

ASSESSMENT OF CHANGES IN GENETIC DIVERSITY OF NILE
TILAPIA, *Oreochromis niloticus* (LINNAEUS, 1758), OF LAKE BARINGO,
KENYA



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DECLARATION

This is to testify that this thesis is my original work and has not been presented for award of degree in any other university.

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DEDICATION

I dedicate this thesis to my late grandmother Lillian Nyato Mkocho (Nguvila) and my late guardian Mr Benny Zillinde. Their love, care and moral support have been the main pillars for my academic success. They will be remembered for their efforts to ensure I achieve higher academic heights through hard work despite the pitfalls I encounter. May the Almighty God have mercy on them and rest their souls in eternal peace.

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LIST OF ABBREVIATIONS AND ACRONYMS

CO1:	Cytochrome Oxidase 1
CR:	Control Region
DNA:	Deoxyribonucleic acid
DNTPs:	Deoxyribonucleoside triphosphates
ddNTPs:	Dideoxynucleotide triphosphates
DnaSP:	DNA Sequence Polymorphism
ESP:	Economic Stimulus Programme
MEGA:	Molecular Evolutionary Genetics Analysis
mtDNA:	Mitochondrial DNA
ML:	Maximum Likelihood
MUSCLE:	Multiple Sequence Alignment
NCBI:	National Center for Biotechnology Information
PAST:	Paleontological Statistical Test
PCA:	Principal Component Analysis
PCR:	Polymerase Chain Reaction

ABSTRACT

The rapid expansion of tilapia aquaculture farming in the Rift Valley Region of Kenya, has led to the transfer of different *Oreochromis* species from farms (ponds) to natural ecosystems such as rivers and lakes. These transfers have negatively impacted on native tilapia species of these systems through competition, hybridization and introgression which consequently has led to the dwindling in number as well as compromising the genetic integrity of the native species. In Lake Baringo, introductions of tilapia from unverified sources and unknown species has been reported in the past but has continued due to the rapid expansion of tilapia farming. The present study aimed at evaluating the current genetic diversity of *Oreochromis* species in Lake Baringo in order to determine the recent species introductions and effects on endemic populations of the Nile tilapia *Oreochromis niloticus baringoensis*. Morphological (morphometric and meristics) and molecular techniques involving mtDNA markers (Cytochrome Oxidase 1, Control region and D-loop genes) were used to identify tilapia species from the lake. The observed morphometric and meristic differences showed variations congruent with two tilapia species *Oreochromis niloticus* and *Oreochromis spilurus*. Genetic studies revealed four haplotypes based on maximum likelihood phylogenetic trees on mtDNA Cytochrome Oxidase 1 and seven haplotypes each for mtDNA Control region and D-loop marker genes. Blasting, alignment and phylogenetic analysis of mtDNA D-loop partial fish sample sequences with the NCBI data base sequences for most cultured tilapia species enabled the identification of the haplotypes as *O. n. baringoensis*, *O. spilurus*, *O. n. vulcani*, *O. niloticus* (unknown) and the hybrids of *O. n. baringoensis* with *O. leucostictus*. These findings confirm indications that increased aquaculture activities around Lake Baringo basin have led to continued species introduction and hybridization in the main lake, thus endangering the native species *O.n baringoensis* which form an important role in commercial fisheries for the communities around the lake.

CHAPTER ONE

1.0. INTRODUCTION

1.1. Background information

The fishes of the genera *Tilapia*, *Oreochromis* and *Sarotherodon* belong to the family Cichlidae and are collectively and commonly known as tilapia (Trewavas, 1983). These fishes are indigenous to tropical and sub-tropical fresh waters of Africa, Mediterranean and Middle East (Trewavas, 1983). The three genera are mainly taxonomically distinguished on the basis of their parental care patterns (Mjoun and Rosentrater, 2010). In the genera *Tilapia* both parents (male and female) guard the eggs, wrigglers, and free-swimming fry and hence they are referred to as biparental caring substrate spawners (Trewavas, 1983; Mjoun and Rosentrater, 2010). On the other hand, in the genus *Oreochromis*, the females incubate the fertilized eggs and the young fry in their mouth hence they are known as arena- spawning maternal mouth brooders (Trewavas, 1983). Cichlids of the genus *Sarotherodon* are paternal, maternal or biparental brooders in which either male or female or both parents, protect and carries the eggs, wrigglers and free swimming fry in their mouth (Mjoun and Rosentrater, 2010; Canonical *et al.*, 2005). All three genera have been introduced in different parts of the world for various purposes such as aquaculture, biological control of aquatic weeds and insects, as aquarium species and to enhance capture fisheries (Canonical *et al.*, 2005).

Out of the three genera, the genus *Oreochromis* is of great economic importance in global fisheries and aquaculture (Bostock *et al.*, 2010) with the Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) ranking first within the genus (Josupeit, 2010; Agnès *et al.*, 1997). This species is also ranked 5th among the most cultured species in the world after grass carp (*Ctenopharyngodon idella*), Silver carp (*Hypophthalmichthys molitrix*), Common carp

(*Cyprinus carpio*) and a Japanese bivalve Asari (*Ruditapes philippinarum*) (FAO, 2012). The global commercial production of this species in 2012 was 3.197 million tonnes with a market value of about \$ 5.3 billion (FAO, 2012). Due to its commercial value, the species has been introduced in almost every tropical and subtropical climate for aquaculture purposes (Nyingi *et al.*, 2009; FAO, 2009; Mjoun and Rosentrater, 2010).

The Nile tilapia fish have been nick named as “aquatic chicken” due to their potential affordable protein source and their ability of being raised in a wide array of environmental systems ranging from subsistence units to large scale farming (Coward and Little, 2001; Gupta and Acosta, 2004). Moreover, these fishes utilize a variety of feed ranging from phytoplankton, periphyton, benthic fauna, detritus and bacterial films (FAO, 2012). The fishes also exhibit trophic plasticity according to the environment and other species they co-exist with (Bwanika *et al.*, 2007). These and other physiological attributes such as early maturation of 5 to 6 months make them popular and suitable for aquaculture (Mjoun and Rosentrater, 2010).

In Kenya, the Nile tilapia is an important source of dietary protein for a large number of people in urban and rural areas. It is also an important means of economic and social empowerment, as it constitutes 90% of the farmed fish (Fisheries Department, 2012) providing employment directly to about 80,000 people. Despite remarkable contribution of the fish to the national economy, the *O. niloticus* status in Kenya’s natural fresh waters is at crossroads due to changes in water biophysical properties, overfishing and exotic species introductions. The dwindling fish catch in Kenyan lakes suggests that the population is endangered in the wild and hence requires conservation measures to prevent extinction of stocks (Britton *et al.*, 2009). Several measures have been taken in trying to overcome the shortage of *O. niloticus* in the Kenyan market; these include, periods of fishing bans,

establishment of restocking programmes as well as establishment of aquaculture farms to substitute fish capture fisheries. For instance the dramatic decrease in tilapia catch in Lake Baringo was reported despite the suspension of fishing activities in the lake from 1993 to April 1994 and in 2002 to allow multiplication of the fish (Hickley *et al.*, 2008). This dramatic decrease in fish catch was attributed to many biophysical factors including overfishing, ecological factors and the introduction of exotic fish species (Hickley *et al.*, 2008; Department of Fisheries, 2009; Omondi *et al.*, 2013)

The Fisheries department of the Ministry of Agriculture and Fisheries of Kenya initiated the Economic Stimulus Programme (ESP) in the year 2009 in order to present aquaculture as a viable economic enterprise and enhance fish protein consumption (Manyala, 2011). In this programme, the Nile tilapia, *Oreochromis niloticus* and the African Sharptooth Catfish, *Clarias gariepinus* were highly promoted for aquaculture (Fisheries department, 2012). The fish farming enterprise under ESP aimed at injecting commercial thinking into fish farming to build up a vibrant aquaculture industry (Munguti *et al.*, 2014). Following the government emphasis on aquaculture farming through ESP, many aquaculture farms were established throughout the country. In the first year of the programme, there were about 27,000 ponds constructed national wide (Musa *et al.*, 2012; Munguti *et al.*, 2014).

Fish farmers in the rift valley region of Kenya have been implementing ESP programme by constructing the fish-ponds along rivers and streams as a way of improving their livelihoods (Ndiwa *et al.*, 2014). It has been observed that these small-scale fish farmers have been breeding a variety of tilapia species with fingerlings from diverse sources within East Africa (Ndiwa *et al.*, 2014). This massive establishment of aquaculture farms along water bodies (rivers, lakes, streams and wetlands) has led to escape of fish from the ponds into large water bodies, hence resulting in mixing of escapees with native species (Ndiwa *et al.*, 2014, Nyingi

and Agnèse, 2007). The repercussions of these mixing have been reported to be competition, hybridization and introgressions. In Lake Baringo, the Omega fish farm around the shores of Ol-kokwe Island was submerged as a result of floods in May 2013 causing the reared fish to escape and mix up with other fish in the lake (Onywere *et al.*, 2014). This farm was built in 2010 for commercial and restocking purposes. The fish farm used brood stock from the lake by purchasing tilapia fingerlings from groups of fishermen (Johnstone, 2011). However, it is not known if the brood stock supplied by fishermen was in fact only from the Lake Baringo or if there might have been fingerlings also from other sources including other aquaculture farms.

Morphological and molecular techniques have been used in characterization of Nile tilapia. Compared to morphological based techniques, DNA based methods have been proven to be ideal in identification of tilapia strains even in mixed populations (Wu and Yang, 2012; D'amato *et al.*, 2007). Until recently, morphological studies of the Nile tilapia in Lake Baringo (Trewavas, 1983; Worthington and Ricardo, 1931; Ssentongo and Mann, 1971) showed *Oreochromis niloticus baringoensis* as the only existing tilapiine fish in the lake. However Nyingi and Agnese, (2007) have shown that the native Baringo tilapia (*O. n. baringoensis*) has been compromised by introductions of *O. leucostictus* through mitochondrial DNA introgression. Hybridization between exotic species with native species of this nature is a major concern for conservation of species in the wild (D'Amato *et al.*, 2007; Canonical *et al.*, 2005). The most remarkable incident in the world of fisheries was the disappearance of *Oreochromis variabilis* (Boulenger, 1906) and *Oreochromis esculentus* (Graham, 1928) from the main Lake Victoria in 1960s (Welcomme, 1966). The main cause of extinction was the introduction of invasive *Oreochromis niloticus* and the rapacious predator, Nile perch (*Lates niloticus*) between 1950s and 1960s, which altered the indigenous cichlids ecosystem (Angienda *et al.*, 2011; Canonical *et al.*, 2005; Mwanja *et al.*, 2010).

These and other factors such as over fishing, water pollution and bad land use policies are believed to have led to the extinction of the two species from the main lake.

The findings regarding hybridization through genetic introgression of Nile tilapia of Lake Baringo by *Oreochromis leucostictus* by Nyingi and Agnèse (2007) is the basis of the present research. The introduced species *O. leucostictus* occur in the Lake Naivasha, which is about 150 km away from Lake Baringo. The present study seeks to establish whether the increased emphasis on aquaculture development in the region has changed the genetic diversity of tilapia in the lake. Furthermore, the study is aimed at finding out whether the introduced *Oreochromis* species from aquaculture continues to endanger endemic species and to what extent introduced species have hybridized with endemic species.

1.2. Statement of the problem

The Baringo tilapia *Oreochromis niloticus baringoensis* is endemic to Lake Baringo. Two main species introductions have recently compromised the genetic integrity of these fishes; one occurred prior to the year 2002 while the other in 2013 (Nyingi and Agnèse, 2007). Apart from these introductions, other factors such as climate change, human encroachments (Siltation) and overfishing have further impacted negatively on the survival and continued existence of *O.n baringoensis* (Britton *et al.*, 2009; Department of Fisheries, 2009; Hickley *et al.*, 2008). Nyingi and Agnèse (2007) described mitochondrial introgressions (mtDNA D-loop) of *O. niloticus* from *O. leucostictus* in fish specimens collected in 2002 from Lake Baringo. The second introduction occurred during the long rains of May 2013 when Omega fish farm located along the shores of Lake Baringo in Ol-Kokwe Island flooded and released fish from ponds into the main lake (Johnstone, 2010).

The fish cultured by Omega Farm were previously destined for aquaculture development through provision of fingerlings for pond culture in the area. Since selection of broodstock

was based on morphology it is difficult to conclude the exact species that were introduced into the lake due to the floods. In this regard, there is a great need for an assessment of the genetic status of the tilapia fishes of this lake in order to determine the changes in genetic diversity in light of the recent introductions.

1.3. Justification

The introduction of tilapia species to the wild through anthropogenic activities has been reported to have detrimental effects on the native tilapia populations (Firmat *et al.*, 2013). Several cases of introduction of alien tilapia species and their subsequent effects to the native tilapiine population have been reported (Van der Waal and Bills, 2000; D'Amato *et al.*, 2007; Mwanja *et al.*, 2010; Firmat *et al.*, 2013). In Lake Baringo, the native tilapia species *Oreochromis niloticus baringoensis* forms an important basis of commercial fishery for the communities living around the lake (Britton *et al.*, 2009). However, factors such as overfishing, species introductions, climate change and siltation have resulted into dwindling in fish catch of this native species.

Therefore there is need to assess the current genetic diversity of Lake Baringo Nile tilapia because of the evidence of mtDNA introgression from *O.leucostictus* to *O.n baringoensis* by Nyingi and Agnèse (2007) and recent spilling of fingerlings from Omega farm in May, 2013. Given that the fish fingerlings reared in the farm prior to May, 2013 floods were obtained by purchasing from local fishermen, it is unsubstantiated if these fingerlings were really from the lake or from other sources as well since no efforts to verify this was made. If in fact other unknown tilapia fish were reared in the farm, there is a great possibility of hybridization with the native species which would have further compromised the genetic integrity of *O. n. baringoensis*. The knowledge of genetic diversity of these fish will be crucial for conservation and management of the native tilapia species. In addition, this study will

provide invaluable information to the policy makers on the effects of aquaculture expansion in order to mitigate future uncontrolled expansion of aquaculture farms.

1.4. Objectives

1.4.1. Overall objective

The overall objective of the study was to undertake an assessment of the recent changes in genetic diversity of tilapia species of the genus *Oreochromis* in Lake Baringo, Kenya for enhanced conservation of the species in the lake.

1.4.2. Specific objectives of the study

- (i) To evaluate the current genetic diversity of *Oreochromis* species in Lake Baringo
- (ii) To determine the effects of introduced *Oreochromis* species on the native species.
- (ii) To investigate the status of hybrids of *Oreochromis niloticus* with *Oreochromis leucostictus* in Lake Baringo
- (iv) To determine the possibility of new hybrids resulting from recent introductions.

1.5. Hypotheses

- (i) Introduction of other *Oreochromis* species in Lake Baringo may have compromised the genetic structure, diversity and integrity of *O.n baringoensis*
- (ii) The introgressed hybrids of *O.n baringoensis* with *O.leucostictus* exist in Lake Baringo.
- (iii) Incidences of hybridization have occurred between native Nile tilapia *O. n. baringoensis* with other introduced *Oreochromis* species

CHAPTER TWO

2.0. REVIEW OF LITERATURE

2.1. Classification, distribution and socioeconomic importance of *Oreochromis niloticus*

2.1.1. Classification and distribution

Oreochromis niloticus belongs to the family Cichlidae, order Perciformes and class Actinopterygii (Nelson, 2004). Apart from *Oreochromis*, this family also contains three other genera: *Sarotherodon*, *Tilapia* and *Danakilia* (Trewavas, 1983). *Sarotherodon* are characterized by small mouth with very small teeth (Trewavas, 1983). They also have slender shafts and spoon-shaped crowns (Trewavas, 1983). The general colour is pale blue on lower flanks to orange or metallic golden yellow on the back (Trewavas, 1983). In the genera *Tilapia*, teeth of the jaws and pharynx are typically coarser than in *Sarotherodon* (Trewavas, 1983). On the other hand *Oreochromis* species are characterized by notched teeth throughout their life (Trewavas, 1966), females are mouth brooders, which mean they hold their eggs in mouth and offspring for a certain period of time (Mjoun and Rosentrater, 2010).

Trewavas (1983), morphologically identified seven sub-species of *Oreochromis niloticus* from western and eastern Africa fresh waters: *Oreochromis niloticus niloticus* from West Africa (Lake Chad basin, river Niger, Benue, Volta, Gambia and Senegal) and the Nile river system. *Oreochromis niloticus edwardianus* from the Lakes Edward, Albert, George (Uganda) Tanganyika (Tanzania and Burundi) and Kivu (Rwanda); *Oreochromis niloticus baringoensis* endemic to Lake Baringo (Kenya), *Oreochromis niloticus sugutae* endemic in River Suguta (Kenya), *Oreochromis niloticus vulcani* from Lake Turkana, (Kenya) *Oreochromis niloticus cancellatus* from Lakes Tana, Zwai and Stefani as well as rivers Ergino and Awash in the Ethiopian rift valley; and *Oreochromis niloticus filoa* from the hot

alkaline springs in the Awash system, Ethiopia. Nyngi *et al.*, (2009) also discovered another endangered new sub species of *Oreochromis niloticus* from Bogoria hotel spring of the Loboï swamp, Kenya. Figure one is a schematic representation of the classification of *Oreochromis niloticus*.

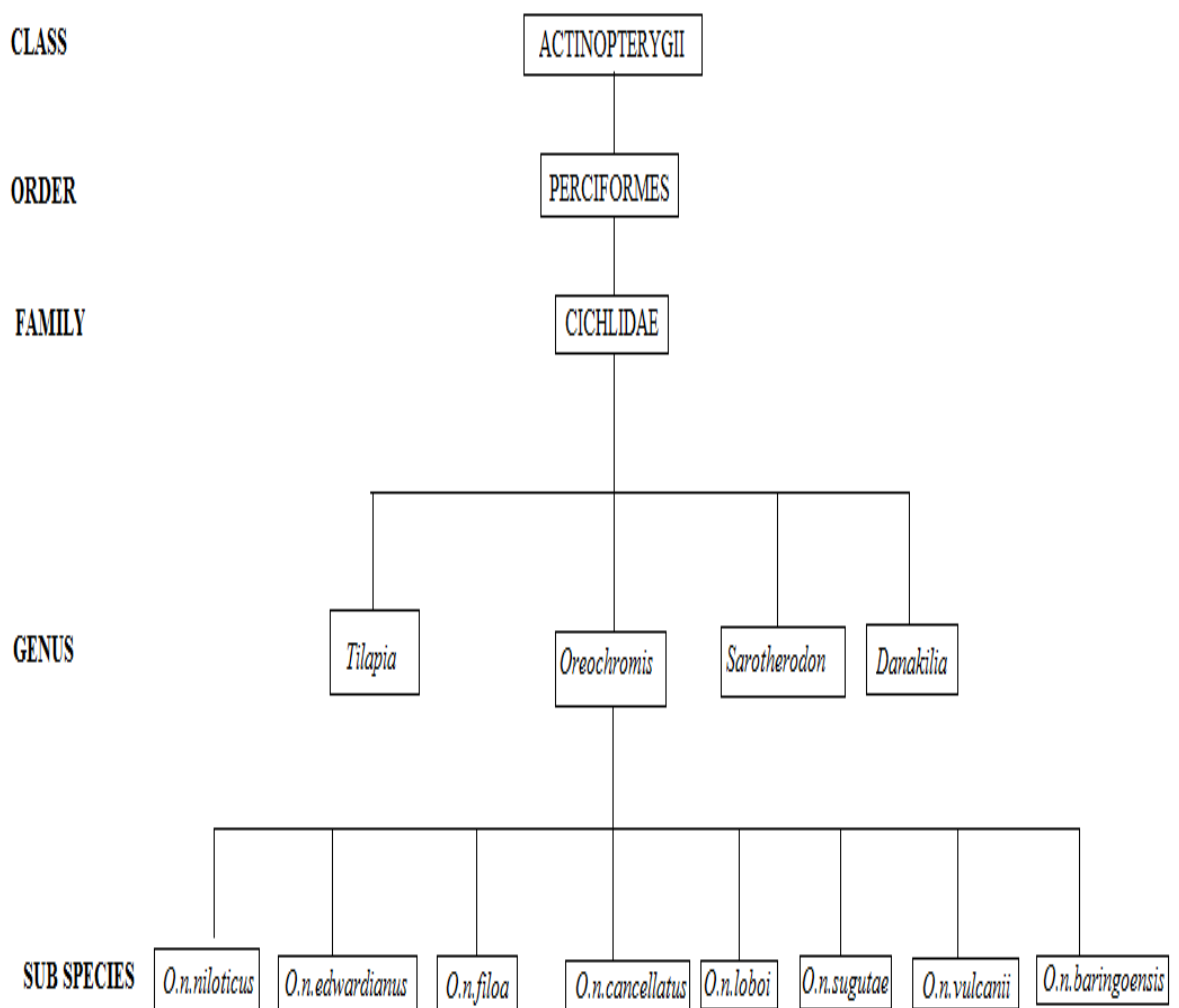


Figure 1: Classification of *Oreochromis niloticus* according to Trewavas, (1983)

2.1.2. Socio economic importance of *Oreochromis niloticus*

The Nile tilapia *Oreochromis niloticus* is commercially the most important Cichlid species in global fisheries and aquaculture (Agnèse *et al.*, 1997). According to Josupeit (2010), *O. niloticus* is the most commonly reared species accounting for about 75% of global tilapia production. The fishes have been introduced to many parts of the world due to their commercial and nutritional value (Agnese *et al.*, 1997). According to Food and Agricultural Organisation (FAO) of the United Nations, the Nile tilapia fish is the 5th most cultured species in the globe and its annual world production from aquaculture in 2012 was about 3.197 million tonnes with a market value of \$ 5.3 billion (FAO, 2012). In Kenya *O. niloticus* constitute 75% of the fish produced through aquaculture (Kaliba *et al.*, 2007; Munguti *et al.*, 2014). This fish and the African catfish (*Clarius gariepinus*) are the two prioritized fishes for aquaculture by the Kenyan government 2009 Economic Stimulus Programme that aimed at creating business opportunities and jobs as well as alleviating food insecurity and poverty through aquaculture (Manyala, 2011; Munguti *et al.*, 2014). In the year 2010, the total fish production from aquaculture in Kenya was 12,000 MT/y representing 7% of the national fish harvest, *Oreochromis niloticus* accounted for 75% of this production (Munguti *et al.*, 2014). The production through aquaculture is expected to shoot to 20,000 MT/y representing 10% of total production valued at USD \$ 22.5 million over by the year 2020 (Munguti *et al.*, 2014). In Lake Baringo, the indigenous tilapia fish *Oreochromis niloticus baringoensis* is an important source of socio-economic development to the communities around the lake (Britton *et al.*, 2009). Previously, the endemic fish formed the basis of fishery in the lake, however owing to changes in biophysical factors such as drought, siltation and species introduction, the fishery is now dominated by lung fish (*Protopterus aethiopicus*) (Department of Fisheries, Kenya , 2009).

2.2. Natural hybridization of *Oreochromis* species with other Cichlids and their potential effects to native species

The increasing anthropogenic activities on earth's ecosystems play a big role in genetic exchange between fish populations and species resulting into genetic plasticity of the species in their native range (Crispo *et al.*, 2011). This has been perpetrated by intentional or unintentional introduction of exotic species that often hybridize with native species resulting into genetic admixture (Firmat *et al.*, 2013). Intentional introductions occur by the alien species being introduced deliberately in a water body to boost fish production while unintentional introductions are those that occur when alien species move to areas outside their geographical range without human intervention (Canonical *et al.*, 2005). There are three categories of invasion-mediated hybridization namely: Hybridization without introgression meaning hybrids beyond F₁ generation are absent; hybridization with widespread introgression but with persistence of pure populations; and complete admixture (Allendorf *et al.*, 2001; Firmat *et al.*, 2013).

Fish species often hybridize probably more than any other animal taxon due to combination of intrinsic factors such as external fertilization and weakness of reproductive barriers or gametic specificity and high susceptibility to secondary contacts between recently evolved forms (Rognon and Guyomard, 2003). Many cases of human mediated translocation and introduction associated with interspecific hybridization appear widespread in a paraphyletic and most widely spread group of African Cichlid called tilapia (Firmat *et al.*, 2013). It has been reported that tilapia species have high propensity to hybridization due to their recent evolutionary radiation caused by incomplete speciation (D'Amato *et al.*, 2007). Due to their high tendency of hybridization, introduced tilapia fish species often hybridize with the native

Cichlids resulting into sterile hybrids or hybrids that back cross with their parental species resulting into introgression (Gregg *et al.*, 1998).

Further, due to increased aquaculture, tilapia fingerlings have been reported to escape into natural aquatic ecosystems where they interact and mix with native populations and hybridize, thus eventually compromising the genetic integrity of native local species.

In Kenya, the genetic integrity of native tilapia in the wild is highly jeopardized by the rapid expansion of aquaculture ponds / farms along water bodies causing transfer of fish from one drainage system to another allowing mixing between populations (Ndiwa *et al.*, 2014). Evidence for this is the widespread expansion of aquaculture ponds / farms facilitated by the Economic Stimulus Program initiated by the Kenyan government in 2009 to expand aquaculture as a viable economic activity (Munguti *et al.*, 2014). The construction of fish ponds and farms in rift valley of Kenya, breeding tilapia species from different sources has resulted into escape, admixture and hybridization of tilapia species (Ndiwa *et al.*, 2014; Nyingi *et al.*, 2007). *Oreochromis niloticus* species hybridization with other Cichlids under experimental setups has been reported by Eknath and Hulata, (2009). Under these experimental conditions in ponds and tanks, *O.niloticus* has been crossed with other *Oreochromis* species producing F₁ with varying sex ratios (table 1).

Table 1: Hybridization of Nile tilapia with other *Oreochromis* species from deliberate crosses in tanks/ponds (Adopted from Eknath and Hulata, 2009)

Female x males	Sex ratio (Female : male)
<i>O.niloticus</i> x <i>O.aureus</i>	Sometimes all male
<i>O.aureus</i> x <i>O.niloticus</i>	1:1-1:3
<i>O.niloticus</i> x <i>O.esculentus</i>	-
<i>O.niloticus</i> x <i>O.leucostictus</i>	Surplus of males
<i>O.leucostictus</i> x <i>O.niloticus</i>	Surplus of males
<i>O.niloticus</i> x <i>O.macrochir</i>	Sometimes all male
<i>O.macrochir</i> x <i>O.niloticus</i>	1:3
<i>O.niloticus</i> x <i>O.mossambicus</i>	Surplus of males
<i>O.mossambicus</i> x <i>O.niloticus</i>	1:1
<i>O.niloticus</i> x <i>O.spilurus niger</i>	Surplus of males
<i>O.spilurus niger</i> x <i>O.niloticus</i>	1:1
<i>O.niloticus</i> x <i>O.urolepis hornorum</i>	Sometimes all male
<i>O.urolepis hornorum</i> x <i>O.niloticus</i>	1:3
<i>O.niloticus</i> x <i>O.variabilis</i>	All male
<i>O.niloticus vulcani</i> x <i>O.aureus</i>	Surplus of males
<i>O.niloticus vulcani</i> x <i>O.urolepis hornorum</i>	Surplus of males
<i>O.urolepis hornorum</i> x <i>O.niloticus vulcani</i>	Surplus of males
<i>O.mossambicus</i> x <i>O.aureus</i>	Sometimes surplus of males
<i>O.niloticus</i> x <i>O.jipe</i>	All male
<i>O.jipe</i> x <i>O.niloticus</i>	1:1

Several cases of hybridization and introgression in the wild of *Oreochromis niloticus* with other cichlids and their subsequent effects have been reported. In Lake Victoria, introduction of *O.niloticus* in the 1960s resulted into interspecific competition with indigenous *Oreochromis* species which further enhanced the likelihood of genetic dilution through hybridization (Kudhongania and Chitamwebwa, 1995). These introductions and the resulting competition and hybridization resulted to the extinction of the Lake Victoria tilapia, *Oreochromis variabilis* and the Singidia tilapia, *Oreochromis esculentus* from Lake Victoria (Mwanja *et al.*, 2010). The remnant populations of these species which formed principal baseline of fisheries in Lake Victoria have occasionally been found in satellite lakes around Lake Victoria and Kyoga and their affluent rivers (Mwanja *et al.*, 2010; Angienda *et al.*,

2011). The hybrids between *O.niloticus* and *O.variabilis* have been observed under experimental conditions producing all males in F1 generation (Eknath and Hulata, 2009). The hybrids of *O.niloticus* with *O.esculentus* have been detected by Mwanja and Kaufman, (1995) from the satellite lakes near Lake Victoria suggesting that no pure stocks of *O.esculentus* exist. However, Angienda *et al.*, (2011) found low nuclear gene transfer from *O.niloticus* to *O.esculentus* in Lake Kanyaboli and Namboyo, Kenya.

In Zimbabwe, the escape of *O.niloticus* from aquaculture farms and ponds has introduced this species into the Limpopo river system where they have hybridized with the indigenous *Oreochromis mossambicus* forming red hybrid population (Canonical *et al.*, 2005; D'Amato *et al.*, 2007). The major effect of these hybridizations has been reported to be loss of genetic integrity of *O. mossambicus* (Van der Waal and Bills, 2000). In Madagascar the *Oreochromis macrochir* (Boungler, 1912) introduced in 1958 disappeared after introduction of *O. niloticus* in the 1960s (Daget and Moreau, 1981). This disappearance was attributed to formation of slow growing and deformed hybrids of the two species (Reinthal and Stiassny, 1991). In Zambia, hybrids of *O.niloticus* with indigenous *Oreochromis andersonii* and *Oreochromis macrochir* in Kafue river was detected by Daines *et al.*, (2014). These hybrids have posed a threat to the conservation and management of the indigenous species in the Kafue river system.

