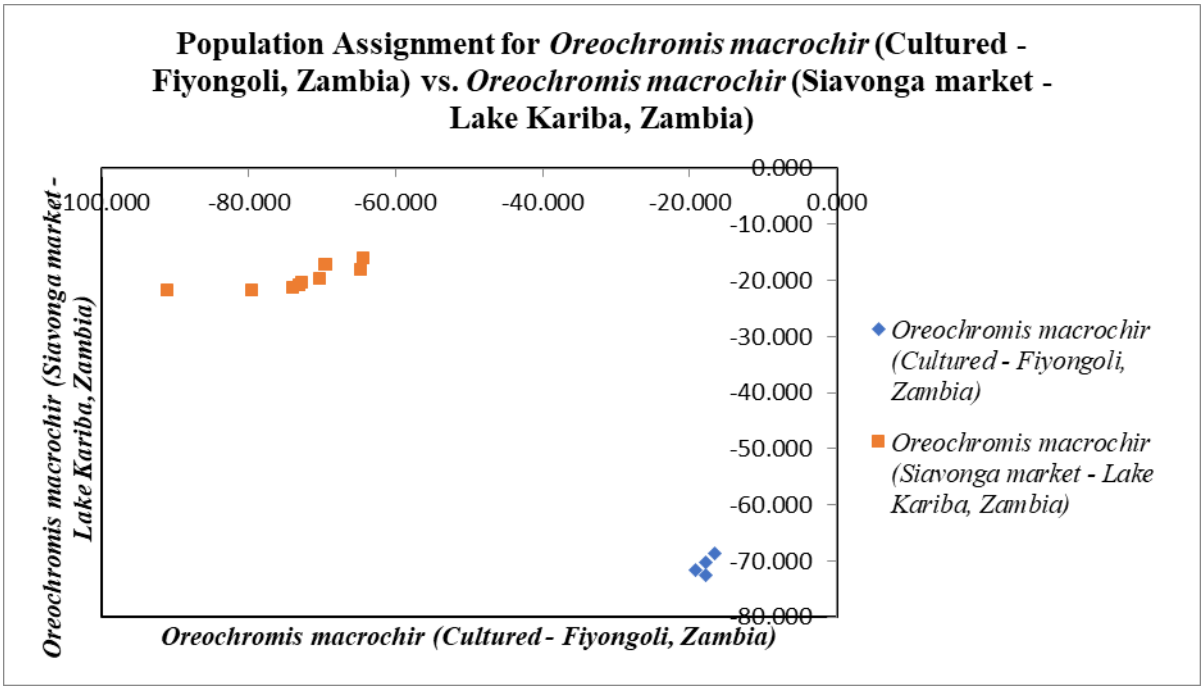

Population Assignment for Oreochromis macrochir (Cultured - Misamfu, Zambia) vs. Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Cultured - Misamfu, Zambia)
-100.000
-80.000
-60.000
-40.000
-20.000
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-90.000
Oreochromis macrochir (Cultured - Misamfu, Zambia)
Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)

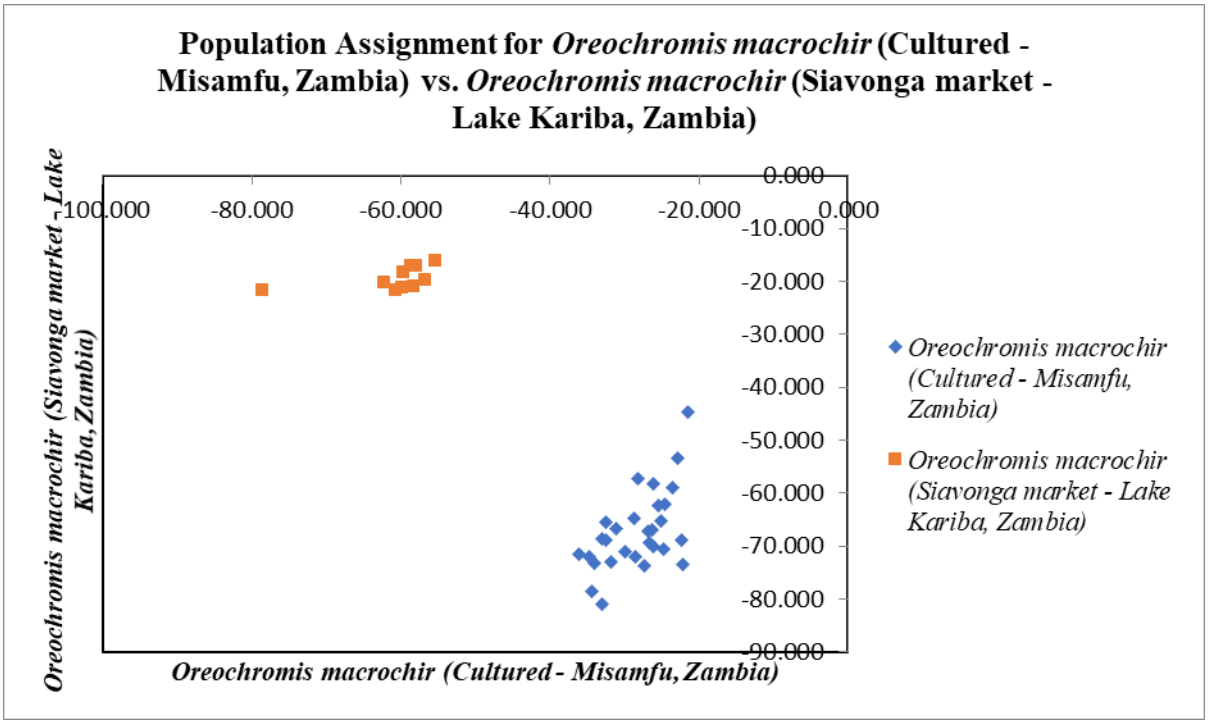

Population Assignment for Oreochromis macrochir (Cultured - NARDC, Zambia) vs. Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Cultured - NARDC, Zambia)
-100.000
-80.000
-60.000
-40.000
-20.000
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-90.000
Oreochromis macrochir (Cultured - NARDC, Zambia)
Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)

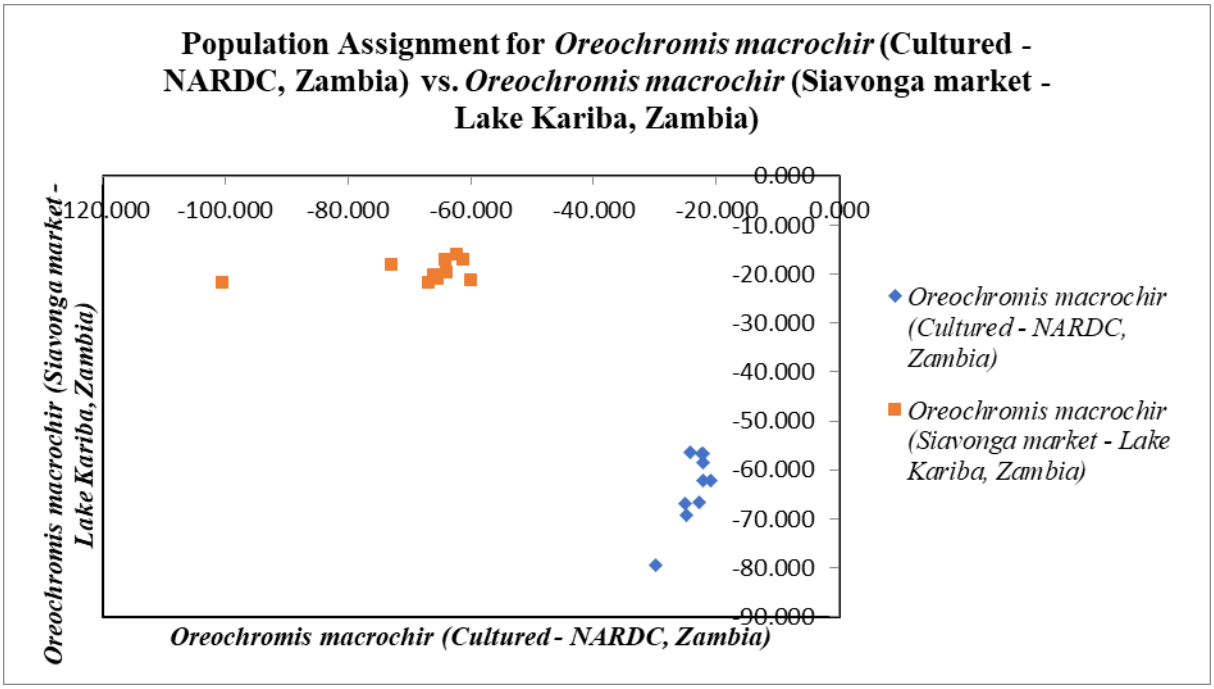

Population Assignment for Oreochromis macrochir (Kashikishi market - Lake Mweru, Zambia) vs. Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Kashikishi market - Lake Mweru, Zambia)
-100.000
-80.000
-60.000
-40.000
-20.000
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-90.000
Oreochromis macrochir (Kashikishi market - Lake Mweru, Zambia)
Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)

