GENETIC BASIS OF HEAT INDUCED SEX DETERMINATION IN NILE TILAPIA (*Oreochromis niloticus* L.), AND GENETIC VARIABILITY OF THE SPECIES LOCAL POPULATIONS

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A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD) IN CELL AND MOLECULAR BIOLOGY

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ABSTRACT

Oreochromis niloticus commonly known as Nile tilapia is the most cultured tilapine cichlid in the sub-Saharan Africa. Most of the culture practices are of mixed sexes with the inherent problem of overpopulation leading to stunted growth. Mono-sex culture of all-male individuals is now encouraged by sex reversal through heat treatment technology. However the method is still at development stage. The aim of the present study was to determine the genetic basis of heat induced sex-determination in *O. niloticus* as an approach towards improving heat treatment technology and to assess the genetic variability of the invasive *O. niloticus* for conservation and management within Lake Victoria Nyanza gulf and the Yala swamp. Juvenile *O. niloticus* were subjected to heat treatment at temperatures ranging from 28°C to 36°C using 26°C as control. There was a positive correlation between treatment temperatures and resultant male sex ratios ($p<0.01$). However, the survival rates of the fry showed a negative correlation with the temperature ($p<0.01$). The optimal temperature that provided for optimal sex reversal towards males and survival rates of the fry was found to be 36±1°C, which gave a male proportion of 86.31% and fry survival rate of 65.25%. To study genetic basis of heat induced sex determination three sex linked markers UNH846, Abur36 and Abur100 were selected, genotyped and sequenced. The marker Abur36 identified the sex of 95% of the heat induced individuals. The putative function of this gene indicated that the gene could be a male biased hormone producer or transcriptional factor whose activity is influenced by elevated temperature within a narrow range before the onset of the temperature sensitive gonadal sexual differentiation. Determination of the genetic diversity and purity of *O. niloticus* was carried out based on eight microsatellite markers and mitochondria DNA control region and compared with that of *O. esculentus*. No admixture between the two species was detected. However there were low levels of nuclear admixture primarily from *O. niloticus* to *O. esculentus*. Genetic diversity of *O. niloticus* (h =0.829, π = 0.008) was higher than that of *O. esculentus* (h = 0.816; π = 0.004). All populations of the two species retained unique haplotypes and alleles in their respective lakes. Pairwise F_{ST} values for population differentiation of both species were all highly significant ($P < 0.001$). Therefore the four populations of *O. niloticus* are potential sources of broodstock choice from which all-male fingerlings could be produced using heat treatment technology.

CHARTER ONE

INTRODUCTION

Nile tilapia (*Oreochromis niloticus*) is a popular culture fish of African origin, native of Sudan. It has been widely introduced world over and it is second only to carp in global production (FAO, 2008). It is cultured either for subsistence or commercial needs where the great majority of the culture practices are semi-intensive (Liti *et al.*, 2005). However overpopulation of the species in confined ponds reduces faunal diversity of the system thereby producing stunted fish population of poor market value. Various methods of population control have been applied such as culture in cages, culture with predators, intermittent harvesting, induction of sterility and mono sex culture of male tilapia.

Mono sex culture of all male tilapia is the most preferred approach of controlling the problem of overpopulation of tilapia in ponds. Several hypotheses have been proposed to explain the sex related differential growth in favour of tilapia males, major ones being anabolism-enhancing effect of androgens (Baroiller *et al.*, 1999), an increase of food utilization efficiency (Liti *et al.*, 2005), a greater reallocation of metabolic energy toward the reproductive function in females as compared to males and a sexual behaviour of females, especially after spawning, that is associated with a large decrease in feeding activity (Fontainhas-Fernandes *et al.*, 2002).

Beginning with the work of Hickling (1960), a variety of methods have been used to produce all-male fingerlings, including interspecific hybridization (Wohlfarth and Hulata, 1983), dietary supplementation of synthetic androgen (Phelps and Popma, 2000; Chakraborty and Banerjee, 2009) production of supermale fish (YY-males) (Majundar and MacAndrew 1986;

Mair *et al.*, 1997) and hand sexing (Hafeez-ur-Rehman *et al.*, 2008). These methods are not entirely reliable e.g. hand sexing method is labour intensive, genetic methods on the other hand are high tech and therefore not easily adaptable by ordinary farmers. The hormonal treatment, though effective is not preferred due to the public's general resentment against the use of hormones in food. Moreover environmental factors such as temperature have been shown to affect phenotypic sex in fish (Baroiller *et al.*, 1995; Desprez and Me´lard, 1998; Abucay *et al.*, 1999; Wang and Tsai, 2000a), and autosomal genes may also influence sex-determination in fish (Mair *et al.*, 1991; Hussain *et al.*, 1994; Sarder *et al.*, 1999, Ospina-Alvarez. and Piferrer, 2008).

The influence of temperature on sex determination in fish has been exploited in production of mono-sex individuals with no inherent problems (Nomura *et al.*, 1998; Baroiller *et al.*, 1995; Pavlidis *et al.*, 2000). The technology also involves simple and inexpensive procedures that can easily be adapted by local farmers. However the method is still not 100% effective as a tool for sex reversal necessitating the understanding of the genetic basis of heat induced sex determination as an approach toward improving the method.

Sex determination in tilapia is ultimately under genetic control. Although genetic factors regulate sex-determination in teleosts, sex chromosomes of tilapia are at an early stage of differentiation and there are no gross morphological differences in any chromosome pair that would identify the X and Y chromosomes (Carrasco *et al.*, 1999). Therefore additional autosomal loci also contribute to sex-determination (Lee *et al.*, 2004). In only one fish (Medaka) has the sex-determining gene (DMY) been identified (Nanda *et al.*, 2002). The gene DMY is expressed only in developing male gonads and is necessary for differentiation of testes. The lack of a definite genetic sex determination system in tilapia may mean that

different genes are implicated as the primary regulators of sexual differentiation. Extrinsic factors like temperature may also influence these genes to skew sex ratio in favour of either male or female individuals (Lee *et al.*, 2004).

Sexual differentiation in *O. niloticus* is triggered by temperature during the critical developmental period (Wibbles *et al.*, 1991; Phelps and Popman 2000; Baroiller *et al.*, 2009). Exposure to elevated temperature for 10 or more days between 9–13 days post fertilization increases the proportion of male individuals (Baroiller *et al.*, 1995; Ospina-Alvarez. and Piferrer, 2008; Angienda *et al.*, 2010).

Gonadal sex differentiation relates to all occurrences during development that lead to the expression of genetic sex via the appropriate phenotype (Piferrer, 2001), and encompasses all the events that take place in the primordial gonad, including the migration of primordial germ cells (PGCs), the establishment of gonadal ridges, and the differentiation of the gonads into testes or ovaries (Brusle´ and Brusle´, 1983). Sex differentiation occurs in two stages: cytological and anatomical (Coward and Bromage, 1998). The former involves the meiotic differentiation of oogonia and oocytes or spermatogonia and spermatocytes from primordial germ cells. The latter involves structural changes into testes or ovaries, including the mitotic proliferation of follicle cells to form an ovarian cavity, appearance of lobular or club-shaped testes, and formation of efferent ducts and blood vessels (Nagahama, 2000). In the genus *Oreochromis*, anatomical differentiation precedes cytological differentiation (Papadaki *et al.*, 2005), while in other species, such as the Medaka, *Oryzias latipes,* the opposite occurs (Kondo *et al.*, 2003).

Although much is known about the process of sex differentiation in fish, the precise mechanisms involved in primary sex determination remain undefined. Sex determination occurs through several mechanisms in teleosts. Primarily, sex determination has a genetic basis, which is determined at fertilization. This genetic mechanism usually occurs in one of two forms: heterogametic male (XY) or heterogametic female (WZ) (Donaldson, 2000).

However, Muller-Belecke and Horstgen-Schwark (1995) suggested that two or more sex determining factors might override the XX–XY mechanism in *O. niloticus*. The less common heterogametic delineate females as WZ and males as ZZ. Shelton (1989) suggests that species with environmental sex determination showing female size advantage are predisposed to evolve the WZ/ZZ mechanism, whereas species with male size advantage are predisposed to evolve the XX/XY mechanism. Knowledge of such a mechanism in a species can reveal the most suitable mechanism for sex reversal.

Lee *et al.*, (2004) working on blue tilapia (*O. aureus*) using microsatellite markers in construction of sex specific linkage maps, showed that the gene for maleness was located in the linkage group 1 (LG1) while the gene for femaleness was in LG3. Studies by Cnaani *et al.*, (2008) on mechanism of sex determination in tilapiine species, identified DNA markers linked to sex determining genes. In the species *Oreochromis karongae,* the sex-determining locus were found on linkage group (LG) 3, whereas in *O. niloticus* the sex-determining locus was on LG1. These microsatellite markers could be used for tracking sex-linked haplotypes in breeding programmes aimed at controlling the sex of fingerlings. Sex linked microsatellite or simple sequence repeats (SSR) located in the promoter regions have been shown to influence gene expression (Chistiakov *et al.*, 2006) thereby affecting fitness, survival and adaptation to environmental conditions. Changes in the base sequences of SSR may alter the open reading

frame thereby affecting gene transcription and translation with the resultant change in the phenotypic sex.

The success of sex reversal by heat treatment as a technology for producing all-male individuals will depend on the appropriate stock choice of populations that are genetically robust and diverse. The multiple introduction of this species into the Lake Victoria (Balirwa *et al.*, 2003) may have had a number of effects on the genetic variability of this species; e.g. diluted founder effect, reduced inbreeding depression and increased genetic variation (Hanfling, 2007). However *O. niloticus* may have also extensively hybridised with native species thereby reducing it genetic diversity (Angienda *et al.* 2011). Therefore to ensure the success of monosex (all-male) culture, it is necessary to have a selection of strains with high genetic diversity. Identification of a good broodstock population of *O. niloticus* for all-male tilapia production within the Lake Victoria region can be achieved through genetic characterization to assess its genetic variability and population genetic structure.

The overall objective of this study was to determine the genetic basis of heat induced sex-determination in *O. niloticus* with am aim of understanding the temperature dependent sex determination to improve on the heat technology and to assess the genetic variability of the species' populations in the Lake Victoria region in identification of the most genetically robust and diverse population for recruitment into aquaculture. This entailed; identifying major sex-determining loci in *O. niloticus* using microsatellite markers, determination of the role of sex linked microsatellites in sex determination in *O. niloticus* and assessment of the genetic variability of *O. niloticus* populations within the Lake Victoria region using both microsatellite and mitochondria DNA markers.

1.1. Statement of the problem and justification of the study

Tilapias are currently the second most farmed group of fish after carps with an annual world production of 2.5 million tons (FAO, 2008). This ranking is due to the advantageous aquaculture traits of the species e.g. faster growth rate, high tolerance to adverse environmental conditions and high feed utilization efficiency. Various strategies utilizing sex reversal can lead to the development of predominantly, male or female populations, or a "super-male" genotype (YY). The primary aim is to take advantage of sexually dimorphic characteristics of the species and control reproduction. Currently male monosex populations are produced mainly by androgen treatments. However hormonal treated is prohibited in most countries where the fish is destined for human consumption. Hormone residues may also impact negatively on water quality and biodiversity. Genetic approaches such are development of supermales (YY-males) though effective, are hampered by very long procedure of producing and identifying putative YY male individuals. Alternative methods such as sex reversal by heat treatment have no inherent problems though still not 100% effective.

Sex determination in *O. niloticus* is more complex than the simple XX/XY mono factorial system. Therefore understanding of the mechanisms of sex determination and the genetic basis of heat induced sex determination in *O. niloticus* is required in attempts to improving heat treatment technology. Identification of sex linked loci and determination of the effect of temperature at the nucleotide base sequence level during temperature sensitive sex differentiation period, would elucidate the role of temperature in sex reversal. The best genotypes for *O. niloticus* aquaculture applications will rely on appropriate stock choice of high genetic diversity with such traits as feed conversion efficiency and growth rate. Combining this approach with sex reversal has a strong potential to enhance aquaculture production, efficiency and sustainability. There is therefore further need to undertake genetic

characterization of the *O. niloticus* genetic resources within the Lake Victoria region to determine its genetic diversity and identify genetically robust populations.

1.2. Hypothesis

i). *Oreochromis niloticus* population within the Nyanza Gulf of Lake Victoria have no genetic basis for temperature sex determination.

ii). There is no genetic variation between the *O. niloticus* populations within the L. Victoria Region.

1.3. General objective

The overall objective of the study was to determine the genetic basis of heat induced sex determination in *O. niloticus* and to study the genetic variability of the species' local populations within the L. Victoria region.

1.4. Specific objectives

The specific objectives of the study were to:

i). Determine optimum temperature at which there is maximum sex reversal towards males.

ii). Determine the effect of temperature (using expressed sequence tag (EST) based sex-linked genes in sex-determination) in *O. niloticus*.

iii). Assess the genetic variability of local populations of *O. niloticus* using microsatellite and mitochondria DNA markers.

CHAPTER TWO

LITERATURE REVIEW

2.1. Cichlids classification and distribution

Tilapia belong to the tribe Tilapiine (subfamily: Tilapiinae) of the family Cichlidae in the order Labroidea of the Perciformes. Trewavas (1983) describes ten genera within the tilapiine, including three major genera, Tilapia, Oreochromis and Sarotherodon which are all represented in the Lake Victoria basin. Tilapia are substrate spawners and both parents guard the young. Oreochromis are maternal mouth brooders and only females carry eggs and young in the mouths. Sarotherodon are biparental mouth brooders, both female and male parents carry eggs and young in their mouth.

Species classification has been largely based on variation in dentition, bone structure, pigmentation, squamation characteristics and general body morphology (Kornfield *et al.*, 1984; Trewavas, 1983). However most of these characters overlap and may fail to unambiguously identify species owing to interpopulation variation and small differences among species (Klett and Meyer, 2002). Molecular techniques have been employed in an attempt to identify and classify Tilapias. Random Amplified Polymorphic DNA (RAPD) finger printing has been used to discriminate tilapia species and their hybrids (Bardakci and Skibinski, 1994; Mwanja *et al.*, 2008; El-Alfy *et al.*, 2009) while mitochondrial DNA markers have been used to identify subspecies of *O. niloticus* (Okumus and Ciftci, 2003; Angienda *et al.*, 2011)

Tilapias are African in their natural range and are thought to have diverged from a complex ancestral mixture, which also included the ancestors of the haplochromines more than

10million years ago (Trewavas, 1983). The *O. niloticus* invaded Lake Victoria in the early 1900s, with the first recordings of the species in the lake occurring in the 1920s (Trewavas 1983). Trewavas postulates that the species may have entered the lake through the Kagera River, following introductions into Lake Bunyonyi from Lake Edward. *O. niloticus* is now the most ecologically and economically dominant tilapiine species in the Lake Victoria region waters in both the main lakes and in most surrounding satellite lakes (Balirwa 1992). Its dominance is attributed to its ecological versatility and trophic virtuosity and ability to withstand limnological changes in Lake Victoria (Balirwa 1992; Sanderson *et al.*, 1995; Opiyo 1994; Balirwa *et al.*, 2003).

Geographical distribution of *O. niloticus* is influenced by both physical and chemical factors e.g. current velocity, depth, temperatures, salinity, alkalinity, oxygen and other dissolved gases. Within their original area of distribution they have colonised a great range of habitats: permanent and temporary rivers, flowing water, large equatorial and sub tropical rivers, swamps, lakes, lagoons and brackish estuaries (Aloo 2003). These varied habitats represent a very wide range of physical parameters (depth, current, velocity, and turbidity) and of temperature and chemical compositions (salinity, pH, dissolved oxygen). The species appears to be very resistant to low dissolved oxygen; it can tolerate values as low as 0.1 p.p.m. This enables it to live and reproduce in swamps and shallow lakes, which become deoxygenated (Chapman *et al.*, 1996; Aloo 2003). Though massive fish kill occasionally occur, it can withstand very high levels of CO_2.