Agnèse *et al.*, (1997) in a survey of genetic differentiation among natural populations of *O.niloticus* in West and East Africa, observed the introgressive hybridization of mtDNA from *Oreochromis aureus* (Steindachner, 1864) to *O.niloticus* in West Africa. Rognon and Guyomard (2003) and Nyingi and Agnèse, (2007) reported the same observation in West Africa. The effects of these introgressions have not as yet been studied extensively.

Nyingi and Agnèsè (2007) demonstrated the natural hybridization through introgression of mtDNA in *O. niloticus* and *O. leucostictus* in Lake Baringo, Kenya using mtDNA D-loop and Microsatellite markers. This study revealed the transfer of mtDNA from *O. leucostictus* endemic to Lake Naivasha to *O. n. baringoensis* without any nuclear gene transfer. Ndiwa *et al.*, (2014) have observed a similar incidence in Loboï swamp hot springs of Kenya where the native tilapia *Oreochromis niloticus* discovered by Nyingi *et al.*, (2009), were found to have been introgressed by mtDNA genes of *Oreochromis leucostictus* from Lake Naivasha. The cause of these introgressions have been attributed to intentional introduction of *O. leucostictus* in this system to boost tilapia catch as way to overcome the nose-diving fish decline in the catchment as well as the expansion of aquaculture in rift valley which utilizes fingerlings from different backgrounds (Nyingi *et al.*, 2007; Ndiwa *et al.*, 2014). The effects of these introgressions on the existence of native species are not known. However, the genetic purity of the native species has been compromised by these introgressions.

Generally, the introduction of exotic species and subsequent formation of hybrids has greater negative impact to the native species as they can result into extinction of parental species due to reproductive isolation (Allendorf *et al.*, 2001). The phenomenon also results into loss of pure species due to transgressive segregation and creation of hybrid swarms (Crispo *et al.*, 2011). Moreover, hybridization affects fitness and reproductive success of the parental population (Muhlfeld *et al.*, 2009) which eventually result into low fecundity which can in turn cause lower fish yields (Amarasinghe and De-Silva, 1996). However, Seehausen, (2004) reported that, natural hybridization could be invaluable in adaptive radiation and evolution of new species through speciation process. Hybridization can also prevent extinction when exchanges of beneficial mutations occur among gene pools (Crispo *et al.*, 2011).

2.3. Conservation status of *Oreochromis* species in Lake Baringo

Lake Baringo is inhabited by nine fish fauna species: the native and endemic Baringo tilapia *Oreochromis niloticus baringoensis* (Trewavas, 1983), Catfish *Clarias gariepinus* (Burchell, 1822), hybrids of *Oreochromis niloticus baringoensis* with *Oreochromis leucostictus* (Nyingi and Agnèse, 2007), Lung fish *Protopterus aethiopicus* (Heckel, 1981), Cyprinids (barbs): *Labeo intermedius* (Banister, 1783), *Labeo cylindricus* (Peters, 1868), (classified as an endangered species) and the two rare species, *Barbus lineomaculatus* (Boulenger, 1983) and two undescribed species (*Barbus* spec ‘Baringo’ and *Aphocheilichthys* spec ‘Baringo’) (Nyingi, 2013; Hickley *et al.*, 2008; Britton *et al.*, 2009). Of these nine species, three species namely *Clarius gariepinus*, *O.n baringoensis* and *Protopterus aethiopicus* are economically the most exploited by the communities around Lake Baringo (Omondi *et al.*, 2013).

The Baringo tilapia *Oreochromis niloticus baringoensis* is endemic to Lake Baringo. The fish has not been evaluated for International Union for Conservation of Nature (IUCN) redlist of threatened species. However, the dwindling in catch returns over the last two decades and compromisation of the fish’s genetic integrity through introduction of other tilapiines in the lake suggest that the population may be threatened (Britton *et al.*, 2009). The trend of tilapia catch in the lake has been declining with time. In 1990s, the fish collected by gill nets was 86% of the total catch, in 2002 the catch stood at 80.04% (Aloo, 2002). In 2004 the fish catch by commercial gill nets contributed only 4% of the total earnings compared to other species in the lake. Britton *et al.*, (2009) observed that the population status of *Oreochromis niloticus* in Lake Baringo was not threatened *per se* but subject to unpredictable and unstable environment, overfishing and introduction of exotic species. Recent studies have revealed that *P. aethiopicus* which was introduced in 1975 is now dominating the Lake Baringo fishery (Omondi *et al.*, 2013).

Different measures have been taken to rescue the disappearance of *O. niloticus* from the Lake Baringo including periodic suspension of fishing by Kenyan Department of fisheries from early 1993 to September 1994 and from November 2001 to January 2004 (Nyingi and Agnès, 2007; Hickley *et al.*, 2008). This was done to allow multiplication of the fish so as to increase catch returns. Another conservation strategy used was establishment of fish culture farms in the lake to improve fish production through aquaculture. However, this strategy has posed conservation threats to the native tilapia species as the fish from aquaculture farms have spilled and mixed with the indigenous fish. This spilling and mixing of fish fingerlings from their captivity violates the Nairobi declaration of a year 2002, on the management of tilapia aquaculture and biodiversity which emphasized on the need to identify and manage wild stocks of important tilapia species (WorldFish Center, 2002).

It has been reported by Canonical *et al.*, (2005) that unmanaged introduction of exotic species has pernicious effect to the native biodiversity. These effects include ecological alterations, hybridization with native species and competition for resources such as food, breeding sites and habitats (Canonical *et al.*, 2005). Further, the introduced species may host pathogens and parasites which can be transmitted to the native species through the excreta released in water (Dabbadie and Lazard, 2010).

2.4. Morphological characterization of the Baringo Tilapia

The traditional distinction of species within family Cichlidae rely on the differences in appearances of characteristics such as body size, shape, colour, number of spines, shape of fins (Wu and Yang, 2012). Morphologically, the Nile tilapia is characterized by a compressed body with caudal peduncle depth equal to its length, cycloid scales, first gill arch with 27-33 gill rakers, spined and soft ray sections of dorsal fin are continuous (Trewavas, 1983). The number of spines in dorsal fin is 16 to 17 spines and 11 to 15 soft rays (Trewavas, 1983). The

anal fin has three spines and 10-11 rays. The caudal fin is truncated and a knob like protuberance is absent on dorsal surface of the snout. Most remarkably, all Nile tilapia species have a caudal fin with dark vertical stripes (Trewavas, 1983). All *O. niloticus* subspecies have overlapping morphological characters and can only be distinguished on the basis of their natural geographical locations. *O. niloticus* can be distinguished from *O. spirulus* by the fact that the latter species has blotches in the caudal fin rather than dark vertical stripes (Trewavas, 1983).

According to Nyingi, (2013), the Baringo tilapia (*Oreochromis niloticus baringoensis*) has basic characteristics of the species. It is however characterized by 7-8 vertical bars in the flank, caudal fin with dark brown vertical stripes. The females and juveniles have pale slate-grey colouration with white belly while their pelvic fins, dorsal fin edge contain dark lappets and the pectoral fins are red to light brown in colour. Meanwhile, the breeding males have dark grey tipped pelvic and anal fins and dusky grey on ventral part of the body.

Morphological characterization of species is often carried out by morphometrics and meristics methods that employ respectively, measurement and counting of morphological (phenotypic) characters respectively, to delineate fish varieties including species, subspecies and populations (Samaradivakara *et al.*, 2012). For *Oreochromis niloticus* the choice of morphological characters follow those used by Trewavas, (1983) and expanded by Vreven *et al.*, (1998). Morphometrics (measurements) and meristics (counts) have been used to differentiate groups of fish by several authors; Arctic char *Salvelinus aepinus* (L) (Doherty and McCarthy, 2004), *Oreochromis niloticus* and *Lates niloticus* (Yakubu and Okunsebor, 2011), *Oreochromis niloticus* (Vreven *et al.*, 1998; Samaradivakara *et al.*, 2011; Hassanien *et al.*, 2011), *Toxotes chatareus* and *Toxotes jaculatrix* fish (Simon *et al.*, 2010).

2.5. Molecular characterization and population genetics of Nile Tilapia

The morphological identification of cichlid species based on differences in body characters such as colour, number of spines, number of scales and number of rays has proven to be subtle in identification of tilapia species especially hybrids (Moralee *et al.*, 2000; Nagl *et al.*, 2001). Compared to traditional morphometric based techniques, DNA based methods have been proven to be ideal in identification of tilapia strains even in mixed populations (Wu and Yang, 2012; D'amato *et al.*, 2007). Since its adoption in the 1980s, different molecular methods have been used in characterization and identification of tilapia species, these include allonzymes (Agnèse *et al.*, 1997; Moralee *et al.*, 2000; Naish and Skibinski, 1997; Gregg *et al.*, 1998; Rognon and Guyomard, 2003; Vreven *et al.*, 1998), Restriction Fragment Length Polymorphism (RFLP) (Agnèse *et al.*, 1997) and Random Amplified Polymorphic DNA (RAPDs) (Ali *et al.*, 2005). Other markers used include, microsatellites (Ndiwa *et al.*, 2014; D'Amato *et al.*, 2007; Kocher *et al.*, 1998; Carleton *et al.*, 2002; Sandeep *et al.*, 2012; Hassanien and Gilbey, 2005; Bezault *et al.*, 2012; Nyingi and Agnèse, 2007), Mitochondrial DNA Control Region (D'Amato *et al.*, 2007; Nyingi and Agnèse, 2007; Wu and Yang, 2012; Firmat *et al.*, 2013; Ndiwa *et al.*, 2014) Cytochrome Oxidase 1 (CO1) (Hebert *et al.*, 2003; Shirak *et al.*, 2009; Ferri *et al.*, 2009; Abumourad, 2011), Cytochrome b (Rognon and Guyomard, 2003), Ribosome 45s and 5s (rDNA) (Martins *et al.*, 2000; El-Serafy *et al.*, 2003).

Of all these markers, the maternally inherited mitochondrial DNA (mtDNA) is mostly used in tilapia studies due to its relatively small size of about 16,625 bp (Yue *et al.*, 2006; He *et al.*, 2010), high mutation rate of its genome and absence of recombination (Seyom and Kornfield, 1992b; Ali *et al.*, 2005). These genetic features make this marker ideal for studies involving population genetic differentiation (Agnèse *et al.*, 1997), hybridization between species

(Firmat *et al.*, 2013), species identification (Wu and Yang, 2012) and Phylogenetics (Nagl *et al.*, 2001). Two mtDNA marker genes Cytochrome Oxidase 1 (CO1) and Control region (CR) have widely being used in genetic studies of tilapia and other animal species.

The mtDNA Cytochrome Oxidase 1 (CO1) marker has been widely used in barcoding of living species. A short stretch of DNA (barcode) in this marker gene is used to allocate an unknown individual into a species (Galtier *et al.*, 2009). The wide applicability of CO1 marker gene in species identification is attributed by mainly two reasons; robustness of its universal primers enabling recovery of its 5' end representative of different groups in the animal kingdom as well as possession of a wider range of phylogenetic signals than any other mitochondrial gene (Hebert *et al.*, 2003). In Nile tilapia *Oreochromis niloticus*, this 1139 bp (Abumourad, 2011) gene has not often been widely used in population studies, however the sequence divergences in this gene makes it ideal for identification of closely allied species in animal phyla including Nile tilapia (Hebert *et al.*, 2003). Wu and Yang, (2012) used COI gene to identify captive and wild tilapia in Hawaii. However the results showed that the gene had low nucleotide diversity mainly due to highly conserved regions of this protein coding gene.

The non coding mitochondrial control region or mtDNA D-loop situated between tRNA –Pro and tRNA-Phe genes is the most polymorphic and fast evolving region in the mitochondrial genome (Wu and Yang, 2012; Yue *et al.*, 2006; He *et al.*, 2011). This 927 bp long (He *et al.*, 2011) gene has been widely used as a marker in Nile tilapia population and phylogenetic studies (Nagl *et al.*, 2001), species identification, determination of presence of hybrids and mtDNA introgression (Nyingi and Agnèse, 2007; D'amato *et al.*, 2007; Wu *et al.*, 2012; Firmat *et al.*, 2013; Ndiwa *et al.*, 2014). The preference of this gene in genetic studies of *Oreochromis niloticus* is attributed to its rapid mutation rate of three to five times more than

other mitochondrial genes making it hyper variable and highly polymorphic (Wu *et al.*, 2012). For instance the comparison of interspecies variation between mitochondrial Control region (mtDNA CR) and Cytochrome C Oxidase 1 (CO1) for captive and wild tilapia in Hawaii revealed that mtDNA CR had higher nucleotide diversity than CO1 (Wu and Yang 2012).

2.6. Molecular techniques for Isolation, Amplification, Visualization, Purification and Sequencing of DNA

2.6.1. DNA Isolation

The procedure for extraction and isolation of genomic DNA from animal tissues including fish involves mainly three steps, which are tissue or cell lysis, DNA precipitation and DNA wash. Lysis involves cutting and maceration of the tissues into small size that can easily be acted by digesting enzymes and extraction buffers (Ndiwa *et al.*, 2014). These tissues with a size of about 10-100mg are normally minced with a sterile blade or scissor. The next step in lysis involves the addition of proteolytic enzyme, Proteinase K for digestion of tissues and buffers containing anionic detergents such as Sodium dodecyl sulphate (SDS), Tris EDTA and STE (Bernatchez *et al.*, 1992; Beaumont, 1994; Pearson and Stirling, 2003). These buffered salts disrupt cell membranes providing favourable pH for the enzymes to work as well as preventing the degradation of DNA by inactivating all other enzymes in the cytosol (Beaumont, 1994). The mixture of minced tissues, Proteinase K and detergents is normally incubated over night in a water bath shaker at a temperature of 55-56°C to provide an optimum condition for the enzyme to work (Pearson and Stirling, 2003).

Extraction of the DNA in the digested tissue has normally been done using organic solvents or special extraction kits. The organic solvents include phenol, chloroform, phenol-chloroform (1:1) or phenol-chloroform isoamyl alcohol (25:24:1) (Bernatchez *et al.*, 1992;

Pearson and Stirling, 2003). To extract DNA, the solvents are mixed with homogenized aqueous solution of the lysed cells or tissues and separation of the two layers through centrifugation. During separation of the aqueous and organic phases, the negatively charged DNA dissolve in the upper aqueous phase instead of the organic layer since the former is more polar than the latter (McMurry, 2003). Nuclear DNA and partially purified mitochondrial DNA can be separated by homogenization and differential centrifugation (Beaumont, 1994; Skibinski, 1994). Meanwhile, proteins with hydrophobic and hydrophilic domains are normally precipitated by phenol forming a white flocculent at the interphase between the two phases (Avison, 2007). The DNA in the aqueous phase is normally precipitated by addition of absolute ethanol which is much less polar than water (Pearson and Stirling, 2003). This means that adding ethanol to the aqueous solution disrupts screening of charges by water increasing electrical attraction between the DNA phosphate groups and any positive ions present in solution resulting into precipitation of the DNA. Purification of the DNA is done using 70% ethanol which break the pellet loose and wash it to remove some of the salts present in the leftover supernatant and bound to DNA pellet making the final DNA cleaner. The purified DNA can be dissolved in water or desired buffers for storage.

Now days the ordinary solvent DNA extraction has been widely replaced by DNA extraction kits which use similar principles as in solvent extraction except that they use special silica gel columns which hook up with the DNA. Different protocols have been established by different kit manufacturing companies for isolation of DNA from different materials. The advantages of these extraction kits over the traditional solvent extraction approach are; high recovery rate of DNA, less harmful, produce DNA with high quantity and quality.

2.6.2. Polymerase Chain reaction (PCR)

PCR is a technique used for enzymatic *in vitro* amplification of a specific DNA sequence in the genome producing billion copies of the targeted DNA segment (Saiki *et al.*, 1988). Prior to the discovery of this approach, amplification of a gene of target involved cloning of a homologous DNA region from every individual in the sample (Skibinski, 1994). Currently, PCR approach can be applied to both nuclear and mitochondrial DNA and could be done with trace amounts of starting tissue in a short period of time without necessarily requiring a host organism for cloning (Skibinski, 1994; Snustad and Simmons, 2006; Klug *et al.*, 2009).

This technique uses two oligonucleotide sequences called primers that flank the opposite ends of the strands allowing the DNA polymerase to copy the DNA segment to be amplified using deoxyribonucleoside triphosphates (dNTPs) (Saiki *et al.*, 1988, Beaumont, 1994). The technique involves repeated cycles of heat denaturation of the DNA, annealing of primers to their complementary sequences and extension of the annealed primers with DNA polymerase.

During denaturation which normally occurs at high temperature between 92-97°C, the hydrogen bonds holding the DNA stands break down allowing the DNA duplex of the fragment to separate (Snustad and Simmons, 2006). This separation of double strands of the DNA (dsDNA) allows the primers to hybridize (anneal) to the 5' of opposite strands of the targeted gene when the temperature is lowered to 50-60°C depending on the length of the primer used.

During the extension step which normally occurs at 72°C, the taq DNA polymerase from *Thermus aquaticus* bacteria (Chien *et al.*, 1976), use the annealed primers as starting point to copy the rest of the fragment as if it were replicating DNA. Since the extended products are complementary and capable of binding the primers, each successive cycle doubles the amount of DNA produced in the previous cycle (Saiki *et al.*, 1988). In this case the PCR

machine is programmed to repeat cycles of denaturation, annealing, extension (figure 2) 30-40 times until sufficient replicates of the targeted gene accumulate to the extent of being visible under the gel.

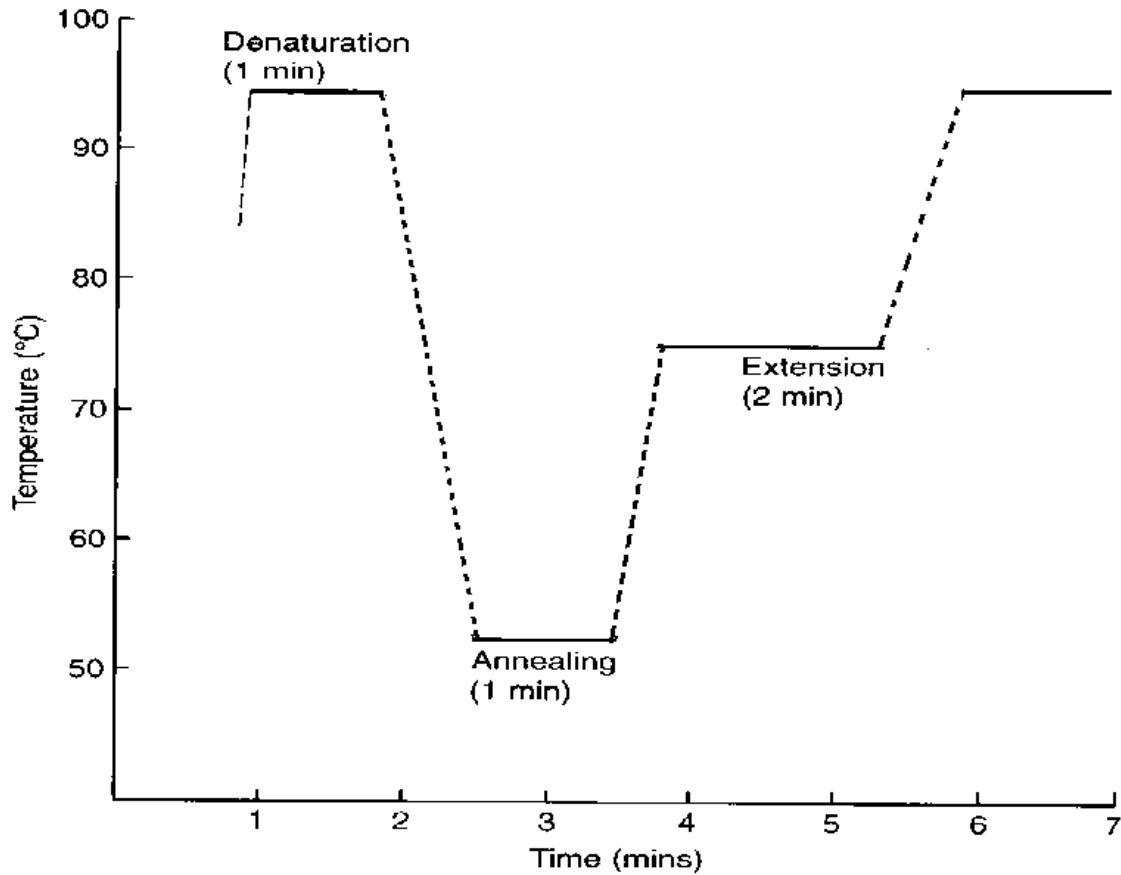


Figure 2: PCR cycle (Adopted from Brown, 2010)

2.6.3. Agarose gel electrophoresis

Gel electrophoresis is a technique that is used to separate proteins and nucleic acids based on their size by the aid of an electric field where negatively charged molecules migrate towards the anode (Yilmaz *et al.*, 2012). The migration rate of the molecules in the gel is dependent on the shape and charge to mass ratio of the fragments (Brown, 2010). In this case, the larger the DNA fragment, the slower it moves and the more compact or super coiled a piece of DNA is, the faster it moves (Sambrook *et al.*, 1989; Brown, 2010).

The Gel electrophoresis method is employed to observe the restriction enzyme digestion, to visualize and determine the size, purity and concentration of extracted DNA or PCR products (Bartlett, 2003). In this technique a gel which refers to the matrix that act as a sieving medium is a cross linked polymer whose composition and porosity is chosen based on the specific weight and composition of the target to be analysed (Brown, 2010; Yilmaz *et al*, 2012).

Normally, nucleic acid electrophoresis uses a seaweed-derived polysaccharide called agarose as an anticonvective medium to resolve the DNA fragments on the basis of their size. Agarose gel is more robust and easy to prepare than other polyacrylamide gels. This and its ability to separate a wide range of fragment size (200-5000bp) make them suitable for resolving the DNA fragments (Bartlett, 2003).

The concentration of agarose gel depends on the sizes of the DNA fragments to be separated with gels ranging between 0.5- 2% (w/v) (Lee *et al.*, 2012). This is achieved by dissolving a weighed amount of agarose powder in a given volume of electrophoresis buffer such as TAE (40mM Tris acetate, 1mM EDTA) or TBE (45mM Tris borate, 1mM EDTA) in an Erlenmeyer flask (Bartlett, 2003; Lee *et al.*, 2012). TAE buffer is more widely used in gel preparation as it facilitates easy recovery of materials from the gel (Bartlett, 2003). The mixture is swirled and heated in a microwave or Bunsen burner until it is completely homogenized.

The DNA intercalating fluorescent stain, ethidium bromide (0.5µg/ml) is normally added to the molten agarose at 60°C before the agarose matrix is cast into a casting tray with combs for solidification. Ethidium bromide is included in the gel matrix to enable fluorescent visualization of the DNA fragments under ultra violet light (U.V). After solidification, the gel is placed in the gel box half filled with buffer and the combs carefully removed to expose the

wells. Each DNA sample is mixed with gel loading dye normally 0.25 % bromophenol blue or 0.25% Xylene or 30% glycerol (Lee *et al.*, 2012). Loading dye helps to track how far the DNA has travelled and also allows the samples to sink into the wells upon loading (Bartlett, 2003; Lee *et al.*, 2012).

The size marker (ladder) is normally co-electrophoresed with DNA samples to estimate the concentration and size of the desired fragment. After loading, more buffer is added to cover the surface of the gel to prevent it from drying during electrophoresis (Bartlett, 2003). The gel box is normally connected to a voltage of between 1-5V/cm where cm is the distance in centimetre between the electrodes in the gel tank (Bartlett, 2003) and the wells are placed towards the negative electrode (Yilmaz *et al.*, 2012).

During electrophoresis, the negative DNA and loading dye migrates from the anode to cathode while ethidium bromide migrates in a reverse direction to meet and couple with the DNA fragments (Yilmaz *et al.*, 2012). According to Sambrook and Russel, (2001), linear DNA fragments migrate through the agarose gel with a velocity that is inversely proportional to log₁₀ of their molecular weight. Once the DNA has separated, the gel is placed under UV light which illuminate and fluoresces the ethidium bromide bound to the DNA indicating the presence of DNA bands (Brown, 2010).

2.6.4. Purification of PCR products

Purification of PCR products is done to get rid of primer dimers, non-specific amplifications, excess buffers, salts, dNTPs and unincorporated primers (Leonard *et al.*, 1998). If these by-products remain unisolated from the PCR products, they could interfere with downstream processes such as DNA sequencing and single nucleotide polymorphism (SNP) analysis (Werle *et al.*, 1994).

There are two major ways of getting rid of unwanted stuffs from the PCR products, these are; gel isolation and direct isolation. Isolation of the band of target through gel excision is the most effective way of obtaining a clean PCR product suitable for downstream reactions. This is done by first running the PCR products on the agarose gel as described in 2.6.3 above. The bands of the targeted gene as referred to the ladder are excised from the gel upon visualization in a UV light box leaving the non-specific amplicons and primer dimmers on the gel. The excised gel slices are melted by heating in a water bath and combined with binding columns made up of silica, which bind the DNA from the molten gel. The remaining agarose and buffers can be washed from the bound DNA by using alcohol followed by DNA elution by water or buffer (Thermo Scientific GeneJET Gel Extraction Kit protocol)

Purification of PCR products by isolation method can be done by simple ethanol precipitation and Exonuclease 1 Shrimp Alkaline phosphatase (ExoSAP) (Leonard *et al.*, 1998). Alcohol precipitation is carried out by adding a certain volume of alcohol in a reaction tube containing the PCR products. This method is ineffective in the sense that non -specific amplicons are incorporated with the targeted fragment. ExoSAP is another simplest way of purifying the PCR products. Its procedure involves addition of the enzyme (ExoSAP) to the PCR products (Werle *et al.*, 1994). In the enzyme-PCR products mixture, Exonuclease 1 removes the left over primers while Shrimp Alkaline phosphatase gets rid of dNTPs (Werle *et al.*, 1994). This method is limited by its inability to remove primer dimmers and non-specific amplifications (Leonard, *et al.*, 1998).

2.6.5. DNA sequencing

This is a technique used to obtain an exact sequence (order) of the bases in a given DNA or RNA fragment. DNA Sequencing is a PCR based reaction, which requires a DNA primer, normal deoxyribonucleoside triphosphate precursors (dATP, dTTP, dCTP and dGTP), and

DNA polymerase enzyme (Brown, 2010). The ability to sequence DNA has been pivotal in understanding genomic organization, gene structure, function, and mechanisms of regulation (Klug *et al.*, 2009).

There are several procedures for DNA sequencing but the most popular being the chain termination method which was first described by Sanger and colleagues in mid 1970s (Klug *et al.*, 2009; Brown, 2010). This sequencing method relies on base specific chain termination in four separate reactions containing four different nucleotides in a DNA make up. The technique uses 2'3' dideoxynucleotide triphosphates (ddNTPs), which lack OH group at the 3' end to terminate the extension of a newly synthesized product every time a corresponding ddNTP is incorporated. Without the 3' OH group needed to form a connection with the next nucleotide no more nucleotides can be added and DNA polymerase falls off (Brown, 2010). The ddNTPs (ddATP, ddTTP, ddCTP and ddGTP) are normally included in small amounts in the reaction mixture of a primer, DNA template and normal dNTPs (Klug *et al.*, 2009; Brown, 2010). This enables termination to happen rarely and stochastically resulting in a cocktail of extension products where every position of a given base would result in a matching product terminated by incorporation of ddNTP at the 3' end (Janitz, 2008). To get the exact DNA sequence, the mixture is loaded on a polyacrylamide slab gel or capillary gel system and the fragments are separated by electrophoresis according to their length (Klug *et al.*, 2009; Brown, 2010).

The Sanger sequencing method has been modified by an automated technique in which the four nucleotides are labelled differently with a fluorescent dye that fluoresces at different wavelength any time a reaction terminates. These fluorescent-labelled nucleotides tend to give peaks on computer screen when excited by laser indicating the exact nucleotide by its colour (Klug *et al.*, 2009). All four chain termination reactions can be performed in the same

tube and run on a single lane and a machine with laser can scan the lane producing the fluorescence output in form of chromatograms.

With technological advancement, different automated pyrosequencing kits have been developed by different biotechnological companies for commercial sequencing. The commonly used automated machine is AB1 capillary sequencer, which employs different versions of instruments. The technique does not require electrophoresis or other fragment separation procedure making it quicker than chain termination sequencing (Brown, 2010).

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Study Site

Lake Baringo, an internationally recognized Ramsar site, is a shallow fresh water lake in the eastern arm of the Great Rift Valley of Kenya between $0^{\circ} 32'$ and $0^{\circ} 45'$ N and $36^{\circ} 00'$ and $36^{\circ} 10'E$ at an elevation of 975m above mean sea level (Ssentongo and Mann, 1971). The lake is of approximately 130 km^2 in size (Omondi *et al.*, 2013) and is situated north of Lake Bogoria and south of Lake Turkana (Figure 3). It is fed by perennial rivers namely: Ol-Arabel, Mukutan, Tangelbei, Endao, Chemeron, Perkerra and Molo (Odada *et al.*, 2006), and is characterized by turbidity and periodic fluctuation of water levels attributed by prolonged periods of drought interspersed with heavy rainfall (Anderson, 2002). The depth of Lake Baringo as with most Rift Valley lakes fluctuates significantly although a mean depth of 3m with the deepest point of 7m at high water levels has been reported (Hickley *et al.*, 2008; Fisheries department, 2009; Omondi *et al.*, 2013). In 2013 however, heavy rainfall resulted into flooding of the Rift Valley lakes between April and June, 2013 which caused increase of the lake's water surface by 88km^2 from 143km^2 in January, 2010 to 231.6km^2 in September, 2013 (Onywere *et al.*, 2014). The lake is a home to diverse fauna and flora and is a major source of fish, water for domestic consumption, irrigation, tourism and transport for people living around (Omondi *et al.*, 2013).