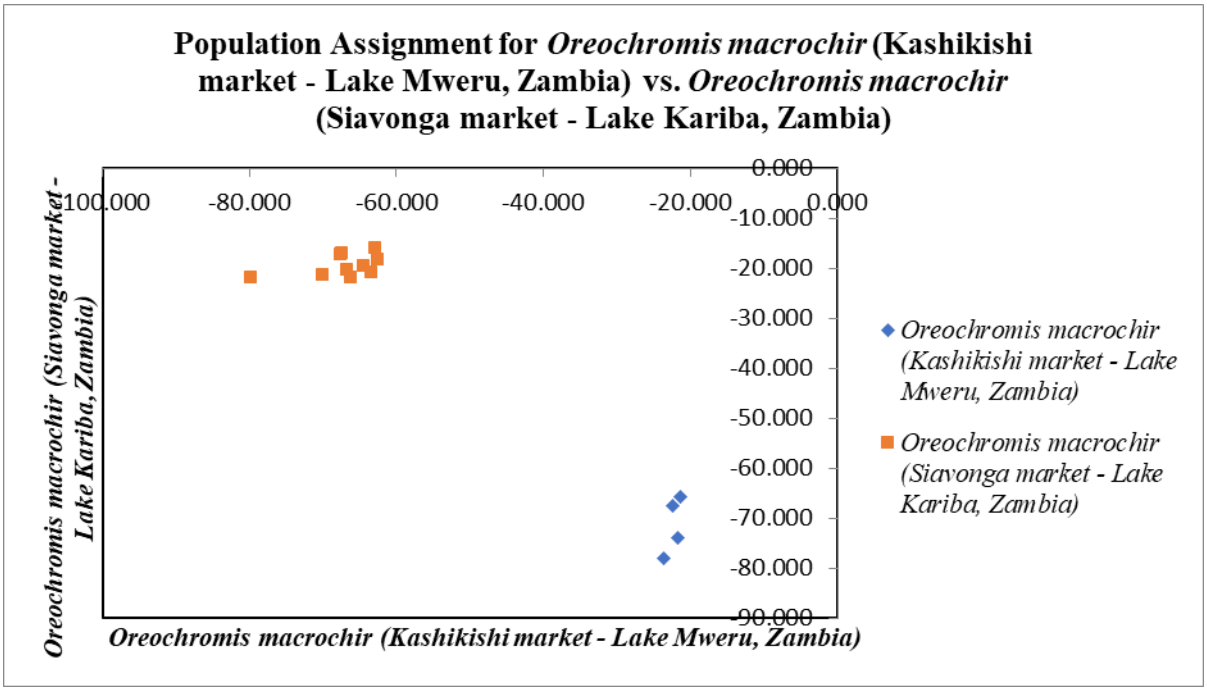

Population Assignment for Oreochromis macrochir (Mwela - Lake Bangweulu, Zambia) vs. Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Mwela - Lake Bangweulu, Zambia)
-120.000
-100.000
-80.000
-60.000
-40.000
-20.000
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-90.000
Oreochromis macrochir (Mwela - Lake Bangweulu, Zambia)
Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)

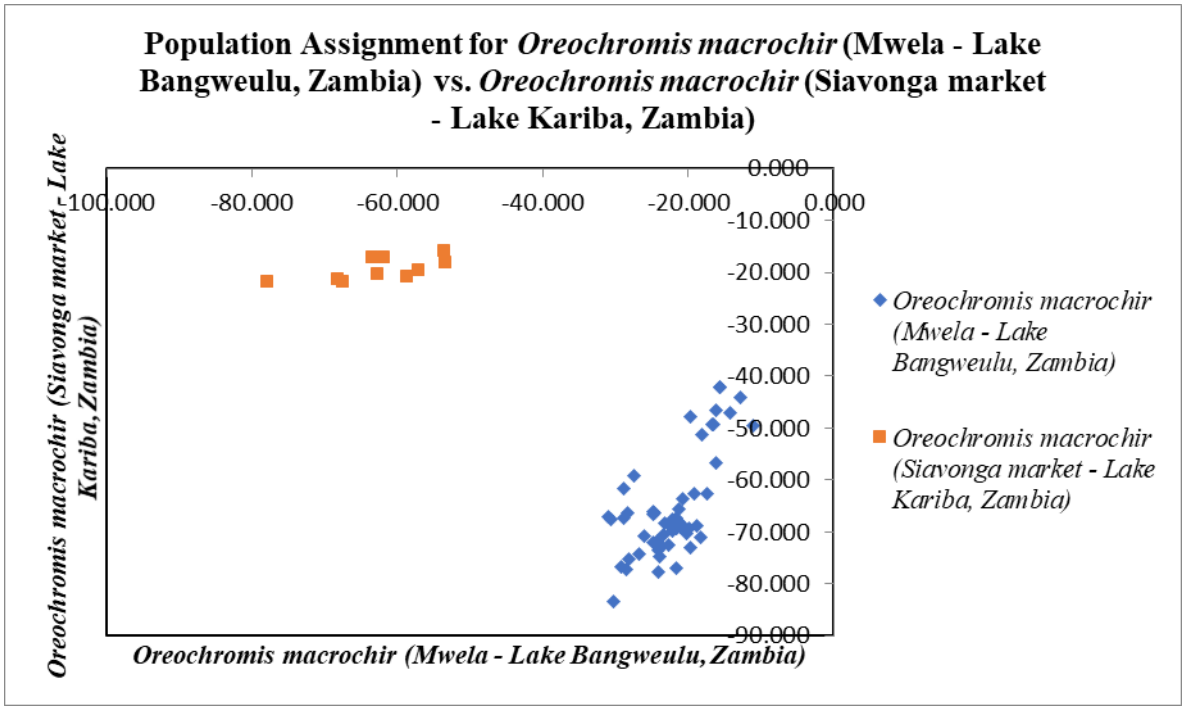

Population Assignment for Oreochromis macrochir (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia) vs. Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-90.000
-100.000
-80.000
-60.000
-40.000
-20.000
0.000
Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
Oreochromis macrochir (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)

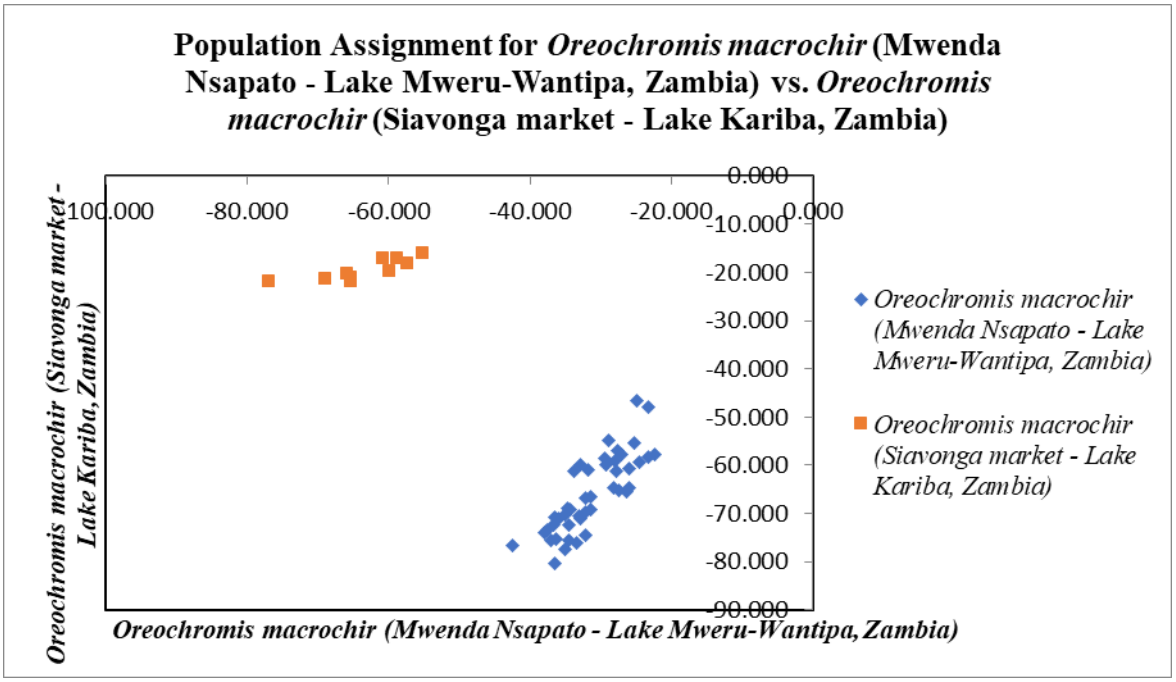

Population Assignment for Oreochromis macrochir (Cultured - Fiyongoli, Zambia) vs. Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-100.000
-80.000
-60.000
-40.000
-20.000
0.000
Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)
Oreochromis macrochir (Cultured - Fiyongoli, Zambia)
Oreochromis macrochir (Cultured - Fiyongoli, Zambia)
Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)