2.2. Sex determination and differentiation in teleosts

Sex-differentiation in fish is controlled ultimately by specific sex-determining genes (Muller-Belecke and Horstgen-Schwark, 1995; Lee *et al.*, 2003), but in contrast with other taxa, sexual development in teleost is protracted and plastic. Genetic and phenotypic ratios do not

necessarily coincide and in several species, interaction between the genome and variable environmental and internal factors may determine sex (Ospina-A' Ivarez and Piferrer, 2008). In addition, the diversity of reproductive strategies shown by teleosts does not help to obtain a clear picture and typical genetic mechanisms of sex-differentiation are inadequate to explain sexual phenotype (Lee *et al.*, 2003).

Sex determination is defined as genetic processes that influence the definition of sex, whereas sex differentiation refers to gonadal development once sex has been determined, i.e. the pathway followed by the undifferentiated or bipotential gonad developing into either an ovarian or testicular tissue. Sex determination in teleosts have genetic basis and in some species environmental factors may influence sex. Genetic sex determination (GSD) involve monogenic (determination of sex by a gene located on a specific chromosome) or polygenic system (determination of sex by accumulative genetic action of all factors involved in its determination e.g. sex chromosomes, influence of autosomal genes and environmental factors as in the case of *O. niloticus*) (Devlin and Nagahama 2002).

In teleosts that show monogenic sex determination system, sex is determined by a gene located on the sex chromosome and genes on other chromosomes have no influence. Most teleost species do not possess morphologically distinct sex chromosomes. However some species e.g. catfish of the genus Liobagrus (*L. marginatus* and *L. styani*) have heteromorphic sex chromosomes (Chen *et al.*, 2008). Two systems exist in species with sex chromosomes: heterogametic males (XY) in species like medaka (*Oryzias latipes*) and heterogametic females (ZW) found in *Oreochromis aureus* (Mair *et al.*, 1991). However variations have been reported in the two sex determination systems: XY system where the two chromosomes are cytogenetically distinguishable and the number of diploid chromosomes is the same in both

an ovary or testis as exhibited in *Dicentrarchus labrax*. (Bla´zquez *et al.* 1998) and (ii) differentiated gonochoristics in which all individual develop gonads with oocytes as an indication of ovarian differentiation but later the oocytes degenerate by apoptosis and gonad masculinization proceeds eventually developing into normal testicles as observed in Zebra fish (Maack and Segner 2003). In hermaphroditic species, individuals posses both male and female gonadal tissue that are either simultaneously or sequentially active (Devlin and Nagahama 2002).

2.3. Effect of temperature on sex-determination

The widely studied environmental factor implicated in sex- differentiation, is temperature. It may alter biochemical pathways of sexual determination and act upon an individual to induce male or female development. Temperature has been shown to influence sex differentiation in several vertebrates such as reptiles and amphibians (Ohtani *et al.*, 2000), and most thermosensitive fish species such as the European sea bass (Pavlidis *et al.*, 2000) and the blue tilapia (Baras *et al.*, 2000). In most thermosensitive fish species, the male proportion raises when temperature increases, and development of ovaries is induced by lower temperatures (Ospina-Álvarez *et al.*, 2008). Baroiller *et al.*, 1999 observed that lower temperatures were leading to a higher proportion of females only when exposed during the first 10 days post hatch (dph), while higher temperatures led to a biased sex ratio towards males when the temperature treatment were started 10 dph. The thermal sensitive period has been observed in many species to range between the middle and the late larval development stages when the gonads are still undifferentiated (Goto-Kazeto *et al.* 2006). Temperature treatment is thus not necessary beyond the critical sensitive period to affect the sex ratio in any significant way (Ospina-Álvarez *et al.*, 2008).

Pavlidis *et al.*, (2000) and Vandeputte *et al.*, (2007), studying the European sea bass, (*Dicentrarchus labrax*), a gonochoristic species with gonads remaining sexually undifferentiated until the end of the first year of life, reported that if the fish is maintained under low temperatures (13^0C or 15^0C) conditions, the sex ratio is consistently skewed in favour of females whereas high temperatures (20°C) treatment resulted in a male biased sex-ratio. Similarly in tilapia, temperature has been shown to exert a significant effect on sex ratio. In *O. mossambicus*, a species with the XY system, as well as in *O. aureus*, a species with ZW system, high temperatures during early development increase male proportion (Desprez and Melard 1998; Wang and Tsai 2000a). In *O. niloticus*, high temperatures generally have a masculinizing effect, which counteracts the genetic influence on sex determination (Baroiller *et al.*, 1999). Temperature also affects growth rate and development of fry that have temperature sex determination (TSD) (Azaza *et al.*, 2008).

In temperature sex-determination (TSD) in fish, gonadal sex is determined by temperature before the onset of gonadal differentiation (Hendry *et al.,* 2002). Brain development has been shown to influence gonadal differentiation in tilapia (Wang and Tsai, 2006). However, Sudhakumari *et al.,* (2005) showed that brain acts merely as a synchronizer in the sex differentiation process initiated by gonadal factors in the *O. niloticus*. The influence of water temperature on the development of central neurotransmitter systems differs according to the developmental period in tilapia (Wang and Tsai, 2000a). Oestrogen-forming aromatase and oestrogen-sensitive networks of neurones developing peri- and post-natally are essential in brain differentiation (Beyer, 1999). Aromatase, a key enzyme for converting androgen to oestrogen (Balthazart *et al.*, 1998), plays a role in neural differentiation and maturation in the brain and its activity and gene expression in neurones developing in the embryonic male brain is greater than in the female brain (Hutchison *et al.*, 1997).

The oestrogen-forming capacity of the male hypothalamus affects brain differentiation at specific steroid-sensitive stages in the ontogeny of mammals (Hutchison *et al.*, 1997). Oestrogen receptor concentration represents an important component of the mechanism of brain sexual differentiation (Hutchison *et al.*, 1997). To study sex-determination in teleosts, it is therefore important to understand the development of central oestrogen-forming aromatase and oestrogen-sensitive neurones, particularly throughout the sex-determining period and the influence of temperature on the expression of oestrogen receptor mRNAs in the developing tilapia brain.

In reptiles, temperature is thought to influence the activity of enzyme aromatase and the synthesis of female estradiol, as well as steroid receptors in both sexes (Pieau and Dorizzi 2004). In fish, temperature diminishes aromatase expression when fries are incubated at masculinizing temperatures (Kitano *et al.*, 2007). In teleost fish, high temperatures suppress the expression of FSH and *Foxl2* (*Foxl2* is a female specific transcription factor that belongs to a family of transcription factors named forkhead, detected only during early stages of gonadal differentiation) (Liu *et al.*, 2007). *Foxl2* possibly act by regulating the transcription gene *Cyp19* (a subset of the cytochrome P gene family that regulates the expression of cytochrome P aromatase whose function is to convert oestrogen to androgen. Thus temperature activates at least four genes: masculinizing temperatures activates genes encoding for 5α-5β reductase and androgen receptors while feminizing temperatures activates genes encoding for aromatase and oestrogen receptors (Pieau and Dorrizi, 2004).

Special proteins, the heatshock proteins (HSP), are a class of functionally related proteins whose expression is induced when cells are exposed to elevated temperatures. Heat shock proteins function as intra-cellular chaperones for other proteins through modulation of protein-

protein interactions such as folding and assisting in the establishment of proper protein conformation (shape) and prevention of unwanted protein aggregation. They also help in transportation of proteins across membranes within the cell (Dietz, 1994; Basu *et al.*, 2002; Fangue *et al.*, 2006). Temperature also has direct influence on the gene products, which are then inactivated or changed in their configuration or component and the synthesis, activation or inhibition of other enzymes or hormones (D'cotta *et al.*, 2001; Devlin and Nagahama 2002). Studies on the role of HSP in temperature dependent sex determination in the American alligator (*Alligator mississipiensis*), have identified HSP as good candidates for temperature sex determination because of their temperature sensitive expression and their ability to modify steroid receptor function (Kohno *et al.*, 2010).

The physiological, cellular and endocrine changes associated with the effect of temperature on sex determination in *O. niloticus* has been studied but the genetic basis of heat induced sex reversal still remains unknown. However there is evidence that like any other teleost, *O. niloticus* has the same temperature response (Baroiller *et al.*, 1995; Baroiller *et al.*, 2009; Angienda *et al.*, 2010). Studies to establish temperature tolerance have shown that the species *O. niloticus* is able to tolerate temperatures ranging from 8-42°C (Wooton, 1990). Temperature preference depends on size, young *O. niloticus* being more tolerant to higher and lower temperatures than adults (Wooton, 1990).

2.4. Genetic basis of sex-determination in vertebrates

The evolution of sex chromosomes can be traced in medaka fishes where the entire male determining region on Y chromosome has been characterised. The Y chromosome is only 5-10 million years old, allowing inferences to be made on the molecular events that have shaped the sex chromosome in fish (Arkhipchuk 1995; Herpin and Scharti 2009). Although genetic

factors regulate sex determination in most fishes, existing evidence show that some teleosts have karyotypically distinct sex chromosomes (Arkhipchuk, 1995; Lee and Kocher, 2007). In most species, however, the sex chromosomes are still in early stages of differentiation, and do not show distinct differences in length or gene content (Lee *et al.*, 2003). Both XY and WZ gonosomal systems have been shown to play a role in sex determination (Devlin and Nagahama, 2002). Autosomal loci also contribute to sex determination in many species (Lee *et al.*, 2003; Lee and Kocher, 2007; Ospina-A´ lvarez and Piferrer, 2008) e.g. stickleback species have unique sex chromosome system with a derived Y-autosome fussion (Ross *et al.*, 2009).

Medaka (*Oryzias latipes*), is the only fish whose genetic sex determination is known. The primary sex determining factor factor (*dmrt1bY*) has been isolated and identified as a duplicate of an autosomal gene that is known to function at downstream position of the sex-determining regulatory gene cascade. The entire male-specific region of the Y-chromosome and adjacent pseudo-autosomal regions have been characterized. Except in medaka where *DMY* (a doublesex / *mab-3* gene) equivalent to the mammalian Sry (sex determining region on Y chromosome) has been described, in no other teleosts has this been described yet (Herpin and Scharti, 2009). However a closely related gene *Dmrt1* has been associated with sex determination in several teleost species e.g. rainbow trout where it is expressed in male gonads and is involved in testicular sexual differentiation. *DMY* (an ortholog gene to *Dmrt1*) in medaka has been proposed as the sex determinig gene of this species (Matsuda *et al.*, 2002). Similarly, in tilapia, *Dmrt1* shows a specific pattern of expression in XY gonads, suggesting an important role for this gene in testicular differentiation, while in XX gonads it is not expressed (Ijiri *et al.*, 2008).

Sex chromosomes of tilapia are relatively undifferentiated and there are no gross morphological differences in any chromosome pair that would identify the sex chromosomes (Kornfield, 1984; Majumdar and McAndrew, 1986; Lee and Kocher 2007). Campos-Ramos *et al.*, (2001) using florescence in-situ hybridization technique, visualized the synaptonemal complex of *O. aureus* and observed incompletely paired segments in the longest bivalent and a smaller bivalent, which they postulated could be the sex-determining regions. An association between loci with deleterious alleles and distorted sex ratios has been reported in an inbred line of *O. aureus* (Shirak *et al.*, 2002).

Studies on gonadal differentiation during ontogenic development indicate that the general changes in developing gonads of various teleosts show similar patterns (Devlin and Nagahama, 2002; Pieau and Dorizzi, 2004; Sudhakumari *et al.*, 2005). However there are slight variations that have been observed mainly on size which is attributed to environmental differences and physiological conditions of the teleost (Mayer and Ryland 1988).

In teleost, oocyte development starts with oogonia. The oogania arise from primordial germ cell near the germinal epithelium. The maturation of oogonia involves the proliferation and progressive development of primodial germ cells. Oocyte growth occurs in two distinct phases the primary growth phase (PGP) and the secondary growth phase (SGP). The PGP is ganadotrophin independent and involves growth of primary oocyte, which is concomitant with nuclear changes. SGP is gonadotrophin dependent and involves deposition of yolk in the oocyte as it grows (Waindi, 1996).

In vertebrates in which the sex is determined by genotype of the zygote, sex chromosome plays a decisive role in inheritance and determination of sex. In the XX/XY sex determining

system, the sex is determined by a testis-determining gene on the Y-chromosome of the heterogametic male or by balance between X-chromosome and autosomes (Ohtani *et al.*, 2000). On the other hand it is presumed that the sex in the ZW/ZZ-system is determined by a putative ovary determining gene on the W-chromosome.

In young fowl, a ZZW-chromosomal set develops the right gonads into testis and the left gonad into an ovotestis (Fritzgerald and Cardona, 1993). This suggests that the sex of fowl is not necessarily determined by an ovary-determining gene.Studies have shown that the male sex of birds in the ZW/ZZ-system is determined by the effect in the disomic state of one or more masculinizing genes on the Z-chromosomes, whereas the female sex is determined by the effect of estrogen rather than a direct gene effect (Mittwoch, 1998).

Sex reversal has been shown in the frog, *Rana rogusa,* to change sex determination system from XX/XY to ZW/ZZ, with concomitant structural changes to the sex chromosome (Miura *et al.,* 1997) indicating that X chromosome has undergone intercalary and terminal pericentric inversions to give rise to two new geographic races, one with male and the other with female heterogamy.

An example of the structural change of the X-chromosome producing sex-reversed females with XY karyotype has been shown in the Wood Lemming (*Myopus schisticolor*) a species of rodent in the Cricetidae family (Fredga *et al.,* 1976). The origin of XY females is explained by loss of function mutation of the *Zfx* gene located at or proximal to a presumed breakpoint for inversion (Lau *et al.*, 1992). In *R. rogusa* the structural changes in the original X gave rise to two kinds of female with XX and XY (ZW) karyotype as reported by Miura *et al.,* (1997).

Studies involving comparison of the function of the putative ovary-determining gene and the original X, have shown that triploids with two evolved XX and ZZ grew into females, whereas all those with the original XX and ZZ grew into males (Mair *et al.*, 1991; Lau *et al.*, 1992). It has been suggested that the putative ovary-determining gene on the evolved X is functionally stronger than those on the original X (Ohtani *et al.,* 2000). In humans, it is known that XY individuals carrying duplications of Xp21 (chromosome X at position 21), referred to as the dosage-sensitive sex reversal (*DSS*) region on the X chromosome, develop as females (Bardoni *et al.*, 1994). McElreavey *et al.*, (1995) have explained that if *DSS* functions as an inhibitor to testis development, the presence of two copies of *DSS* may be sufficient to overcome the suppression by the gene *SRY*. Swain *et al.*, (1998) has shown that *Dax-1* gene in the mouse is responsible for *DSS* and that *Dax-1* and *SRY* function antagonistically to each other, with increasing expression of the *Dax-1* leading to female development and increasing activity of *SRY* to male development. They also explained that the Dax-1 gene functions more as an anti-testis gene rather than an ovary determinant. A homologue of *Dax-1* gene in *R. rogosa* would explain the coexistence of different sex determining gene.

A number of gene families that are involved in the sex determination in other vertebrate species are similar to those in fish, suggesting that sex determination pathways are highly conserved (von Hofsten and Olsson, 2005). A couple of genes involved in sex determination in mammals have also been identified in teleosts but there functions have not been completely elucidated. For instance, in mammals the expression of the gene Wt1 has been described in chick urogenital system (Miles *et al.*, 2003). In teleost fish two Wt1 genes, Wt1a and Wt1b have been identified (Klu¨ver *et al.*, 2009). The presence of the two genes have been found at high expression levels in gonads and kidney of adult zebrafish (Bollig *et al.*, 2006). In *O. niloticus*, Wt1b has been excluded as the candidate gene for sex determination (Lee and

Kocher, 2007). Another gene that has been identified to play a role in sex determination and differentiation in vertebrates and tilapia is *Sox9*. In tilapia the expression of *Sox9* gene is similar in both XX and XY gonads but positively regulated in XY gonads after sexual differentiation (Ijiri *et al.*, 2008).