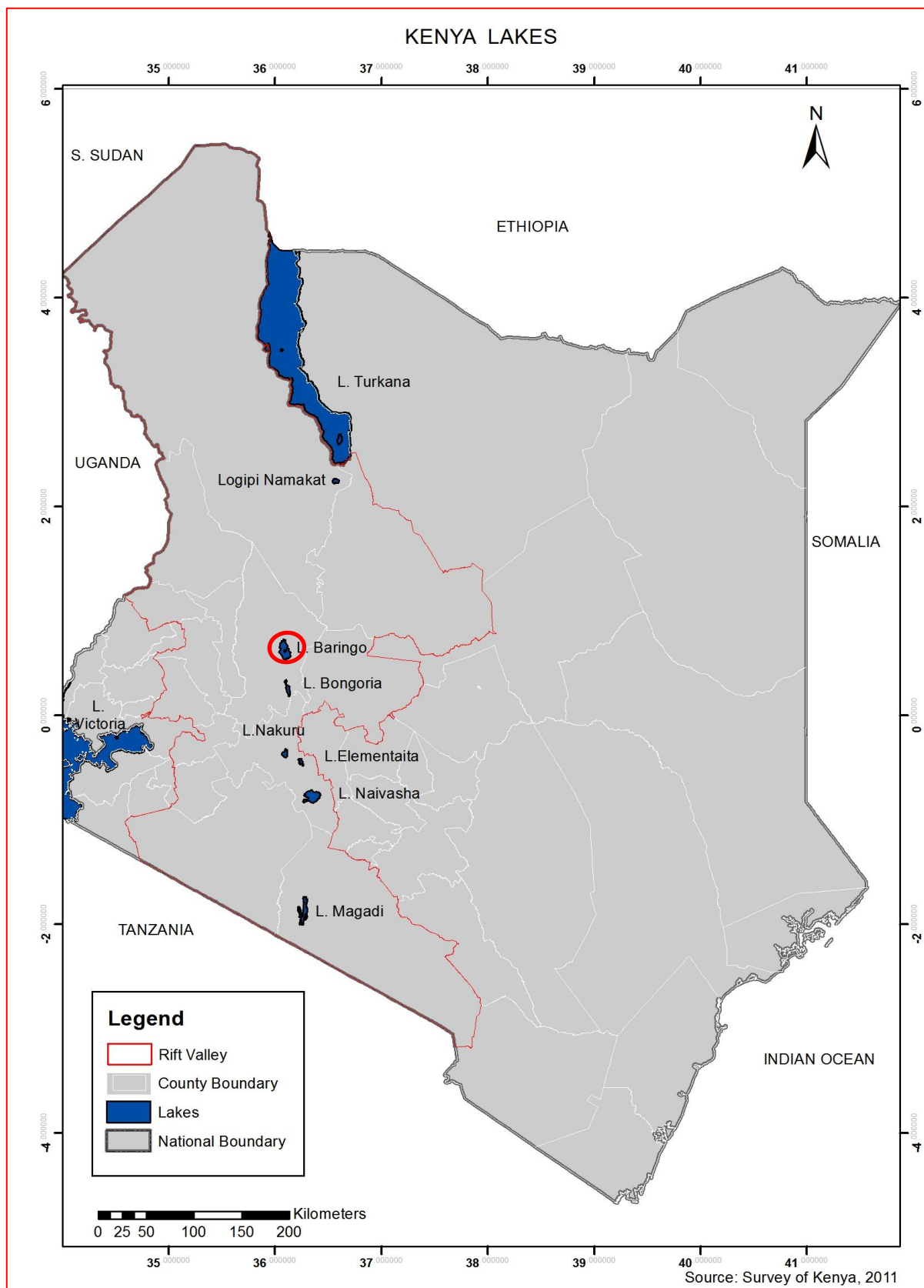


Figure 3: Map of Kenya showing major lakes and the location of Lake Baringo

3.2. Fish collection and sampling

Ninety eight fish specimens were collected randomly from three sites of Lake Baringo (south, central and north) using monofilament gill nets in May and October, 2013. The three sites were stratified into three localities for each except for north, which had two sites as shown in table 2 and figure 4. The sites were chosen on the basis of their proximity to the river mouth except for northern part of the lake which has neither inlet nor outlet rivers. The coordinates at which the samples were collected were marked by Geographical Positioning System (GPS) to ensure that the samples were collected from the same points every time sampling was done. These GPS coordinates were also used to plot the sampled points on the map in figure 4. The monofilament gill nets were set at the selected site and hauled after three hours. After capture, the standard length (SL) of each fish was measured and the specimen tagged for identification purposes. Photographs of some of the captured tagged fish specimens from the selected study sites are shown in plates 1 to 17.

Table 2: Fishing sites in Lake Baringo and the catalogue numbers for the preserved specimens at National Museums of Kenya

SECTION OF THE LAKE	LOCALITY	CO-ORDINATES	NMK Catalogue number
South	Molo river mouth (S2)	N0°31'27.2" E36°05'04.9"	FW/3722/1-7 FW/3725/1-4
	Salabani (S3)	N0°33'56.3"E36°03'22.8"	FW/3722/1-12
	Block Hotel (S1)	N0°36'38", E36°01'25.6"	FW/3713/1-3 FW/3760/1-3
Central	Nosuguro (C2)	N0°36'33.4" E36°07'32.8"	FW/3702/1-8, FW/3712/1-5, FW/3758/1-15
	Longicharo (C3)	N0°39'02.2" E36°06'05.1"	FW/3759/1-16
	Ng'enyin (C1)	N0°38'19.1" E36°02'08.6"	FW/3761/1-8, FW/3723/1
North	Katuwit (N1)	N0°43'25.8" E36°03'42.1"	FW/3724/1-5 FW/3762/1-8
	Komolion (N2)	N0°41'31.8" E36°04'47.5"	FW/3763/1

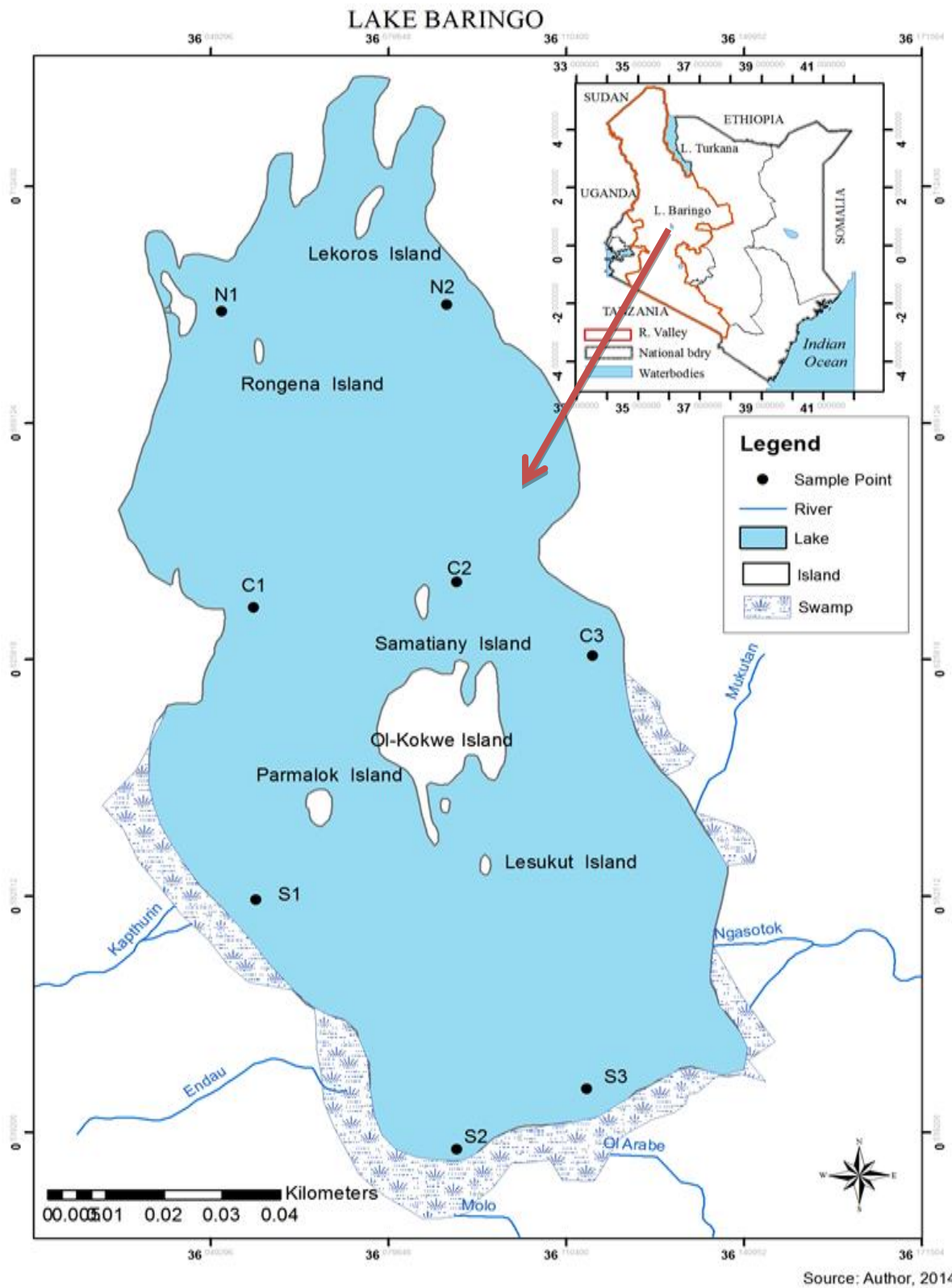


Figure 4: A map of Lake Baringo showing the sampling sites: S1, S2 and S3; C1, C2 and C3; N1 and N2 represents the sampling sites for southern, central and northern parts of the lake respectively.

3.2.1. Tissue preparation

A fragment of muscle tissue close to the dorsal fin was excised from each fresh fish sample, tagged and preserved in 95% analytical ethanol in eppendorf vials. These specimens were transported to the laboratory at National Museums of Kenya and refrigerated at -20°C to prevent DNA in the tissues from being degraded by endonucleases. The remaining whole voucher fish specimen was immediately fixed in 10% formalin in the field. The samples were then packed accordingly at a collection site and transported to the Ichthyology Section of the National Museums of Kenya where formalin was washed off using tap water after five days of preservation and then preserved in 70% ethanol for long-term curation as reference material. The catalogue numbers of these are listed on Table 2. Morphological analyses were carried out on these ethanol preserved specimens.

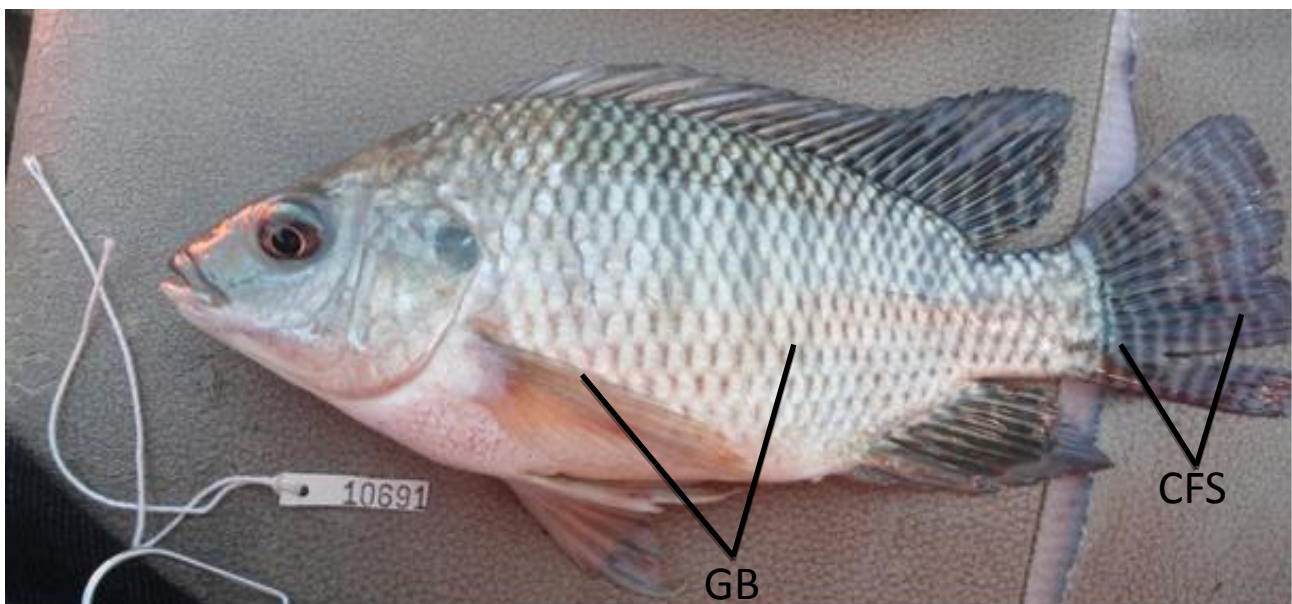


Plate 1: *Oreochromis niloticus* (10691) from Salabani (S1) south east of Lake Baringo (SL= 157.35 mm) (GB- Grey brownish trunk without vertical bars, CFS- Caudal fin with dark vertical stripes)

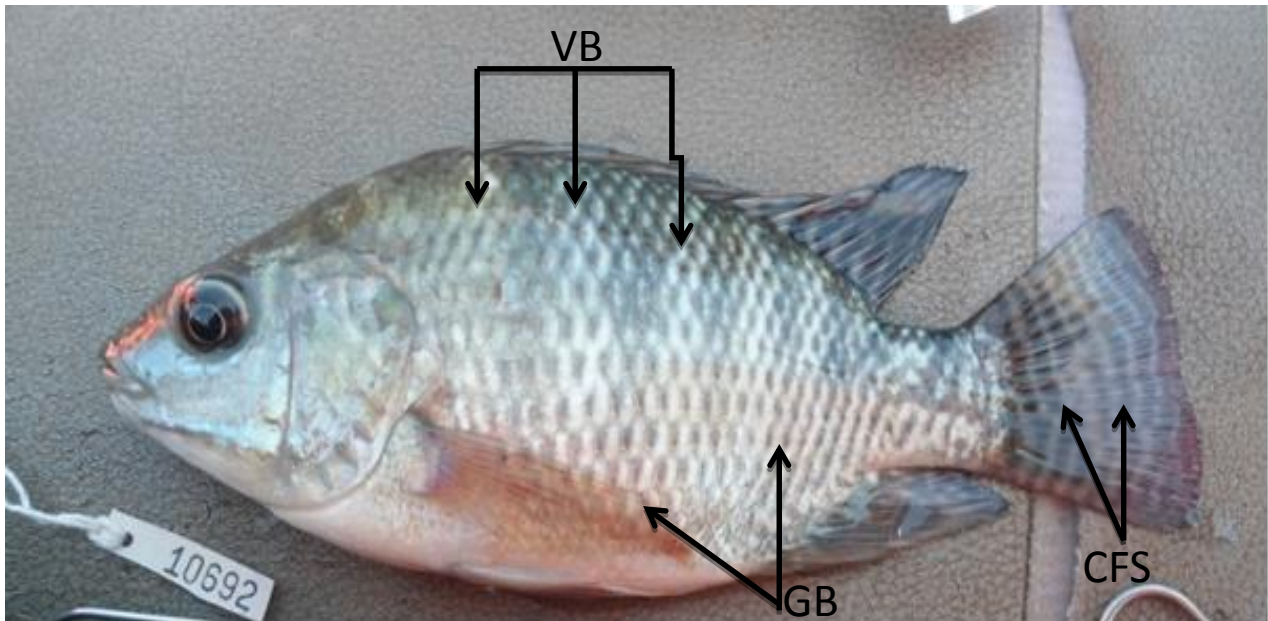


Plate 2: *Oreochromis niloticus* (10692) from Salabani (S1) south east of Lake Baringo (SL= 137.15 mm) (VB- Vertical bars, GB- Grey brownish trunk without vertical bars, CFS-Caudal fin with dark vertical stripes)

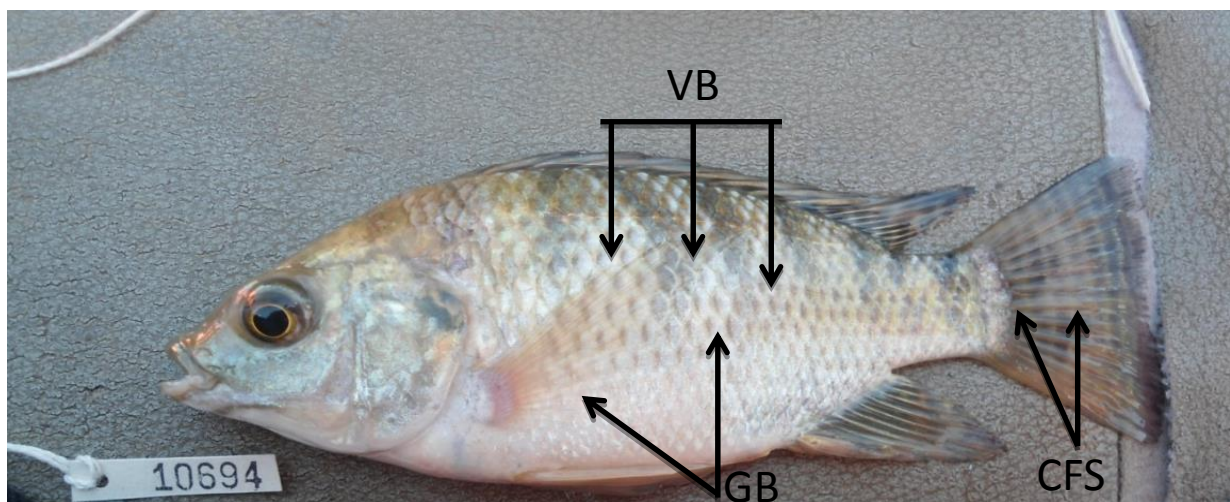


Plate 3: *Oreochromis niloticus* (10694) from Salabani (S1) south east of Lake Baringo (SL= 118.30 mm) (VB- Vertical bars, GB- Grey brownish trunk, CFS-Caudal fin with dark brown vertical stripes)

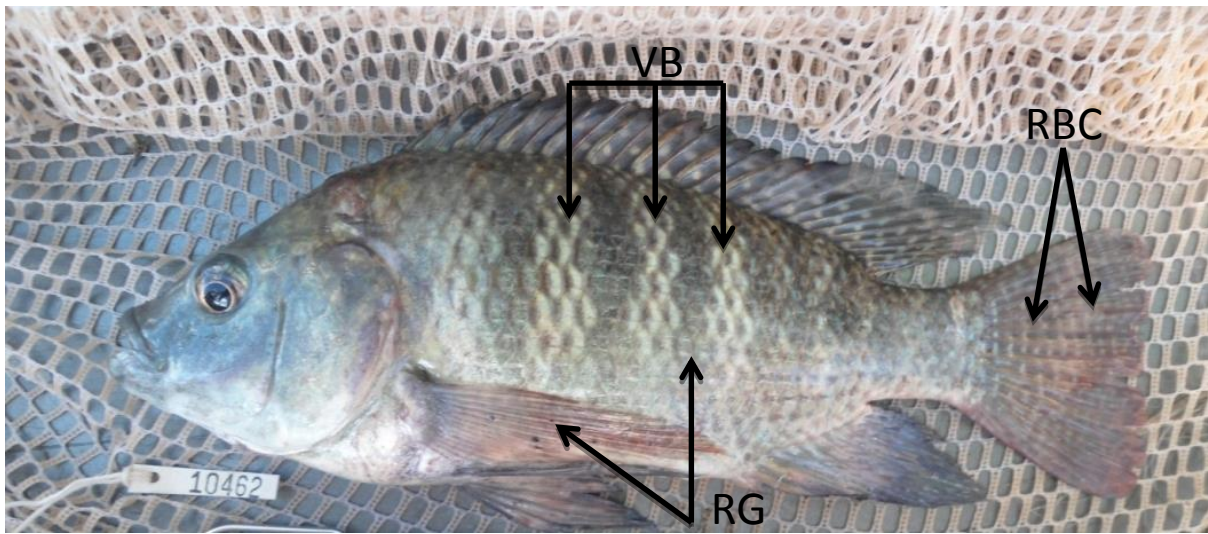


Plate 4: *Oreochromis spirulus* (10462) from Block Hotel (S1) South west of Lake Baringo (SL= 170.15 mm) (VB- Vertical bars, RBC- Reddish brown caudal fin with no dark vertical stripes, RG- Reddish darkish grey trunk)

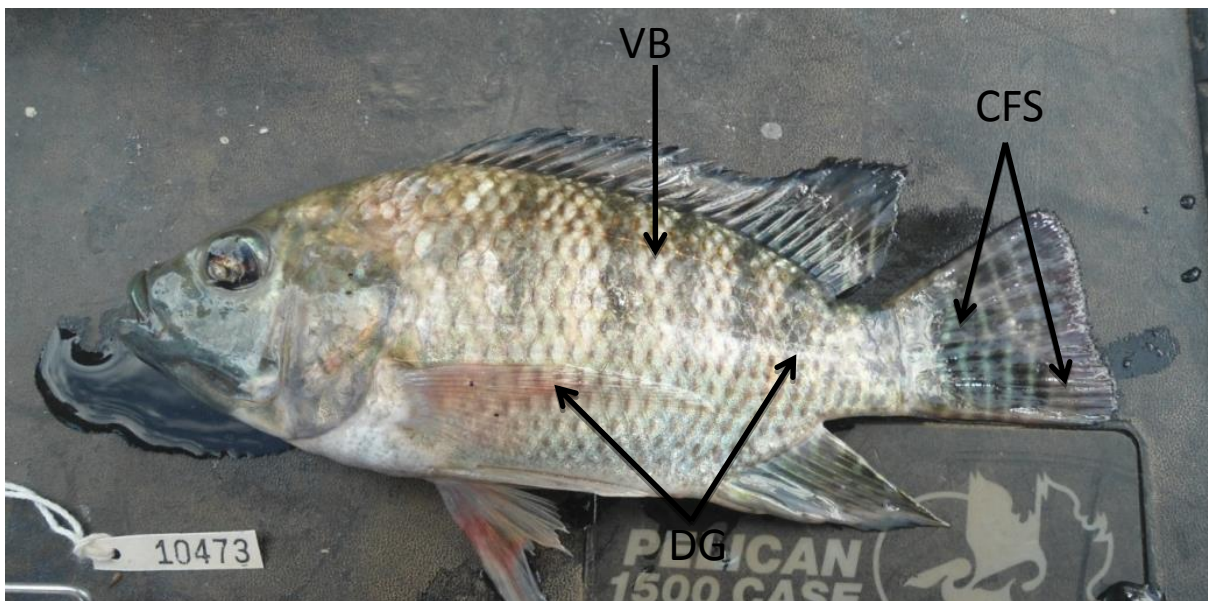


Plate 5: *Oreochromis niloticus* (10473) from Ng'enyin (C1) central west part of Lake Baringo (SL=118.15). (VB- Vertical bars, DG- Dark grey colour on body trunk, CFS-Caudal fin with dark vertical stripes)

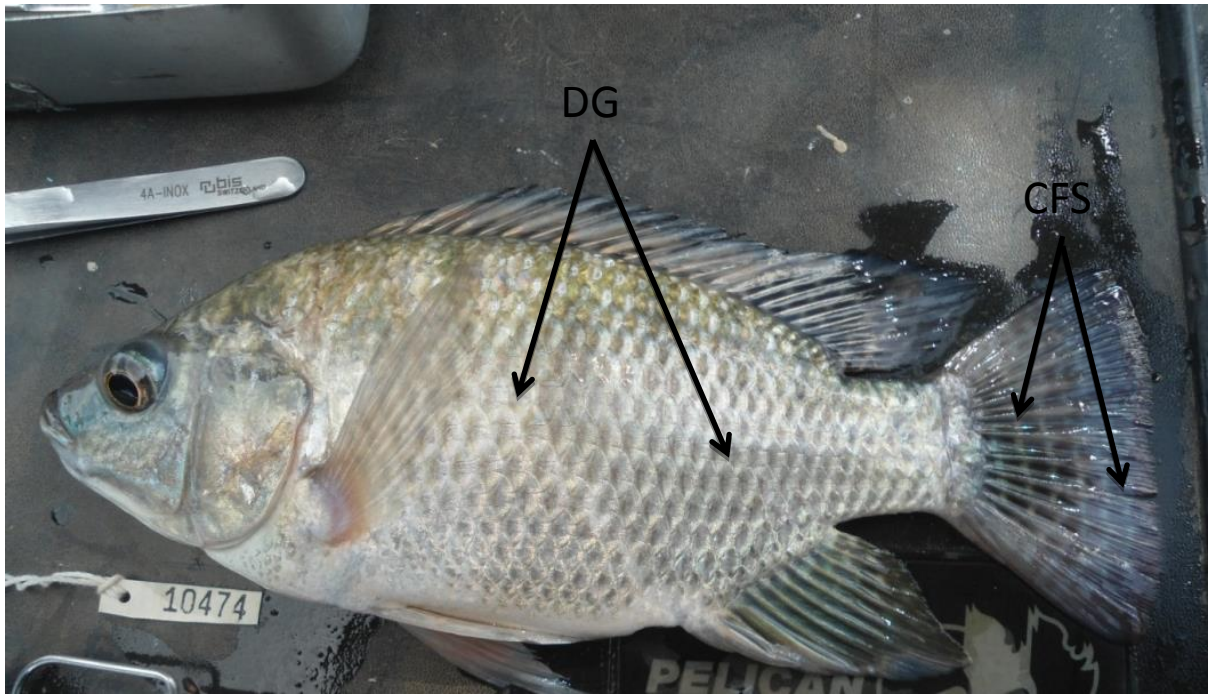


Plate 6: *Oreochromis* sp (10474) from Ng'enyin (C1) Central west part of Lake Baringo (SL= 154.35mm) (DG-Dark grey colour on body trunk with no vertical bars, CFS- Caudal fin with greyish vertical stripes)

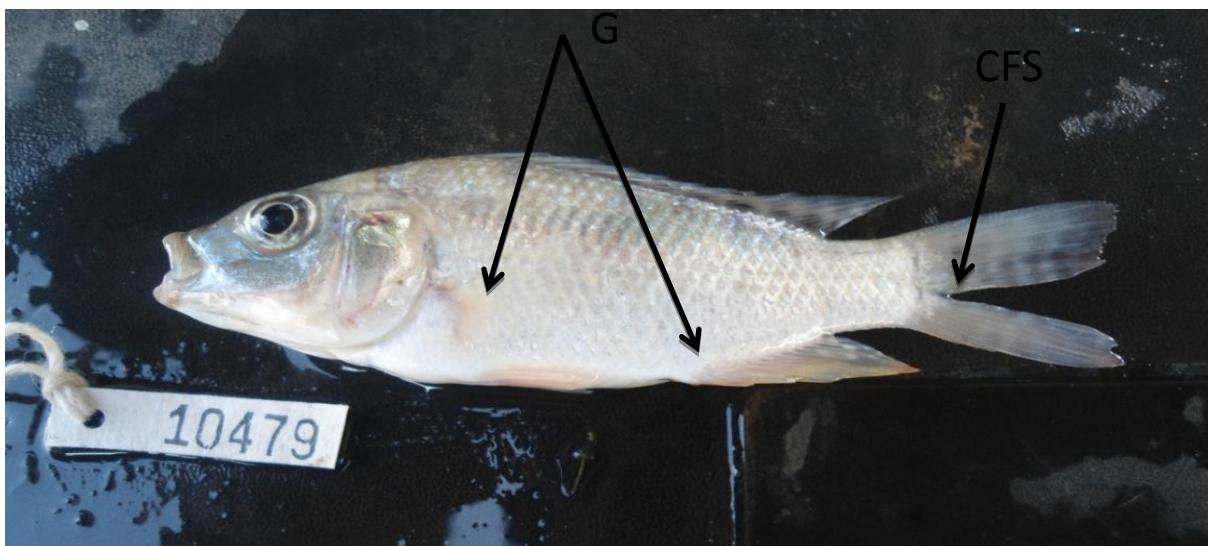


Plate 7: *Oreochromis* sp (10479) from Ng'enyin (C1) Central west part of Lake Baringo (SL= 65.30 mm) (G- Grey colour on body trunk with no vertical bars, CFS- Darkish grey caudal fin with dark vertical stripes)