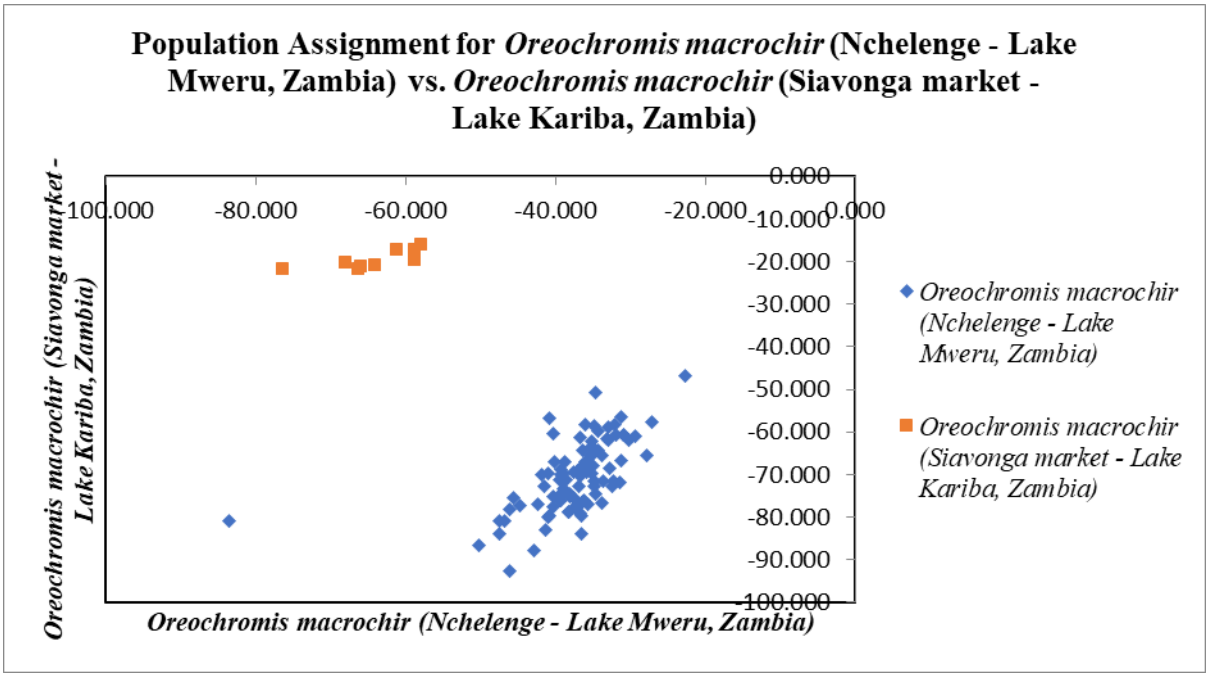

Population Assignment for Oreochromis macrochir (Cultured - Misamfu, Zambia) vs. Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)
Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)
Oreochromis macrochir (Cultured - Misamfu, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-90.000
-100.000
-80.000
-60.000
-40.000
-20.000
0.000
Oreochromis macrochir (Cultured - Misamfu, Zambia)
Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)

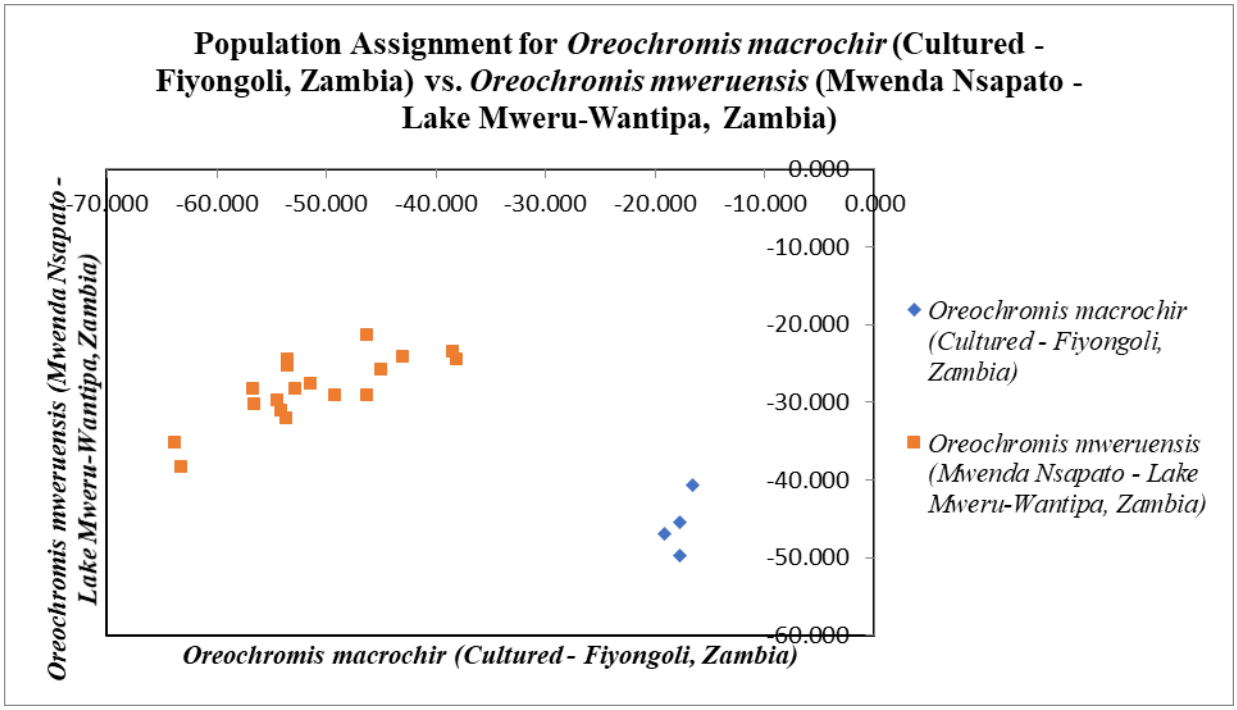

Population Assignment for Oreochromis macrochir (Cultured - NARDC, Zambia) vs. Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)
Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)
Oreochromis macrochir (Cultured - NARDC, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-90.000
-120.000
-100.000
-80.000
-60.000
-40.000
-20.000
0.000
Oreochromis macrochir (Cultured - NARDC, Zambia)
Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)

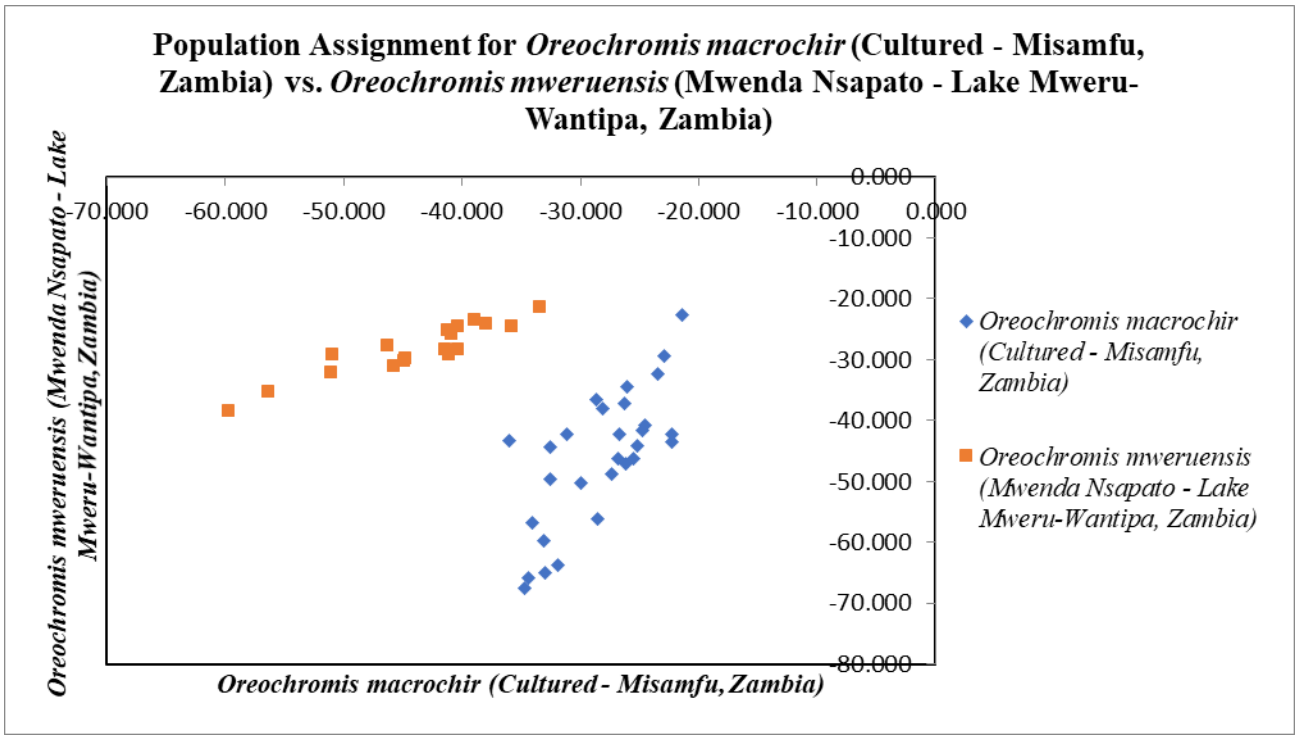

Population Assignment for Oreochromis macrochir (Kashikishi market - Lake Mweru, Zambia) vs. Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-90.000
-100.000
-80.000
-60.000
-40.000
-20.000
0.000
Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)
Oreochromis macrochir (Kashikishi market - Lake Mweru, Zambia)
Oreochromis macrochir (Kashikishi market - Lake Mweru, Zambia)
Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)