Another well studied gene is *Cyp19*, whose expression has been shown in several teleost species including zebrafish, European bass and tilapia. The gne codes for the enzyme cytochrome P450 aromatase (P450arom), an important enzyme in the conversion of androgens to estrogens. The proportion of androgens and estrogens is important for sexual differentiation in many teleost species. P450 regulates the amount of estrogens (mainly 17b-estradiol) and keeps its levels within the required level for ovarian differentiation (Fenske and Segner 2004). In fish, two genes code for aromatase enzymes: *Cyp19a1* and *Cyp19a2* (Liu *et al.*, 2007). The gene *Cyp19a1* is restricted to the gonads, and is involved in sexual gonadal differentiation, while *Cyp19a2* is mainly expressed in the brain, and is indirectly involved in sexual differentiation, through the hypothalamus-hypophysis-gonad axis (Sawyer *et al.*, 2006).

2.5. Hormonal regulation of sex-determination

The sex steroid 17ß-estradiol is responsible for inducing and maintaining ovarian development and its levels is always higher in females than in males. Testicular development is mainly regulated by the androgen 11-ketotestosterone (11KT). According Bogart (1987) and Baroiller *et al.*, (1999), sex determination is based on the ratio between 11KT and 17ß-estradiol in such away that high levels of 11KT induces musculinization whereas excess of 17ß-estradiol induces feminization. This has been observed in several species of teleosts including the Eurasian perch (Rougeot *et al.*, 2007)

The balance between the two steroids is regulated by the enzyme P450arom (which converts androgens to estrogens) expressed by the gene Cyp19 (aromatase). For instance, in Zebra fish, inhibition or induction of P450arom activity during the period of gonadal development leads to a change in sexual gonadal differentiation (Fenske and Segner 2004). Studies by Sudhakumari et al., (2005) on expression of P450arom in the in brain and gonads of *O. niloticus* suggested their involvement in sex differentiation whereby expression of P450 aromatase was observed throughout in the developing testis with varied pattern and only appeared in the first few days during ovarian differentiation (Pieau and Dorrizi 2004).

The mechanism of action of steroids in gonadal differentiation in fish is mediated by binding to specific receptors (Devlin and Nagahama 2002). Androgens exert their effect by binding and activating the androgen receptor (Ar), while estrogens exert their action by means of two receptors: estrogen receptors alpha (ERα) and beta (ERβ). The levels of steroid receptors are themselves influenced by either androgens or estrogens, which affects the expression of the gene that codes for the Ar and ERs respectively. This positive control ensures that enough amounts of receptors are available to respond to the presence of steroids (Hossain *et al.*, 2008).

Sex steroids have been shown to regulate sexual differentiation at expression level of the genes involved in sexual differentiation. Both positive and negative regulation by sex steroids of the gene Cyp19 (aromatase) have been reported in fish, the regulation usually depends on the tissue (Filby *et al.*, 2007). For instance, in the rainbow trout, treatment with 17β-estradiol diminishes the expression of *Dmrt1* gene in male gonads during differentiation. This decrease indicates an indirect action of estrogens on the regulation of the expression of this gene (Marchand *et al.*, 2000).

Temperature has been shown to act in the same way as estradiol, and therefore, like estradiol, it regulates the production of steroid hormones, particularly that of aromatase enzyme as an integral part of the sex determining cascade (Pieau and Dorrizi, 2004). Steroid hormones are suspected to regulate the sex determining gene like those of Y chromosome in the early evolutionary stages of species. Pieau (1996) showed that temperature has dramatic influence on the metabolic pathway of hormones through activation of at least four genes; the gene encoding for aromatase receptors and oestrogen receptors at special female producing temperatures, and genes encoding for 5α-5β reductase receptors and androgen receptors at male producing temperatures.

Baron *et al.*, (2002) showed that it is possible to change the sex of fish by exposing them to heterologous sexual steroids during a sensitive stage of their life cycle. In fish, two distinct pituitary gonadotropins (GtH I and GtH II) have been purified and characterized from tilapia (Melamed *et al.*, 2000) and salmonids (Itoh *et al.*, 1990; Kato *et al.*, 1993; Tyler *et al.*, 1997). From their chemical characteristics, it is clear that GtH I and GtH II are related to the tetrapod gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) respectively (Tyler *et al.,* 1997). In salmonid species, it has been established that GTH I mediates vitellogenesis while GTH II regulates oocyte maturation and ovulation as does LH (Kobayashi *et al.,* 1997; Tyler *et al.,* 1997). At around 26 days after fertilization, the gonads of the juvenile Rainbow trout undergo sex differentiation and gonadotropin-releasing hormone (GnRH) neurons appear in the preoptic area of the brain and also in the pituitary (Chyb *et al.*, 1999). The GnRH stimulates synthesis and release of the pituitary LH-like gonadotropin (GtH II) as well as stimulating release of growth hormone (Melamed *et al.,* 1996).

In salmonids, the presence of an additional GtH I has been confirmed. It appears to be parallel to the mammalian FSH in both structure and function. It has also been reported to be the main stimulator of initial gonadal development, steroidogenesis, and vitellogenensis (Itoh *et al.*, 1990; Kato *et al.*, 1993; Tyler *et al.,* 1997). Gonadotrophin subunit,GtHα, has also been found in *O. niloticus* (Fan *et al.*, 2003), leading to the concept of dual GtHs in many teleosts, although a notable exception is the African catfish in which only GtHII has been found (Melamed *et al.,* 2000).

In tilapia, evidence for the presence of GtHII, with a distinct function, was provided by a study in which levels of GtH II increased in spawning *O. niloticus* around the time of actual spawning (Fan *et al.*, 2003). The gene encoding GtH I β subunit has been isolated and cloned and shown to encode homologue amino acids with those of other teleosts (Tyler et al., 1997). It has also been shown that the mRNA levels of the two GtH β subunits are altered differentially in female tilapia with varying degrees of relative gonadal size (Fan *et al.*, 2003). In vitro experiments by Melamed *et al.*, (2000) have shown that this may be due to the differential effects of gonadal steroids on the expression of these two genes.

Another special group of proteins that have been shown to play pivotal role in sexual differentiation in teleosts, are the heat shock proteins (Hsp) (Place *et al.*, 2004). Hsps are a ubiquitous and highly conserved group of proteins that have been found in nearly all organisms, including fishes (Basu *et al.*, 2002). They have special functions in adaptation of an animal to external stress, e.g. temperature (Hofman, 2005). They also play a role in proper folding, oligomerisation, compartmentalisation, translation and activation of other cellular proteins (Dietz *et al.*, 1994; Hofman, 2005).

One of the major classes of heat shock proteins induced in cells in response to thermal stress is the hsp90 family (Dietz *et al.*, 1994). Evidence suggests that hsp90 interacts with specific target proteins (e.g. kinases) and plays an important role in steroid-receptor fidelity (Pan *et al.*, 2000). Though these proteins are mainly for the adaptation of salmons to thermal stress (DuBeau *et al.*, 1998), they also have a role in the sex-determination, for example in loggerhead turtle (*Caretta caretta*). Two hsp of molecular weights 42 and 46kDa were found to be differentially expressed in the urinogenital tissue of developing females embryos reared at 32^0C but absent in the urinogenital systems of developing male embryos reared at 26^0C (Harry *et al.*, 1990). The author assumed that these proteins might mediate post transcriptional steps necessary for sexual development.

Another class of hsps is the 70kDa. These proteins have general 'housekeeping' functions within the cell, related to ensuring proper folding, transport and degradation of cellular proteins (Dietz *et al.*, 1994). In addition to their constitutive functions, hsp70 are induced when animals are exposed to temperature changes and environmental pollutants (Williams *et al.*, 1996; Vijayan *et al.*, 1997). Hsp70 bind to heat shock damaged proteins to prevent them from aggregating, and to provide them with an opportunity to re-fold to their native form when the insult has abated (Hofmann *et al.*, 2000). The proteins (hsp70s) are also considered as indicators of stress given that they are induced in fish following exposure to various stressors (Iwama *et al.*, 2004). However, lack of hsp70 response after exposure to thermal stress have been reported in Antartic fish, *Trematomus bernacchi*, (Hofmann *et al.*, 2000) and Northern Atlantic cod, *Gadus morhua,* (Zakhartsev *et al.*, 2005). The absence of an hsp response to thermal stress has also been shown in Hydra due to reduced hsp70 mRNA stability (Brennecke *et al.*, 1998) and in Antarctic notothenoid fishes due to altered expression of the inducible hsp70 gene (Place *et al.*, 2004).

2.6. The role of simple sequence repeat (SSR) in sex determination and population genetics

Microsatellite or simple sequence repeats represent a unique type of tandemly repeated genomic sequences, which are abundantly distributed across genomes and demonstrate high levels of allele polymorphism (Chistiakov *et al.*, 2006). Microsatellites can be found anywhere in the genome, both in protein-coding and noncoding DNA (Chistiakov *et al.*, 2006). Di- and tetranucleotide motifs are mostly clustered in noncoding regions (Toth *et al.*, 2000). In contrast to other types of repeat motifs, triplets are found in both coding and non-coding genomic regions with a high frequency (Morgante *et al.*, 2002). SSR contribute to DNA structure, chromatin organization, regulation of DNA recombination, transcription and translation, gene expression and cell cycle dynamics (Chistiakov *et al.*, 2006).

SSRs located in promoter regions do influence gene expression level. Loci containing tandem repeats within either a coding sequence or a promoter are hypermutable. Altered numbers of repeats thus cause switches in the reading frame of translation or changes in the level of promoter activity, while SSR situated in the 3′-UTR (untranslated region) affect gene expression through their influence on the stability of transcribed products. This role was found for a GA (guanine – adenine) rich repetitive DNA segment in the 3′-UTR of the rat polymeric immunoglobulin receptor gene (Fabregat *et al.*, 2001). SSR variation produces either drastic or quantitative variations in gene expression. Because of genomic overabundance and high mutability of SSRs (Chistiakov *et al.*, 2006), changes in SSR array size may serve as a rich source of variation in fitness-related traits in natural populations (Streelman and Kocher, 2002). Its role is especially important for population survival and adaptation of tilapia to varying environmental conditions (Blankenship *et al.*, 2002).

Following the identification of microsatellite-containing short sub-sequence of a transcribed spliced protein-coding nucleotide sequence known as expressed sequence tags (ESTs), it is possible to predict the functions of the sequences. In order to predict the putative functions of SSR-containing genes, the sequences are compared to the database of amino acid sequences in the public domain. UniProt/Swiss-Prot, a central database of protein sequence and function, is an annotated protein sequence database which is helpful for predictiting the amino acids and proteins coded from nucleotide base sequences.

A number of studies have found associations of DNA markers with sex in tilapia species and their hybrids; e.g. two recent studies mapped the location of 13 genes known to be involved in the sex determination pathway in tilapia (Cnaani *et al.*, 2004). Although some of these genes are linked to sex associated markers (Shirak *et al.*, 2002), they are excluded as major sex-determining genes, since sex-determination can not be fully ascribed to these genes (Shirak *et al.*, 2006; Lee and Kocher, 2007). Although some genes have been described as sex-related, Herpin and Scharti (2009) working on Medaka fish to determine the mechanism of sex determination and the evolution of Y chromosome, showed that *MRT1bY* can be considered as a master sex determination gene though non has been utilized in aquaculture.

Cnaani *et al.*, (2008) identified DNA markers linked to sex determining genes in six closely related species of tilapiine fishes. In two of the species, *Oreochromis karongae* and *Tilapia mariae,* the sex-determining locus was found on linkage group (LG) 3 with females being heterogametic (WZ-ZZ system), whereas in *O. niloticus* and *Tilapia zillii* the sex-determining locus was on LG1 with males being heterogametic (XX-XY system).

Studies on the expression patterns of various transcription factors along the Hypothalamus pituitary gonadal axis (HPG) may elucidate the mechanism of sex determination in the *O. niloticus*. For example, ontogenic expression patterns of nuclear receptors and cytochrome P450 aromatases in brain and gonads of the *O. niloticus* suggest their involvement in sex differentiation (Sudhakumari *et al.*, 2005). Expression of P450 aromatase was observed throughout in the developing testis with varied pattern while in developing ovary it was evident till 15 days post hatch and reappeared only after 90 days post hatch. According to Sudhakumari *et al.*, (2005), this observation suggested that brain acts merely as a synchronizer in the sex differentiation process initiated by gonadal cues/factors in the *O. niloticus*.

Gene expression studies have also shown that simple sequence repeats (SSR) located in promoter regions can influence gene expression (Mansour *et al.*, 1998). These SSRs can affect the sequence of the binding site and influence the affinity for the binding of the corresponding regulatory transcription factor. The CT-elements lying close to the transcription start point of these genes have been shown to be the target sequence for binding transcription factors (Edwards *et al.*, 1998). Such an effect has been described for a dinucleotide (CA/GT)n microsatellite in the *O. niloticus* prolactin 1 (PRL1) promoter (Streelman and Kocher, 2002). Transcribed microsatellites located in 5′ untranslated regions can form specific and unusual DNA structures. In this case, the length of the repeat region can affect the translation level from the target mRNA. The role of SSR or expressed sequence on sex determination in fish tag has not been elucidated.

Microsatellites are highly variable and considered to be selectively neutral, allowing for the assumption that the estimated amount of sequence divergence between units of interest is

directly proportional to the length of time since separation. They are co-dominant, inherited in a Mendelian fashion and tandem arrays of very short repeating motifs of 2-8 DNA bases that can be repeated up to ~100 times at a locus (Wright and Bentzen 1994). They are among the fastest evolving genetic markers, with 10-3-10-4 mutations/generation (Goldstein *et al.*, 1995). Their high polymorphism, and PCR based analysis has made them one of the most popular genetic markers (Wright and Bentzen 1994).

With current molecular methods it is possible to score microsatellite length polymorphisms in large numbers of individuals for genetic analyses within and between populations. Some microsatellite loci have very high numbers of alleles per locus (>20) (Tessiere *et al.*, 1997; Hansen *et al.*, 2001), making them very useful for applications such as parent-offspring identification in mixed populations, while others have lower numbers of alleles and may be more suited for population genetics and phylogeny (O'Connell and Wright, 1997; Estoup and Angers, 1998). Primers developed for one species will often cross-amplify microsatellite loci in closely related species (Estoup and Angers, 1998; Sanetra *et al.*, 2009). In the present study primers developed for the Cichlid *Astotilapia burtoni* (Abur) easily amplified loci from those of related species. For instance the published primers Abur36 and Abur100 from *A. burtoni* cross-amplified *O. niloticus* microsatellite loci (Renn *et al.*, 2004; Sanetra *et al.*, 2009), since the two species are related and belong to the same family, and primers developed for rainbow trout have successfully been used on salmonids (Morris *et al.*, 1996).

Microsatellite markers have a number of advantages over other molecular markers such as allozymes and mitochondria DNA markers. Microsatellite loci are typically short, easy to isolate and amplify by PCR and can be highly polymorphic. Sample DNA can be isolated quickly because labour-intensive phenol-chloroform steps can generally be eliminated in

favour of a simpler form of DNA extraction such as the sodium precipitation extraction method. The high variability or polymorphism observed in microsatellites is quite advantageous in studies such as population genetics and can also be used to differentiate between sexes. Another advantage of using microsatellites in genetic studies is that only small amounts of tissues are required for typing microsatellites and these markers can be assayed using non-lethal tissues such as fin clips and archived scale samples. This might facilitate retrospective analysis of depleted population where individuals in such populations are to be conserved and are therefore suitable for genetic analysis of endangered species. For applications where a large number of loci are required, such as genome mapping or identification of Quantitative Trait Loci (QTL), microsatellites offer a powerful alternative to marker systems such as RAPD (Jackson *et al.*, 1998).

There are two main techniques for microsatellite analysis. The first one requires probing complete digests of nuclear DNA with simple sequence repeats (di-, tri-, or tetra- nucleotide repeats). Alternatively, they are genotyped by the PCR, using primers targeted to the unique sequences flanking the microsatellite motif according to the methods of Ziegle *et al.*, (1992). The resulting PCR products are separated according to size by gel electrophoresis using either agarose gels or more commonly (higher resolution) denaturing SDS polyacrylamide gels. This amplification presents a significant advantage over other non-PCR based methods because it allows the use of relatively small amounts of tissue, including that from preserved otoliths, scales, larvae, and small fry. Despite the advantages of microsatellite markers they are not without constraints. One of the main problems is the presence of "null alleles" (Jarne and Lagoda, 1996). Null alleles occur when mutations take place in the primer binding regions of the microsatellite locus, not in the microsatellite DNA itself. The presence of null alleles at a

locus causes scoring problems, in particular in individual based analyses such as relatedness estimation and assignment tests (Hansen, 2003).