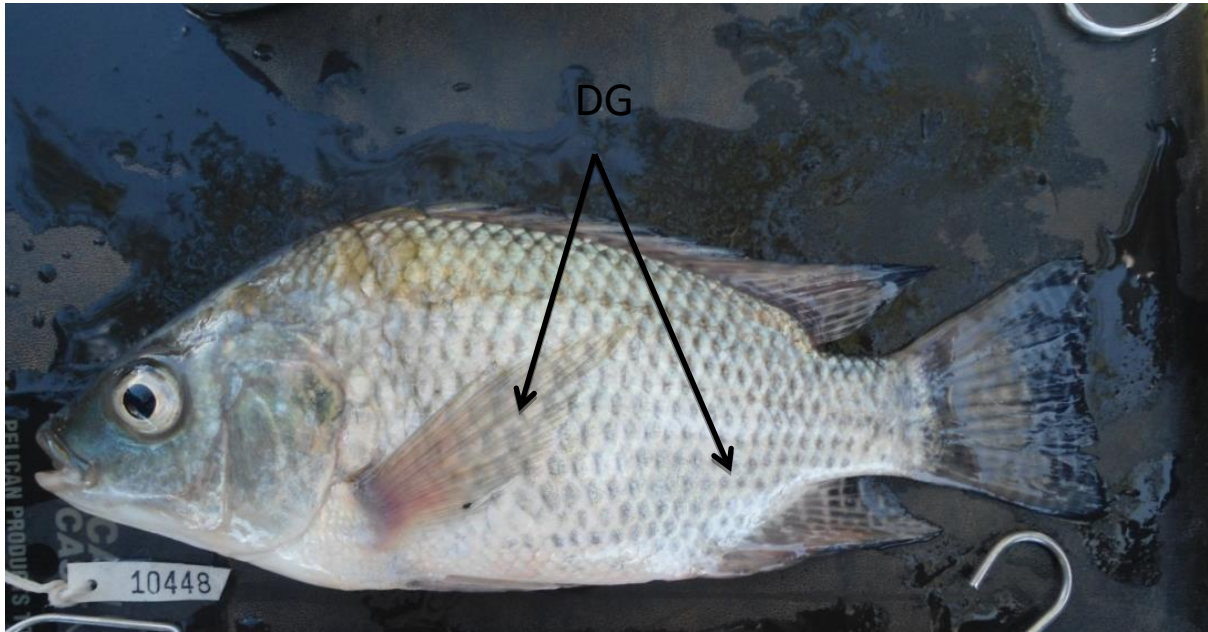


Plate 8: *Oreochromis* sp (10448) from Longicharo (C2) Central part of Lake Baringo (SL= 149.75 mm) (DG- Dark grey trunk with no vertical bars)

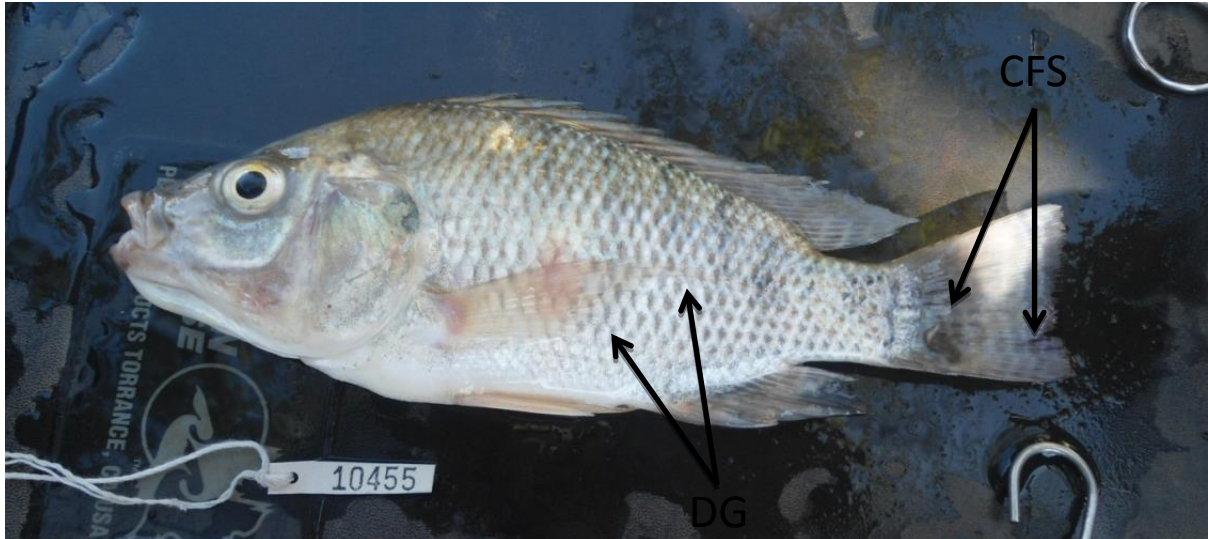


Plate 9: *Oreochromis* sp (10455) from Longicharo (C2) Central part of Lake Baringo (SL= 134.60 mm) (DG-Dark greyish trunk lacking vertical bars, Caudal fin with inconspicuous vertical stripes)

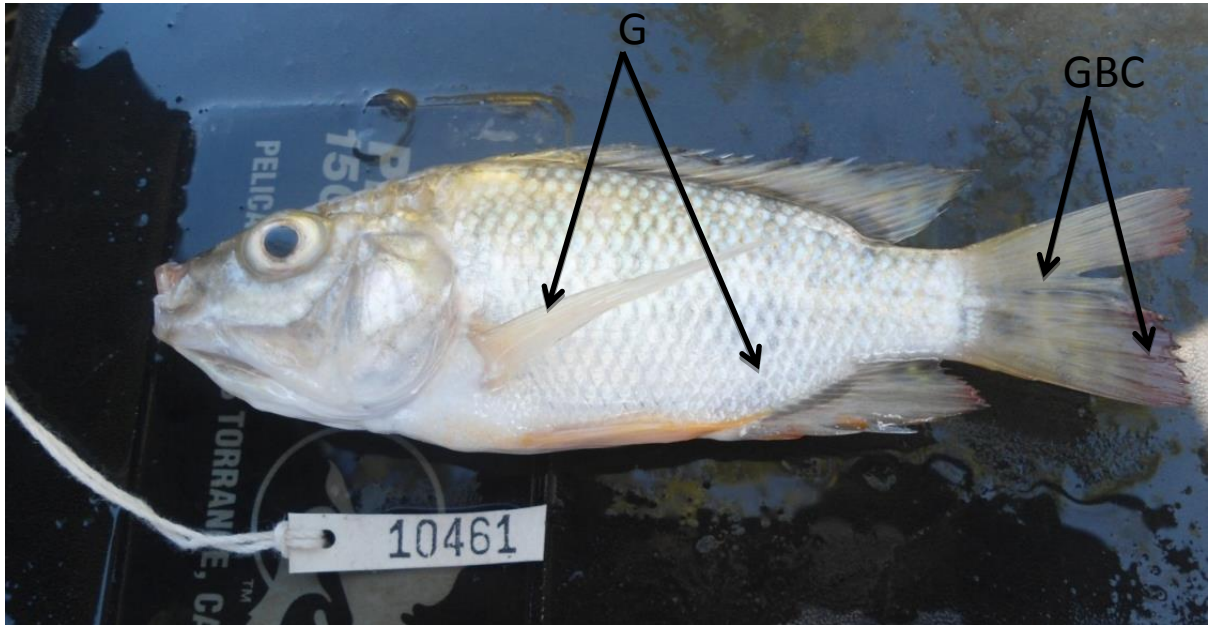


Plate 10: *Oreochromis* sp (10461) from Longicharo (C2) Central part of Lake Baringo (SL= 85.55 mm) (G-Grey colour on body trunk with no vertical bars, GBC- Greyish brown caudal fin with no vertical stripes)

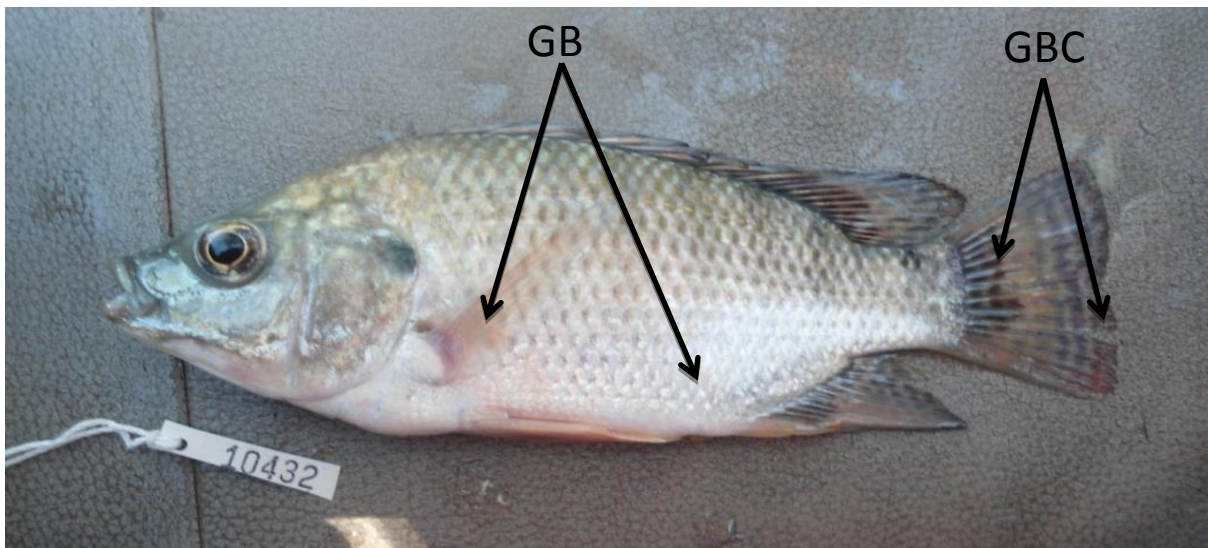


Plate 11: *Oreochromis niloticus* (10432) from Nosuguro (C3) Central west part of Lake Baringo (SL=137.35 mm) (GB- Greyish brown trunk with no vertical bars, GBC- Greyish brown caudal fin with dark vertical stripes)

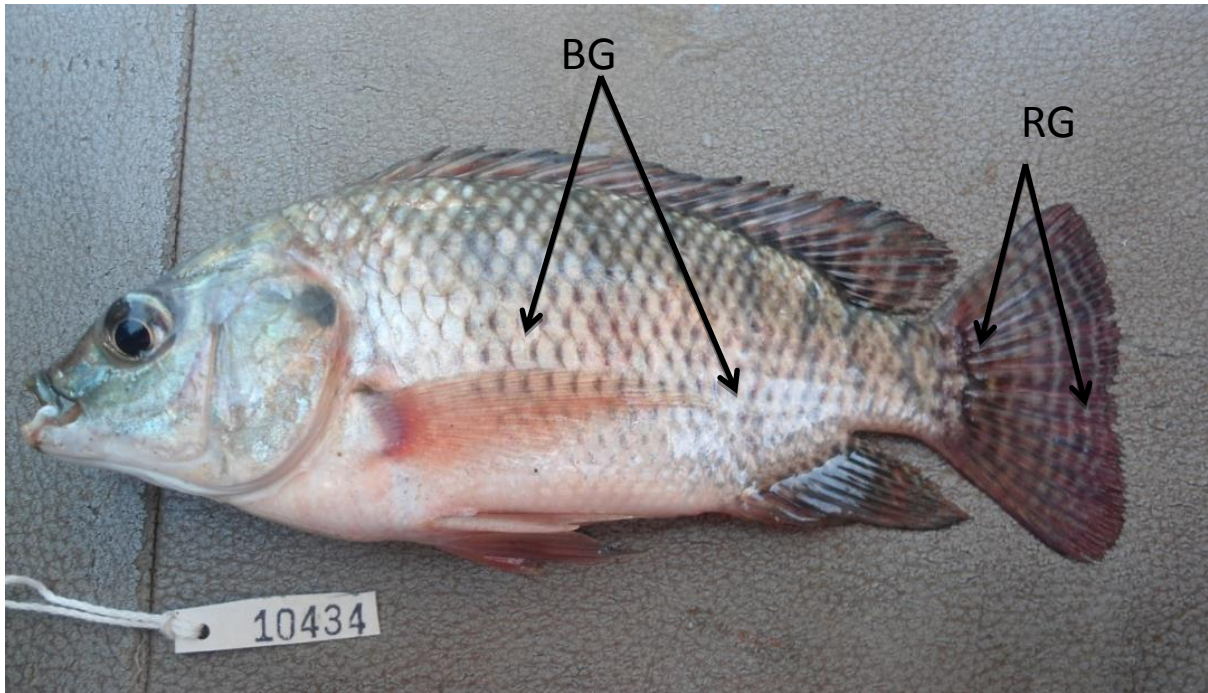


Plate 12: *Oreochromis niloticus* (10434) from Nosuguro (C3) Central west part of Lake Baringo (SL= 129.05 mm) (BG-Brownish grey trunk with no vertical bars, RG-Reddish grey caudal fin with dark brown vertical stripes)

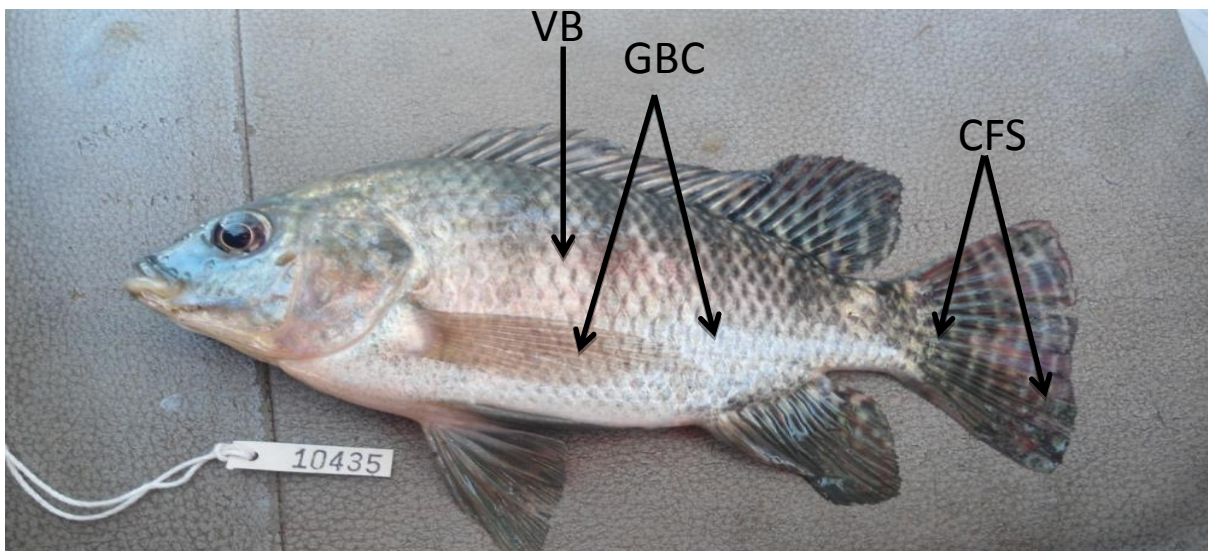


Plate 13: *Oreochromis niloticus* (10435) from Nosuguro (C3) Central west part of Lake Baringo (SL= 143.70 mm) (VB- Vertical bar, GBC- Grey brownish colour, CFS- Caudal fin with dark vertical stripes)

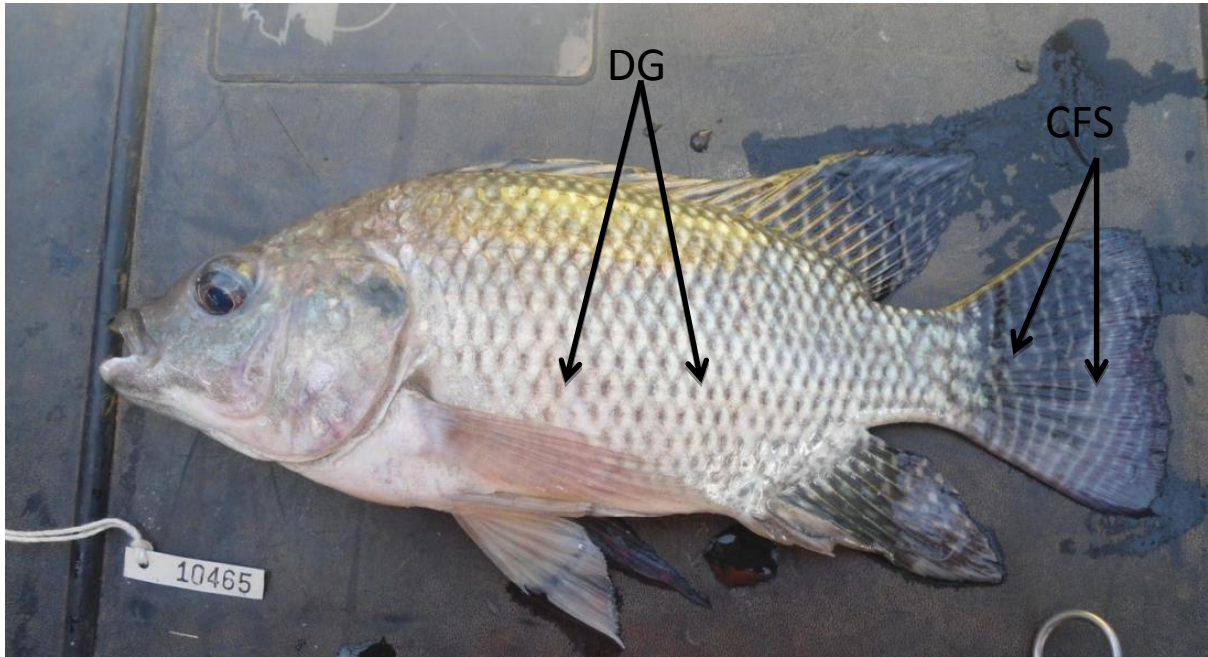


Plate 14: *Oreochromis niloticus* (10465) from Katuwit (N1) North West of Lake Baringo (SL= 160.20 mm) (DG- Dark greyish trunk without vertical bars, CFS- Caudal fin with dark vertical stripes)

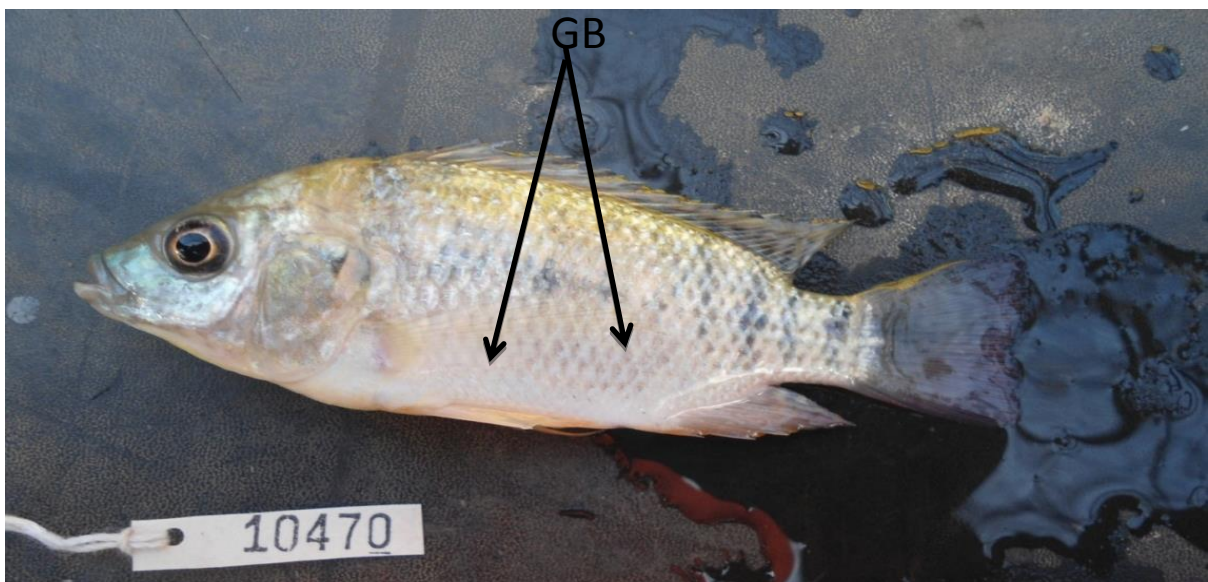


Plate 15: *Oreochromis* sp (10470) from Katuwit (N1) North West of Lake Baringo (SL= 80.90 mm) GB-Grey brownish trunk lacking vertical bars, Caudal fin with inconspicuous vertical stripes)

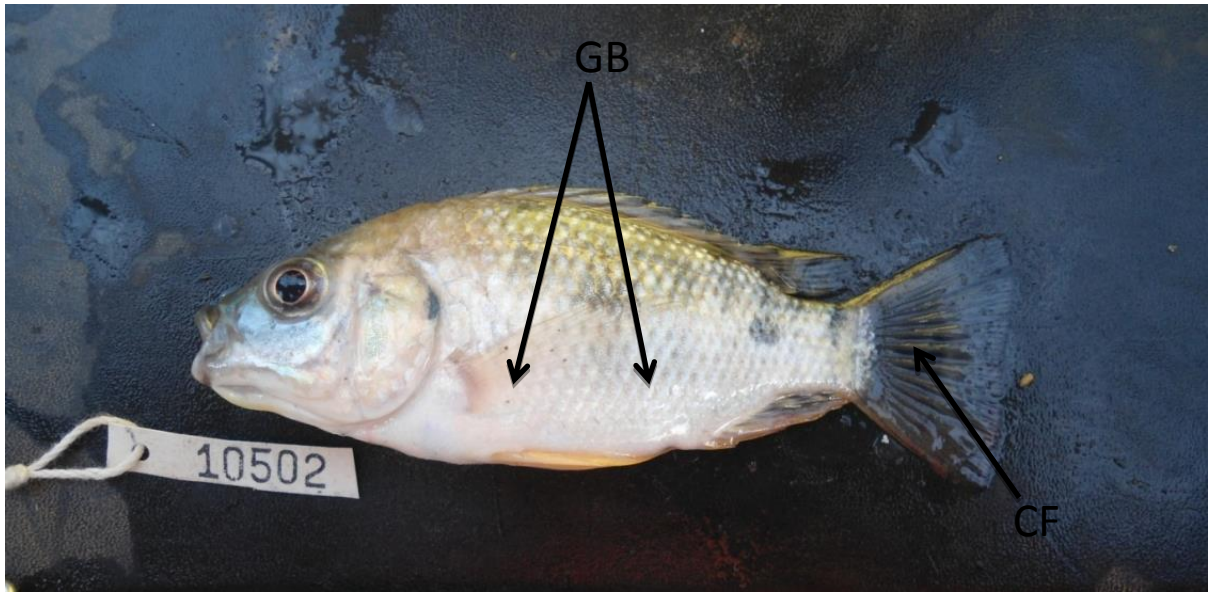


Plate 16: *Oreochromis* sp (10502) from Katuwit (N1) North West of Lake Baringo (SL= 75.75 mm) (Grey brownish trunk without vertical bars, CF- caudal fin without vertical stripes)

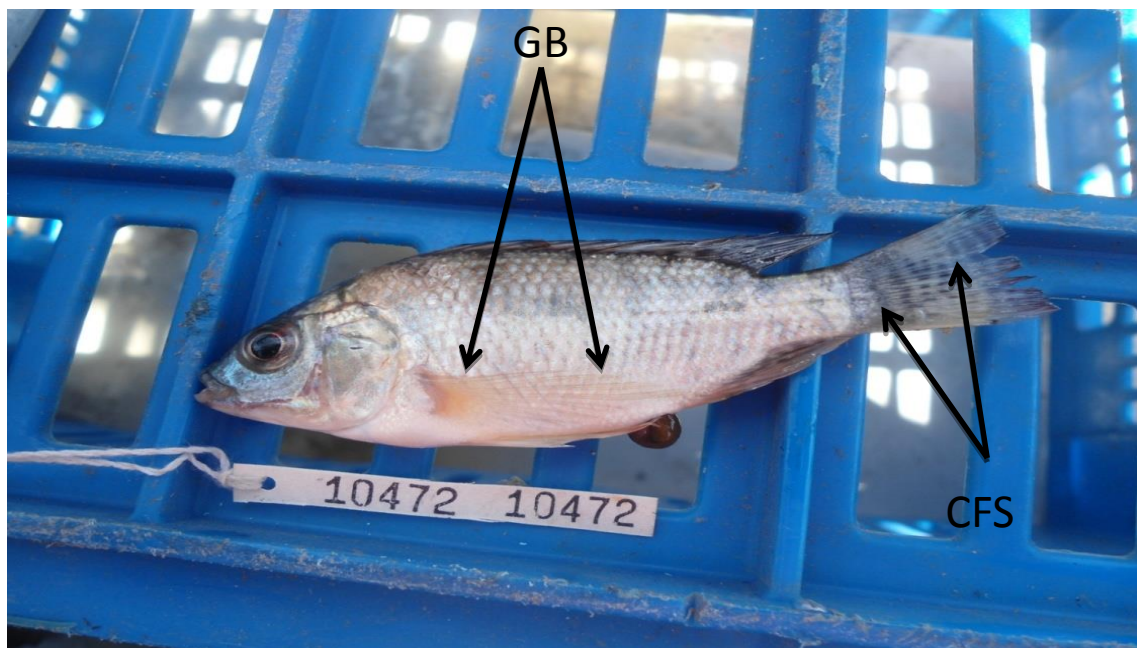


Plate 17: *Oreochromis* sp (10472) from Komolion (N2) North east of Lake Baringo (SL= 72 mm) (GB- Grey brown trunk without vertical bars, CFS- Dark greyish caudal fin with dark vertical stripes)

3.3. Morphological studies

The whole fish voucher specimens were identified morphologically as *Oreochromis* species using characteristics described by Trewavas (1983). In order to assess the taxonomic status of the specimens at species level, 22 morphometrics and 7 meristic characters were measured and counted respectively following the method described by Vreven *et al.*, (1998) and Samaradivakara *et al.*, (2012). The Morphometrics shown in figure 5 were measured using a digital calliper and recorded to the nearest 0.01 mm. The morphometrics numbered 1-22 (figure 5) are described in section 3.3.1 below.

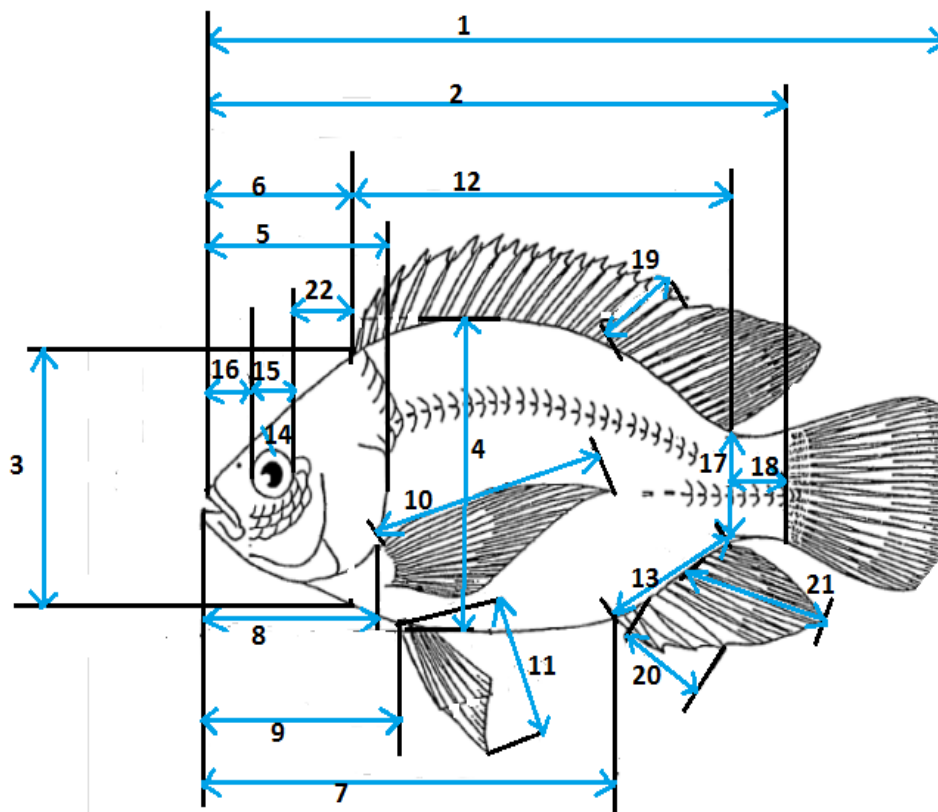


Figure 5: Morphometrics of Lake Baringo Tilapia (Adopted from Vreven *et al.*, 1998)

3.3.1. Description of Morphometric measurements

1. Total length (TL): Distance measured from the tip of the mouth/ snout to the tip of the caudal fin.
2. Standard Length (SL): Distance from the anterior edge of the upper lip to base of the caudal fin.
3. Head Depth (HD): Broadest distance measured perpendicular to the Standard length from the upper part of the head to its base.
4. Body Height (BH): Measured perpendicular to standard length, from the anterior base of the anal fin and the dorsal fin (broadest part of the body).
5. Head Length (HL): Measured in the horizontal plane as the shortest distance between the most posterior part of the opercula edge, and the projection from the edge of the upper lip
6. Pre-dorsal Distance (Pre-D): Distance between the start of the dorsal fin and anterior edge of the upper lip.
7. Pre-anal Distance (Pre-A): Shortest distance from the start of the anal fin to the anterior edge of the upper lip.
8. Pre-pectoral Distance (Pre-PECT): Distance measured horizontally from the base of the pectoral fin to the tip of the upper lip.
9. Pre-ventral Distance (Pre-V): Distance measured horizontally from the base of the ventral fin to the tip of the upper lip.
10. Pectoral Fin Length (PFL): Distance between base of pectoral fin and distal tip.
11. Ventral Fin Length (VFL): Distance between the base of ventral fin and its distal tip.
12. Dorsal Fin Base Length (DBFL): Distance from the first dorsal spine to the last dorsal ray.
13. Anal fin length: Distance measured from the first anal spine to the last anal ray.
14. Inter-orbital Distance (IOD): Shortest distance between the bony edge of both orbits.

15. Eye Diameter (ED): Shortest distance between the skins around both eyes.
16. Snout Length (SNL): Measured in the horizontal plane as the shortest distance between the anterior border of the eye and the anterior edge of the upper jaw.
17. Caudal Peduncle Depth (CPD) – Measured as the least depth of the caudal peduncle.
18. Caudal Peduncle Length (CPL) - Measured from the tip of the anal fin base to the start of the caudal fin.
19. Greatest Dorsal Spine Length (DSL): Distance measured from the base to the tip of the longest dorsal spine.
20. 3rd Anal Spine (ASL): Distance measured from the base to the tip of the 3rd anal spine.
21. Longest Anal Ray (LAR): Distance measured from the base to the tip of the longest anal ray.
22. Post orbital length: Distance measured from the eye orbit to the first spine of the dorsal fin.