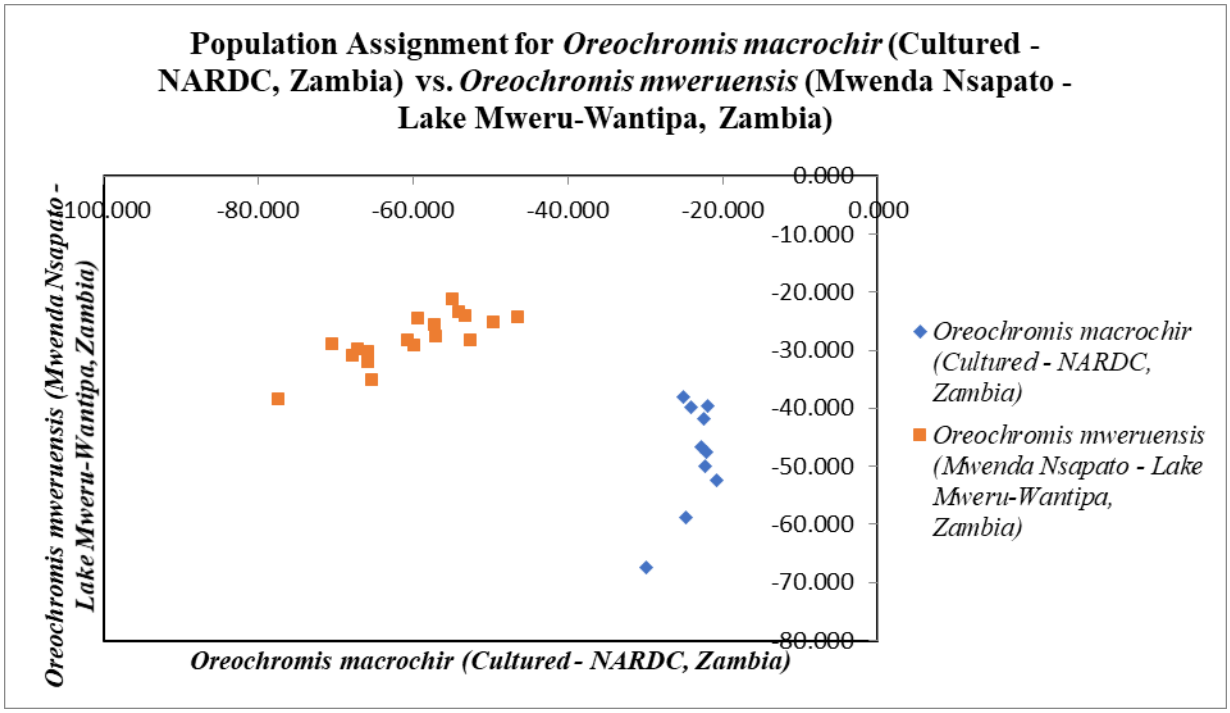

Population Assignment for Oreochromis macrochir (Mwela - Lake Bangweulu, Zambia) vs. Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-90.000
-100.000
-80.000
-60.000
-40.000
-20.000
0.000
Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)
Oreochromis macrochir (Mwela - Lake Bangweulu, Zambia)
Oreochromis macrochir (Mwela - Lake Bangweulu, Zambia)
Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)

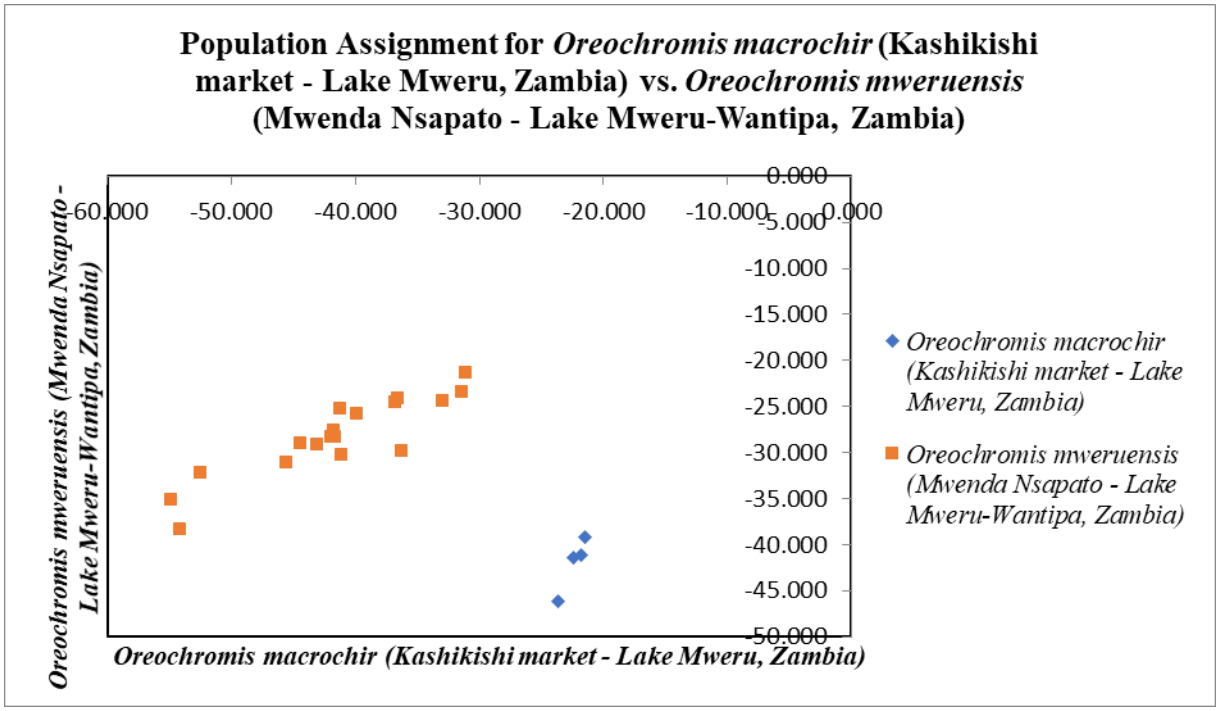

Population Assignment for Oreochromis macrochir (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia) vs. Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-90.000
-100.000
-80.000
-60.000
-40.000
-20.000
0.000
Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)
Oreochromis macrochir (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
Oreochromis macrochir (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)

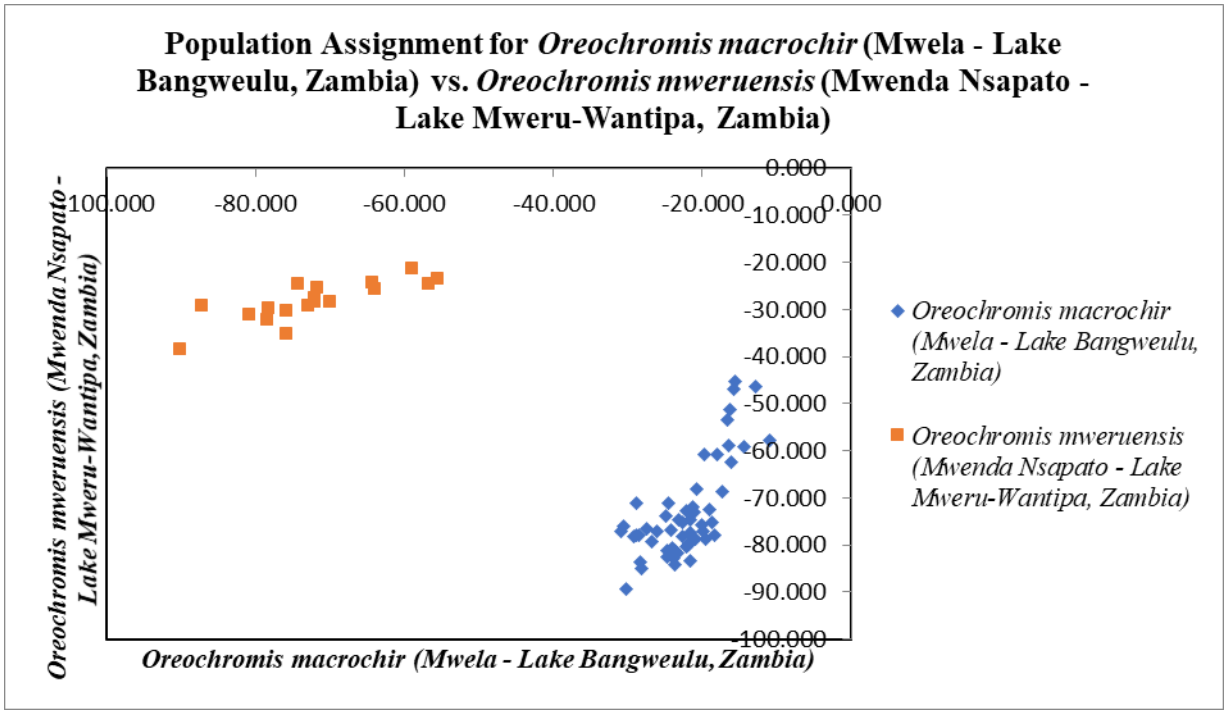

Population Assignment for Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia) vs. Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-90.000
-100.000
-100.000
-80.000
-60.000
-40.000
-20.000
0.000
Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)
Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)