An important disadvantage of microsatellite alleles is that amplification of an allele by PCR often generates bands of 1 to 2 base pairs apart known as stutter or shadow bands that is common in dinucleotide microsatellites. They appear as a result of incomplete denaturation of the amplified products or strand impairing during amplification. However, trinucleotide and tetranucleotide microsatellite typically exhibit little or no stuttering.

Nuclear DNA exhibits the greatest variability of all genetic markers related to fisheries science. Main applications in fisheries and aquaculture are in phylogenetics and phylogeography (DeWoody and Avise, 2000; Hansen *et al.,* 2003), population genetic structure (Shaklee and Bentzen 1998; Abila *et al.*, 2004; Angienda *et al.*, 2011) and conservation of biodiversity (Reilly *et al.,* 1999). Application of microsatellite markers are also in hybridization and stocking impacts (Hansen *et al.,* 2001; Ruzzante *et al.,* 2001) and inbreeding (Tessier *et al.*, 1997). Microsatellites have also become popular in genome mapping, gene flow and effective population size analysis (Hallerman, 2003; Withler *et al.*, 2004).

2.7. Mitochondria DNA

Mitochondria genome of vertebrates is a single small, double stranded circular DNA molecule of 1.6kb contained in the mitochondria. Up to several thousand copies of the DNA are found per cell compared to only two copies in most nuclear DNA. Mitochondria of vertebrates contain 13 genes coding for proteins. The protein coding genes are six sub-units of NADH dehydrogenase (ND 1, 2, 3, 4L, 5 and 6), cytochrome b, three sub-units of cytochrome c

oxidase (CO, I, II, III) and two sub-units of ATP synthetase (ATPase 6 and 8). It also contains two genes coding for ribosomal RNAs (small 12s and large 16S rRNA), 22 genes coding for tRNAs and one major non-coding control region (the D-loop) (Brown, 1981). The D-loop is a 1.2kb that contains the initiation sites for mtDNA replication and RNA transcription. Based on the distribution of the variable nucleotide positions and differential nucleotide frequencies in the different parts of the control region, it can be divided into three domains. Domain I and III are more variable while domain II is more conserved. The advantages of the use of mtDNA over nucleus DNA in phylogenetics is that; first, mitochondria is maternally inherited. All mtDNA is clonally inherited from the mother. Mitochondria are located in the cytoplasm and only the egg contributes cytoplasm to the zygote. Such maternally derived molecules do not recombine genetically in progeny. Therefore, unlike nuclear DNA which gets reconstituted in each generation during meiosis, the only alterations to mtDNA can only be an accidental change caused by mutation, copying errors or other accidents. Secondly, mtDNA especially the D-loop region has high rate of evolutionary change due to high synonymous substitution rate of the control region (Sturmbauer and Meyer, 1992). The basis of the higher rate of mtDNA evolution is due to transitional differences (Brown, 1981). In closely related cichlids, substitution outnumbers deletion and addition (Sturmbauer and Meyer, 1992). Because mutations accumulate fastest here (Meyer *et al.*, 1990), the control region is the molecule of choice for the study of population genetics and phylogenetic relationships in recently radiated species like the tilapiines.

2.8. Effect of introducing O. niloticus and other fishes in Lake Victoria

The introduction of the *O. niloticus* and Nile perch into the Lake Victoria region have altered the original ecosystem and consequently led to the extinction of hundreds of indigenous Lake Victoria fish species. For instance *O. esculentus* (Singida tilapia) and *O. variabilis* have virtually disappeared from Lake Victoria ((Lowe-McConnell 2000; Balirwa *et al.*, 2003). The

disappearance of *O. esculentus* from Lake Victoria and its major decline throughout the region has led to its classification as a 'critically endangered' species in the IUCN red list (Twongo *et al.*, 2006).

The aquatic ecosystem of L. Victoria has been greatly modified by overfishing, increased human activity in the drainage basin, and the introduction of exotic species. The exotic species rapidly displaced many of the indigenous haplochromine cichlids from the lake, driving hundreds of the species to extinction. In addition to the Haplochromine cichlids, two endemic Tilapiine species, *Oreochromis variabilis* and *O. esculentus*, were extirpated from L. Victoria, and their only remaining natural refugia are satellite lakes near L. Victoria (Ogutu-Ohwayo, 1990). In a majority of satellite lakes, the remaining populations of the endemic L. Victoria tilapiines are threatened due to occurrence of exotic tilapiines.

Certain ecological, genetic and physiological characteristics make *O. niloticus* a successful invader. They are omnivorous, grow faster and larger than native tilapias and have the ability to exclude native fish from feeding and breeding grounds (Lowe-McConnell 2000). They also easily hybridize with the native species (Canonico *et al.*, 2005). As with other cichlidae, tilapiines have undergone a recent evolutionary radiation. The Recent or incomplete speciation processes allow them to hybridize readily (Wohlfarth and Hulata 1983; Trewavas 1983). Moreover, hybridization in the natural environment is poorly documented; it has been recorded for the sympatric *O. niloticus* and *O. aureus* in Western Africa, in Mozambique where escapee *O. niloticus* from aquaculture farms hybridised with Mozambique tilapia (D' Amato *et al.*, 2007) and a case of extinction by hybridization in Lake Victoria where *O. variabilis* was out-competed and disappeared after introduction of Nile and Mozambique tilapia.

Such introgression has been shown to hasten the decline in genetic diversity of the native tilapia species (Dowling 1992) and led to a reduction in the fitness of native species (Rhymer and Simberloff 1996; Fitzsimmons 2001). A recent study of native westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) in the upper Flathead River system, Montana, USA and British Columbia, Canada, revealed that even small degrees of hybridization (e.g. 20%) with introduced rainbow trout (*Oncorhynchus mykiss*) markedly reduced fitness of males and females of the native species, with reproductive success sharply declining (Muhlfeld *et al.*, 2009). However studies on the genetic isolation between populations of sculpins within natural hybrid zones that formed between one invading species and two separate populations of residence species showed that selection forces promoted introgression of about 30% of the loci (Nolte *et al.*, 2009). This implied that hybridization increased the fitness of the admixed individuals.

The introduction of Nile perch also contributed significantly to the disappearance of native species. In the 1980's the native *Oreochromis* species of Lake Victoria experienced unprecedented rate of extinction from the adverse effects of the exotic Nile perch. The loss was attributed predation (Ogutu – Ohwayo, 1990) that mainly led to the quick disappearance of the native *Oreochromis* species. Despite the threats of the introduced *O. niloticus*, remnant populations of the native tilapia species *O. esculentus* and *O. variabilis* still persist in peripheral water bodies such as small lakes and dam reservoirs in the Lake Victoria catchment (Loiselle 1996; Aloo 2003; Angienda *et al.*, 2011).

Lake Victoria satellite lakes were formed through series of drying and refilling processes that characterized the geology of Lake Victoria (Kaufman 1992; Johnson *et al.*, 1996). The three satellite lakes located within the Yala Swamp in Western Kenya are Kanyaboli, Sare and

Namboyo. Lake Kanyaboli is approximately 10.5 km^2 with an average depth of 2.5 m and a maximum depth of 4.5 m). It is the largest and most remote from Lake Victoria (Crafter *et al.* 1992). It is separated from Lake Victoria by extensive papyrus swamps. It has an average dissolved oxygen level of 7.3 mg O_2 l^{-1} (Aloo 2003). Native *O. esculentus* coexists with introduced *O. niloticus* in this lake and population size of *O. esculentus* was found to be high (an average catch per canoe of 26 kg; Opiyo 1994). No records of Nile perch have been documented in Lake Kanyaboli.

Lake Sare is continuous with Lake Victoria and discharges its water directly into the lake. It is about 5 km^2 in area, 5 m deep at its centre and has an average dissolved oxygen level of 8.1 mg O_2 l^{-1}. *O. esculentus* no longer exist in this lake probably due to predation pressure by Nile perch, which is established there (Aloo 2003).

Lake Namboyo is a small lake of about 0.01 km^2 with an average depth of about 17 m, and has an average dissolved oxygen level of 4.8 mg O_2 l^{-1}. *O. esculentus* coexists with *O. niloticus* in the lake. Nile perch does not exist in this lake (Aloo, 2003).

The Yala swamp is Kenya's largest freshwater wetland and covers about 175 km^2 along the north-western shores of Lake Victoria. About 23 Km^2 has so far been converted for commercial agriculture purposes. The swamp is bounded by Hwiro and Nzoia River to the North and Yala River to the south. It is separated from L. Victoria by a sand bar through which the Yala River cuts in many delta outflows into the Lake. It is a valley swamp formed in Pleistocene by water level changes and river flow reversal that led to deposition of silts, infilling and colonization by rooted plants. The common rooted plants of the swamp are *Cyperus papyrus* and *Phragmites mauritianus* (Aloo, 2003). It is host to a number of

indigenous fish species e.g. *Clarias gariepinus*, *Protopterus aethiopicus*, *Labeo victorianus* and Barbus spp. The dissolved oxygen levels are very low, about 4 mg O_2 l^{-1} (Aloo, 2003).

These satellite lakes play a crucial role in the conservation and future survival of the native *O. esculentus* and *O. variabilis*, since they function as refugia (Ogutu-Ohwayo 1990; Kaufman and Ochumba 1993; Maithya 1998; Mwanja *et al.* 2001). A recent study of six haplochromine cichlids of Lake Kanyaboli indicated that the region may provide an opportunity for the conservation of endangered species and genetic diversity that is threatened by both exotics and anthropogenic factors affecting Lake Victoria haplochromines (Abila *et al.*, 2004, 2008).

2.9. Genetic structure of O. niloticus in Lake Victoria

Few studies have previously been carried out to determine the genetic structure of the *O. niloticus* within L. Victoria basin. Agnèse *et al.*, (1999) resolved the genetic structure of *O. esculentus* by comparing its genetic composition with that of *O. niloticus* based on three microsatellite markers and 24 allozymes and suggested purity of *O. esculentus.* However studies by Mwanja *et al.*, (2008) revealed a slight level of genetic admixture between the two species within Lake Kanyaboli based on random applified DNA polymorphism (RAPD).

Knowledge of the genetic make up of the *O. niloticus* will be crucial for the future development of sustainable aquacultural strains, conservation, and management of this important L. Victoria genetic resource. Identification of diverse populations may provide knowledge on genetic diversity, novel genetic, physiological, behavioural and other characteristics that are associated with differences in life-history traits such as growth rates, fecundity, abundance and disease resistance (Bert 1993; Stepien 1995). Such traits could be exploited to develop suitable aquaculture strains of this native species through marker assisted

selection (MAS). The characterization of genetic variation within and among populations is important for the interpretation, understanding and effective management of fish species. Resolving population structure, detecting hybridization, defining management units and identifying invasive species has been identified as the key elements of conservation genetics (DeSalle and Amato 2004). To secure the future of *O. niloticus* as an excellent fishery species, it will be important to determine whether *O. niloticus* has hybridized with the native *O. esculentus* species. This would permit identification of genetically pure and distinct populations of *O. niloticus* that can be used to develop genetically robust strains. Genetic diversity among the populations of *O. niloticus* can be exploited through MAS to develop and identify unique aquaculture strains.

It is therefore important to explore the extent of genetic variation and gene introgression between *O. niloticus* and *O. esculentus* found in the Lake Victoria region (L. Victoria, satellite lakes: Sare, Namboyo and Kanyaboli). Studies on gene differentiation and gene flow may reveal how genetic diversity between Lake Victoria and satellite lakes compare. Population genetic theory would predict that since only fewer individuals were introduced (small founder effect) into the satellite lakes, a population bottleneck was introduced in the process leading to a loss of genetic diversity in the satellite lakes.

The future of tilapia stock improvement will rely on appropriate stock choice, development of sound management techniques and selective breeding. The basis of this approach is the ability to characterize and monitor tilapia genetic resources both in their natural environment and under culture, provide a sound knowledge of the genetic characteristics of each stock and to examine the effects of management practices on the gene pools of each stock.

CHAPTER THREE

MATERIALS AND METHODS

3.0. Sampling sites

The study was carried out in the Nyanza Gulf, the eastern most portion of Lake Victoria. The gulf has an area of approximately 1920 km^2 and a total length of approximately 60 km, and is shallow, with a mean depth of only 6 m. The Yala swamp lies immediately to the east of the Gulf, ($0^0$00'S - $0^0$30'S; $34^0$30'E - $35^0$15'E, 1134 m above sea level) and is bordered to the north by the Nzoia River and to the south by the Yala River (Fig. 1). Three main lakes are found in the Yala wetlands: Kanyaboli (00°04'30"N; 34°09'36"E), Sare (00°02'36"S; 34°03'32"E) and Namboyo (00°00'23"N; 34°05'09"E) located between lakes kanyaboli and Sare.

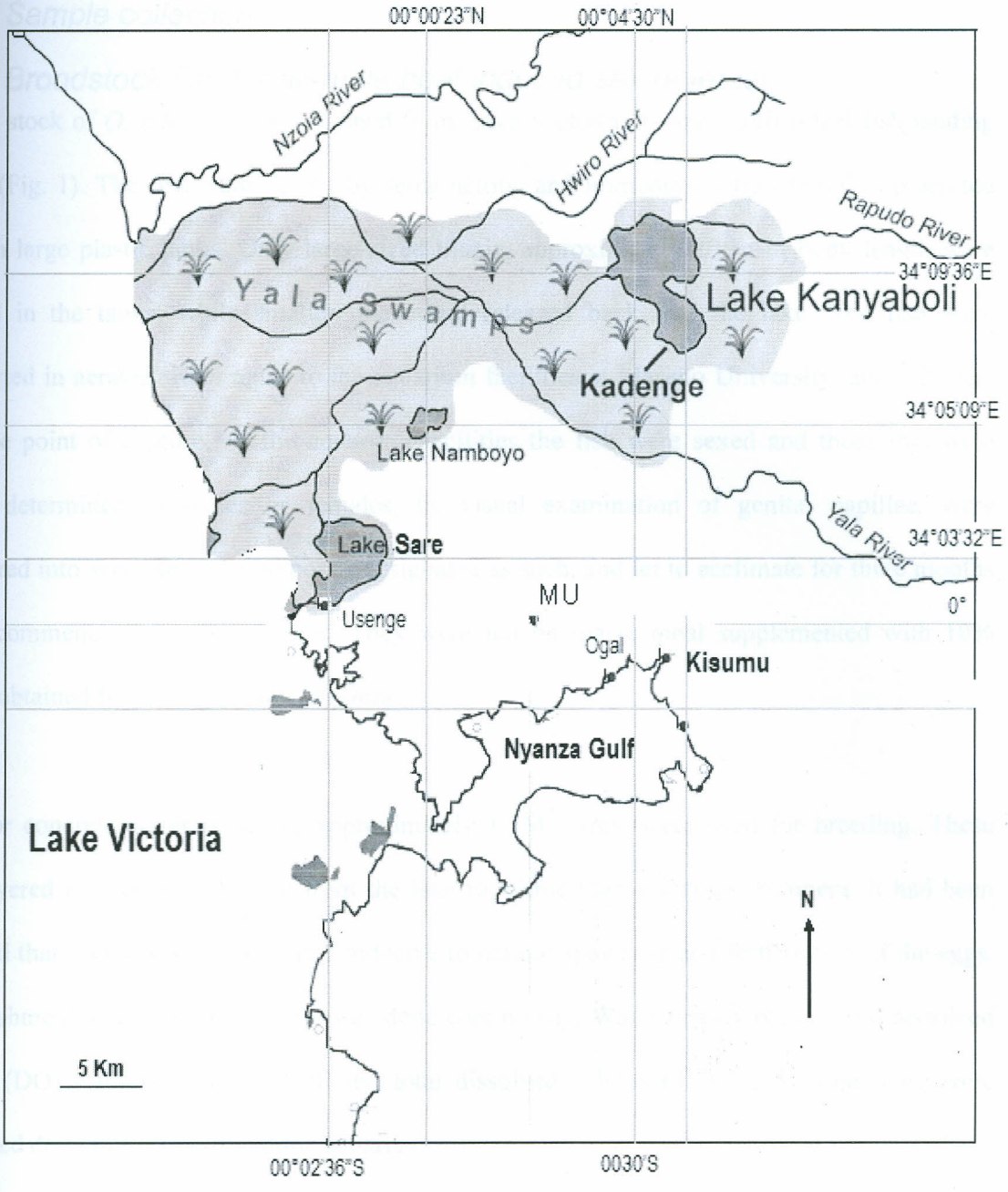


Fig. 1. Map of Lake Victoria showing the position of the Nyanza Gulf and the Yala Swamp within which are the satellite lakes Kanyaboli, Sare and Namboyo (Re-drawn from Crafter *et al*., 1992).