The counted meristic characters were the number of dorsal fin spines (DFS), number of dorsal fin rays (DFR), number of anal fin spines (AFS), number of anal fin rays (AFR), number of lateral line scales (LLS), scales from lateral line to the dorsal fin (LLDor), and scales from lateral line to the ventral fin. The morphometric measurements and meristic counts were recorded in a sheet in appendix 1 before analysis.

3.3.2. Morphological data analyses

Morphometrics and meristic data were analysed separately using Paleontological Statistical Test (PAST) software (Hammer *et al.*, 2001) since the former is continuous and more susceptible to more environmentally induced variability while the latter is discrete and fixed early in the developmental stages (Simon *et al.*, 2010; Samaradivakara *et al.*, 2012). To eliminate the effects of size differences, the morphometric measurements were transformed

into proportions of standard and head length respectively. Caudal peduncle length was expressed as a ratio of caudal peduncle depth. The morphometric proportions were further log transformed by PAST software and thereafter analysed by principle component analysis (PCA), which falls under multivariate statistics (Hammer *et al.*, 2001). The meristic data were analysed in the same way as Morphometric data except that the former data were not log transformed. Significant differences between locations of the most variable morphometric characters were tested by Mann-Whitney U-test in PAST soft software.

3.4. Molecular genetic studies

This step involved extraction of DNA from 81 fish samples whose site of collection, number of the captured fish and sample tags are shown in table 3. Other steps involved in this step were amplification of three marker genes (Cytochrome Oxidase 1, Mitochondrial Control region and mitochondrial D-loop) through Polymerase Chain Reaction (PCR), Purification of the amplified products and Sequencing. Before DNA extraction, the tissue samples were exposed in air to remove ethanol which could affect the DNA extraction process.

Table 3: List of specimens of Lake Baringo sampled tilapia fish that were used in DNA extraction and their codes used.

LAKE BARINGO SAMPLING SITE	NUMBER OF SAMPLES	SPECIMEN CODE
Salabani (South)	12	BS428, BS429, BS430, BS691, BS692, BS693, BS694, BS695, BS696, BS697, BS698, BS699.
Nosuguro (Central)	22	BN431, BN432, BN433, BN434, BN435, BN436, BN437, BN438, BN439, BN440, BN442, BN441, BN443, BN444, BN445, BN630, BN631, BN632, BN636, BN639, BN640, BN641.
Longicharo (Central)	16	BLR446, BLR447, BLR448, BLR449, BLR450, BLR451, BLR452, BLR453, BLR454, BLR455, BLR456, BLR457, BLR458, BLR459, BLR460, BLR461.
Ng'enyin (Central)	9	BNG473, BNG474, BNG475, BNG476, BNG477, BNG479, BNG648, BNG478, BNG480
Block Hotel (South)	6	BBH452, BBH463, BBH464, BBH618, BBH627, BBH629.
Molo river mouth (South)	4	BER310, BER611, BLB674, BLB675
Katuwit (North)	11	BKT465, BKT468, BKT471, BKT664, BKT466, BKT467, BKT469, BKT470, BKT502, BKT658, BKT659.
Komolion (North)	1	BKO472

Key

BS - Baringo Salabani, BN -Baringo Nosuguro-, BLR -Baringo Longicharo, BNG -Baringo Ng'enyin, BBH- Baringo Block Hotel, BER -Baringo Endao River mouth, BLB -Baringo Lobi Bridge, BKT -Baringo Katuwit, BKO- Baringo Komolion

3.4.1. DNA isolation from fish tissues

A fragment of about 50 mg of fish tissue was removed from each specimen stored in vials and macerated into pieces before being digested in a water bath at 56°C over night in a vial containing 20µl (10 mM/ml) proteinase K in a mixture of 200µl of Sodium Chloride-Tris-EDTA (STE) buffer (0.1M NaCl, 1mM Tris-EDTA, pH= 8) and 75µl (1%) of sodium dodecyl sulphate (SDS). In this extraction mixture, the role of Proteinase K was to digest tissue proteins while STE buffer was used to dehydrate the fish sample tissues. On the other hand sodium dodecyl sulphate (SDS) salt was used to lyse the fish tissue cells to release DNA.

Total DNA was extracted from the digested samples using ZymoBead™ Genomic DNA kit (D3004) following the manufacturers' protocol as described below:

- (i) **Separation of DNA from cellular debris:** 800µl of Genomic Lysis Buffer was added to each volume of digested sample tissue then thoroughly mixed by vortexing for 6 seconds at room temperature. The lysate was placed in a microcentrifuge and centrifuged at a speed of 13,000 x g for 5 minutes to separate DNA from other cellular debris.
- (ii) **DNA binding:** The supernatant containing DNA from step (i) above was transferred into a clean microcentrifuge tube upon which 10µl of DNA binding silica Zymobeads™ slurry was added to adsorb genomic DNA suspended in the supernatant. The contents were there after mixed by inversion and incubated at room temperature for 5 minutes to give time for the DNA to hook up with the positively charged silica beads, after which the tube was then centrifuged at 1500 x g for one minute to separate the DNA on beads from other cellular debris.

- (iii) **Digestion of remaining debris:** The supernatant from step (ii) above was discarded and then 200µl of Genomic Lysis buffer added to the pellet (Zymobeads™ with bound DNA) to lyse any undigested tissue. The mixture was then homogenized by pipetting up and down to mix DNA from the lysed tissue with the DNA containing beads. The mixture was further centrifuged at 1500 x g for 1 minute to isolate the DNA containing beads from other debris.
- (iv) **DNA washing:** This stage used two buffers, DNA Pre-wash and genomic DNA (g-DNA) wash buffer. Each centrifugation to remove residue involved 1,500 x g spins. The supernatant from step iii above was discarded and 200µl of DNA Pre-wash buffer added to the pellet (Zymobeads™ with bound DNA); and pellets further re-suspended by vortexing and centrifuged at room temperature. This stage was followed by two washes of the pellet with genomic DNA (g-DNA) wash buffer and centrifuged as above.
- (v) **DNA elution:** After the second wash, and discarding of the supernatant, 70µl of elution buffer was added to the pellet to separate DNA from the beads. This was carried out through up and down pipetting using a micropipette. Finally, the mixture was centrifuged at 13,000 x g for one minute to separate DNA from the Zymobeads.
- (vi) **DNA storage:** The supernatant containing DNA was transferred to a new eppendorf tube and stored at -20°C in freezer until PCR amplification.

3.4.2 Gel electrophoresis and visualization of fish genomic DNA

Agarose gel (1.2% w/v) was made by suspending 0.6g of agarose in 50ml of pH adjusting TAE buffer (40mM Tris-Acetate and 1mM Ethylenediaminetetraacetic acid (EDTA) pH= 8) in a 150ml Erlenmeyer flask. The mixture was homogenized by boiling in a microwave at

100°C for 2 minutes. The gel solution was then left to cool for 15 minutes after which 3µl of DNA intercalating Ethidium bromide was added and thoroughly mixed by swirling the flask. The agarose-ethidium bromide mixture was then poured in a casting tray with 2 combs each having 11 wells then left to cool at room temperature for 20 minutes. The solidified agarose gel was placed in a gel box which was filled with TAE buffer, and the combs carefully removed to make the wells visible.

Five microliters (5µl) of 1kb HyperLadder™ was loaded into the first lane of the gel. 5µl of each DNA sample was mixed with 1µl of loading dye on a parafilm. The loading dye was added to enable the DNA sink into the well as well as to gauge how far the gel had run. The dye-DNA solution of each sample was then loaded into each well, thereafter the gel box was covered with a lid and its terminals connected to the electrophoretic machine set at 80V for 1 hour. The resolved DNA bands were observed under UV light in AH diagnostic UVP MultiDoc-It Digital Imaging System.

3.4.3. DNA amplification by PCR

3.4.3.1. Cytochrome Oxidase 1 (CO1) PCR amplification

A fragment of 700bp (0.7kb) in the 5' region of the mtDNA Cytochrome Oxidase C subunit 1 (CO1) was amplified by Polymerase Chain Reaction (PCR) using a pair of broad targeting primers VFd1_t1 (Forward) and VRd1_t1 (Reverse) whose primer sequences were 5'TGT AAA ACG ACG GCC AGT TCT CAA CA ACC ACA ARG AYA TYG G 3' (Forward) and CAG GAA ACA GCT ATG ACT AGA CTT CTG GGT GGC CRA ARA AYC A -3' (reverse) respectively (Ivanova *et al.*, 2007). The Polymerase Chain Reaction was carried out in PTC-200™ programmable Thermal Controller machine (MJ Research, INC) in a total volume of 25µl containing 5µl of 5X MyTaq Reaction buffer containing 5mM dNTPs, 15mM MgCl₂, stabilizers and enhancers, 1µl (10pmol) of each primer, 0.3µl of DNA Polymerase,

3µl of DNA template and 14.7µl of nuclease-free water. The reaction mixture was mixed thoroughly by vortexing and PCR was performed under the following conditions: initial denaturation was carried out for 1 minute at 95°C followed by 5 cycles of denaturation at 95°C for 30s, annealing at 50°C for 40 s and extension at 72°C for 1 minute. This was followed by 35 cycles of denaturation at 95°C for 30s, annealing at 55°C for 40s and extension at 72°C for 1 minute. The amplification was finalized by extension step of 72°C for 5 minutes.

3.4.3.2. mtDNA Control region amplification

A fragment of 450 bp (0.45 kb) mtDNA CR in the 5' region was amplified by Polymerase Chain Reaction (PCR) according to D'Amato *et al.*, (2007), using a pair of primer H16498 (Meyer *et al.*, 1990) and L19 (Bernatchez *et al.*, 1992) whose sequences were 5' CCT GAA GTA GGA ACC AGA TG -3' and 5' CCA CTA GCT CCC AAA GCT A -3' respectively. The PCR mixture and amplification conditions were as described in 3.4.3.1 above except that the denaturation, annealing and extension were carried out in 40 cycles and annealing temperature for the primers was 55°C.

3.4.3.3. mtDNA D-loop amplification

A 450 bp fragment in the 5' region of mtDNA D-loop was amplified in each sample using two primers: 5' ACC CCT AGC TCC CAA AGC TA -3' (Forward) and 5' CCT GAA GTA GGA ACC AGA TG -3' (Reverse) (Ndiwa *et al.*, 2014). The PCR mixture and amplification conditions were as described in 3.4.3.1 above, except that the annealing temperature for the primers was 56°C. Nuclease free water was used as negative control in all PCR reactions to make sure that no contamination occurred during the reaction.

3.4.3.4. Visualization of PCR Products

This step was done after the completion of PCR cycles. It involved loading the PCR products on agarose gel and observing them under UV light. In this step, 1.5% (w/v) agarose gel was made as described in 3.4.2 above, except that 0.75g of agarose was dissolved in 50ml of TAE buffer. After solidification of the gel, 2 μ l of PCR products were loaded into each well of 1.5% (w/v) of agarose / ethidium bromide gel in TAE buffer with 1kb HyperLadder™ (Bioline). The gel box was then connected to 80 V of electricity for 1 hour to separate the PCR products based on their size. The resolved bands were observed under UV light as described in 3.4.2 above.

3.4.4. Gel Purification of PCR Products

The PCR products were purified using Thermo Scientific GeneJET Gel Extraction Kit following manufactures' protocol as follows;

- (i) **Slicing and dissolution of the gel:** The gel slice containing the DNA fragment of target specimen was excised using a clean scalpel and then placed into a 1.5 mL eppendorf tube. Two hundred microliters (200 μ l) Binding Buffer was added to the gel in the tube, followed by incubation of the gel mixture at 60°C for 10 minutes to dissolve the gel.
- (ii) **DNA binding to the column:** The solubilised gel solution from step i above was loaded to the DNA binding silica GeneJET purification column and centrifuged at 8000 g for 1 minute to separate DNA from the binding buffer. The flow-through (buffer) was then discarded leaving an empty collection tube with a GeneJET column with DNA.

- (iii) **DNA washing:** Seven hundred microliters (700µl) of Wash buffer was loaded to the GeneJET purification column followed by centrifugation at 8000g for 1 minute. The flow-through was then discarded and the column placed back to the same collection tube. The empty GeneJET purification column with bound DNA was centrifuged at 8000g for 1 minute to completely remove residual wash buffer. Centrifugation of empty column was essential to avoid any residual ethanol in the purified DNA solution as the presence of ethanol in the DNA sample could inhibit downstream enzymatic reactions.
- (iv) **DNA elution:** The GeneJET purification column with DNA washed in step iii above was transferred into a clean labelled 1.5 mL microcentrifuge. This was followed by addition of 25µl of Elution buffer to the centre of the purification column membrane. After addition, the mixture was incubated at room temperature for 2 minutes before centrifugation to increase DNA yield. The Elution buffer in a column was then centrifuged at 12,000g for 2 minutes. Finally the GeneJET purification column was discarded and the purified PCR products in the tube stored at -20°C for sequencing.

3.4.5. Sequencing

The purified products were sequenced commercially at Inqaba biotec South Africa using BigDye Terminator v.3.1 Cycle Sequencing kit following the manufacturers' protocol on AB1 3500 XL genetic analyser.

3.4.6. Molecular Data Analyses

The raw mtDNA COI, mtDNA CR and partial mtDNA D-loop sequences were edited and aligned manually using BioEdit Software (Hall, 1999). This soft was used to align the forward and reverse sequences in one direction for the purpose of editing the ambiguities that

occurred during sequencing. The edited mtDNA CO1, mtDNA CR and mtDNA D-loop sequences were separately aligned with the published Genbank (NCBI) sequences of other *Oreochromis* species through Basic Local Alignment Search tool (BLAST) at a query cover between 99-100% and maximum identity of 98-100% in order to compare the query sequences at hand with the data base of sequences (<http://blast.ncbi.nlm.nih.gov>). The sequences with maximum likelihood were aligned by MUSCLE software (Edgar, 2004) using Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 (Tamura *et al.*, 2013) in order to align the sequences according to their similarities and differences. The genetic distance based on number of substitutions per site was determined based on Kimura 2-Parameter model (Kimura, 1980). Meanwhile, Genetic diversity between sequences for each marker gene was calculated using DnaSP version 5 software (Librado and Rozas, 2009). Pair wise sequence divergences between mtDNA haplotypes were calculated by MEGA v.6.0 using Kimura two parallel model (Kimura, 1980). Maximum Likelihood Phylogenetic trees for CO1, mtDNA CR and mtDNA D-loop sequences were constructed using MEGA 6.0 software based on Kimura 2-Parameter model to depict phylogenetic relationships between the haplotypes. The branching order of the trees was tested at 500 bootstrap replications (Felsenstein, 1985). The resulting phylogenetic trees were edited using Figtree version 1.4.2 software.

CHAPTER FOUR

4.0. RESULTS

4.1. Morphological identification of the sampled fish

Fish collected from Lake Baringo were characterized by long spiny dorsal fins with rayed sections, dorsal spines located at the anterior part and soft rays located posteriorly. Other features included, presence of nostril on each side, uninterrupted lateral line with the first section being slightly above and curved following the dorsal profile of the body, laterally compressed body with cycloid scales (Plate 1-17). These morphological features were congruent to the features for the family Cichlidae and genus *Oreochromis*. The colour of these fishes ranged from grey- brownish (e.g. plate 1, 2 and 3) to dark-grey (e.g. plate 6 and 8) with some fish having vertical bars on their trunks (e.g. Plate 4 and 5). Some of the sampled fish were dark grey with reddish-brown caudal fins harmonious to *Oreochromis spilurus*. Eighty-two out of 98 sampled fish had caudal fin with dark stripes (e.g. Plate 12 and 13) portraying the characteristic features of *Oreochromis niloticus* species described by Trewavas (1983). On the other hand, 16 out of 98 fish had no stripes on the caudal fin, indicating that the fishes were not *Oreochromis niloticus* (e.g. Plate 7 and 10). From these morphological observations it can be inferred that the sampled fish had more than one *Oreochromis* species. In order to verify this, morphometric and meristic data had to be analysed to delineate species based on their differences in body morphometry and meristic counts.

4.2. Morphometrics

Morphometrically, the Standard length (SL) of the fish samples collected from Lake Baringo ranged from 47.95 mm to 187.60 mm.

4.2.1. Principal Component Analysis

The Principal Component Analysis (PCA) of 22 morphometric characters for 98 fish specimens of Lake Baringo tilapia collected from three different locations of the lake (south, central and north) is shown in table 4. The principal components (PC 1, 2 and 3) were used to measure the percentage variance of the sampled fish by computing the similarities and differences of the 22 morphometrics as eigen vectors (Components). Eigen values were used to measure the variance accounted for by each principal components. In this case, the higher the eigen value the smaller the principal component and the higher the percentage variance of the morphometric characters as shown in table 4. The three Principal Components obtained had the variance of 97.304% for Principal Component I (PC1), 0.586% for Principal Component II (PC 2) and 0.381% for Principal Component III (PC 3). The eigen values for the respective Principle component are as shown in table 4.

Table 4: Summary of Principal Components, Eigen values and % Variance for 98 sampled tilapia fish

Principal Component (PC)	Eigen Value	% Variance
1	0.517	97.304
2	0.003	0.586
3	0.002	0.381

4.2.2. Character variation expressed by Loadings

The character variation measured by loadings for the 22 morphometric characters is shown in table 5. All loadings in PC1 were positive indicating the presence of size variability in the

measured fish samples. In this case, the first Principal Component was not taken into account as it is a size determining factor (Vreven *et al.*, 1998). PC2 had both negative and positive coefficients with caudal peduncle length, caudal peduncle depth, post orbital length, anal fin base length, pectoral fin length, ventral fin length, dorsal fin base length, longest anal ray length, dorsal spine length and anal fin spine length having positive coefficients depicting high correlation in shape (Table 5). On the other hand, standard length, head length, snout length, eye Diameter, inter orbital depth, head depth, Pre-dorsal length, pre anal length, pre pectoral length and pre-ventral length had negative coefficients depicting high negative correlation in shape (Table 5). Variables that showed high variation under PC 2 were snout length and longest anal ray (figure 6). Principal Component 3 loadings had also mixed coefficients (Positive and negative) with post orbit length, anal fin base length and dorsal Fin base length showing high positive correlation while pectoral fin length, dorsal spine length and anal spine length showed high negative correlation (Table 5). On the other hand, anal spine length, dorsal spine length and anal fin base length accounted more for the observed variance under PC3 (figure 7).

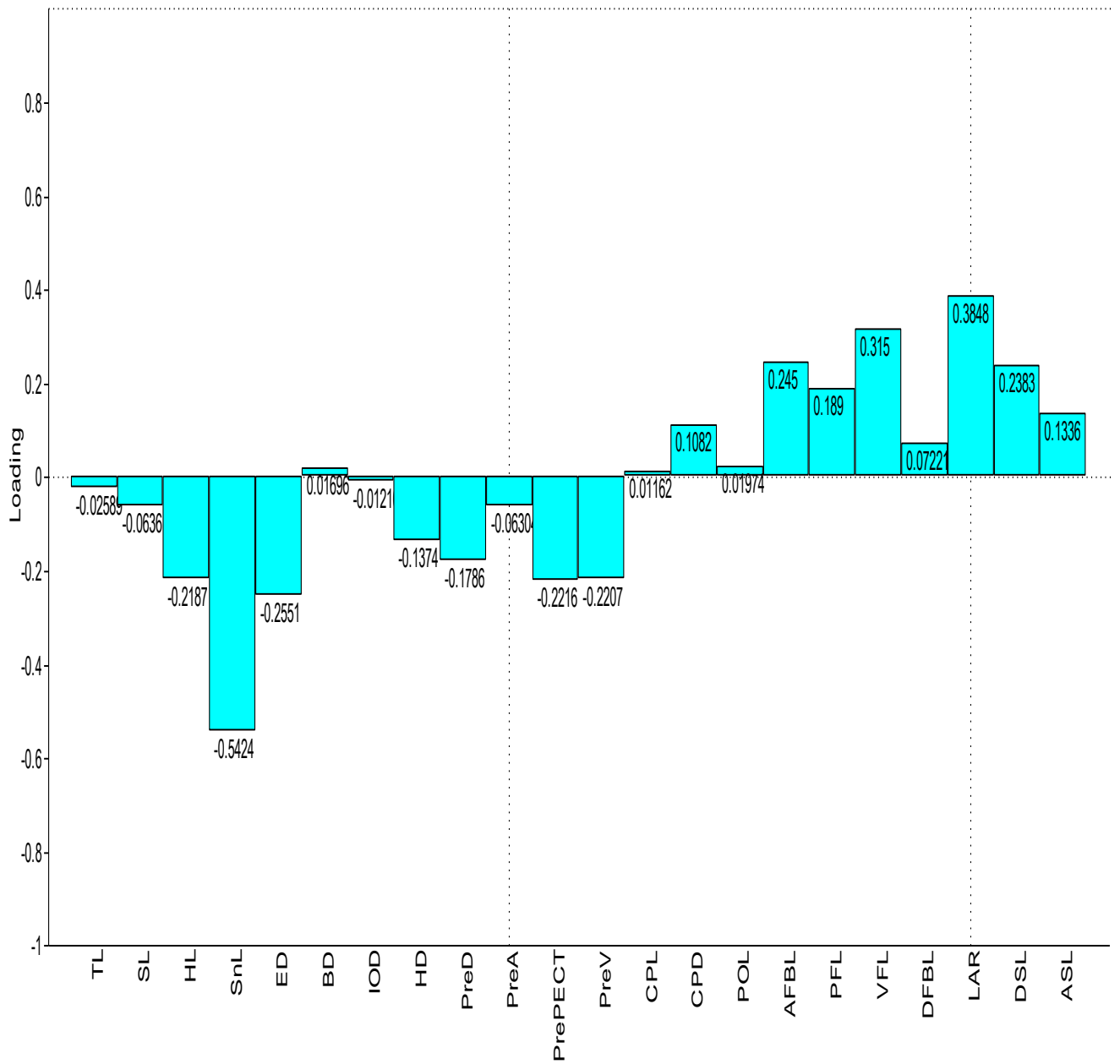


Figure 6: Loadings of PC 2 showing variation of different morphometric characters for Lake Baringo tilapia fish

Table 5: Principal Component Loadings for the morphometric characters of Lake Baringo sampled fish on log transformed data as analysed by PAST software under multivariate PCA

Variables	PC1	PC2	PC3
Total length (TL)	0.2042	-0.02589	0.08954
Standard length (SL)	0.2058	-0.0636	0.09419
Head length (HL)	0.1997	-0.2187	0.009196
Snout length (SnL)	0.2295	-0.5424	-0.1239
Eye diameter (ED)	0.1464	-0.2551	-0.1375
Body depth (BD)	0.2246	0.01696	0.1441
Inter orbital depth (IOD)	0.2324	0.011216	0.0891
Head depth (HD)	0.2224	-0.1374	0.1123
Pre-dorsal length (Pre-D)	0.197	-0.1786	0.03541
Pre-anal length (Pre-A)	0.2121	-0.06304	0.037664
Pre-pectoral fin length (Per-PECT)	0.1947	-0.2216	-0.04953
Pre-ventral fin length (Pre-v)	0.197	-0.2207	-0.04942
Caudal peduncle length (CPL)	0.2114	0.01162	0.08797
Caudal peduncle depth (CPD)	0.2158	0.1082	0.1122
Post orbital length (POL)	0.2181	0.01974	0.1802
Anal fin base length (AFBL)	0.216	0.245	0.2705
Pectoral fin length (PFL)	0.2245	0.189	-0.0807
Ventral fin length (VFL)	0.2237	0.315	0.01867
Dorsal fin base length (DFBL)	0.2163	0.07221	0.1843
Length of longest anal ray (LAR)	0.2469	0.3848	0.03583
Dorsal fin spine length (DSL)	0.2332	0.2383	-0.3734
Anal fin spine length (ASL)	0.1986	0.1336	-0.7697

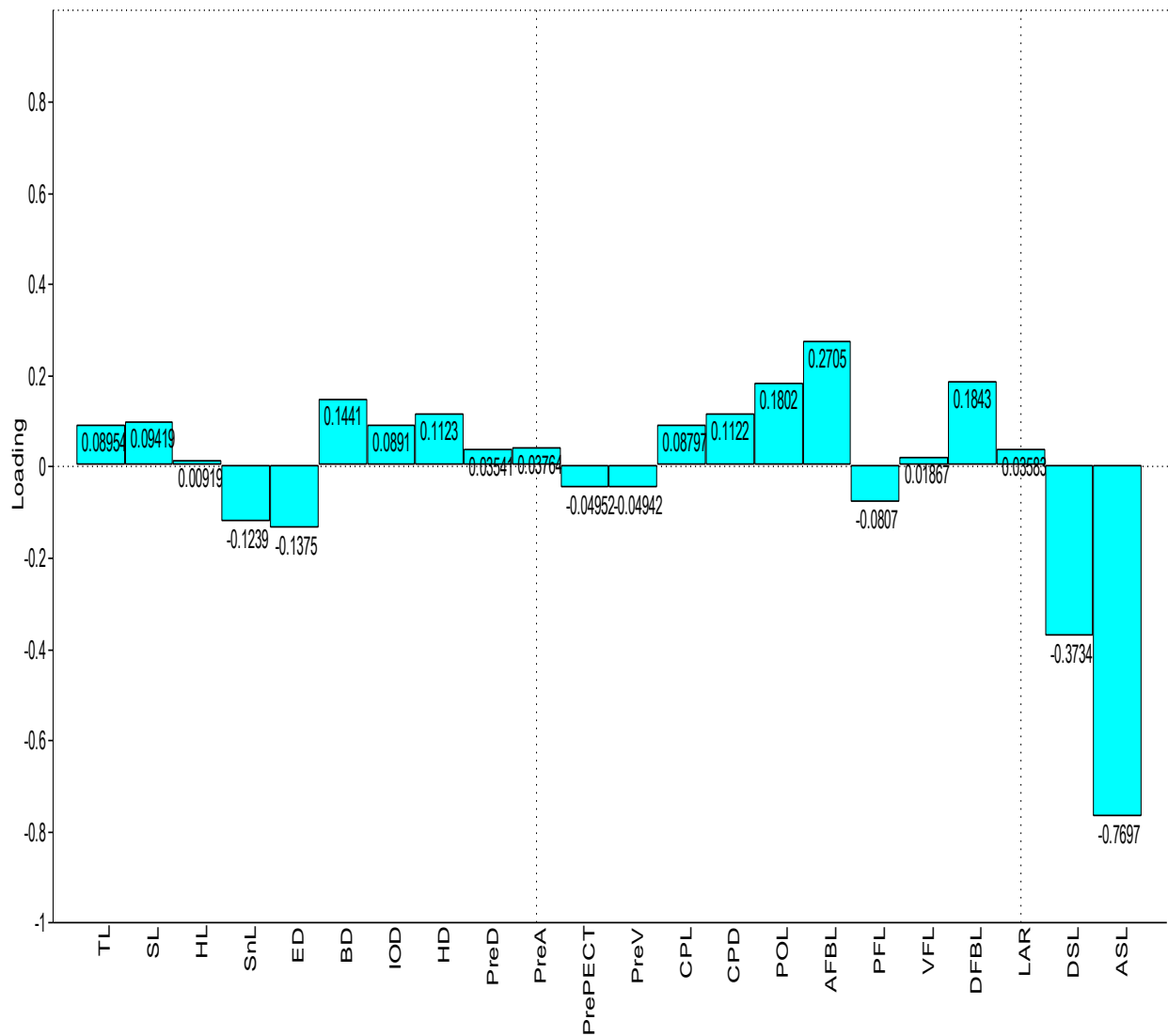


Figure 7: Loadings for PC 3 showing variability of different morphometric parameters for Lake Baringo tilapia fish

4.2.3. Comparative analyses of the variable characters of the fish samples collected

from southern, central and northern parts of Lake Baringo using Mann-Whitney U-test

Further analysis using Mann-Whitney test revealed significant differences for the length of longest anal ray (LAR) between samples collected from the southern (N1) and central parts (N2) of the lake (N1=31, N2=53, U=473.5, P<0.05). On the other hand there was no significant difference for this morphometric parameter observed between samples collected

from the southern and northern parts of the lake ($N_1=31$, $N_2=14$, $U=215.5$, $P>0.05$) Further, a significant difference was observed for this character for the samples collected from central and north ($N_1=53$, $N_2=14$, $P<0.05$).

Snout length (SnL) showed a significant difference between the fish samples collected from the southern and central parts of the lake ($N_1=31$, $N_2=53$, $P<0.05$). Similarly, a significant difference was observed for the character between the samples collected from the central and northern part of the lake ($N_1=53$, $N_2=14$, $P<0.05$). Like in LAR, the SnL showed no significant difference for the samples collected in the southern and northern parts of the lake ($N_1=31$, $N_2=14$, $U=202.5$, $P>0.05$).

No significant difference was observed for anal spine length between samples collected from the southern and northern parts of the lake ($N_1=31$, $N_2=14$, $U=196$, $P>0.05$). On the contrary, a significant difference was observed between the samples collected from south and central ($N_1=31$, $N_2=53$, $U=492$, $P<0.05$) as well as those from central and northern parts of the lake ($N_1=53$, $N_2=14$, $U=198.5$, $P<0.05$). Similarly, analysis of the dorsal spine length showed significant differences between the fish samples collected from the southern and central part of the lake ($N_1=31$, $N_2=53$, $U=497$, $P<0.05$). Similar observations were recorded between samples collected from central and north part of the lake ($N_1=53$, $N_2=14$, $U=199.5$, $P<0.05$). Like other previous observations, no significant difference in dorsal spine length was observed between the samples collected from the south and north ($N_1=31$, $N_2=14$, $U=209.5$, $P>0.05$).