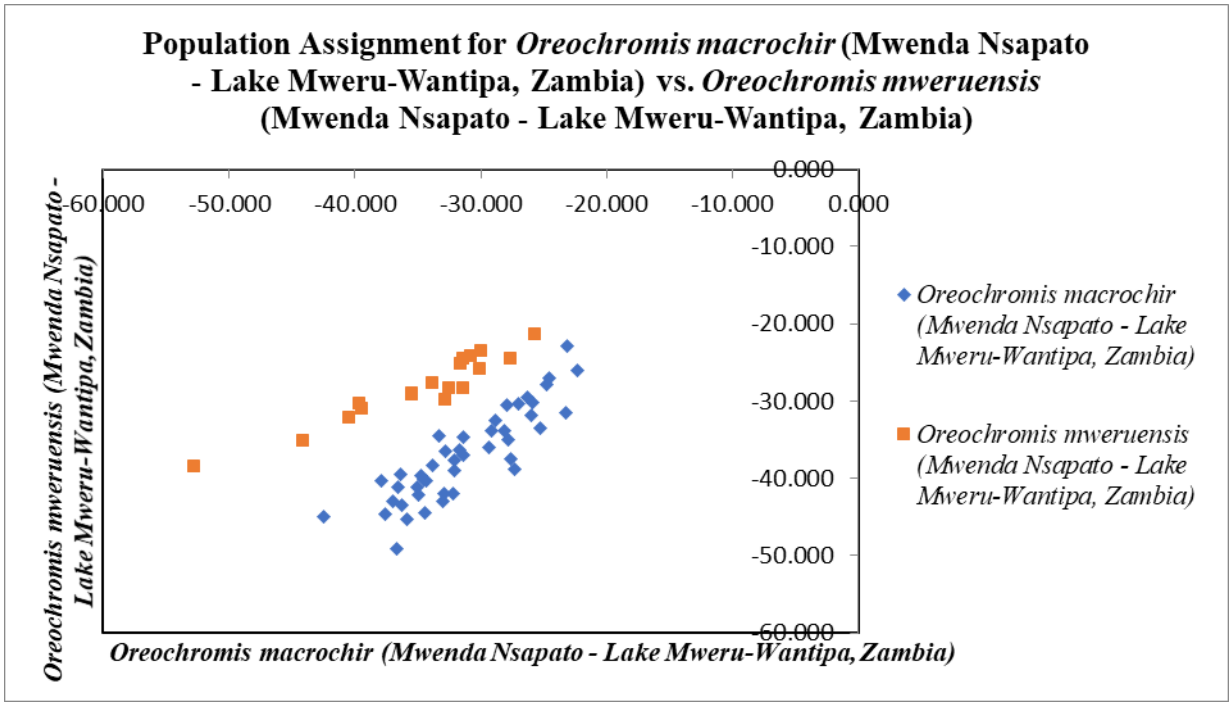

Population Assignment for Oreochromis macrochir (Cultured - Fiyongoli, Zambia) vs. Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-60.000
-50.000
-40.000
-30.000
-20.000
-10.000
0.000
Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
Oreochromis macrochir (Cultured - Fiyongoli, Zambia)
Oreochromis macrochir (Cultured - Fiyongoli, Zambia)
Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)


Population Assignment for Oreochromis macrochir (Cultured - Misamfu, Zambia) vs. Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-70.000
-60.000
-50.000
-40.000
-30.000
-20.000
-10.000
0.000
Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
Oreochromis macrochir (Cultured - Misamfu, Zambia)
Oreochromis macrochir (Cultured - Misamfu, Zambia)
Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)

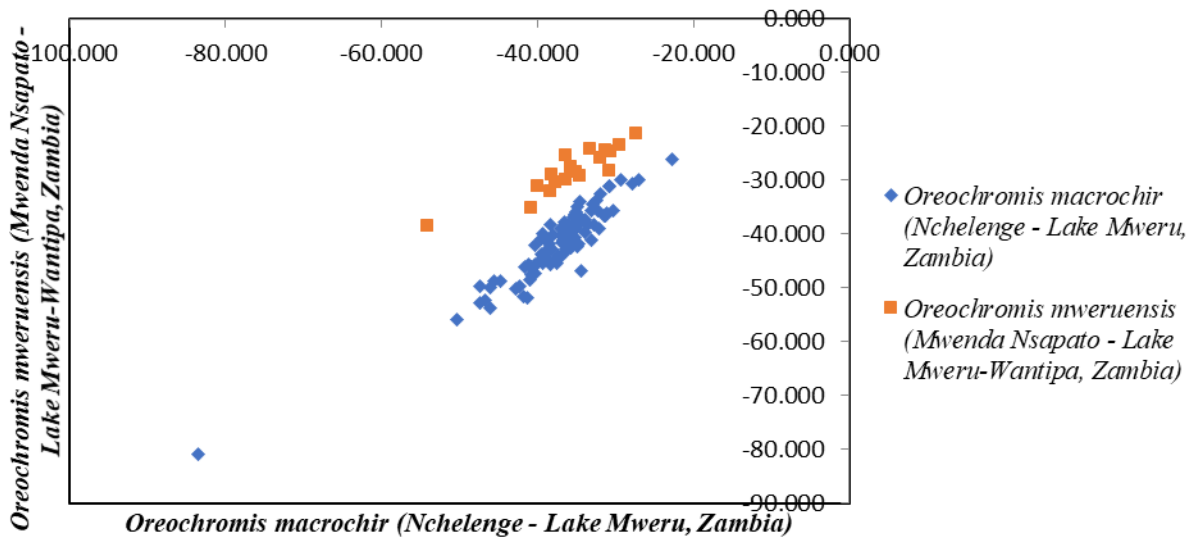

Population Assignment for Oreochromis macrochir (Cultured - NARDC, Zambia) vs. Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-100.000
-80.000
-60.000
-40.000
-20.000
0.000
Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
Oreochromis macrochir (Cultured - NARDC, Zambia)
Oreochromis macrochir (Cultured - NARDC, Zambia)
Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)


Population Assignment for Oreochromis macrochir (Kashikishi market - Lake Mweru, Zambia) vs. Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
0.000
-5.000
-10.000
-15.000
-20.000
-25.000
-30.000
-35.000
-40.000
-45.000
-50.000
-60.000
-50.000
-40.000
-30.000
-20.000
-10.000
0.000
Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
Oreochromis macrochir (Kashikishi market - Lake Mweru, Zambia)
Oreochromis macrochir (Kashikishi market - Lake Mweru, Zambia)
Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)

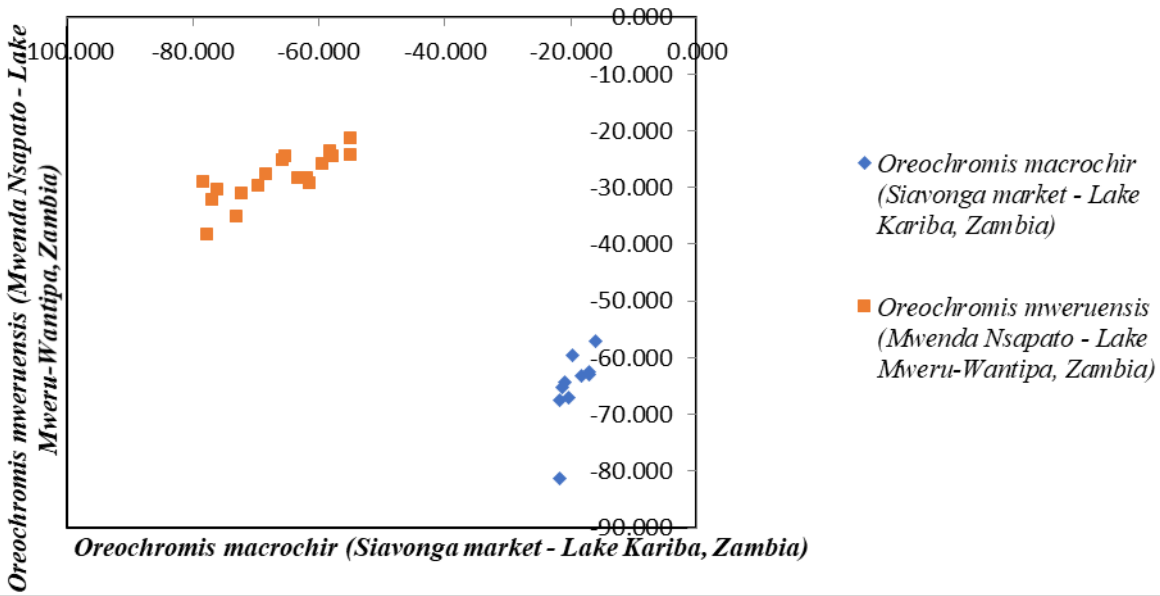

Population Assignment for Oreochromis macrochir (Mwela - Lake Bangweulu, Zambia) vs. Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-90.000
-100.000
-100.000
-80.000
-60.000
-40.000
-20.000
0.000
Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
Oreochromis macrochir (Mwela - Lake Bangweulu, Zambia)
Oreochromis macrochir (Mwela - Lake Bangweulu, Zambia)
Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)