3.1.0. Sample collection:

3.1.1. Broodstock fish for all-male heat induced sex reversal

A fresh stock of *O. niloticus* was obtained from Lake Victoria, Nyanza Gulf (Ogal fish landing beach) (Fig. 1). The fish were caught by seine netting and immediately transferred into aerated water in large plastic tanks. Only large sized tilapia, approximately 20cm in body length were retained in the tanks, while smaller ones were released back into the lake. The fish were transported in aerated water tanks to the aquarium facilities at Maseno University, about 20 Km from the point of capture. At the aquarium facilities the fish were sexed and those that were clearly determined as males or females, by visual examination of genital papillae, were transferred into separate concrete ponds designated as such, and let to acclimate for three months before commencing the experiments. They were fed on maize meal supplemented with 10% protein obtained from *Rastniobola argentae*.

Out-door concrete water ponds of approximately $1.5M^3$ sizes, were used for breeding. These were layered at bottom with sand from the lake to mimic the natural environment. It had been observed that such a system is more conducive to natural spawning and fertilization of the eggs. Replenishment and aeration of water was done continously. Water quality parameters: dissolved oxygen (DO), salinity, pH, conductivity, total dissolved solutes (TDS) and temperature were monitored daily in the ponds and the aquaria.

Sexually mature males and females were stocked in the ratio of two females to one male and let to reproduce freely. Hatched embryos at yolk sac stage were collected by scooping from the bottom of the tanks using plastic cups, pooled into one lot and transferred into indoor glass aquaria ($1.5M^3$). They were observed daily for yolk absorption. One day after yolk absorption was considered as day one post yolk sac stage of development and light feeding on juvenile feeds was also commenced at this time.

3.1.2. Fishes for genetic variability studies

Adult fish samples (minimum body length of both species: 15 cm) of *O. niloticus* and *O. esculentus* we collected by gill netting. *O. niloticus* was collected from Lake Victoria and the three satellite lakes. *Oreochromis esculentus* samples were only collected from two satellite lakes, Kanyaboli and Namboyo. Thirty individuals of each species were collected from each lake. The collected specimens comprised of approximately equal ratio of male and female for both species at each sampling site (*O. niloticus*: Lake Victoria—male:female = 13:17, Lake Kanyaboli—16:14, Lake Namboyo—15:15, Lake Sare—12:18; *O. esculentus*: Lake Kanyaboli—15:15, Lake Namboyo—14:16). Sexing was conducted by visual inspection of the genital papillae area of every sampled fish in the field. The fishes were fully developed enough to sex them precisely. The two species were readily identified in the field based on morphology: *O. esculentus* are distinguished from *O. niloticus* by being smaller in body size with small heads and whitish coloured ventrally and reddish coloured dorsally (Fig. 2). It is almost impossible to identify hybrids between the species based on morphology (Lowe-McConnell 2000). Fin clips were obtained for genetic analysis and immediately preserved in eppendorf vials containing 95 % ethanol.

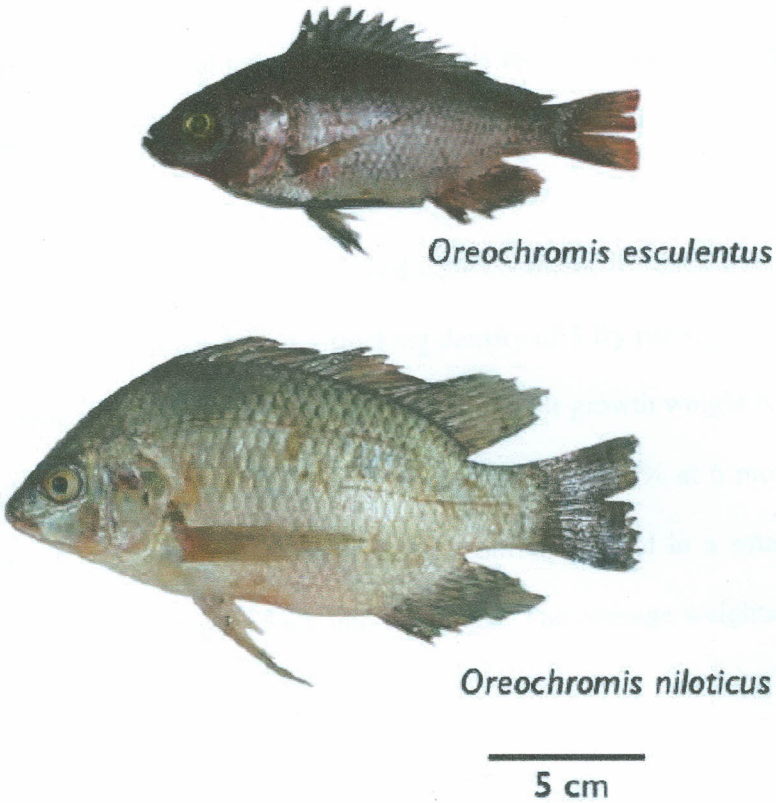


Fig. 2. Photographs of *O. niloticus* and *O. esculentus* shows the differences in shape, colour and size of the two study species.

3.2. Heat treatment for sex reversal

Glass aquaria measuring 1.5 M^3 were used for heat treatments. These were fitted with constant temperature thermostat heaters, aerator pumps, sand-fine gravel filters systems and mercury thermometers. The aquaria were filled with equal volumes (40 litres) of water and thermostats set at respective temperatures: 26°C (control), 28°C, 30°C 32°C, 34°C, 36°C and 38°C. The temperatures were allowed to stabilize for several days before introducing the fry into the aquaria. One hundred fry at 10 days post yolk sac were introduced into each aquarium for the various treatment temperatures. Heat treatments were conducted for 10 days after which the thermostats were switched off and the aquaria allowed to cool down to room temperature. During heat treatments, the fry were observed daily and any deaths recorded. The final numbers of the fry in each aquarium were taken and recorded for at least 24 hrs (during which the system was

adjusting to normal room temperature) post heat treatment. These were used to calculate survival rates in order to determine the optimal temperature that gave the maximum sex reversal towards males with optimal survival rates.

At 15 days post heat treatment, the fry that survived heat treatment for each temperature were transferred into separate fry holding ponds at a stocking density of 5 fry per sq. metre where they continued to receive graded feed portions commensurate with their growth weight regimens, from a maximum of 5% per kg body weight at 1 month to a minimum of 3% at 6 months. At two monthly intervals samples of about 10 fry were drawn from each pond in a small quantity of water and quickly weighed and returned back into the ponds. The average weights were used to determine the feed portions.

3.3. Sexing the fingerlings

Few samples were obtained at 4 months post heat treatment for sexing and at 6 months all samples where obtained and sexing done by both squash and histological examination of the gonads. Gonad quashes were fixed and stained in aceto-orcein fix stain (Waindi, 1994) and histological preparations were fixed in Bouin's fixative and stained with eoisin-haematoxylin. Male individuals were identified by the presence of developing seminiferous tubules and spermatocyes, while females were identified by the presence of oocytes (Fig. 3). The numbers of males and females were recorded for each temperature treatment. Sex ratios were calculated as percentages of the numbers of male or female fry sexed as such.

3.4. Genomic DNA extraction by NaCl precipitation

Approximately $2mm^3$ fish fin tissue preserved in 90% ethanol was used as the source of genomic DNA. Genomic DNA was extracted by proteinase K (10 mg/ml) digestion followed by NaCl precipitation using the method of Bruford *et al.*, (1998).

The tissue was placed in an eppendorf tube containing 270µl extraction buffer, 80µl 10% sodiumdodecylsulfate (SDS) and 20µl 1% protenase K and incubated at 56^0C overnight. The extract was then centrifuged for 5minutes at 13000 rpm and the supernatant transferred into a fresh eppendorf tube. 180µl 5M NaCl was added to the supernatant and mixed gently. The sample was afterwards centrifuged at 13000 rpm for 5 minutes and the supernatant transferred into another eppendorf tube. 420µl ice cold isopropanol was then added to the supernatant mixed gently and centrifuged for 5 minutes at 13000 rpm and the residues discarded. Washing of the pellete obtained at the bottom of the eppendorf tube with 250µl 70% ethanol followed by vortexing and centrifuging at 13000 rpm for 5 minutes. After washing, the pellete was left to dry for 15-20 minutes in the water bath. The dry pellete (DNA) was finally eluted with double distilled water and stored at -20^0C.

3.5.1. DNA amplification via Polymerase Chain Reaction (PCR)

PCR amplification was performed in a reaction volume of 20 µl, which comprised 1X PCR buffer, 25 µM of each dNTP, 0.5 µM of each of the forward and reverse primers, 0.1 U *Taq* polymerase (Genaxxon) and 100–200 ng of DNA template (Table 1). The following thermal conditions were used: an initial denaturation phase at 94^0C for 5 min followed by 35 cycles with a denaturation phase at 94^0C for 30 s, an annealing phase at 49–58^0C for 30 s, an extension phase at 72^0C for 90 s, followed by a final extension phase at 72^0C for 10 min in Perkin Elmer GeneAmp PCR 9700 (Norwalk, CT).

Amplification reactions for microsatellite and mitochondria DNA were carried out using the published primers listed in Table 2.

Table 1. PCR master mix for one DNA amplification reaction

Reagent	**Volume (µl)**	**Quantity**
Double distilled water	12.5	
10X PCR buffer	2	
dNTPs	2	25 µM
Primer (forward)	1	0.5 µM
Primer (reverse)	1	0.5 µM
Taq Polymerase DNA	0.5	0.1 U
Template DNA	1	100 ng
Total	20.0	

Table 2 Primers sequence for PCR amplification of microsatellite and mitochondria DNA (obtained from Sanetra *et al.*, 2009)

Sex linked microsatellite primers	***Sequence* (5' – 3')**
Abur36	Fwd TCTCTGAAGGTGAGCCACAG
	Rvs ACGCTGCCCCTCTTCATC
Abur100	Fwd CATTCCAATCCCCTGTGC
	Rvs CTGCTCCAACTCTCCTGTCC
UNH846	Fwd CACAAAGATGTCTAAACATGT
	Rvs GAATTTGACAGTTTGTTGT
Non-sex linked microsatellite primers	
Abur30	Fwd GGTGAGCTGCATACCACAA
	Rvs GTCAGCTGTGAGCGTCTGG
Abur51	Fwd GGATGGCATGGACTCAGAAG
	Rvs TTTTCACTTTCCCCATCTCAC
Abur110	Fwd CGAAGGGAGATGATAGGGAAG
	Rvs AGTAGTACTTGCACAGAAGGAG
Abur28	Fwd CGAAGCGCTTTAGTGGTTTC
	Rvs CATCGCTCAGCTTTCTCCTC
Abur41	Fwd TAAGTTTCTGGGCCTTGCTG
	Rvs TGAAGGAAAGAAGCCCAAGC
Abur18	Fwd CACGTGACGTCCTGATGAAC
	Rvs GCCTACCTGTGGGATACAGC
Abur25	Fwd CGAACCTCAAACGTCCTCT
	Rvs CTGCTCTGATGGAGTCCAAG
Abur4	Fwd TTAAGGCCACCGTAGATTCC
	Rvs. CTGGTAGCCTGGAGGTCAGC
Mitochondria DNA primer	
L-Pro (Forward)	CCTGAAGTAGGAACCAGATG
12s5R (Reverse)	GGCGGATACTTGCATGT

3.5.2. Gel Electrophoresis

The PCR amplification products were loaded onto ethidium stained 1% agarose gel and electrophoresed at 76 volts for 15 -20 minutes. The DNA was visualised by running the gel through GelDoc UV illuminator (BioRad^{R}). Presence of bands on the gel indicated successful amplification of the DNA.

3.5.3. Genotyping microsatellite DNA

Sex reversed *O. niloticus* were tested using three sex linked microsatellite loci that were amplified with the primer; Abur100, Abur36, and UNH846, while genetic variability of the species was tested using eight polymorphic microsatellite loci from eight independent chromosomes and amplified with the primers: Abur30, Abur51, Abur110, Abur28, Abur4, Abur18, Abur25 and Abur41. The forward primers were labeled with a fluorescent dye (6-FAM, HEX or NED). Reactions were carried out in 20µL volumes which comprised 1X PCR buffer, 25 µM of each dNTP, 0.5 µM of each of the forward and reverse primers, 0.1U *Taq* polymerase (Genaxxon) and 100-200 ng of DNA using the same PCR conditions as for mtDNA amplification (annealing temperature of 55^{0}C). PCR products were diluted in formamide HiDi and electrophoresed in an ABI 3130xl automated sequencer. Fragment sizes were compared to ROX 500bp size standard (ABI) as determined using GENOTYPER® software (Applied Biosystems).

3.5.4. Microsatellite and mitochondria DNA purification

After size confirmation by gel electrophoresis, enzymatic pre-sequencing treatment of PCR products was done for all PCR products that showed single sharp bands. The total volume of the reaction was 5 µL, which comprises 0.8 U/µL of Exonuclease I (Fermentas) and 0.16 U/µL FastAP (Shrimp Alkaline Phosphatase, Fermentas).

3.5.5. PCR cycle sequencing reaction

Upon DNA purification, 1 µl of the purified PCR product was used as template in the cycle sequence reaction. The reaction mixture comprised 1.0µl of forward primer, 1.5µl Terminator Reaction Mix (Big Dye) and 6.5µl double distilled water. The PCR cycle reaction conditions were as follows: denaturation step at 94^0C for 4 minutes, 35 cycles of 55^0C annealing temperature for 15 seconds and an extension temperature of 60^0C for 4 minute. The product was then stored at 4^0C.

3.5.6. Sequencing (Sex linked microsatellite) and mitochondria DNA

Sex-linked genes were amplified using primers Abur100, Abur36 (the Abur primers were homologous to the loci in the linkage group 1 (LG1) of the *O. niloticus* and UNH846. Approximately 850 bp of mitochondrial DNA control region was amplified using primers L-Pro-F (Meyer *et al.* 1994) and 12S5R (5' GGC GGA TAC TTG CAT GT 3'). PCR amplification was performed in a reaction volume of 20.0 µL, which comprises 1X PCR buffer, 25 µM of each dNTP, 0.5 µM of each of the forward and reverse primers, 0.1U *Taq* polymerase (Genaxxon) and 100-200 ng of DNA template, under the following thermal conditions: an initial denaturation phase at 94°C for 5 min followed by 35 cycles with a denaturation phase at 94 °C for 30 s, an annealing phase at 55 °C for 30 s, an extension phase at 72 °C for 90 s and a final extension phase at 72 °C for 10 min in Perkin Elmer GeneAmp PCR 9700 (Norwalk, CT). After size confirmation by gel electrophoresis (Fig 15 and Fig. 16), amplified PCR products were purified enzymatically with Exonuclease I (Fermentas) and FastAP (Shrimp Alkaline Phosphatase, Fermentas), following manufacturer's protocol. The purified gene fragments were subjected to direct sequencing in the forward and reverse directions using the same forward and reverse primers as in the PCR and the BigDye Terminator 3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems). All reactions for DNA sequencing were run on an ABI 3130xl automated DNA sequencer (Applied

Biosystems). Forward and reverse sequences were assembled in SEQUENCHER version 4.2.2., edited using CHROMAS ver. 2.0 computer software and aligned in CLUSTAL W ver. 1.83 (Fig. 6) (Thompson *et al.*, 1994).

3.6. Statistical analysis

3.6.1. Sex reversal and survival

STATA statistical analysis tool was used for analysis of variance (ANOVA) between treatment temperature and the resultant mean of sex reversed male individuals ($P<0.05$) and to determine the correlation between the survival rates and treatment temperatures. Correlation analyses between temperature and survival were done at significance level of $P<0.01$. G test was used for analysis of the genetic proportions of sex linked markers in male and female individuals.