4.2.4. Variation in Morphometrics revealed by PC2 vs PC3 scatter diagram

A scatter plot of Principal Component 2 (PC2) against Principal Component 3 (PC3) in figure 8 shows high degree of overlapping of fish samples from the three different locations of the lake. The overlap is more or less equally distributed throughout the negative and positive sectors of PC2 and PC3 graph. From the scatter plot, out of 98 samples used 7 specimens from the central and 3 specimens from northern parts of the lake were more morphometrically distinct from the specimens collected in the southern part of the lake (figure 8). On the other hand, 9 specimens collected from the south were morphometrically distinct from those collected from central parts of the lake. Noteworthy from figure 8 is that 4 specimens from the central part of the lake are located on the far negative sector of PC3 while 9 specimens from the southern part of the lake are far located on the positive sector of this component. The rest had overlapping features hence clustered at the centre of the scatter plot. These observations are agreeable with the Mann-Whitney test of significance described in 4.2.3 above which has shown close similarity in morphometric characters between samples collected from the south and north and significantly different from samples collected from the central part of the lake. Further, the clustering pattern of the fish specimens collected from southern and northern parts of the lake reflect the level of similarity revealed by Mann-Whitney U-test for the characters that have caused more variation described in 4.2.3 above. Thus, the distribution of fish specimens shown in figure 8 reveals that the tilapia composition in Lake Baringo is not homogenous but rather may be comprised of more than one species or sub species.

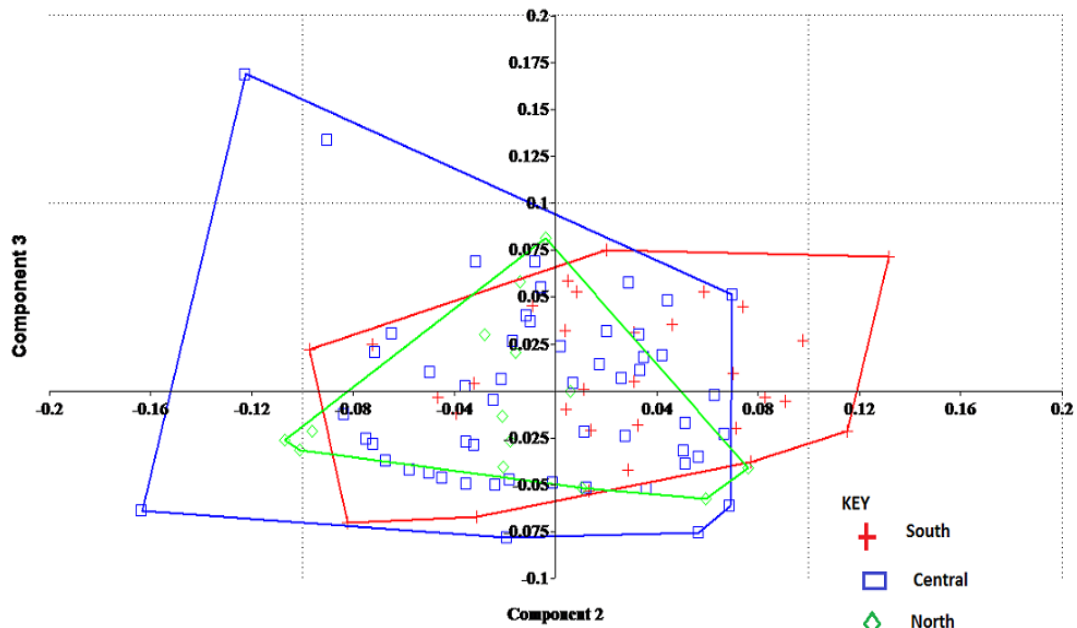


Figure 8: Scatter Plot of PC2 Vs PC3 scores for 22 log transformed morphometric data for Lake Baringo tilapia fish.

4.3. Meristic variability of Lake Baringo tilapia fish

4.3.1. Variation observed in meristic counts

Results for analysis of 7 meristic characters studied from 98 fish specimens are summarized in table 6; the frequency of each meristic character is enclosed in brackets. Dorsal fin spine counts ranged between XV-XIX. The ranges for dorsal fin rays were X-XIII, anal fin spines III-IV, anal fin rays VII-X and lateral line scales 31-36. In addition, all specimens had 4 and 11 scales from the lateral line to dorsal fin and from the lateral line to ventral fin respectively. From table 6, four meristic characters namely dorsal fin spines, dorsal fin rays; anal fin rays and lateral line scales were variable in number for the observed specimens. The dorsal fin spines recorded the highest number of counts (XVII) with a frequency of 46 (47%) followed by XVI spines with a frequency of 40 (41%). Furthermore, 1 (1%) fish had exceptionally high dorsal fin spine counts of XIX.

The dorsal fin rays recorded the highest number of counts (11) observed in 54 (55.10%) of the fish, while the least number of counts (13) was observed in 1(1%) fish. In case of anal fin rays, 66 (67.34%) fish had 9 rays and the least ray count was 7 counted in 3 fish specimens. The lateral line scales were variable among the observed fish samples with scale count of 33 showing the highest frequency from 48 (48.97%) fish specimens followed by 32 scale counts observed in 27 (27.55%) fish specimens. Moreover, four and five fish specimens recorded the lateral line scale counts of 35 and 36, respectively.

Fewer differences were observed in the number of anal fin spines, with III spines being more common (95 specimens equivalent to 97%) and IV spines showing low occurrence (3 specimens equivalent to 3%). Other meristic characters i.e. scale from lateral line to dorsal fin and scales from lateral line to ventral fin were constant for all observed specimens. The characters observed in this study (table 6) indicated that the fish samples belong to either *Oreochromis niloticus* or *Oreochromis spilurus* species.

Table 6: Meristic counts for the 98 tilapia fish specimen with the frequency of occurrence of each character indicated in the brackets

Characters	Number and Frequency
Dorsal fin spines	XV (7) XVI(40) XVII(46) XVIII(4) XIX(1)
Dorsal fin rays	X (14) XI (54) XII(29) XIII(1)
Anal fin spines	III (95) IV (3)
Anal fin rays	VII (3) VIII (7) IX (66) X (22)
Lateral line scales	31(6) 32 (27) 33(48) 34 (8) 35 (4) 36 (5)
Scales from lateral line to dorsal fin	4 (98)
Scales from lateral line to ventral fin	11 (98)

4.3.2. Principal Component Analysis for Meristic counts

Principal Component Analysis on non-log transformed 7 meristic counts for 98 Lake Baringo Tilapia specimens from three different locations (south, central and north) are presented in table 7 below. The principal components (PC 1, 2 and 3) were used to measure the percentage variance of the sampled fish by computing similarities and differences of the 7 meristic characters of 98 fish samples as eigen vectors (Components). Eigen values were used to measure the variance accounted for by each component. In this case, the higher the eigen value the smaller the principal component and the higher the percentage variance of the morphometric characters as shown in table 7. Three Principal Components had the Eigen values of 1.315 for PC 1, 0.588 for PC 2 and 0.389 for PC 3. Principal Component 1 (PC 1) accounted for 51.06%, Principal Component 2 (PC 2) for 22.839% and Principal Component 3 (PC 3) for 15.131% of the observed Variance. These variations indicate that there was differentiation on meristic counts for the observed specimens.

Table 7: Summary of the Principal Components with the eigenvalue and percentage variance respectively for meristic counts of tilapia fish samples collected from the three sites of Lake Baringo.

Principal Component	Eigenvalue	% Variation
1	1.315	51.06
2	0.588	22.839
3	0.389	15.131

4.3.3. Variation in Meristics expressed by Loadings

The loadings for the 7 meristic counts observed in 98 fish specimens are as shown in table 8. Principal Components 1 values were all positive with the lateral line scales showing the highest loading value of 0.9543 indicating that there was high variation in the number of LLS among the observed specimens. On the other hand, there was no variation observed in the

number of scales from the lateral line to the dorsal and ventral fins respectively. This implies that all specimens had 4 scales from the lateral line to the dorsal fin and 11 scales from the lateral line to the ventral fin as shown in table 8. Principal Component 2 and 3 had results with mixed signs (positive and negative) with the dorsal fin spines showing a more negative loading value under PCII and anal fin rays showing a more positive loading value under PCIII (table 8). This observation implies that there was high variation in dorsal fin spine counts and low variation in the number of anal fin spines among the observed voucher specimens.

Table 8: Principal Component Loadings for meristic counts of Lake Baringo Tilapia fish

Meristic character	PCI	PCII	PCIII
Dorsal fin spines (DFS)	0.167	-0.8677	-0.2528
Dorsal fin rays (DFR)	0.2127	0.2772	0.45544
Anal fin spines (AFS)	0.00668	0.04213	-0.01525
Anal fin rays (AFR)	0.1266	-0.3853	0.83368
Lateral line scales	0.9543	0.1415	-0.1681
Scales from lateral line to the dorsal fin	0	0	0
Scales from lateral line to the ventral fin	0	0	0

4.3.4. Variation in Meristic counts revealed by PCI vs PC2 scatter diagram

A scatter plot on raw meristic data is shown in figure 9. There was no clear separation of meristic characters between the samples from the southern, central and northern parts of Lake Baringo. However the PCA biplot and loadings revealed that the difference in number of lateral line scales and dorsal fin spines contributed more to the observed variation in the graph. For instance, the specimen indicated on the far right negative side of PC 1 in figure 9 had a unique number of dorsal fin spines (XIX).

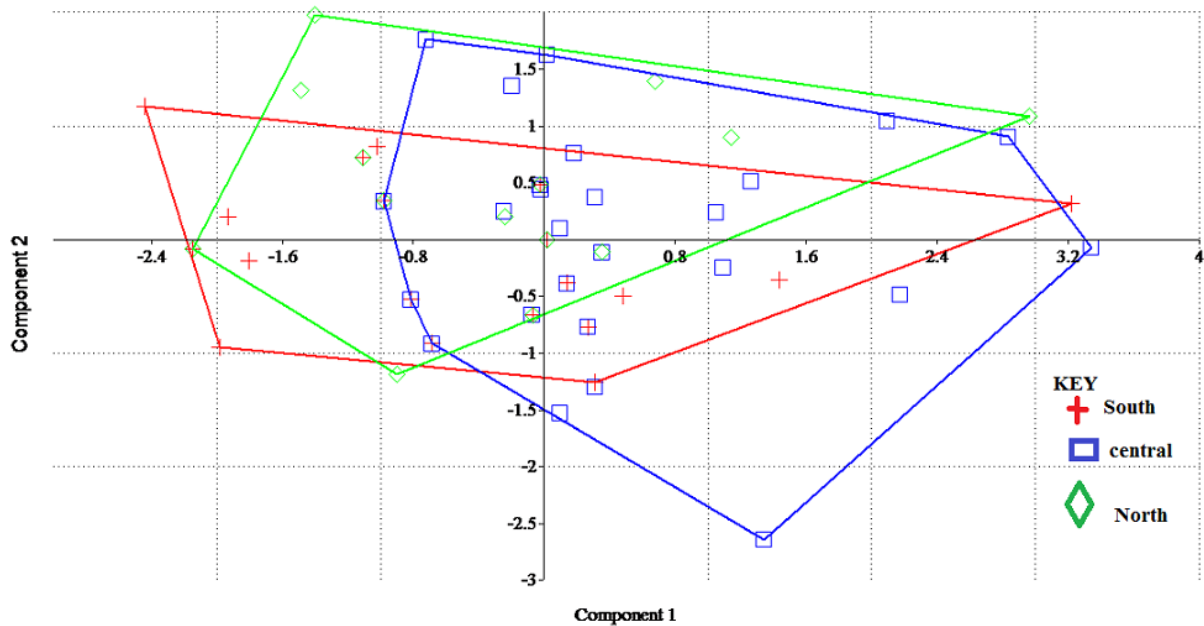


Figure 9: Scatter Plot of PC1 Vs PC2 scores for 7 meristic counts of tilapia fish collected from three sites of Lake Baringo. The scatter plots represents each fish sample collected from the corresponding site.

4.4. Genetic diversity and molecular identification of Lake Baringo tilapia fish

4.4.1. Genetic diversity revealed by mtDNA CO1

4.4.1.1. Visualization of mtDNA CO1 PCR products

The gel profile for the PCR products of mtDNA CO1 gene is shown in figure 10. The results revealed that 98% of the samples visualized under U.V light tested positive for mtDNA CO1 gene and had an expected fragment of 700bp. The sites and codes for the loaded samples in figure 10 are shown in table 3.

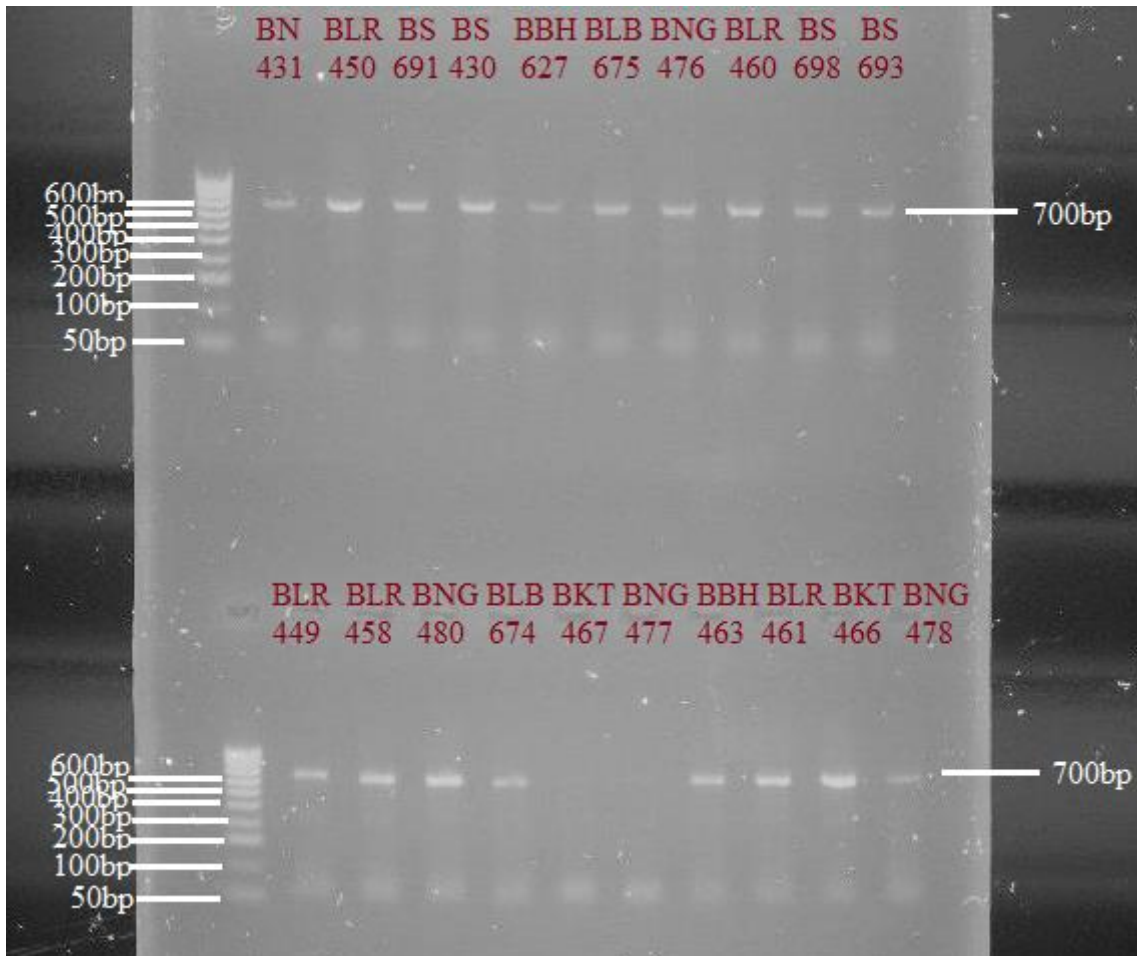


Figure 10: Electrophoretic gel profile for mtDNA CO1 PCR products for sampled Lake Baringo tilapia fish with 1kb HyperLadder™

4.4.1.2. Genetic diversity of tilapia fish based on DnaSP analysis of mtDNA CO1 sequences

Thirty mtDNA CO1 partial sequences were obtained after sequencing the purified products. Analysis of these mtDNA CO1 sequences by DNA sequence Polymorphism software (DnaSP) produced four different haplotypes. This analysis further revealed that, the number of polymorphic sites (S), haplotype gene diversity (Hd), nucleotide diversity (Pi) and average number of nucleotide differences (K) for mtDNA CO1 sequences were 52, 0.729, 0.02378 and 14.221, respectively.

4.4.1.3. Genetic diversity of tilapia fish based on Phylogenetic tree Analysis of mtDNA

CO1 sequences

The Maximum Likelihood (ML) Phylogenetic tree in figure 11 was constructed using 30 mtDNA CO1 partial sequences together with sequences for *Oreochromis niloticus*, *Oreochromis aureus*, *Oreochromis urolepis* and *Oreochromis mossambicus* from the gene bank whose accession numbers were AKC789552, GU477630, HM067614 and HQ219153 respectively. The ML phylogenetic analysis by MEGA 6 software produced four different haplotypes/ clusters (groups) with bootstrap values ranging from 62-100 (figure 11). The ML tree was rooted by a far distant elasmobranch species *Echinorhinus brucus* (Accession number, KJ864923) to form a clear phylogenetic base. From the maximum likelihood tree it was revealed that 3 out of 30 mtDNA CO1 samples (10%) formed a cluster (haplotype) with bootstrap value of 100. This cluster (haplotype) was not identified as blasting of this group against the NCBI database sequences at query cover of 99-100% and Maximum Identity of 98-100% produced no species identical to this haplotype. However, from the Maximum Likelihood tree this group formed a sister clade to *Oreochromis urolepis* indicating that the haplotype was phylogenetically closer to this species than *Oreochromis niloticus*, *Oreochromis aureus* and *Oreochromis mossambicus* (figure 11).

The second cluster (haplotype) from the root in figure 11 with a bootstrap value of 100 had 10 out of 30 (33%) identical mtDNA CO1 samples. This haplotype was not identified through gene bank blast but is a sister clade to cluster (haplotype) number three from the root (bootstrap value of 87) which was identified as *Oreochromis niloticus* species. Clusters 2 and 3 from the root of the tree were phylogenetically closer to *Oreochromis aureus* (Sister Clades) and distant to *Oreochromis mossambicus*. Another notable observation from figure 11 is that, cluster 3 (bootstrap 87 with 11 samples) from the root was identical to *O. niloticus*. This cluster sub branched to form a fourth cluster (6 out of 30 samples) with bootstrap value

of 62. This cluster was not identified through gene bank blast but is suspected to be a subspecies of *O.niloticus*.

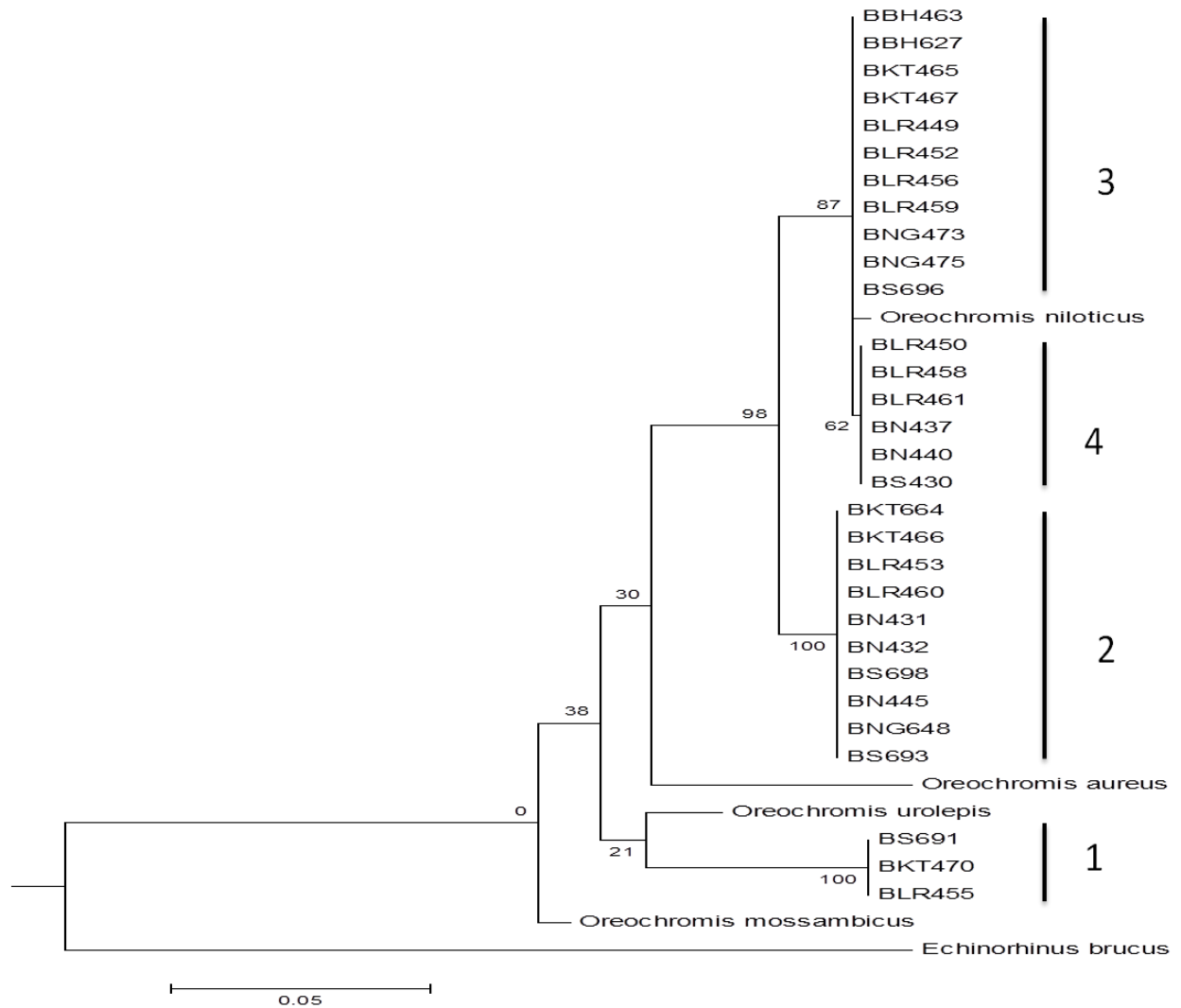


Figure 11: Maximum Likelihood consensus tree of mtDNA Cytochrome Oxidase 1 haplotypes based on Kimura's two-parameter sequence divergence between haplotypes of Lake Baringo tilapia specimens. Numbers in the nodes indicate bootstrap values based on 100 replicates. Bar represents 0.05 units of percentage divergence.

4.4.1.4. Genetic distance between CO1 haplotypes

The genetic distance between haplotypes calculated by MEGA 6 was congruent to the phylogenetic arrangement of the haplotypes from the root in figure 11. Table 9 revealed that haplotype 1 is genetically distant from other haplotypes. Haplotype 2 and 3 were genetically close to each other compared to haplotype 1. Meanwhile the genetic divergence between haplotype 3 and 2 with that of haplotype 4 and 2 had a difference of 0.001 substitutions per site. Haplotype 4 and 3 were more phylogenetically related with the least genetic distance of 0.02 nucleotide substitutions per site (Table 9).

Table 9: Genetic distance matrix between different tilapia haplotypes based on mtDNA Cytochrome Oxidase 1 sequences. The values were obtained by analysing the sequences using MEGA Version 6 software and computed by Kimura 2-parameter Model. The haplotypes were derived from figure 11 arranged from the root to the top of the tree accordingly.

Haplotype	1	2	3
1			
2	0.080		
3	0.076	0.027	
4	0.074	0.026	0.002

4.4.2. Genetic diversity as per mtDNA Control region (CR) marker gene

4.4.2.1. Gel electrophoresis of mtDNA CR PCR products

Visualization of electrophoresed mtDNA CR PCR products with 1kb HyperLadder™ under U.V light revealed 98% positive bands with a targeted gene size of 450bp (Figure 12). The gel sample codes in figure 12 and their site of collection are shown in table 3. The non-

specific amplifications ahead of the targeted gene were removed through gel purification procedure explained in section 3.4.3.

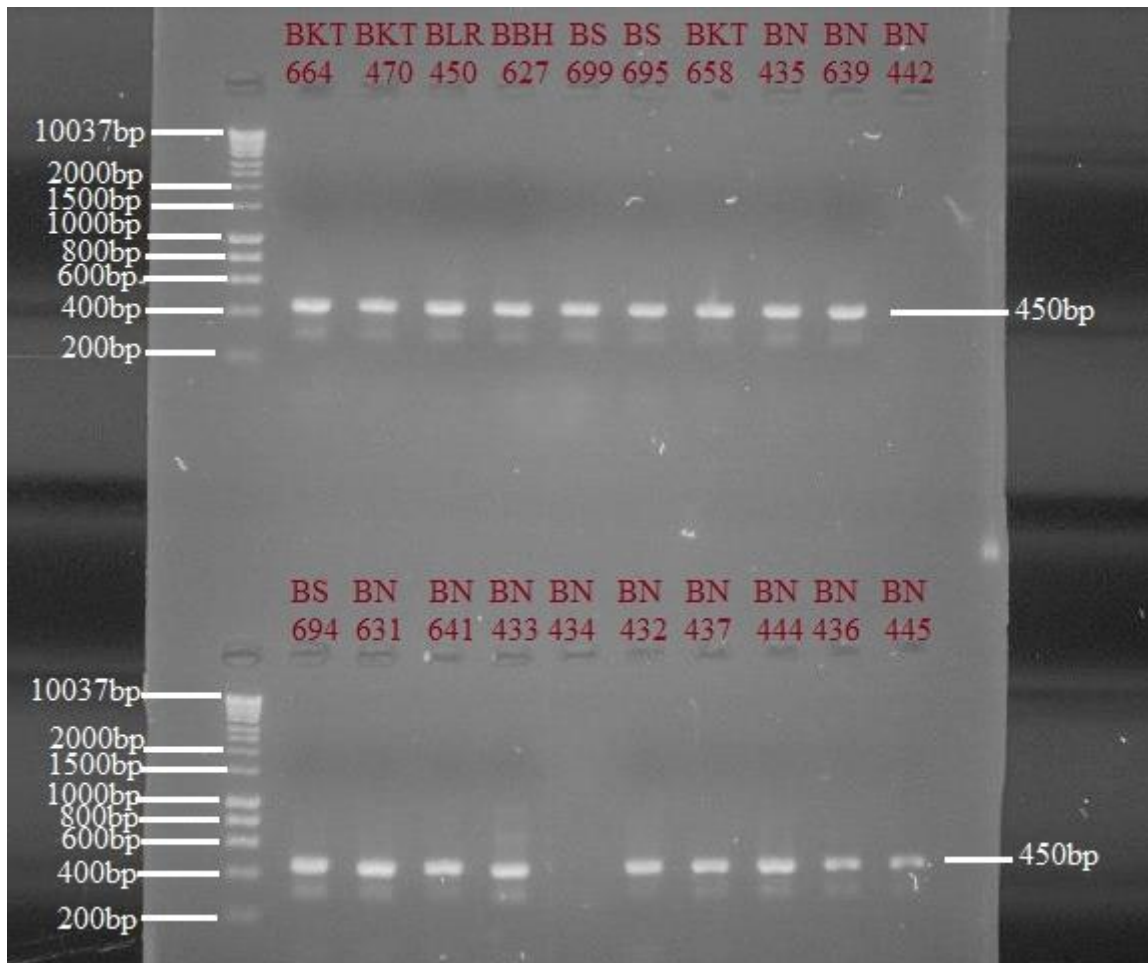


Figure 12: Electrophoretic gel profile for mtDNA CR PCR products for sampled Lake Baringo tilapia fish with 1kb HyperLadder™

4.4.2.2. Genetic diversity of sampled tilapia fish based on DnaSP analysis of mtDNA CR sequences

Fifty partial mtDNA Control region sequences were obtained after sequencing the purified PCR products at Inqaba Biotech Company in South Africa. DnaSP analysis of the edited sequences produced seven different haplotypes with 49 polymorphic sites (S), nucleotide

diversity (P_i) of 0.04639, haplotype gene diversity (H_d) of 0.817 and average number of nucleotide differences (K) of 16.331.

4.4.2.3. Maximum Likelihood Phylogenetic analysis of mtDNA CR sequences for sampled Lake Baringo tilapia fish

The Maximum Likelihood Phylogenetic analysis of 50 mtDNA partial sequences in figure 13 like DnaSP analysis produced seven different haplotypes (groups/ clusters) with bootstrap values ranging from 67-100. Blasting the sequences of these groups against the published sequences in NCBI data base at a query cover of 99-100% and maximum identity of 98-100% produced no species identical to these sequences probably due to the fact that mtDNA CR marker gene has rarely been used in identification of Kenyan tilapia populations.

The first cluster (haplotype) from the base of the ML tree with bootstrap value of 100 in figure 13 had 5 samples out of 50 (10%). This haplotype was phylogenetically distant and genetically different from other groups as it sits at the base of the tree acting as a root. Cluster (haplotype) two from the base of the tree with bootstrap value of 100 had 18 samples making 56% of all 50 samples. This group sub branched to form the third cluster (haplotype) with a bootstrap value of 65 comprised of 3 samples equivalent to 6% of all 50 analysed samples. Further, cluster (haplotype) number four with bootstrap value of 99 from the base of the tree is comprised of 11 (22%) samples. This group is a sister clade to cluster six and seven as they both arise from the same node with bootstrap value of 70. Moreover, cluster (haplotype) 6 with bootstrap value of 67 with 3 (6%) samples sub branched to form cluster number 5 with bootstrap value of 98% with 3 (6%) samples. This implies that samples in clusters five and six were phylogenetically and genetically closer than samples from other groups. Cluster number 7 from the root of the tree (bootstrap value 95) had 10 (20%) samples. This haplotype group was phylogenetically distant from other groups as shown in figure

13.