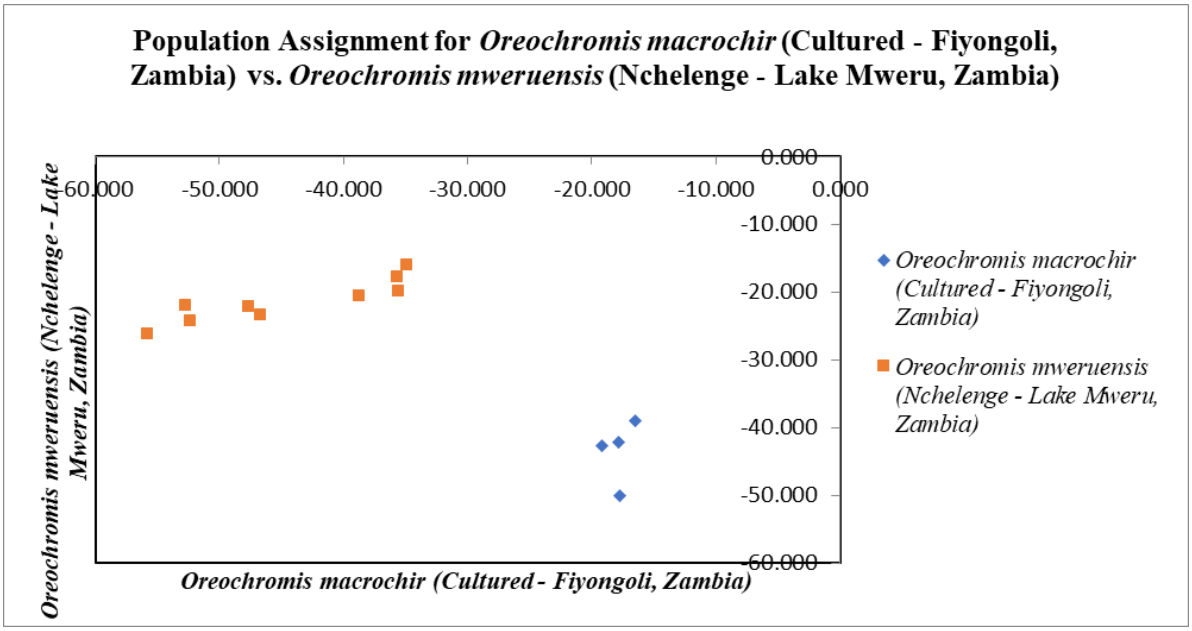

Population Assignment for Oreochromis macrochir (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia) vs. Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-60.000
-50.000
-40.000
-30.000
-20.000
-10.000
0.000
Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
Oreochromis macrochir (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
Oreochromis macrochir (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)

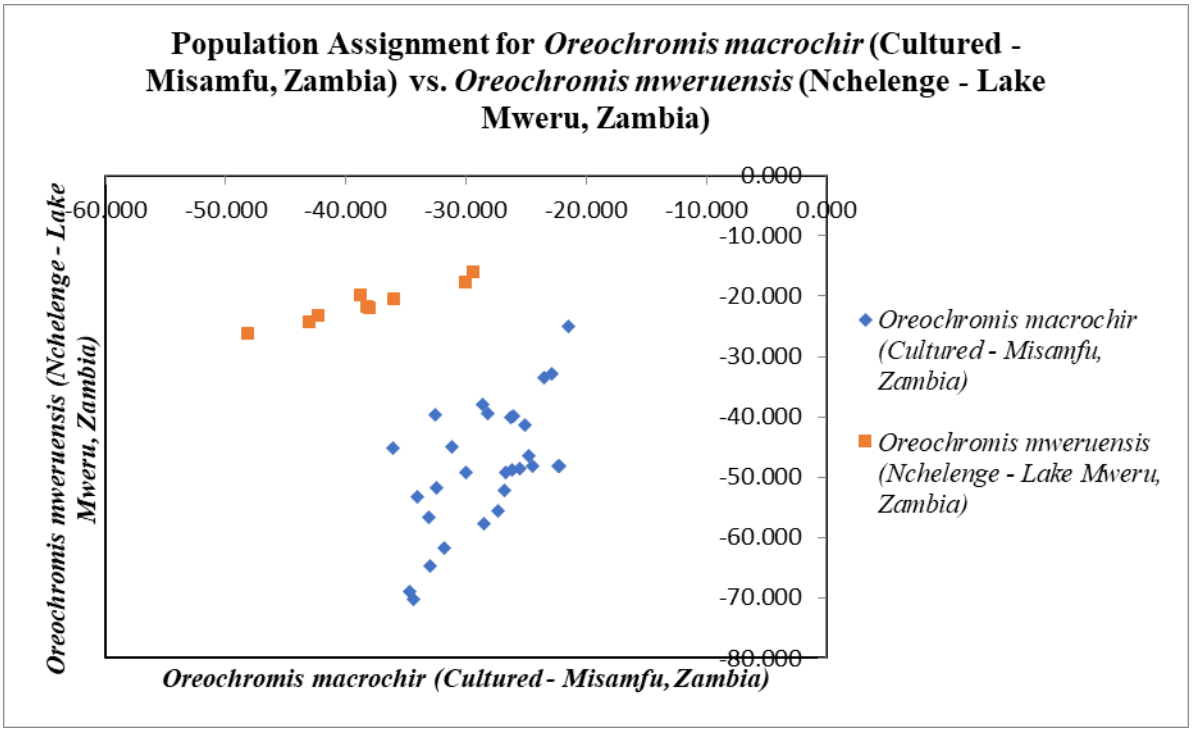
Population Assignment for *Oreochromis macrochir* (Nchelenge - Lake Mweru, Zambia) vs. *Oreochromis mweruensis* (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)