3.6.2. Genetic variability of *O. niloticus*

3.6.3. Mitochondrial DNA control region

The number of polymorphic sites, number of mtDNA haplotypes, haplotype diversity (h) and nucleotide diversity (π) were calculated for each population as well as for the entire pooled population of each species using ARLEQUIN 3.01 (Excoffier *et al.* 2005). TCS 1.21 (Clement *et al.* 2000), which utilizes the statistical parsimony method described in Templeton *et al.* (1992), was used to construct the haplotype network to investigate the phylogenetic relationships among the mtDNA haplotypes of each species. Deletion mutations were treated as a fifth state because they were observed consistently in seven different haplotypes. Ambiguous connections in the haplotype network generated by TCS were resolved following the criteria outlined in Crandall and Templeton (1993).

To examine intra- and inter-specific genetic differentiation between populations, exact tests for population differentiation (Raymond and Rousset 1995) as well as calculation of pair-wise estimates of F_{ST} (Weir and Cockerham 1984) were carried out using ARLEQUIN. The 95% significant levels for pair-wise intra- and inter-specific population comparisons were conservatively adjusted using a Bonferroni correction (Bland and Altman, 1995)

3.6.4. Microsatellites

Allele frequencies for each locus and population were calculated after analysis with MICRO-CHECKER (van Oosterhout *et al.* 2004) was performed to check against errors due to null alleles, drop out and stutter using the Brookfield (1996) equation at 95% confidence level. The number of alleles per locus (N_a), observed (H_0) and the expected heterozygosity (H_E), multilocus tests for Hardy-Weinberg Equilibrium (HWE), F_{IS} estimates, tests for linkage disequilibrium among pairs of loci and intra- and inter-specific population genetic differentiation were performed using GENEPOP ver. 4 (Rousset 2008). Genetic variation was analyzed in two dimensional individual based factorial correspondence analyses (FCA) in GENETIX (Belkhir *et al.* 1996). Genetic admixture between the *O. niloticus* and *O. esculentus* at microsatellite loci was assessed using an individual-based Bayesian cluster approach as implemented in Structure 2.1 under a model of admixed ancestry among populations and correlated allele frequencies (Pritchard *et al.* 2000; Falush *et al.* 2003). MCMC was run for 100 000 generations to estimate Q. A 90% probability interval was used for individual admixture values. Analysis was run eight independent times to check for convergence on similar values. The eight runs arrived at almost identical values of ln (P |D) and Q ($\pm \leq 0.001$).

CHAPTER FOUR

RESULTS

4.4.1. Sex reversal

The effects of heat treatments at various temperatures on sex ratios of the fingerlings indicated that the sensitive temperature that significantly skewed the sex ratio of *O. niloticus* in favour of male individuals was 36^0C ($P < 0.05$), providing the most optimal results of 86.31% males. However there was no significant difference in the sex ratio of both male and female individuals between the controls reared at 26^0C and heat treated individuals at temperatures ranging from 28 to 34^0C, indicating that sex reversal is triggered within a narrow limit of 36 ± 1^0C (Fig.4).

4.4.2. Survival

Contrary to treatment temperature where sex ratio was skewed at a specific temperature, survival rates showed a negative correlation (-0.963) with higher temperatures being unfavourable to the survival of the fry. The maximum temperatures that provide for optimization of the survival (at the optimal sex reversal in favour of males) was 36 ± 1^0C providing a survival rate of 65.25% of the fry. Increasing treatment temperatures to 38 ± 1^0C provided a dismal survival rate of 19 ± 0.2 (Fig. 5).

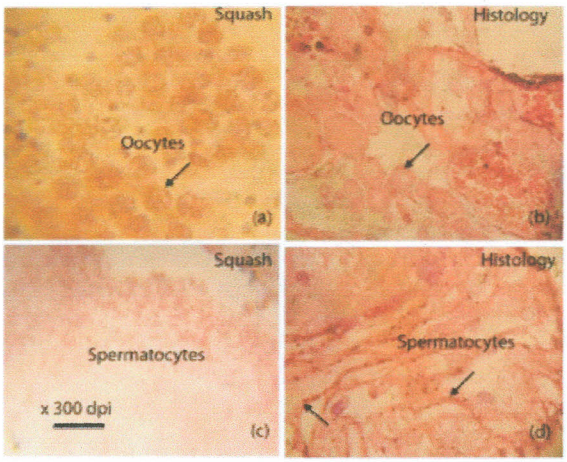


Fig. 3. Micrograph of *O. niloticus* fingerling gonads squash (a) and histology preparations (b) showing the evidence of ovarian development with primary stage oocytes (a–arrows) and secondary stage (b–arrows) oocytes, and evidence of testis with spermatocytes and seminiferous tubules (d–arrows) and clusters of spermatocyes (c)

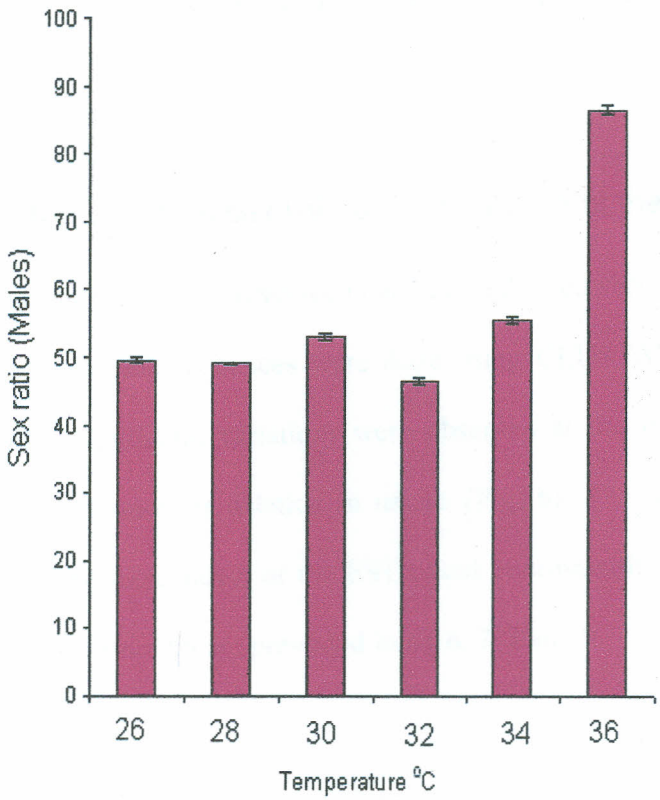


Fig. 4. Sex ratio of *O. niloticus* fingerlings following treatment of the fry at the indicated temperatures, 26^{0}C being control.

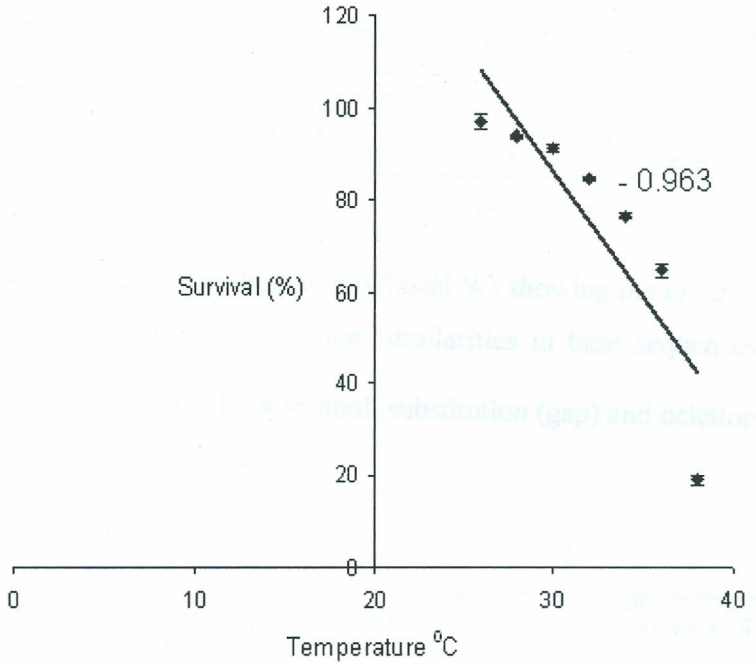


Fig. 5. Survival ratio of *O. niloticus* fingerlings at indicated heat treatment temperatures, showing a negative correlation (-0.963) between the treatment temperature and the resultant survival rates.

4.4 3. Effect of heat treatment on sex linked microsatellite at the nucleotide base level

The nucleotide base sequence of the sex reversed *O. niloticus* showed variation from controls. Alignment of the generated base sequences were done using CLUSTAL W version 1.83 (Thompson *et al.*, 1994) Significant variations were observed at the locus, Abur36, The variations were either substitution or deletion in nature (Fig. 6). The interruptions of the repeat sequences (di and tri nucleotides) of the EST based microsatellites of the sex linked microsatellites Abur 36 and Abur100 are presented in fig 6, 7, 8 and 9.

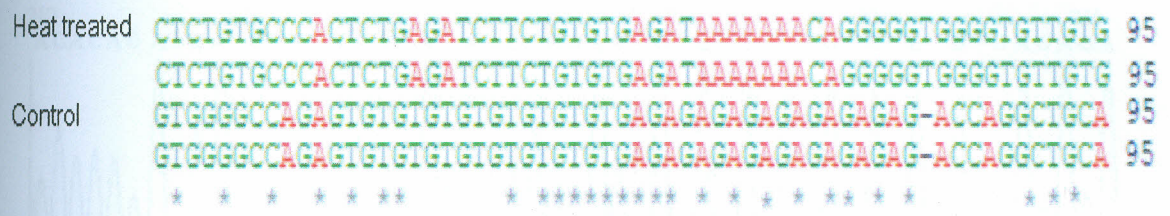


Fig. 6. Microsattelite sequence alignment (Clustal W) showing the effect of heat treatment on the sex-linked gene in *O. niloticus* where similarities in base sequences between the heat treated and control are denoted by ∗ symbol, substitution (gap) and deletion mutations (-).

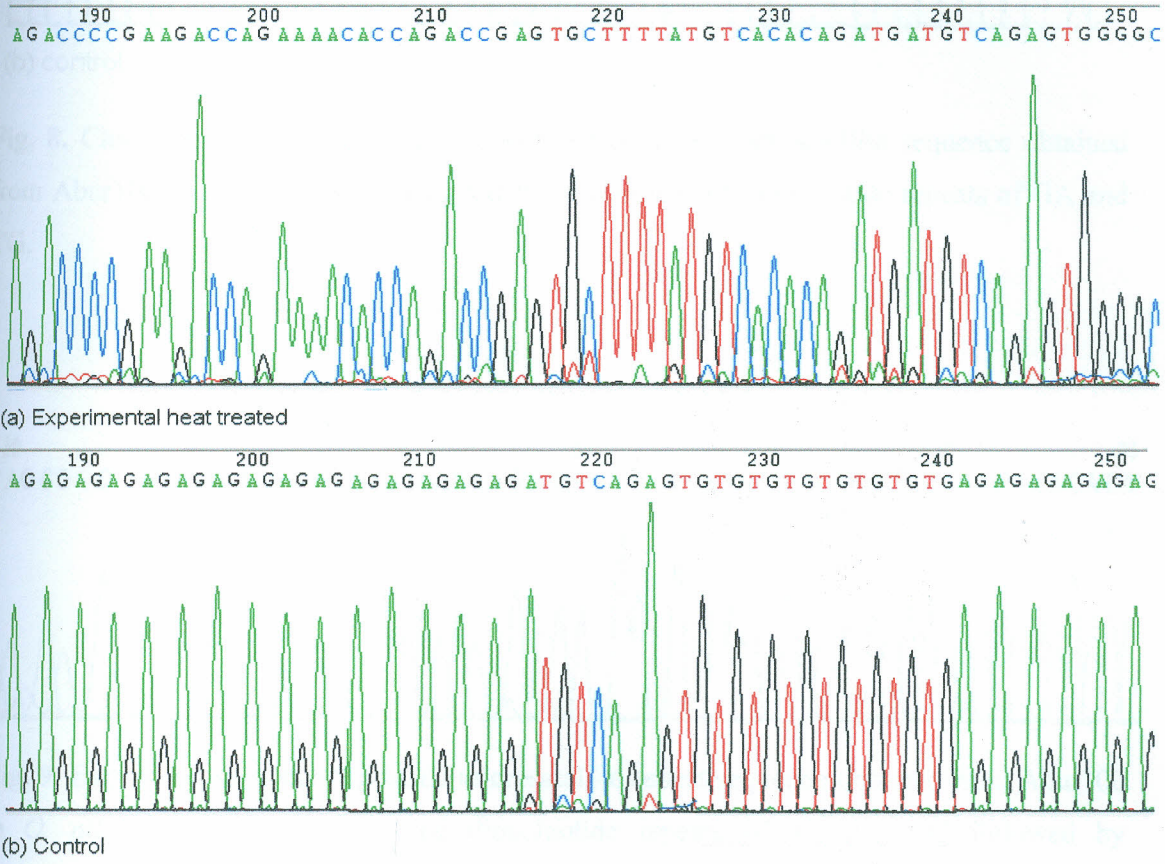


Fig. 7. Chromatograph of heat treated *O. niloticus* sequences showing highly interrupted expression sequence tag based microsatellite (a) and repeat sequence of AG and TG (b) of control obtained from Abur36.

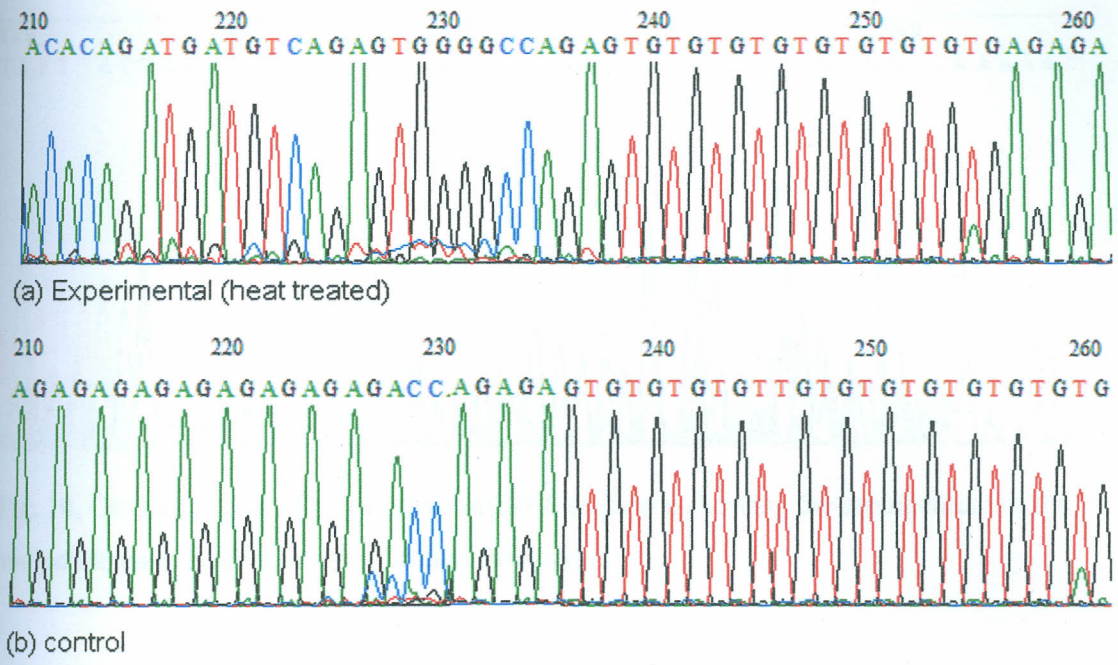


Fig. 8. Chromatograph of expression sequence tag based microsatellite sequence obtained from Abur100 in *O. niloticus* showing slight interruptions of dinucleotide repeats of GA and TG.