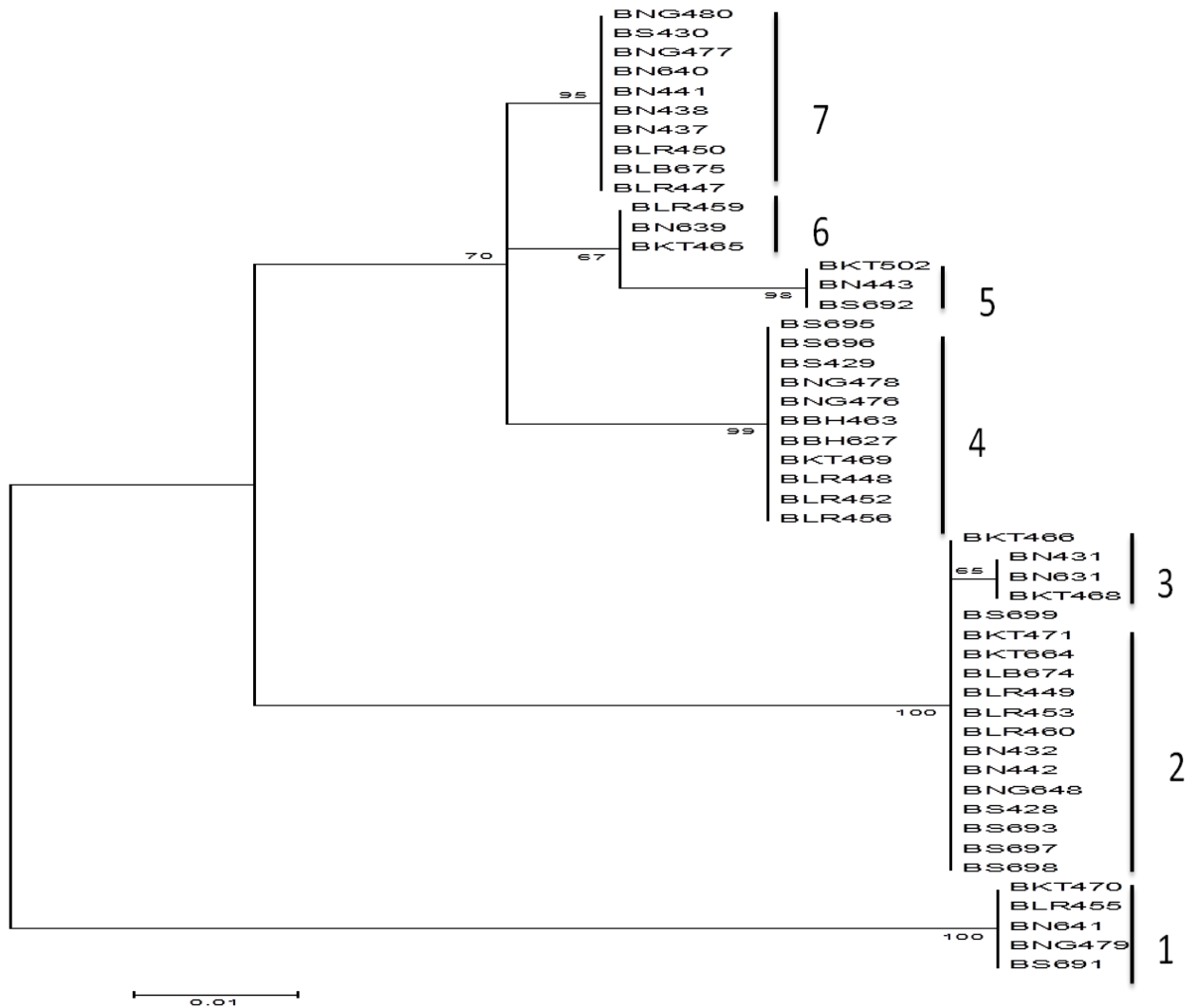


Figure 13: Maximum Likelihood consensus tree of mtDNA Control region haplotypes based on Kimura's two-parameter sequence divergence between haplotypes of Lake Baringo Tilapiines. Numbers in the nodes indicate bootstrap values based on 100 replicates. Bar represents 0.01 units of percent divergence.

NB: The terms Cluster, group and haplotype have been used interchangeably to represent fish samples with similar mtDNA CR sequences (Maternally inherited).

4.4.2.4. Pairwise genetic distance between mtDNA CR haplotypes

The pairwise genetic distance between the haplotype groups observed in figure 13 is shown in table 10. Haplotype one is genetically distant from other groups with high number of nucleotide substitution per site in all pairs. Table 10 reveals that this haplotype is more genetically distant from haplotype 3 with a pairwise distance of 0.117 substitutions per site. Haplotype 2 was genetically close to haplotype 3 with a least pairwise genetic distance of 0.003 and genetically distant to haplotype 1 and 5 whose values were 0.114 and 0.69 respectively. Meanwhile, haplotype 3 was genetically closer to haplotype 6 with a genetic distance of 0.063 and genetically distant to haplotype 1 and 5 whose pairwise genetic distances were 0.117 and 0.072 respectively. Haplotype 4 was genetically distant to haplotype 1 and haplotype 5 with pairwise genetic values of 0.097 and 0.035 respectively. Further, haplotype 5 and 6 were genetically closer to haplotype 7 with pairwise genetic distance values of 0.017 and 0.011. Generally the pairwise genetic distance was congruent to the arrangement order of haplotype groups/ Clusters in figure 13.

Table 10: Genetic distance matrix between different haplotypes based on mtDNA Control Region sequences obtained by MEGA Version 6 and analysed by Kimura 2-parameter Model. The haplotypes were derived from figure 13 arranged from the root to the top of the tree accordingly

Haplotype	1	2	3	4	5	6
1						
2	0.114					
3	0.117	0.003				
4	0.101	0.066	0.069			
5	0.097	0.069	0.072	0.035		
6	0.094	0.060	0.063	0.023	0.011	
7	0.094	0.063	0.066	0.020	0.017	0.011

4.4.3. Genetic diversity revealed by mtDNA D-loop

4.4.3.1. Gel electrophoresis of mtDNA D-loop PCR products

Visualization of electrophoresed mtDNA D-loop PCR products with 1kb HyperLadder™ under U.V light revealed 99% positive bands all having a targeted gene size of 450bp (Figure 14). The gel sample codes in figure 14 and their site of collection are shown in table 3. The non specific amplifications ahead of the targeted gene were removed through gel purification procedure explained in section 3.4.3.

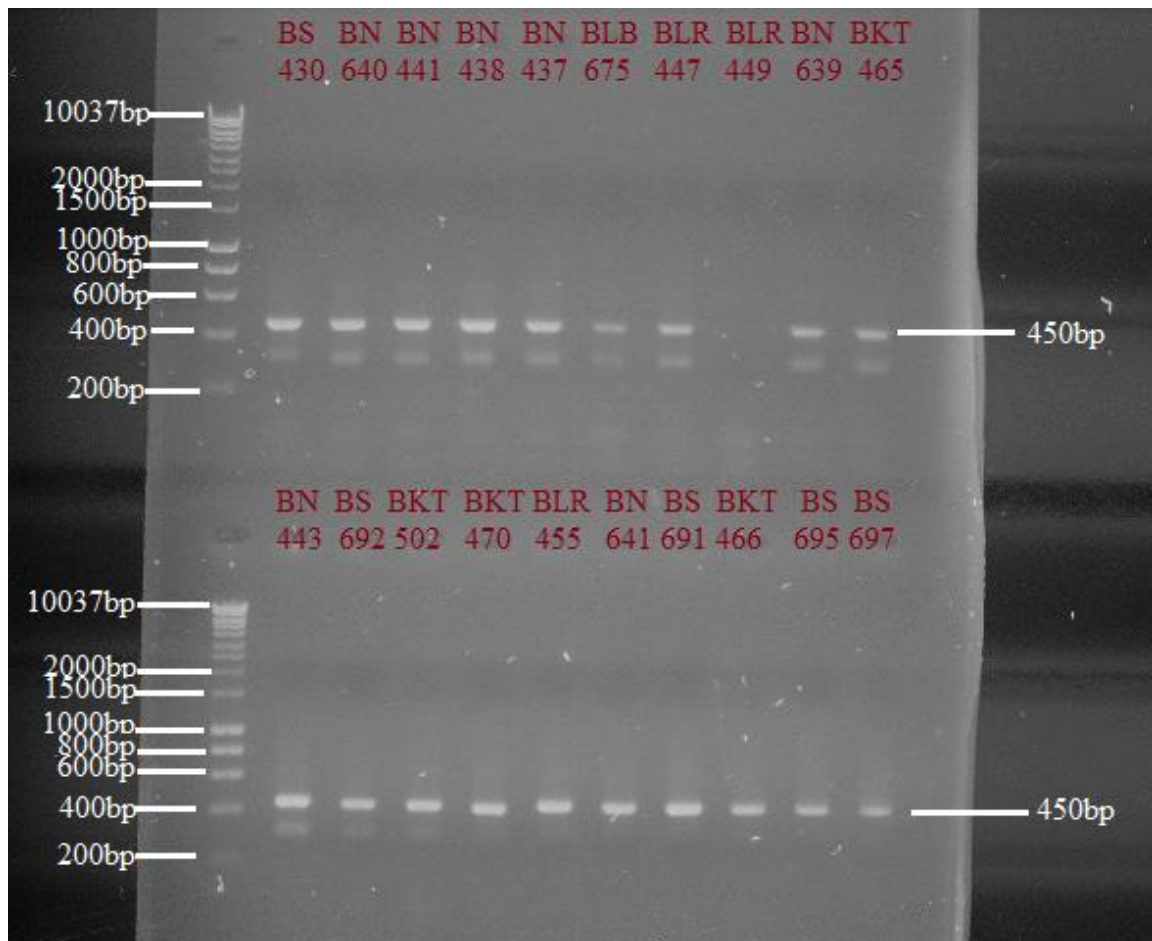


Figure 14: Electrophoretic gel profile for mtDNA D-loop PCR products for sampled Lake Baringo tilapia fish with 1kb HyperLadder™

4.4.3.2. Genetic diversity of sampled tilapia fish based on DnaSP analysis of mtDNA D-loop sequences

Twenty three partial mtDNA D-loop sequences were obtained after sequencing the purified PCR products at Inqaba Biotech Company in South Africa. The genetic polymorphism between the sequences was revealed by DnaSP software. The following diversity parameters were scored from the D-loop sequences; 7 haplotypes with 48 polymorphic sites (S), nucleotide diversity (Pi) of 0.04845, haplotype gene diversity (Hd) of 0.870 and average number of nucleotide differences (K) of 17.538.

4.4.3.3. Maximum Likelihood Phylogenetic analysis of mtDNA D-loop sequences for sampled Lake Baringo tilapia fish

Maximum Likelihood Phylogenetic analysis of 23 mtDNA D-loop sequences produced seven different haplotypes with bootstrap values ranging from 61-100 (figure 15). The first haplotype at the base of the tree had a bootstrap value of 100 and consisted of 3 out of 23 samples (13%). This group was phylogenetically different from other haplotypes as it formed the root of the ML tree. The second haplotype from the root had a bootstrap value of 100 and consisted of 5 out of 23 (21.7%) samples and it sub branched to form a haplotype with bootstrap value of 61 consisting of 3 (13%) samples. Furthermore, the fourth haplotype had a bootstrap value of 67% with 2 samples (8.7%), the grouped was a sister clade to group six (bootstrap 96%) and group 7 (bootstrap 100%). This group sub branched to form another haplotype with bootstrap value of 67 which had only 1 sample (4.3%) (BKT 502). Haplotype 6 from the base of the table with bootstrap value of 96 consisted of 4 samples equivalent to 17.4% of the 23 samples analysed. Moreover, the last group from the ML tree base had a bootstrap value of 100 and consisted of 5 samples forming 21.7% of the 23 samples analysed. The three last clusters from the root were sister clades as they all branched from the same node with bootstrap value of 73.

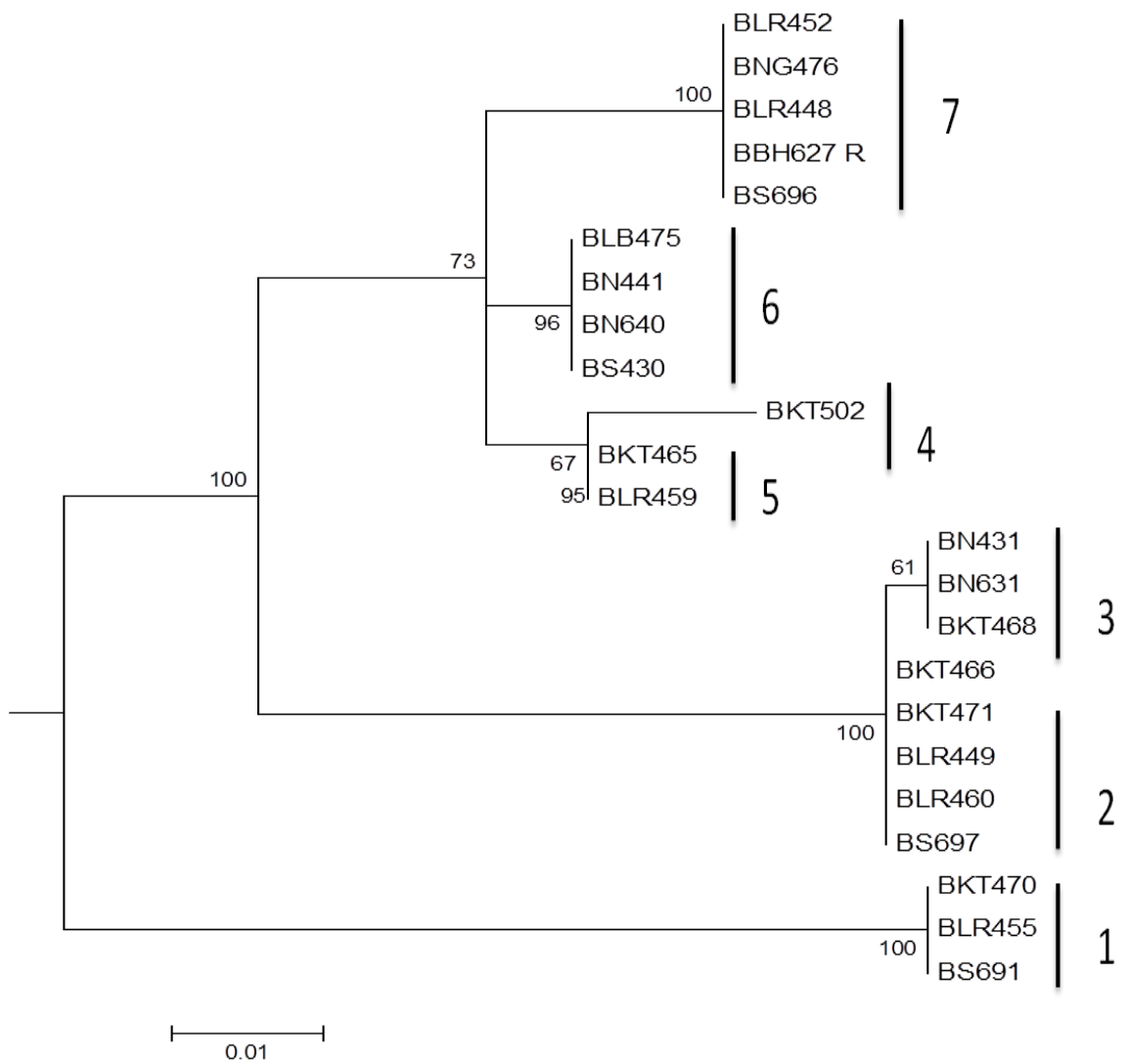


Figure 15: Maximum Likelihood consensus tree of mtDNA D-loop haplotypes based on Kimura's two-parameter sequence divergence between haplotypes of Lake Baringo tilapia specimens. Numbers in the nodes indicate bootstrap values based on 100 replicates. Bar represents 0.01 units of percentage divergence.

4.4.3.4. Pairwise genetic distance between the mtDNA D-loop haplotypes

The pairwise genetic distance between different haplotype observed in Fig. 15 is shown in table 11. The highest pairwise genetic distance of 0.111 was observed between haplotypes 1 and 3. Haplotype 2 was more genetically distant to haplotype 1 by a pairwise distance of 0.107 and closely related to haplotype 3 (0.003). Furthermore, haplotype 3 was genetically close to haplotype 4 (0.061), and distant to haplotype 1 (0.088). On the other hand, haplotype 4 was genetically distant to haplotype 1, and close to haplotype 5 and 6 with each haplotype having smallest pairwise distance of 0.011. Additionally, haplotype 5 was genetically close to haplotype 6, and genetically distant to haplotype 1 with pairwise scores of 0.017 and 0.091 respectively. Likewise, haplotype 6 was genetically similar to haplotype 7 (0.02), and genetically distant to haplotype 1 (0.088). Lastly, haplotype 7 was far distant to haplotype 1 by a pairwise distance factor of 0.094.

Table 11: Genetic distance matrix between different haplotypes based on mtDNA D-loop sequences of Lake Baringo tilapia fish. These values were obtained by MEGA Version 6 analysis and computed by Kimura 2-parameter Model. The haplotypes were derived from figure 15 arranged from the root to the top of the tree accordingly

Haplotype	1	2	3	4	5	6
1						
2	0.107					
3	0.111	0.003				
4	0.088	0.058	0.061			
5	0.091	0.067	0.070	0.011		
6	0.088	0.061	0.064	0.011	0.017	
7	0.094	0.064	0.067	0.023	0.034	0.020

4.4.4. Identification of mtDNA D-loop haplotypes with reference to NCBI data base sequences

A total of 23 mtDNA D-loop partial sequences (excluding samples suspected to be hybrids in Appendix 2) were blasted against the published sequences in the NCBI data base at a query cover of 99-100% and maximum identity of 98-100%. The names and accession numbers of the species obtained from the gene bank after mtDNA D-loop sample sequences blast are presented in appendix 2. The gene bank sequences that showed maximum likelihood to the blasted sequences together with other sequences of tilapia species mostly used in aquaculture are presented in appendix 3. These sequences together with the 23 mtDNA D-loop sample sequences were aligned by MEGA 6 software in order to construct a Maximum Likelihood (ML) phylogenetic tree (Fig. 16) for the purpose of identifying different mtDNA haplotypes observed in figure 15. The clustering pattern of the sample sequences with those from gene bank in figure 16 enabled identification of tilapia mtDNA D-loop samples and their haplotypes at specific and sub specific levels. These identified samples were also used as references for identification of mtDNA CO1 and CR haplotypes whose clustering pattern was similar to mtDNA D-loop samples (Table 13).

The Maximum Likelihood tree for these sequences (Samples + NCBI data) was rooted with *Oreochromis aureus* (Accession number GU980727) which is a sister species to *O.niloticus*. Results of analysis based on ML phylogenetic tree in figure 16 indicated presence of seven different haplotypes five of which were identified at species and subspecies levels. The clusters of the seven identified haplotypes had bootstrap values (measuring the branching pattern of a ML phylogenetic tree) ranging from 45-100 (figure 16). The D-loop samples with codes BLB475, BN441, BN640 and BS430 clustered together (aligned) with *Oreochromis niloticus baringoensis* sequence from the gene bank (Accession number EF016708) to form a cluster with bootstrap value of 95. This implies that the above mtDNA D-loop samples

originated from *Oreochromis niloticus baringoensis* species. In the second haplotype group with bootstrap value of 90, the mtDNA D-loop sample with a code BKT502 was also identified as *O.niloticus baringoensis* (Accession number AJ237397). The mtDNA D-loop sample sequences with codes BKT465 and BLR459 aligned with *Oreochromis niloticus vulcani* sequences from the gene bank with accession number EF016694 to form a haplotype group number 3 with bootstrap value of 68. Therefore all samples in this group were identified as *Oreochromis niloticus vulcani*. Further, cluster number 4 (bootstrap value 99) in figure 16 with sample codes BS696, BNG476, BLR452, BBH627 and BLR448 were identified as *Oreochromis niloticus* (unknown) (Accession number, AF296474). The Phylogeographic origin of this species is not known, however the subspecies is likely to be *Oreochromis niloticus niloticus* from Lake Victoria basin. In cluster number 5 (bootstrap value 99), the mtDNA D-loop samples with codes BKT470, BLR455 and BS691 were closely related to the gene bank sequences for *Oreochromis niloticus baringoensis* (Accession number EF016700) and *Oreochromis leucostictus* (Accession number EF016702) with bootstrap value of 63. These samples are likely to be the mtDNA introgressed hybrids of *O.niloticus baringoensis* with *O.leucostictus*.

Additionally, the sixth and seventh haplotypes from the root with bootstraps values of 88 and 64 respectively emerged from the *Oreochromis spilurus* node with bootstrap value of 99. In this case, the mtDNA D-loop samples with codes BKT466, BKT471, BLR460, BS697 and BLR449 in group 6 (Bootstraps value 84) were definitely from *Oreochromis spilurus* (Accession number EU431000). Additionally, the samples with codes BN431, BN631 and BKT466 in cluster 7 from the root (Bootstrap value of 64) were suspected to be from a subspecies of *O.spilurus*.

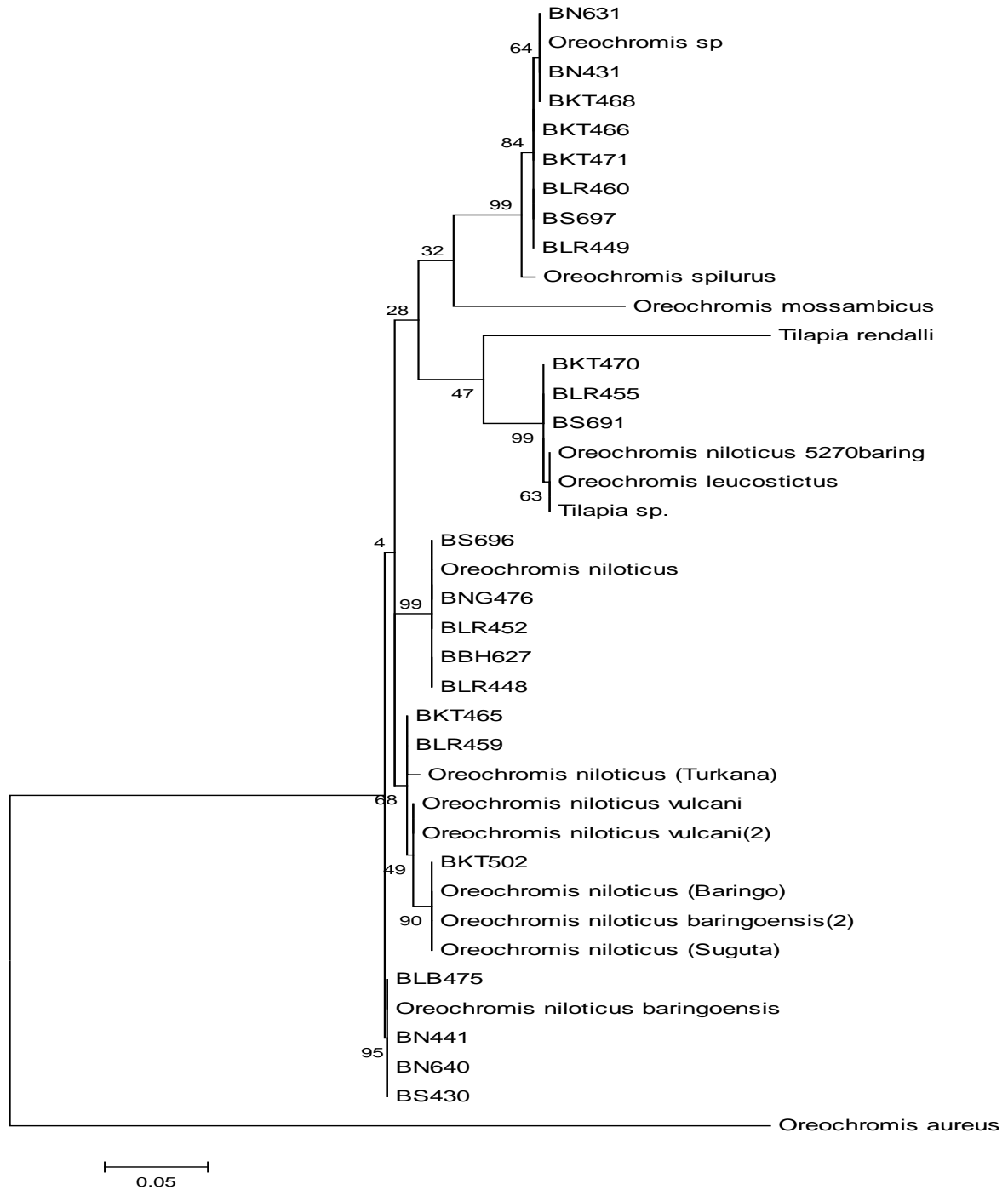


Figure 16: Maximum Likelihood consensus tree of mtDNA D-loop haplotypes (Samples + NCBI sequences) based on Kimura's two-parameter sequence divergence between haplotypes of *Oreochromis species*. Numbers in the nodes indicate bootstrap values based on 100 replicates. Bar represents 0.05 units of percentage divergence.

4.4.5. Comparison of mtDNA CO1, mtDNA CR and mtDNA D-loop genetic diversity by DnaSP

The DNA polymorphism for the three marker genes (mtDNA CO1, mtDNA CR and mtDNA D-loop) entailing number of sequences (N), Number of polymorphic sites (S) Number of haplotypes (H) (Tajima, 1983), Nucleotide diversity (Pi) (Nei, 1987), Haplotype gene diversity (Hd), and Average number of nucleotide differences (k) are compared in table 12. DnaSP analysis of mtDNA CO1 marker gene produced four different Baringo tilapia haplotypes, while the highly polymorphic markers mtDNA CR gene and mtDNA D-loop produced seven different haplotypes. The two marker genes mtDNA D-loop and CR sequences were more polymorphic compared to mtDNA CO1 with respect to haplotype diversity (HD), nucleotide diversity (Pi) and average number of nucleotide differences (k). Of the three marker genes, mtDNA D-loop recorded highest values for haplotype diversity, nucleotide diversity and number of nucleotide difference. These results show that mtDNA CO1 sequences had little genetic diversity compared to mtDNA CR and mtDNA loop sequences.

Table 12: mtDNA CO1, mtDNA CR and mtDNA D-loop partial sequences variability observed in specimens of Lake Baringo tilapia

Gene	N	S	H	Hd	Pi	K
mtDNA CO1	30	52	4	0.729	0.02378	14.221
mtDNA CR	50	49	7	0.817	0.04639	16.331
mtDNA D- loop	23	48	7	0.870	0.04845	17.538

Key; N: Number of sequences, S: Number of Polymorphic sites, H: Number of haplotypes, Hd: Haplotype diversity, Pi: Nucleotide diversity, K: Number of nucleotide differences

4.4.6. Comparison of clustering pattern of mtDNA CO1, mtDNA CR and mtDNA D-loop samples in their respective ML phylogenetic trees.

Table 13 shows the similarity of haplotype clustering pattern for the mtDNA CO1, mtDNA CR and mtDNA D-loop samples from their respective ML phylogenetic trees. The sample codes, group number (entailing the haplotype/cluster) from the root of ML tree and the bootstrap value (BV) of each cluster are presented in figures 11, 13 and 15 respectively. From table 13, the clustering pattern of mtDNA CO1, CR and D-loop samples from figure 11, 13 and 15 respectively was found to be almost the same for the three marker genes. The maximum likelihood trees for the three marker genes in figure 11, 13 and 15 contain the samples coded BKT470, BLR455 and BS691 in their first clusters from the base of their respective trees. These specimens were identified as introgressed hybrids of *O.n baringoensis* with *O.leucostictus* in figure 16. It is therefore undoubtedly that the CR samples coded BN641 and BNG479 which were not sequenced in mtDNA CO1 and mtDNA CR belong to this group. Furthermore, the second groups in the ML trees for all the three markers contained similar samples coded BKT466 and BKT 460. CO1 and CR had similar samples coded BKT464, BLR453, BN432, BNG648 and BS693. Meanwhile CR and D-loop had similar samples coded BKT471 and BLR449. These and other remaining samples were observed to be closely related to *Oreochromis spilurus* based on ML tree in figure 16.

Group three ML tree samples for mtDNA CR and mtDNA D-loop were the same with codes BN431, BN631 and BKT468. This haplotype group was not identified, but is suspected to be a subspecies of *O.spirulus* as described in 4.4.4 above. Group four for mtDNA CO1 and mtDNA CR ML trees and group 7 for D-loop ML tree had identical samples whose codes are shown on the table 13. These samples were identified as *Oreochromis niloticus* in figure 16 but its subspecies and phylogeographical origin is unknown. Samples in cluster 5 of mtDNA CR and mtDNA D-loop ML trees were the same with sample codes BKT502, BN443 and

BS693; the samples were identified as *O.n baringoensis*. Likewise, cluster 6 and cluster 4 for CR and D-loop respectively had similar samples coded BLR459 and BKT465. This haplotype group was identified in figure 16 as *Oreochromis niloticus vulcani* from Lake Turkana. Clusters 3, 7 and 6 in ML trees for CO1, CR and D-loop had similar samples with codes BLR450 and BS430. The samples with codes BLB 475 and BN441 were same for both CR and D-loop ML trees. These coded samples belonged to the native Lake Baringo tilapia *Oreochromis niloticus baringoensis* as shown in table 13.