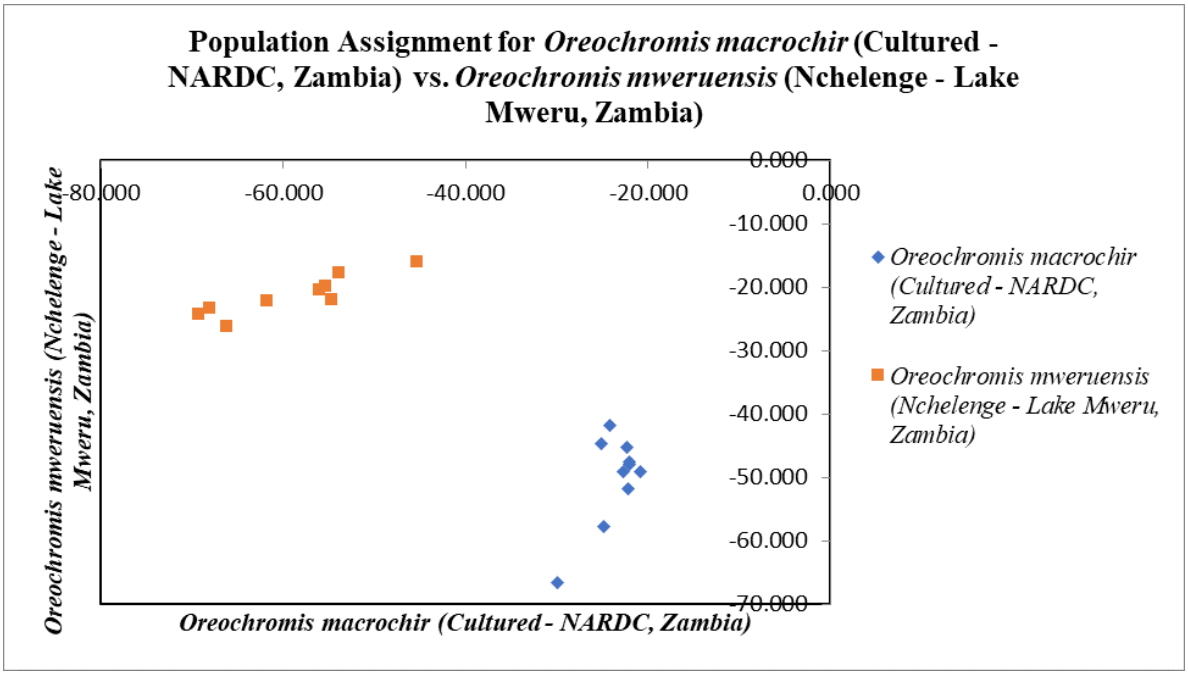


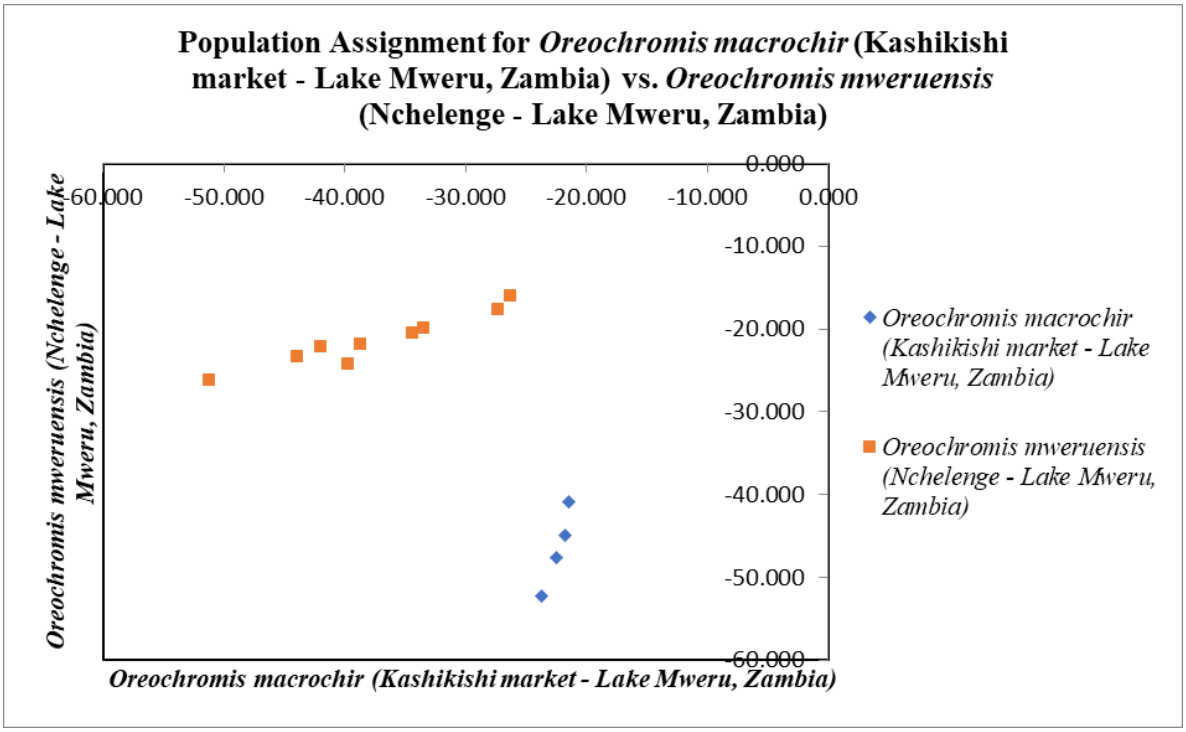
Population Assignment for *Oreochromis macrochir* (Siavonga market - Lake Kariba, Zambia) vs. *Oreochromis mweruensis* (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)

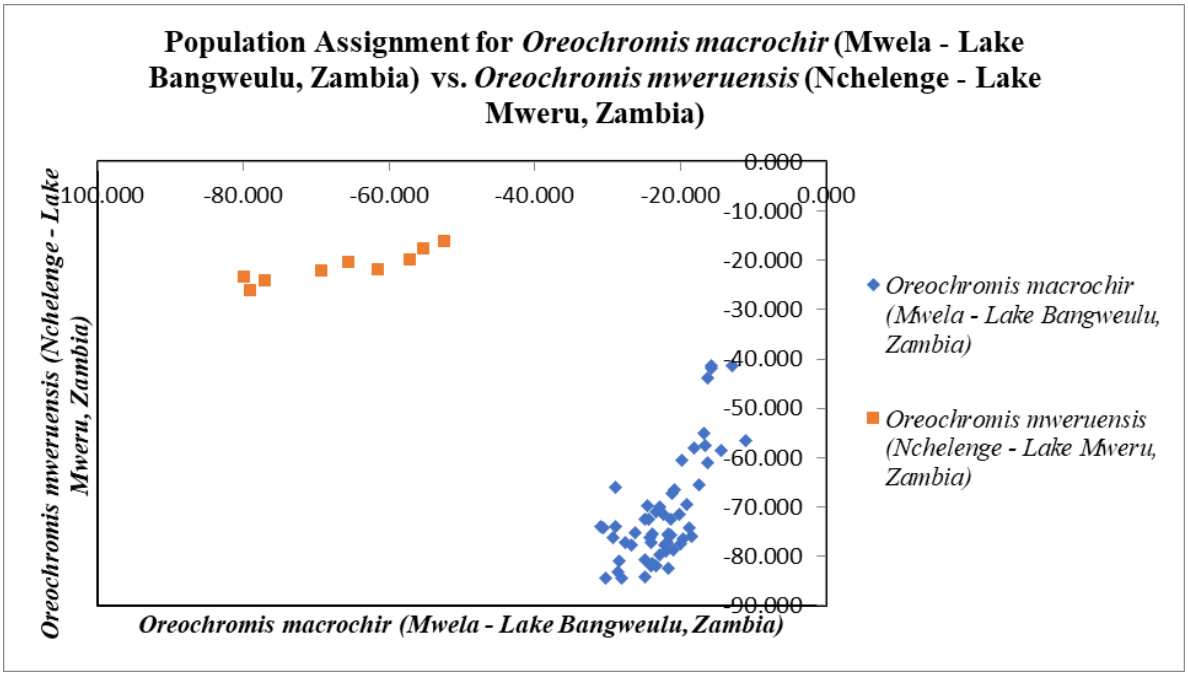


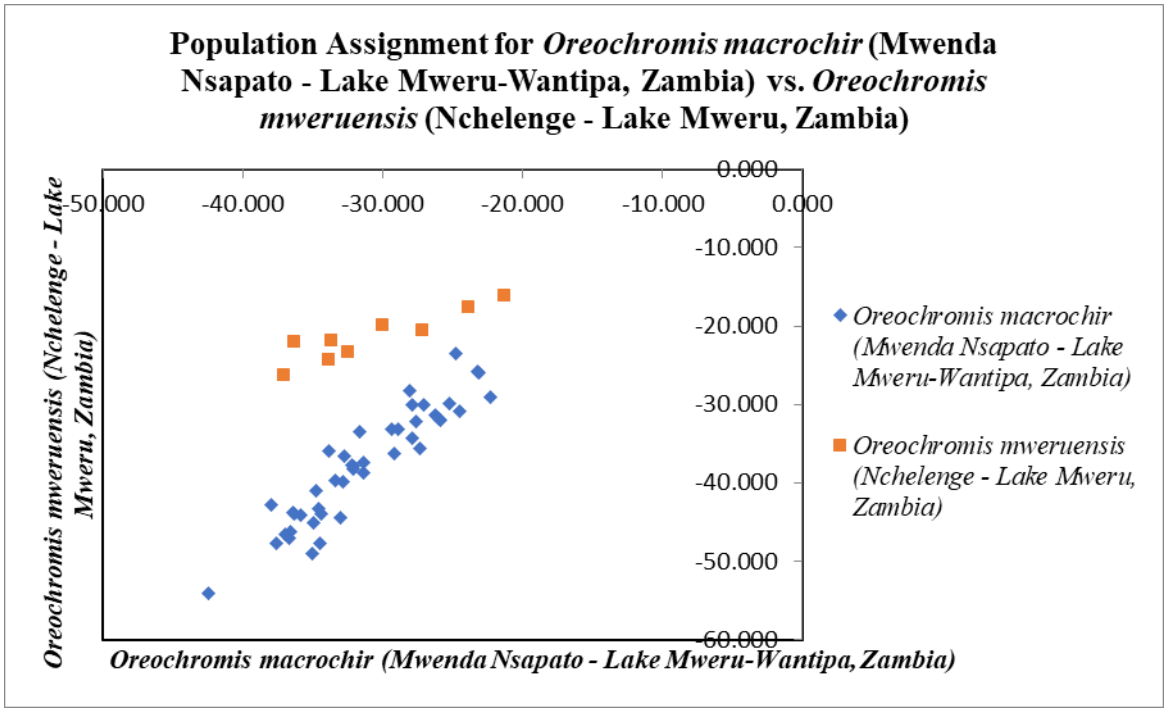

Population Assignment for Oreochromis macrochir (Cultured - Fiyongoli, Zambia) vs. Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-60.000
-50.000
-40.000
-30.000
-20.000
-10.000
0.000
Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Cultured - Fiyongoli, Zambia)
Oreochromis macrochir (Cultured - Fiyongoli, Zambia)
Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)