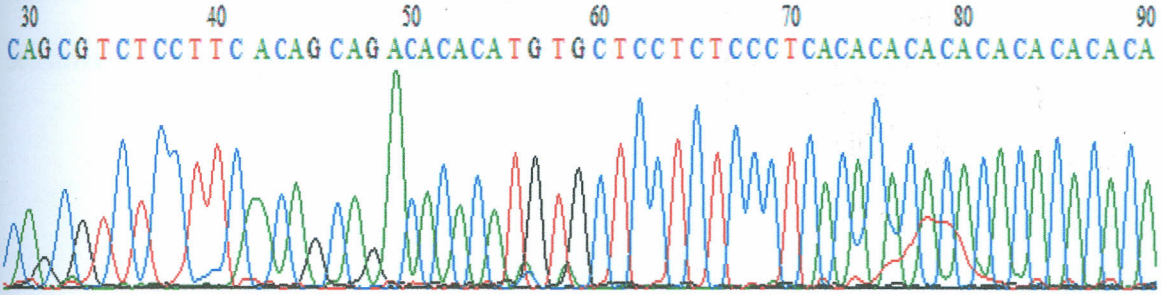


Fig. 9. Expression sequence tag based microsatellite repeat sequence obtained from Abur100 in *O. niloticus* showing interrupted dinucluotide repeats of TC and TG followed by continueous repeats of CA.

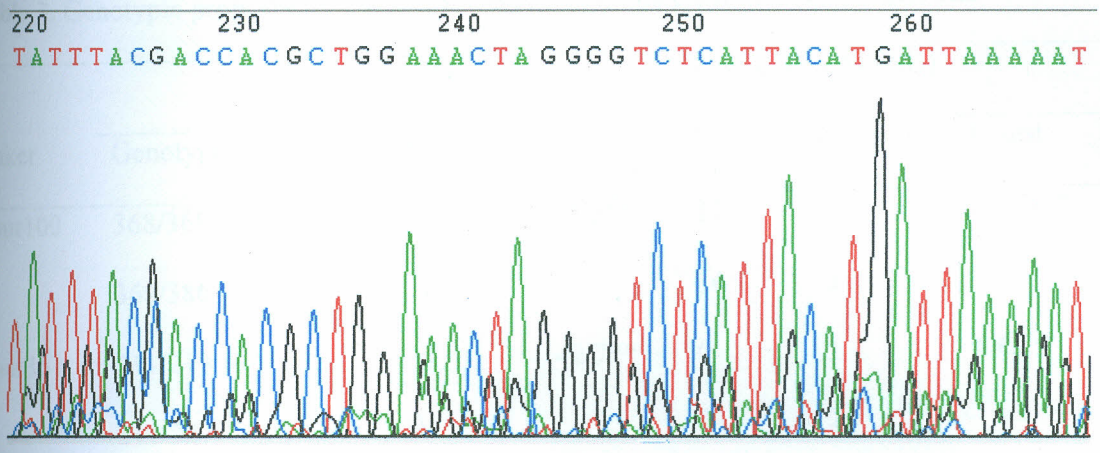


Fig. 10. Chromatogram of *O. niloticus* mtDNA sequence showing the arrangement of the nucleotide base sequences constituting a haplotype.

4.4.4. Genetic proportion of sex linked genes

The genetic proportions of sex linked genes are presented in Table 3. Following G-test, Abur36 predicted 95% phenotypic sex of the sex reversed *O. niloticus* individuals at $P<0.05$ significance level following Bonferroni correction (Bland and Altman, 1995). This result indicated that this locus (Abur36) is highly influenced by temperature during temperature sensitive sex differentiation periods.

Table 3. Genotypic proportion of sex linked markers in male and female *O. niloticus.*

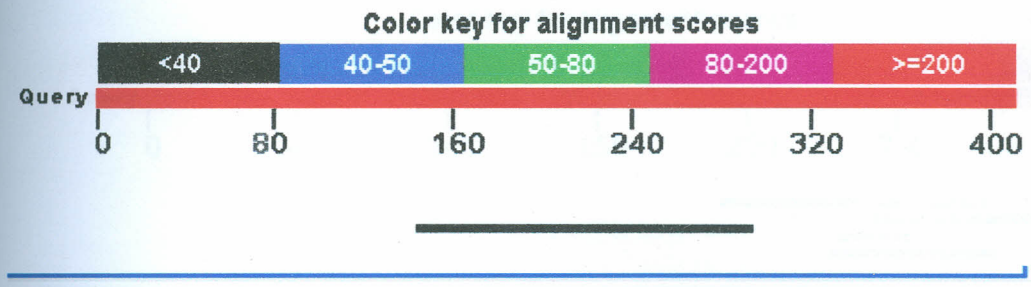
		Control (GSD)			Sex-reversed (TSD)		
Maker	Genotype	Male	Female	G-test	Male	Female	G-test
Abur100	368/368	2	6	15.3^{NS}	11	5	34.45**
	368/386	9	16		14	3	
Abur36	211/213	5	12	42.7**	0	1	68.23*
	211/227	11	0		21	2	
	227/227	6	1		9	0	
UNH846	179/209	2	7	20.15^{NS}	4	6	23.11^{NS}
	179/205	9	14		16	0	

*$P < 0.05$, **$P < 0.01$, NS (Not significant)

GSD (genetic sex determination); TSD (temperature sex determination)

4.4.5. Prediction of the putative function of the sex linked EST based microsatellite

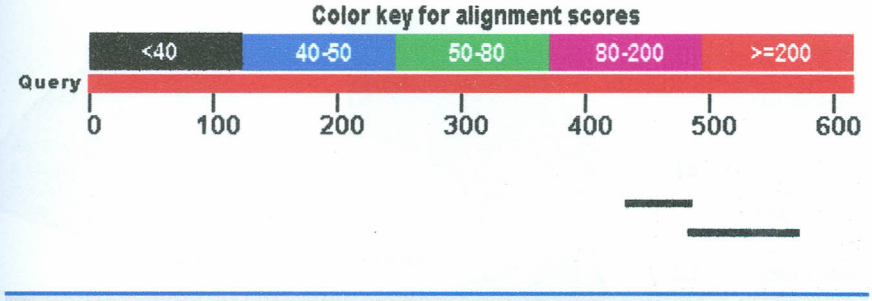
The putative functions of the expression sequence tag (EST) based microsatellites (Abur36) that predicted 95% sex of sex reversed *O. niloticus* , were predicted by comparing the sequencence to the public protein database. The outputs of these predictions are presented in Fig. 11, 12 and 13. The predicted protein functions were male biased hormones (Fig. 12) and transcription factors (For example androgen receptor β, SRY transcriptional facor, DM related transcriptional factor DMRTb2) suggesting that the locus Abur36 is activated or altered in a way that it skews sex ratio in favour of male individuals. The predicted transcription factors including cytochrome P450 (Fig. 13) a novel type of P450c17 that has been shown to be responsible for C21-steroid biosynthesis in the fish ovary and head kidney, Zhou *et al.*, 2007, are summarised in Fig. 14.



Sequences producing significant alignments:

Accession	Description	Total score	Query coverage	E value
BAB20082.1	androgen receptor beta *[Oreochromis niloticus]*	21.2	21%	3.1

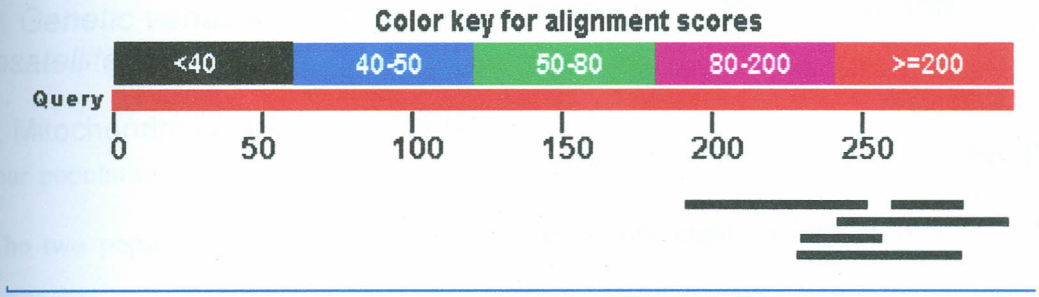
Fig. 11. Distribution of one Blast Hits of sequence generated from the locus Abur36 of a heat treated *O. niloticus* individual predicting the presence of androgen receptor β. A main factor involved in sex determination.



Sequences producing significant alignments:

Accession	Description	Total score	Query coverage	E value
AAO74158.2	DM-related transcriptional factor Dmrt2b *[Oreochromis niloticus]*	21.2	8%	5.5
ACJ03597.1	heat shock protein 70 *[Oreochromis niloticus]*	20.4	14%	9.3

Fig. 12 Distribution of two Blast Hits of sequence generated from the locus Abur36 of the heat treated *O. niloticus* individual predicting the involvement of Hsp 70 and DM-related transcription factor.



Sequences producing significant alignments:		Score (Bits)	E Value
gb|ABM22397.1|	brain super conserved receptor 2 [*Oreochromis*. ...	20.4	2.8
dbj|BAB16107.1|	gonadotropin receptor II [*Oreochromis niloticus*]	20.0	3.6
gb|ACM45609.1|	hormone-sensitive lipase [*Oreochromis niloticus*]	19.2	6.2
dbj|BAF75924.1|	cytochrome P450 C-17 [*Oreochromis niloticus*]	19.2	6.2
gb|ABG11743.1|	SRY-box containing transcription factor 5 [*Ore*...	18.9	8.1

Fig. 13. Distribution of five Blast Hits of sequence generated from the locus Abur36 of the heat treated *O. niloticus* individuals.

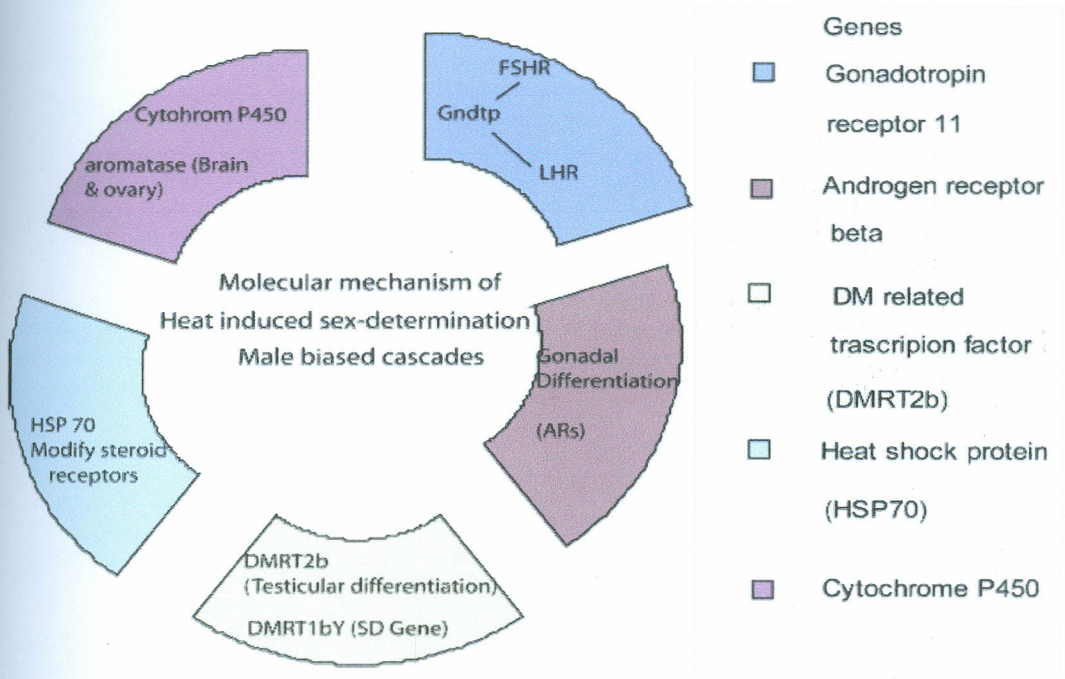


Fig. 14. Predicted protein function of the simple sequence repeat from the EST based Abur36 locus in the sex reversed *O. niloticus* individuals.

4.5.0. Genetic variability of O. niloticus based on mitochondria and microsatellite DNA

4.5.1. Mitochondrial DNA control region

The four populations of *O. niloticus* contained 37 different mtDNA-haplotypes (900bp, Fig 15). The two populations of *O. esculentus* contained only eight haplotypes (Fig. 17). No mtDNA haplotypes were shared between the two different species. The haplotype networks of *O. Niloticus* and *O. esculentus* can be connected by six mutational steps (*O. niloticus*-H1 to *O. esculentus*-H5). Every population of both species had unique haplotypes geographically restricted to particular lakes (Fig. 17, 18 and 19). The most common *O. niloticus* haplotype (*O. niloticus*-H1) was only present in the satellite lakes.

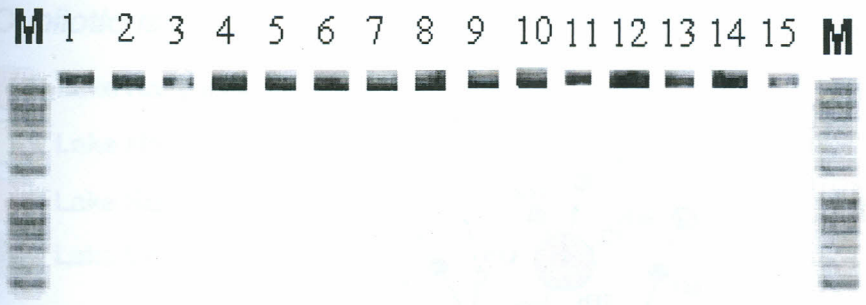


Fig. 15. Micrograph showing sharp mtDNA bands (1 – 15) on agarose gel stained with ethidium bromide and run alongside a 1Kb molecular marker (M).

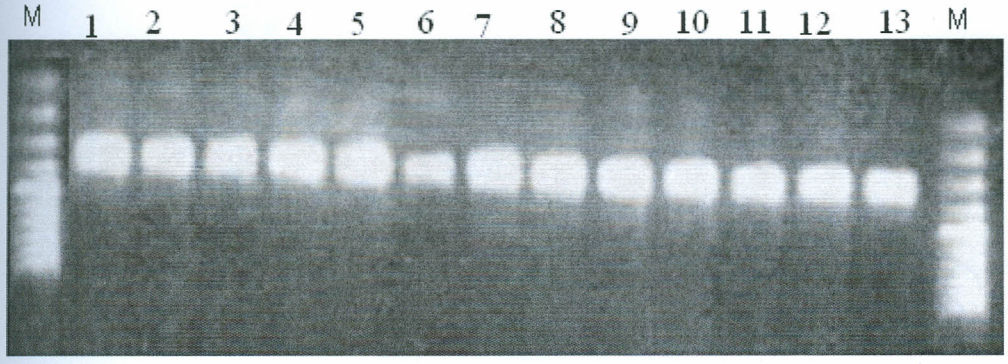


Fig. 16. Micrograph showing sharp microsatellite DNA bands (1 – 13) of *O. niloticus* samples on agarose gel stained with ethidium bromide and run alongside a 1Kb molecular marker (M)