Table 13: Comparative analysis of clustering pattern of mtDNA CO1, mtDNA CR and mtDNA D-loop samples as resolved by their respective Maximum Likelihood phylogenetic trees

mtDNA CO1 samples	mtDNA CR samples	mtDNA D-loop samples	Species/ Sub species
BKT470, BLR455,BS691	BKT 470,BLR455, BS 691,BN 641,BNG 479	BKT470, BLR455, BS 691	<i>O.n baringoensis x O.leucostictus</i>
BKT664, BKT466, BLR453, BLR460, BN431, BN432, BS698, BN445, BNG648, BS693	BKT466, BS699, BKT471, BKT664,BLB674, BLR449, BLR453,BLR460, BN432, BN442,BNG648,BS428,BS693, BS697,BS698	BKT466,BKT471 BLR449,BLR460 BS697	<i>O.spilurus</i>
	BN431,BN631,BKT468	BN431,BN631,B KT468	<i>Oreochromis spirulus</i>
BBH463,BBH627,B KT465BKT467,BLR 449,BLR452BLR456 ,BLR459,BNG473B NG475,BS696	BS695,BS696,BS429,BNG478 ,BNG476,BBH463,BBH627,B KT469,BLR448,BLR452,BLR 456	BLR452,BNG476 ,BLR448,BBH62 7, BS696	<i>Oreochromis niloticus (unknown)</i>
	BKT502, BN443,BS692	BKT502	<i>O. n baringoensis</i>
	BLR459,BN639,BKT465	BLR459,BKT465	<i>O. n vulcani</i>
BLR450,BLR 458,BLR461BN437, BN440,BS430	BNG480, BS430,BNG477, BN640,BN441, BN438,BN437, BLR450,BLB475, BLR447	BLB475,BN441, BN640,BS430	<i>O.n baringoensis</i>

The clustering pattern of the samples and haplotypes for the three mitochondrial marker genes mtDNA CO1, CR and D-loop in table 13 shows that the analysed sequences resolved in a similar way in all markers used. This implies that all three markers gave almost same results for the analysed fish samples.

CHAPTER FIVE

5.0. DISCUSSION

5.1. Morphological identification of Lake Baringo Cichlids

Cichlids caught in Lake Baringo exhibited morphological characteristics of the genus *Oreochromis*. These characteristics were in agreement with previously described features for this genus by Trewavas (1983) and Nyingi (2013). This study also found that some specimens had vertical dark stripes on the caudal fin which according to Trewavas, (1983) portrayed a characteristic feature for *O.niloticus*. Other specimens recorded no stripes on their caudal fins indicating that they were not *O. niloticus* species. The second tilapia species was found to be the Sabaki tilapia *Oreochromis spilurus*. The latter differed morphologically in the number of dorsal and anal fin spines, lateral line scales, snout length and anal spine length. This is the first report to document the presence of this species in Lake Baringo

Morphometrically, the characters measured under PC2 and PC3 had mixed coefficients indicative of shape variation rather than variation in size. This observation is in harmony with the findings by Jalicoeur and Mosimann (1960), who observed that, any component having all coefficients of the same sign was indicative of size variation where as any component having both positive and negative coefficients was indicative of shape variation.

The presence of both *O.niloticus* and *O.spilurus* in Lake Baringo was meristically evidenced by the fact that the sampled fish specimens had anal and dorsal spine numbers ranging from III-IV and XV-XIX, respectively. This is in agreement with the findings by Trewavas (1983), who showed that, presence of III anal and XV-XVIII dorsal spines is a shared characteristic feature for *O.niloticus* and *O.spilurus* species. However, presence of IV anal and XIX dorsal spines is a feature for the subspecies *O.spilurus niger*. The presence of *O.spilurus* in the lake

was also evidenced by the presence of dark grey fish specimens with reddish brown caudal fin lacking dark vertical stripes, which according to Nyingi (2013) is a characteristic feature of this species. Previous morphological studies on Lake Baringo tilapiines by Worthington and Ricardo, (1931); Ssentongo and Mann, (1971) and Trewavas, (1983) did not reveal the existence of *O.spirulus* or their subspecies in the lake. Even recent genetic studies by Nyingi and Agnèse, (2007) and Ndiwa *et al.*, (2014) did not document the existence of this species in the lake. In this regard, the species must have recently been introduced from the spilling of fingerlings from the Omega fish farm in May, 2013. This fish farm was rearing tilapia fingerlings whose source and species are not known since prior genetic characterization was not done prior to rearing. The floods of May 2013 caused the fish farm to submerge thereby releasing the fingerlings to the lake.

The present study did not morphologically delineate *O. niloticus* at sub species level mainly due to overlapping of meristic counts from the observed specimens. This is agreeable with the findings by Samaradivakara *et al.*, (2012) in which meristic measures did not show sufficient divergence among tilapia populations in Sri Lanka. The inability to delineate the fish samples at sub specific level was probably due to the fact that, many *O.niloticus* sub species have overlapping narrow ranged meristic characters which make them hard to delineate when mixed. This reason is agreeable with the findings by Vidalis *et al.*, (1994), who asserted that, the meristic characters have narrow variable range because divergence of these characters from a standard range could be fatal to the individual. This could be the reason why closely related species or sub species have a narrow variable range of meristic counts.

5.2. Identification of the current Cichlid species of Lake Baringo through mtDNA analysis

The mtDNA analysis of fish DNA sequences revealed the continued existence of *Oreochromis niloticus baringoensis* in Lake Baringo. The observation of this *O. niloticus* subspecies by this study is congruent to the findings by Worthington and Ricardo, (1931); Ssentongo and Mann, (1971) and Trewavas, (1983). The authors morphologically identified *O. n. baringoensis* as the only tilapiine endemic to Lake Baringo. Further studies by Nyingi and Agnèse, (2007) using mtDNA D-loop markers also revealed the continued existence of this sub species in the lake.

This study also revealed that *O. n. baringoensis* population in Lake Baringo is under continuous threat and declining very fast. This is mainly due to the introduction of other *Oreochromis* species through increased aquaculture activities within the lake and associated rivers. The present study identified 10 out 50 (20%) sample specimens through mtDNA CR and 4 out 23 (17.4%) mtDNA D-loop samples belonging to this species. The percentage of pure stocks of *O. n. baringoensis* observed in this study is far much lower than that observed by Nyingi and Agnèse, (2007) and Ndiwa *et al.*, (2014). Nyingi and Agnèse (2007) using mtDNA D-loop marker observed that 22 out of 30 (73.3%) sampled tilapia specimens from the lake were of *O. niloticus baringoensis*, while 8 out of 30 samples (26.7%) samples formed a haplotype identical to *O. leucostictus* from Lake Naivasha. Furthermore, Ndiwa *et al.*, (2014) observed that 8 (50%) out of 16 mtDNA D-loop tilapia fish samples from Lake Baringo were introgressed with foreign genes from *O. leucostictus*. The findings of this study show that the number of pure *O. niloticus baringoensis* of this haplotype is declining rapidly. This study has also revealed that, *O. n. baringoensis* is now being outnumbered by *Oreochromis spilurus* which according to this study is now becoming dominant tilapiine in

the lake. The reasons for the dwindling in number of *O. n baringoensis* as revealed by this study could be hybridization and niche competition with the introduced species (*O.leucostictus*, *O. spilurus*, *O.niloticus* sp. and *O. n vulcani*). These introductions of alien species to the lake are likely to pose a critical problem for conservation of the marginalised *O.n baringoensis*, which may become genetically swamped by ecologically dominant species (Mwanja and Kaufman, 1995). Continued co-existence of introduced morphs with native species and subsequent formation of intermediate morphs is likely to play a major role in complete disappearance of pure *O. niloticus baringoensis* species. Amarasinghe and De Silva (1996) reported that, long term existence of hybrids may reduce fitness due to reproductive isolation whose repercussion could be dwindling in total fisheries productivity.

Introduction of alien *Oreochromis* species of this nature in the past resulted into rapid replacement and eventual extinction of species in the wild suggesting that the species at hand is prone to extinction. For example, the introduction of *O.niloticus* in Lake Victoria in 1950s after a decline in production of native tilapiines resulted into disappearance of *Oreochromis esculentus* and *Oreochromis variabilis* from the main Lake Victoria (Mwanja *et al.*, 2010; Angienda *et al.*, 2011). The two species have been relegated to the satellite lakes surrounding Lake Victoria (Mwanja *et al.*, 2010). Meanwhile, Daget *et al.*, (1981) reported the complete extinction of *Oreochromis macrochir* in a Madagascan lake 10 years after introduction of *O. niloticus*. In Limpopo river system, *Oreochromis mossambicus* has been greatly replaced or genetically polluted by introduction of *O. niloticus* (Van der Waal, 2000; Firmat *et al.*, 2013; D'Amato *et al.*, 2007). The disappearance of the mentioned species is hypothesized to have been mainly caused by hybridization, behavioural versatility, and ecological viability of *O. niloticus*, which possess power to outcompete other species in utilizing resources and breeding niches (Nyingi and Agnèse 2007; Mwanja *et al.*, 2010). In this case if rapid

measures will not be taken to prevent further introductions, *O. n baringoensis* will ultimately disappear in the wild.

Oreochromis niloticus vulcani is another *O.niloticus* haplotype that this study has identified. The presence of this haplotype in Lake Baringo is not in agreement with previous studies. The species is known to inhabit Lake Turkana and its affluent streams (Trewavas, 1983; Nyingi, 2013). Therefore, existence of this species in Lake Baringo was not expected. Previous studies on Lake Baringo tilapia diversity by Worthington and Ricardo (1931), Ssentongo and Mann (1971), Trewavas, 1983 and Nyingi and Agnèse (2007) did not show the presence of this species or their hybrids to inhabit the lake. Therefore, presence of this haplotype is surprising as there is no physical connection between Lake Baringo and Lake Turkana where the species is confined. Further, there has been no formal documentation of introduction of this species to Lake Baringo. This would mean that the species could have been deliberately or accidentally introduced into the lake after 2007 through anthropogenic activities, especially through fishing and increasing aquaculture activities in the Central Rift valley region.

Oreochromis niloticus (unknown) is another haplotype that was observed through mtDNA analysis in this study. The Phylogeographic origin of the species is not known, however the species could be a Nile tilapia *Oreochromis niloticus niloticus* commonly known as ‘‘Ngege’’ (Nyingi, 2013). The discovery of this haplotype in Lake Baringo is not in harmony with the findings of previous studies by Nyingi and Agnèse, (2007) and Ndiwa *et al.*, (2014) and no formal documentation has been reported on deliberate introduction of this haplotype to Lake Baringo. Since this haplotype is distributed in Lake Victoria basin (Nyingi, 2013), it is likely that the species made their way to Lake Baringo through aquaculture as there is no

connection between Lake Baringo and Lake Victoria basin. This must have happened through rearing fingerlings obtained from Lake Victoria basin.

In this study, 11 out of 50 (22%) mtDNA CR sequences formed cluster of this haplotype subspecies. Likewise, 5 out of 23 (21.74) mtDNA D-loop sequences depicted this haplotype. Since, this haplotype was not previously identified to be present in the lake; its presence in the lake could pose severe threats to the native tilapia species *Oreochromis niloticus baringoensis*. Introduction of this subspecies (*O.niloticus niloticus niloticus*) to boost fishery catch in Lake Victoria in 1950s resulted in the expulsion of native tilapiines (*Oreochromis esculentus* and *O. variabilis*) from the main lake due to competition and hybridization (Mwanja *et al.*, 2010). No hybrids of this species with *O.n baringoensis* were observed in this study. This scenario could be similar to that of the satellite Lakes Kanyaboli and Namboyo of Kenya, where no hybrids were observed between co-existing species (*O. niloticus* and *O. leucostictus*), though low levels of genetic admixture were observed (Angienda *et al.*, 2011). This implies that, given more time, this rapacious subspecies could form hybrids with other *O. n baringoensis* and eventually eliminate them from the lake. This could be possible since *O. niloticus* species have high propensity to hybridization, as they have been observed to hybridize easily with other *Oreochromis* species (Van der Waal, 2000; D'Amato *et al.*, 2007; Daines *et al.*, 2014).

The present study has also revealed the continued existence of the mtDNA introgressed hybrids of *O.n baringoensis* with *O.leucostictus*. The haplotype was observed in five out of the eight sampled sites of this sympatric lake. The presence of these mtDNA introgressed hybrids is in harmony with the discovery made by Nyingi and Agnèse (2007) and the observation made by Ndiwa *et al.*, (2014) using mtDNA D-loop markers. It has been shown by Nyingi and Agnèse, (2007) that *O. niloticus baringoensis* and *O. leucostictus* are non-

sympatric species with the latter being native to Lake Naivasha, which is located about 150 km from Lake Baringo (Harper *et al.*, 1990). *O. leucostictus* is believed to have been introduced into Lake Baringo around the year 2000's to boost the dwindling tilapia catch thought to have been caused by heavy siltation, overfishing and reduced rainfall (Nyingi and Agnèse, 2007). This mixing caused the introgression of mtDNA genes from *O.leucostictus* to *O.n baringoensis* resulting in formation of introgressed hybrids. Similar scenario involving transfer of mtDNA genes from *Oreochromis aureus* to *Oreochromis niloticus* in West Africa was observed by Rognon and Guyomard (2002). However, in West Africa, the two species are sympatric and their hybridization is ancient dating back to the colonization of *O.niloticus* in the region through River Nile (Rognon and Guyomard, 2002).

This study also revealed that a slight decrease had occurred in the number of hybrids of *O.n baringoensis* with *O. leucostictus* compared to the findings of the previous studies by Nyingi and Agnèse, (2007) and Ndiwa *et al.*, (2014). In this study, 10% of mtDNA CO1 and mtDNA CR samples clustered to form this haplotype while in mtDNA D-loop 13% of the samples formed this group (table 13). Previous findings by Nyingi and Agnèse (2007) and Ndiwa *et al.*, (2014) on Lake Baringo Cichlids using mtDNA- D loop, revealed 26.7% and 50% of the sample catch respectively for this hybrid.

The continued existence of *O.n baringoensis* x *O.leucostictus* hybrids in the lake has caused severe repercussions to the native species as well as other tilapia populations existing in the Rift valley wetlands. A recent study by Ndiwa *et al.*, (2014) using mtDNA D-loop marker has revealed the existence of *O.leucostictus* haplotype previously confirmed to be present in Lake Baringo (Nyingi and Agnèse 2007) in three hot spring populations of Lobo swamp near Lake Bogoria, Kenya. These introgressions must have occurred after the year 2007 since the study conducted by Nyingi and Agnèse (2007) on genetic evidence of unknown and endangered

natural populations of *Oreochromis niloticus* in these swamps revealed no mtDNA gene transfer from *O.leucostictus* to these tilapiines. Therefore the mtDNA gene transfer from *O.leucostictus* to *O.niloticus* observed by Ndiwa *et al.*, (2014) in Loboï swamp must have been caused by introduction of *O. n baringoensis/O.leucostictus* hybrids from Lake Baringo through aquaculture. This observation was supported by existence of fish ponds around the swamp rearing fish obtained from Omega farm located in Ol-kokwe Island in Lake Baringo before 2013 floods (Ndiwa *et al.*, 2014). This farm was used as a reservoir source of fingerlings for aquaculture farms around Lake Baringo basin (Johnstone, 2011). It is therefore likely that these hybrids have been introduced to other unstudied water bodies around Lake Baringo basin.

Another important finding in this study was the presence of a haplotype similar to *Oreochromis spilurus* which had never been documented or reported by previous studies to inhabit the lake. All the three marker genes used in this study discerned this haplotype from the samples. The haplotype was also morphologically identified by the presence of XVIII to XIX dorsal fin and IV anal fin spines depicting characteristic features of *O.spilurus*. In order to explain how *O.spilurus* made their way to Lake Baringo, it is important to understand the natural distribution of this species in Kenya. According to Trewavas (1983), there are three subspecies of *O. spilurus* namely *O. spilurus spilurus*, *O. spilurus niger* and *O. spilurus percivali*. These sub species are distributed along Kenyan Athi river system, which includes Voi system, Lungard's falls, Kibwezi tributary, Tsavo River rising from the slopes of Mount Kilimanjaro. Other sites where the species is found include the ponds at Sagana which is located at the southern foot of Mount Kenya in Kirinyaga County, and Lake Naivasha where they were stocked in early 1930s (Elder *et al.*, 1971). These species have also been widely cultured in fish ponds across Kenya. Ndiwa (2011) reported the culture of *O. spilurus spilurus* and *O. spilurus niger* in Kianda, Lusoi and Gathini dams in Nyeri county, Kenya.

Since *O. spilurus* does not occur naturally in Lake Baringo, two propositions could account for its introduction. One likely proposition is that, the species (*Oreochromis spilurus niger*) from Lake Naivasha could have been introduced together with *O. leucostictus* (Nyingi and Agnèse, 2007) in 2000s to boost tilapia catch. This reason makes sense because the two species share the same sympatric geographical location (Lake Naivasha). Since the transfer of these fish into Lake Baringo was not selective, it is therefore likely that *O. spilurus niger* or their hybrids with *O. leucostictus* were introduced at the same time. However, this proposition is doubtful since the haplotypes of this species were neither observed by Nyingi and Agnèse (2007) nor Ndiwa *et al.*, (2014). Another credible proposition is that the species could have been introduced after 2007 by the fingerlings spilling from the aquaculture farms and ponds located along water bodies in Lake Baringo basin.

The hybrids of *O. niloticus baringoensis* with *O. spilurus* were not observed in this study. However, given more time, the species could hybridize. *O. spilurus* females have experimentally been reported to hybridize with *O. niloticus* males producing morphs with a sex ratio of 1:1 while *O. niloticus* (females) x *O. spilurus* (males) produced more males (Eknath and Hulata, 2009). In Kenya, the hybrids of *O. spilurus* with *tilapia zillii* were first observed in Sagana fish farm by Whitehead (1962). Other hybrids of this species with *O. niloticus* in Tana River system were reported by Omondi *et al.*, (2001).

5.3. Morphological and genetic diversity of Lake Baringo Cichlids

The current study revealed that fish specimens collected from the selected sites of the lake differed morphometrically with respect to the length of the snout, longest anal ray, dorsal fin spines and anal fin spines. However, no significant difference was observed in length of these characters for the samples collected from the southern and northern parts of the lake. On the other hand, the samples collected from the central part of the lake differed significantly from

those collected in the southern and northern parts of the lake in terms of length of the snout, longest anal ray dorsal fin spines and anal fin spines. This was also supported by principal component analyses of Morphometrics and Meristic characters in table 4 and 7 respectively which showed high degree of variation for the analysed samples with respect to Principal Component one indicating high morphological diversity of Lake Baringo tilapiines.

Previous morphological studies by Omoniyi and Agbon, (2008) and Dynes *et al.*, 1999 revealed that morphometric characters such as body depth, caudal peduncle depth and snout length vary among species as a result of environmental conditions especially water temperature, salinity and nutrients. However, the morphometric differences observed in this study between the fish samples collected from the central part with those collected from the southern and northern parts of the lake can be explained by the recent introduction of fish fingerlings. These introductions occurred during the floods of May 2013 when the Omega fish farm located in Ol-kokwe Island in the central part of Lake Baringo submerged releasing the fingerlings into the lake. The fish farm was established in the year 2010 to produce fingerlings for local fish farmers by utilizing brood stock from the lake (Johnstone, 2011). Since, the broodstock utilized in the fish farm was reported to have been supplied by local fishermen without prior genetic characterization or ascertainment of species it is likely different tilapia species were reared in the farm. Since the spilling of fingerlings occurred in the central part of the lake, it is likely that the tilapia mixing affected the species diversity at the central part of the lake than southern and northern parts. This could be the most possible explanation for the observed morphological differences observed between the samples collected from the central with those collected from the southern and northern parts of the lake. Moreover, environmental factors are unlikely to have influenced the observed differences mainly due to the fact that Lake Baringo is a relatively small lake (about 140km²), and lack geographical barrier to prevent the movement of fish within the lake.

Environmental effects have been found to play a big role in morphological differences in situation where similar species are subjected to different environmental conditions; For instance, *O.n filoa* and *O.n cancellatus* were found to be genetically related (Seyoum and Kornfield, 1992; Agnèse *et al.*, 1997) but phenotypically different (Nyingi *et al.*, 2009) because the former inhabit hot springs, while the latter is found in the rivers and lakes.

In this study, the mtDNA analysis for Lake Baringo tilapia fish samples showed higher genetic diversity for mtDNA Control Region and D-loop than Cytochrome Oxidase I marker gene. The first two marker genes produced seven different haplotypes while the latter gene produced only four haplotypes. The low genetic diversity shown by mtDNA COI gene compared to the other two marker genes is in agreement with the findings by Wu and Yang, (2012) who found that mtDNA COI marker gene had lower genetic variability than mtDNA CR marker gene in delineating the tilapia population. In addition, mtDNA COI is a protein coding gene which is highly conserved and less prone to mutation (Abumourad, 2011). Therefore mtDNA COI gene is not a sufficient marker gene for delineating tilapia species.

The mtDNA CR and D-loop produced seven haplotypes (figure 16 and table 13) five of which were identified as *O.n baringoensis*, *O. n vulcani*, *O.niloticus (unknown)*, *O. n baringoensis /O.leucostictus* and *O. spilurus*. Two previous studies by Nyingi and Agnèse (2007) and Ndiwa *et al* (2014) on Lake Baringo tilapia diversity using mtDNA D-loop marker gene revealed 2 haplotypes. The haplotypes observed by these studies were *O. n baringoensis* and the mtDNA introgressed hybrid of *O. n baringoensis* with *O. leucostictus*. Therefore, three additional haplotypes observed in this study are new and must have been introduced in the lake after 2007 through aquaculture.

Comparing the current findings with previous population genetics studies of Lake Baringo tilapia using mtDNA D-loop sequences analysed by DnaSP, it was observed that genetic

diversity of these tilapiines had changed greatly (table 11). The number of polymorphic sites (S) and haplotypes (H) (n=23) for D-loop were 48 and 7, respectively. This is in contrast with the findings by Ndiwa *et al.*, (2014) where the two diversity-measuring parameters were 37 and 4 respectively (n=15) for the same marker gene. However, other variability parameters were observed to remain more or less the same with the number of nucleotide differences (K), and nucleotide diversity (Pi) for the present study (17.54 and 0.048), against 18.06 and 0.051 observed by Ndiwa *et al.* (2014). This increase in values of H and S indicates that the diversity of Lake Baringo tilapia has increased. The reason for Ndiwa *et al.* (2014), to observe fewer haplotypes in Lake Baringo tilapia population compared to this study could be probably due to low number of sampled specimens (15) and the sampling area. Another reason is that the researcher collected the samples in May, 2012 prior to the spilling of the Omega fish farm in May, 2013 while this study was conducted after the submergence of the fish farm.

5.4. Conclusions and Recommendations

5.4.1. Conclusions

- (1) This study using mtDNA revealed that Lake Baringo is currently hosting five different tilapia haplotypes which are *O.niloticus baringoensis*, *O.spirulus*, *O.n vulcani*, *O. niloticus* (Unknown) as well as the introgressed hybrids of *O.n baringoensis* with *O.leucostictus*. Two haplotypes could not be confirmed but are suspected to be subspecies of *O.spirulus* and a hybrid of *O.n baringoensis* x *O. n suguta*.
- (2) The genetic structure, integrity and diversity of the native *O.n baringoensis* has been compromised by the introduction of other *Oreochromis* species.

(3) The number of mtDNA introgressed hybrids of *O.n baringoensis* with *O.leucostictus* has slightly decreased.

(4) MtDNA Control region (mtDNA CR) and mtDNA D-loop have higher genetic polymorphism than mtDNA CO1 genetic markers, hence are the best to use in genetic diversity studies of cichlids.

5.4.2. Recommendations

5.4.2.1. Recommendations from the study

- Microsatellite markers should be used to find out if the hybridization between introduced and native species *Oreochromis niloticus baringoensis* involved nuclear transfer of genes.
- A survey should be done in all fish ponds and farms around catchment areas to find out the tilapia species being used.
- Further comparative analyses between Lake Baringo tilapiines and other tilapiines from Lake Turkana, Lake Victoria and Sagana fish farm should be done to prove the exact source of the species discovered by the present study.

5.4.2.2. Conservation recommendations

- The lessons learned from species introductions in Kenya and elsewhere in tropics should be incorporated in fisheries and development policies.
- Environmental Impact Assessment, documentation of species and risk assessment of stocked exotic species should be done prior to introduction of any species in lakes, rivers and swamps.

- A policy should be formulated to prevent establishment of aquaculture farms closer to the water bodies.
- Ecological studies should be carried out to find out the effects brought to by introduction of the species in Lake Baringo.
- In situation where the endemic pure species co-exist with other introduced species, the native species can be selectively augmented by nursing the fingerling in artificial nurseries adjacent to the native lakes with eventual release back to these waters.
- Since fish farmers have been breeding different tilapia species with fingerlings obtained from diverse sources within East Africa *in situ* approach to conserve the native species should be done by restricting the transfer of fish from different phylogeographically isolated places.

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APPENDICES

Appendix 1: Sample of Data Sheet used in the lab to record both Morphometrics and Meristic characters

Reg. Nr		Identifier		Collector
I.D		Locality		
Date	TL	SL	HL	SnL
Observations				ED
				BD
				HL
				Pre-D
				HD
				Pre-A
				Pre-PECT
				Pre-V
				IoD
				CPL
				CPD
				PoL
				AFL
				PFL
				VFL
				DSL
				ASL
				DFL
				Meristics
				DFS
				DFR
				AFS
				AFR
				LLS
				LLDor
				LLVent

Appendix 2: MtDNA D-loop sample blast against NCBI data base for tilapia fish

Sample code	Query Cover	Maximum Identity	Species	Accession number
BBH627	99	100	<i>O.niloticus</i>	AF296474.1
BKT465	100	99	<i>O.n vulcani</i>	AF296472.1
BKT466	100 100	100 99	<i>Oreochromis sp</i> <i>O.spilurus</i>	AF296477.1 EU431000.2
BKT468	100 100	99 98	<i>Oreochromis sp</i> <i>O.spilurus</i>	KJ648488.1 EU431000.2
BKT470	100	99	<i>Tilapia sp</i>	AF296495.1
BKT471	100 100	99 99	<i>Oreochromis sp</i> <i>O.spilurus</i>	AF296477.1 EU431000.2
BKT502	100 100	99 99	<i>O.n baringoensis</i> isolate <i>Oreochromis niloticus vulcani</i>	AJ237397.1 AF296472.1
BLB475	100 100	99 99	<i>Oreochromis niloticus</i> strain Egypt <i>Oreochromis niloticus</i> isolate	GU477625.1 AF296471.1
BLR448	100	99	<i>Oreochromis niloticus</i> isolate mitochondrial D-loop	AF296474.1
BLR449	100 100	99 99	<i>Oreochromis sp.</i> <i>Oreochromis spilurus</i> D-loop	AF296477.1 EU431000.2
BLR452	100	99	<i>Oreochromis niloticus</i> isolate	AF296474.1
BLR455	100	99	<i>Tilapia sp.</i>	AF296495.1
BLR459	100	99	<i>Oreochromis niloticus vulcani</i>	AF296472.1
BLR460	100 100	99 99	<i>Oreochromis sp</i> <i>Oreochromis spilurus</i> D-loop	AF296477.1 EU431000.2
BN431	100 100	99 98	<i>Oreochromis sp</i> <i>Oreochromis spilurus</i> D-loop	KJ648488.1 EU431000.2
BN441	100 100	99 99	<i>Oreochromis niloticus</i> strain Egypt <i>mtDNA</i>	GU477625.1 AF296471.1
BN631	100 100	100 99	<i>Oreochromis sp</i> <i>Oreochromis spilurus</i> D-loop	KJ648488.1 EU431000.2
BN640	100 100	99 99	<i>Oreochromis niloticus</i> strain Egypt <i>Oreochromis niloticus</i> isolate	GU477625.1 AF296471.1
BN641	100	99	<i>Tilapia sp</i>	AF296495.1
BNG476	100	99	<i>Oreochromis niloticus</i> isolate	AF296474.1
BS430	100 100	99 99	<i>Oreochromis niloticus</i> strain Egypt <i>Oreochromis niloticus</i>	GU477625.1 AF328848.1
BS691	100	99	<i>Tilapia sp</i>	AF296495.1
BS696	100	99	<i>Oreochromis niloticus</i> isolate	AF296474.1
BS697	100 100	99 98	<i>Oreochromis sp</i> <i>Oreochromis spilurus</i> D-loop	AF296477.1 EU431000.2

Appendix 3: Gene Bank data sequences that were used as reference for the identification of Lake Baringo observed haplotypes

Species	Marker	NCBI Accession Number
<i>Oreochromis aureus</i>	mtDNA D-loop	GU980727
<i>Oreochromis leucostictus</i>	mtDNA D-loop	EF016702
<i>Oreochromis mossambicus</i>	mtDNA D-loop	AY833459
<i>Oreochromis niloticus vulcani</i>	mtDNA D-loop	AF296472
<i>Oreochromis n baringoensis</i> (2)	mtDNA D loop	AJ237397
<i>Oreochromis niloticus baringoensis</i>	mtDNA D- loop	EF016708
<i>Oreochromis niloticus 5270baring</i>	mtDNA D-loop	EF016700
<i>Oreochromis niloticus sugutae</i>	mtDNA D- loop	EF016714
<i>Oreochromis niloticus niloticus</i>	mtDNA D-loop	AF296474
<i>Oreochromis sp</i>	mtDNA D-loop	KJ648488
<i>Tilapia sp</i>	mtDNA D-loop	AF296495
<i>Oreochromis spilurus</i>	mtDNA D-loop	EU431000
<i>Tilapia rendali</i>	mtDNA CR	AF328854
<i>Oreochromis niloticus (Turkana)</i>	mtDNA D- loop	EF016694
<i>Oreochromis aureus</i>	Complete genome	GU477630