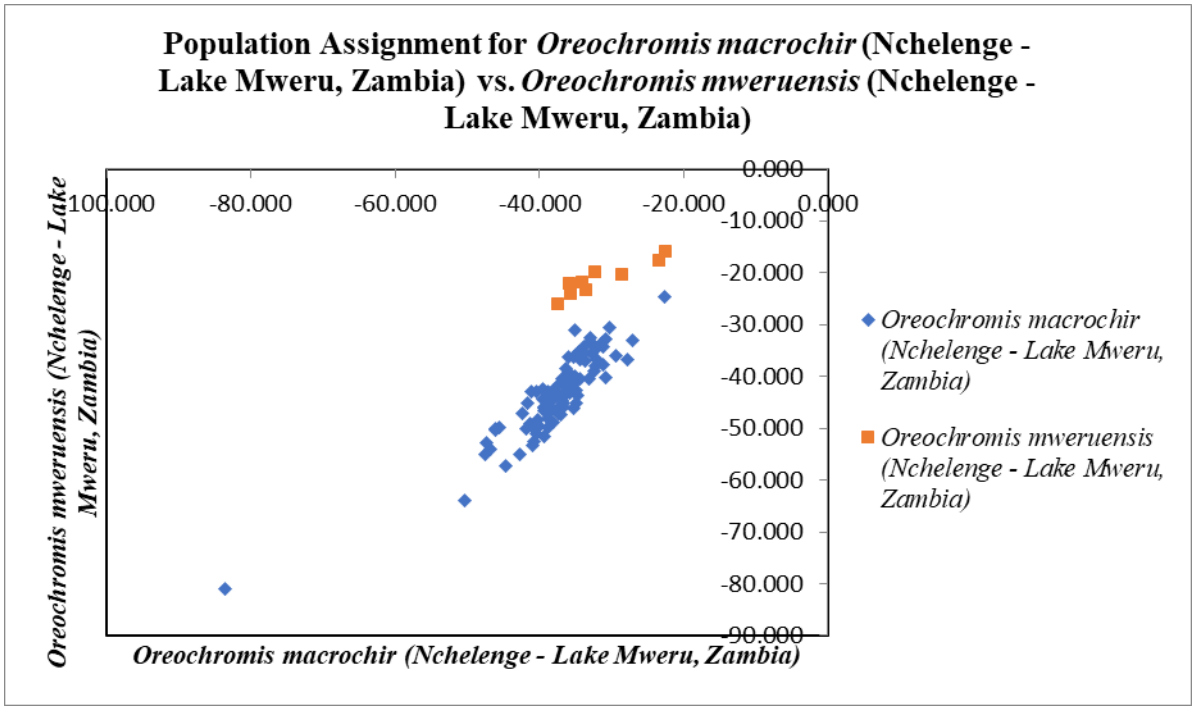

Population Assignment for Oreochromis macrochir (Cultured - Misamfu, Zambia) vs. Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-60.000
-50.000
-40.000
-30.000
-20.000
-10.000
0.000
Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Cultured - Misamfu, Zambia)
Oreochromis macrochir (Cultured - Misamfu, Zambia)
Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)

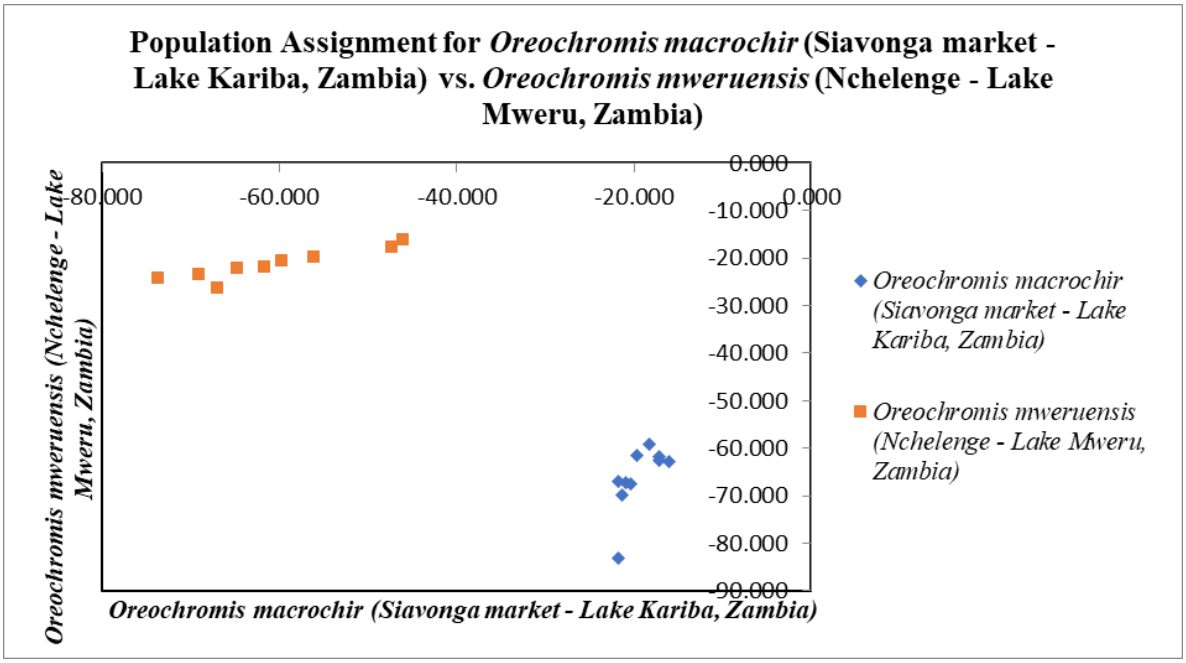

Population Assignment for Oreochromis macrochir (Cultured - NARDC, Zambia) vs. Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-60.000
-40.000
-20.000
0.000
Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Cultured - NARDC, Zambia)
Oreochromis macrochir (Cultured - NARDC, Zambia)
Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)

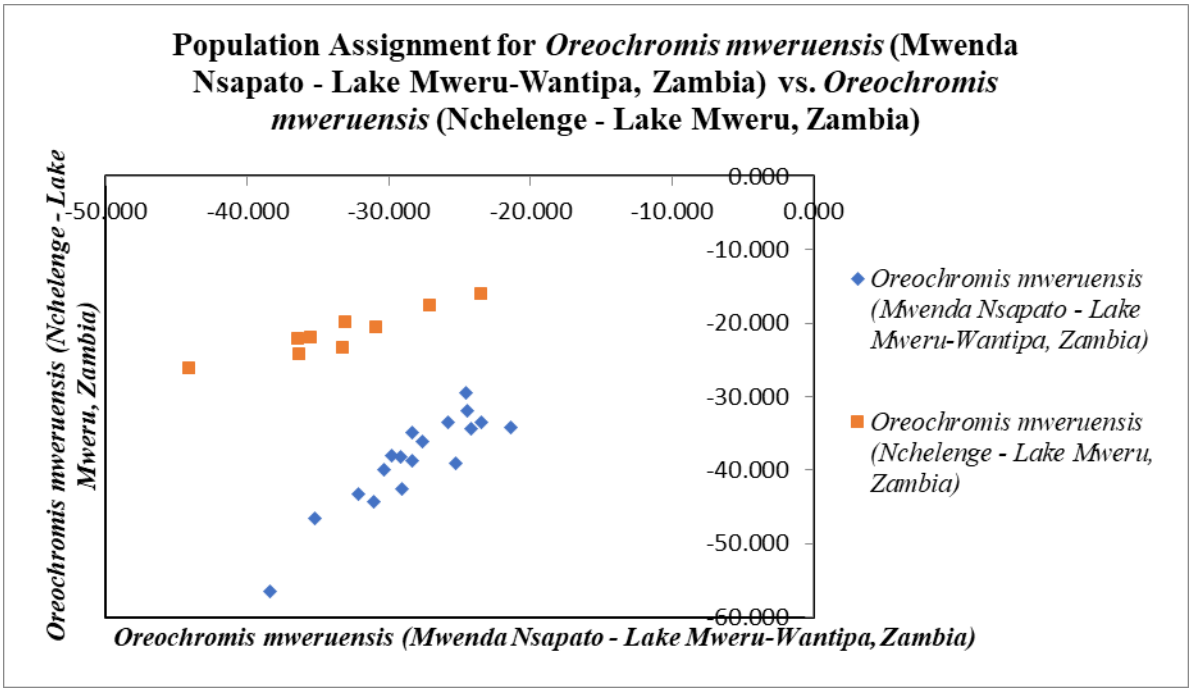

Population Assignment for Oreochromis macrochir (Kashikishi market - Lake Mweru, Zambia) vs. Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-60.000
-50.000
-40.000
-30.000
-20.000
-10.000
0.000
Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Kashikishi market - Lake Mweru, Zambia)
Oreochromis macrochir (Kashikishi market - Lake Mweru, Zambia)
Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)


Population Assignment for Oreochromis macrochir (Mwela - Lake Bangweulu, Zambia) vs. Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)
Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Mwela - Lake Bangweulu, Zambia)
-100.000
-80.000
-60.000
-40.000
-20.000
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-90.000
Oreochromis macrochir (Mwela - Lake Bangweulu, Zambia)
Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)


Population Assignment for Oreochromis macrochir (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia) vs. Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)
Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
-50.000
-40.000
-30.000
-20.000
-10.000
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
Oreochromis macrochir (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)


Population Assignment for Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia) vs. Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-90.000
-100.000
-80.000
-60.000
-40.000
-20.000
0.000
Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Nchelenge - Lake Mweru, Zambia)
Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)


Population Assignment for Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia) vs. Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
-70.000
-80.000
-90.000
-80.000
-60.000
-40.000
-20.000
0.000
Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)
Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)
Oreochromis macrochir (Siavonga market - Lake Kariba, Zambia)
Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)


Population Assignment for Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia) vs. Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)
Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)
-50.000
-40.000
-30.000
-20.000
-10.000
0.000
0.000
-10.000
-20.000
-30.000
-40.000
-50.000
-60.000
Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
Oreochromis mweruensis (Mwenda Nsapato - Lake Mweru-Wantipa, Zambia)
Oreochromis mweruensis (Nchelenge - Lake Mweru, Zambia)