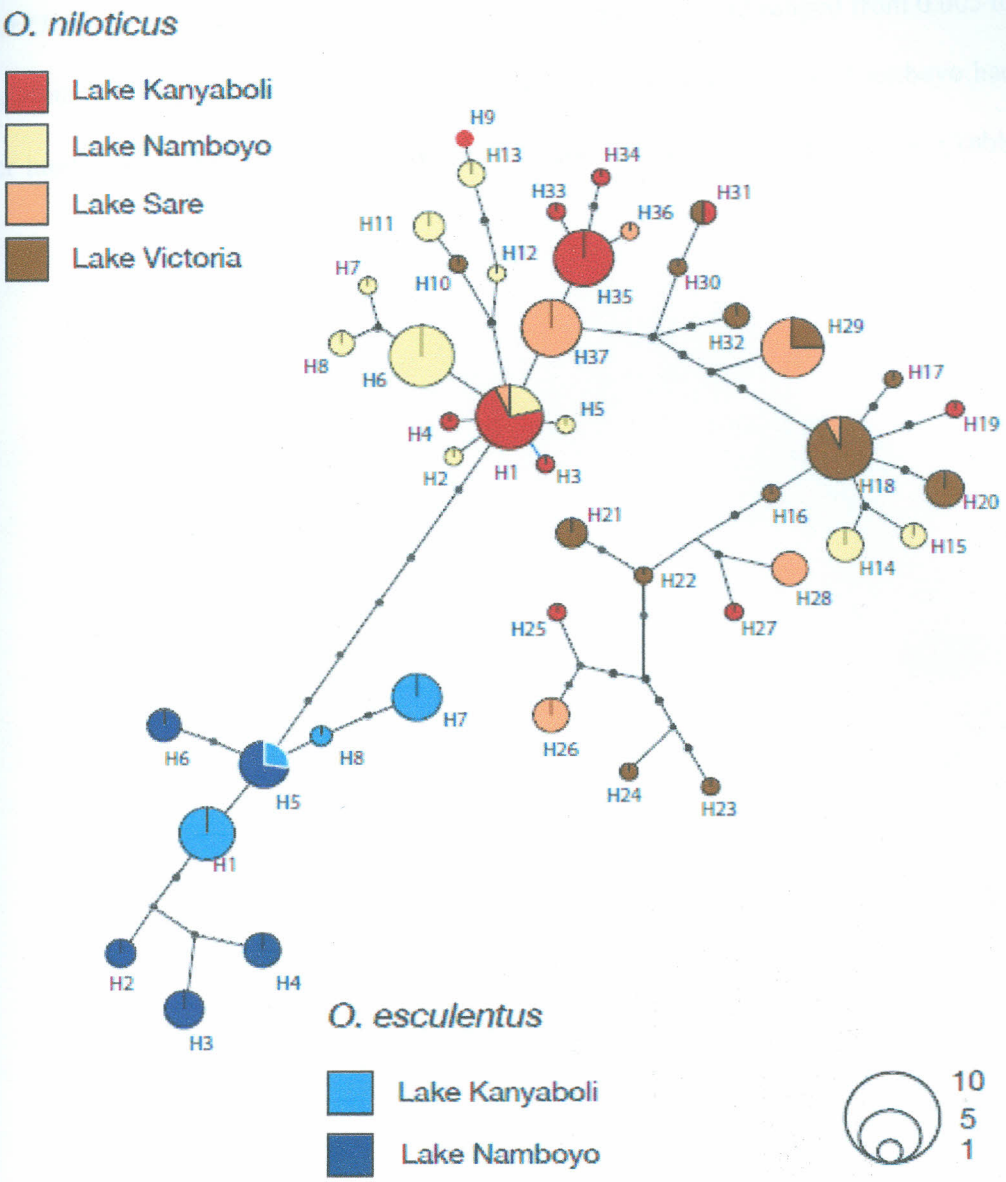


Fig. 17. Haplotype networks of *O. niloticus* and *O. esculentus* based on the mtDNA control region (850 bp).

Each node in the network represents a single mutational step between haplotypes. Small, circles indicate intermediate haplotypes that are not present in the samples, but are inferred mutations in these networks.

Haplotype diversity for *O. niloticus* was highest in Lake Victoria (0.829) but only slightly lower in the three satellite lakes (0.770 to 0.800) (Table 4 and Appendix 2). Nucleotide

diversity for *O. niloticus* was 0.008 for the Lake Victoria population and ranged from 0.005 to 0.012 in satellite lake populations. In the two *O. esculentus* populations, Lake Namboyo had both higher haplotype (0.816) and nucleotide (0.004) diversity than Lake Kanyaboli (Table 4).

Table 4. Summary of the level of genetic diversity in four *O. niloticus* and two *O. esculentus* populations at both mtDNA control region and eight microsatellite loci.

	Species	*O. niloticus*				*O. esculentus*	
	Lake	L. Victoria	L. Namboyo	L. Kanyaboli	L. Sare	L. Kanyaboli	L. Namboyo
mtDNA	N	30	30	30	30	30	30
	Number of haplotypes	13	11	11	7	4	5
	No. of polymorphic sites	27	24	30	35	4	7
	Haplotype diversity (*h*)	0.829	0.8	0.77	0.77	0.655	0.816
	Nucleotide diversity (π)	0.008	0.005	0.006	0.012	0.002	0.004
Microsatellites	N	24	24	24	24	24	24
	N_a	7.625 (± 2.134)	7 (± 3.505)	8.375 (± 2.875)	7.25 (±1.982)	8.125 (± 4.155)	7.125 (± 3.137)
	H-W tests (*P*)	0.386 (± 0.022)	0.368 (± 0.024)	**0.000 (± 0.000)**	**0.000 (±0.000)**	0.476(± 0.029)	0.305 (± 0.020)
	H_E	0.733 (± 0.116)	0.713 (± 0.133)	0.788 (± 0.077)	0.734 (±0.135)	0.771(± 0.129)	0.745 (± 0.104)
	H_O	0.768 (± 0.138)	0.783 (± 0.146)	0.726 (± 0.145)	0.697 (±0.114)	0.810 (± 0.209)	0.795 (± 0.110)
	F_{IS}	-0.0504	-0.1012	0.0810	0.0497	-0.0514	-0.0720

N: sample sizes; N_a: observed mean number of alleles across eight loci; H_E: mean expected heterozygosity and H_O: mean observed heterozygosity. F_{IS}: inbreeding coefficient. Bold means significant departure from HWE

Population differentiation was highly significant between all pairs of populations within and between species (Table 5). F_{ST} values for intra-specific comparisons among the four *O. niloticus* populations ranged from 0.164 to 0.221 and 0.244 for between the two *O. esculentus* populations. F_{ST} values for inter-specific population comparisons ranged from 0.177 to 0.287 (Table 5).

Table 5. Population differentiation between four populations of *Oreochromis niloticus* and two populations of *O. esculentus* (grey region) from the mtDNA control region as well as eight microsatellite loci.

	Victoria (*O. n*)	Namboyo (*O. n*)	Kanyaboli (*O. n*)	Sare (*O. n*)	Kanyaboli (*O. e*)	L. Namboyo (*O. e*)
Victoria (*O. n*)	-	0.051	0.069	0.05	0.147	0.174
Namboyo (*O. n*)	0.185	-	0.02	0.03	0.137	0.163
Kanyaboli (*O. n*)	0.199	0.188	-	0.034	0.122	0.142
Sare (*O. n*)	0.164	0.212	0.221	-	0.151	0.165
Kanyaboli (*O. e*)	0.257	0.272	0.287	0.287	-	0.057
Namboyo (*O. e*)	0.177	0.192	0.207	0.207	0.244	-

The pairwise F_{ST} values between populations were calculated according to Weir & Cockerham (1984). Numbers above the diagonal represent F_{ST} values based on microsatellites, and numbers below the diagonal represent F_{ST} values based on mtDNA control region. The pairwise comparisons from mtDNA and microstellites were all significant ($P < 0.001$) after a Bonferroni correction.

4.5.2. Microsatellite loci

All populations of both species were in Hardy-Weinberg equilibrium except for two populations of *O. niloticus* (Table 4), which may be non-panmictic. The inbreeding coefficient (F_{IS}) ranged from -0.0504 to 0.0810 across all populations. No evidence for linkage disequilibrium between pairs of loci was found in the populations, suggesting that all loci can be treated as independent markers.

The number of alleles per locus ranged from 4 to 15 within populations of *O. niloticus* and 6 to 13 within populations of *O. esculentus*. For *O. niloticus*, Lake Namboyo has the highest mean observed heterozygosity (H_o) (0.783 ± 0.146) while for the *O. esculentus*, Lake Kanyaboli has higher H_o than Lake Namboyo (0.81 ± 0.209) (Table 4). Similar to the results of the mtDNA control region, all populations of both species had private alleles. The highest numbers of private alleles were observed in Lake Namboyo for *O. niloticus* (12) while for the *O. esculentus*, Lake Kanyaboli has a higher number of private alleles (5) than Lake Namboyo (2) (Figure. 18 and Appendix 3). Exact tests showed highly significant population genetic differentiation within and between the species (Table 5).

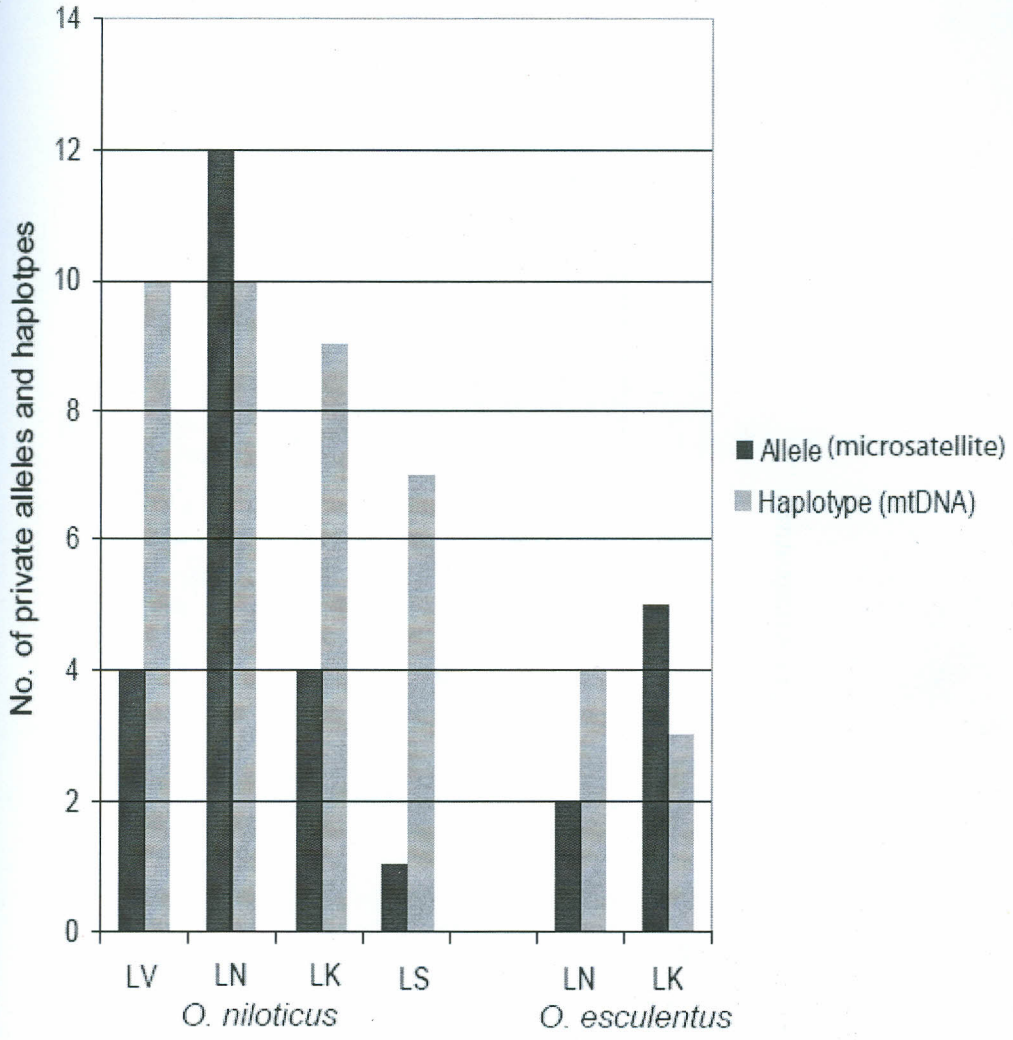


Fig. 18. Number of unique microsatellite alleles and mtDNA haplotpyes of *O. niloticus* and *O. esculentus* present in Lake Victoria (LV) and three satellite lakes Kanyaboli (LK), Sare (LS) and Namboyo (LN).

Factorial correspondence analysis (FCA) of eight microsatellite genotypes revealed two distinct genetic groups separated along the *x*-axis (6.83%), representing the two species. The y-axis (3.15%) separates individual microsatellite genotypes within populations (Fig. 19).

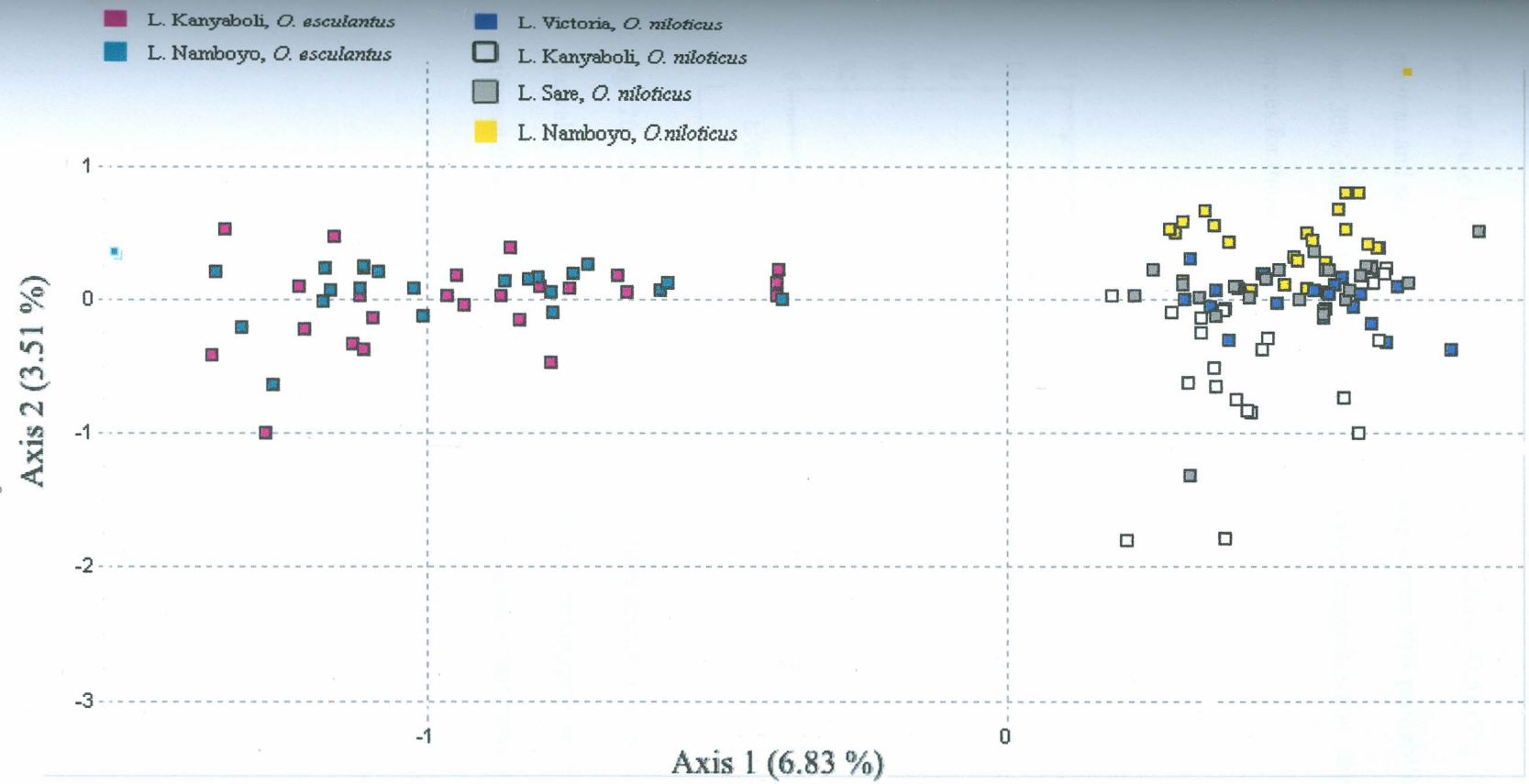


Fig. 19. Factorial correspondence analysis of microsatellite allele variation in *O. niloticus* and *O. esculentus*.

To assess genetic admixture between *O. niloticus* and *O. esculentus* at microsatellite loci, we set $K = 2$ to identify the proportion of membership (Q) towards each of the two species. Therefore Q close to 1 means the sampled genome is mostly *O. niloticus* while Q close to 0 means little or none of the sampled genome is *O. niloticus* and thus is *O. esculentus*. There were no pure hybrid genotypes found (i.e. Q values ~0.5) (Fig. 20). Two individuals of *O. niloticus* and six individuals of *O. esculentus* whose 90% probability intervals extend by more than 30% out of the typical species Q value suggest some degree of admixture between species for those individuals (Fig. 20).

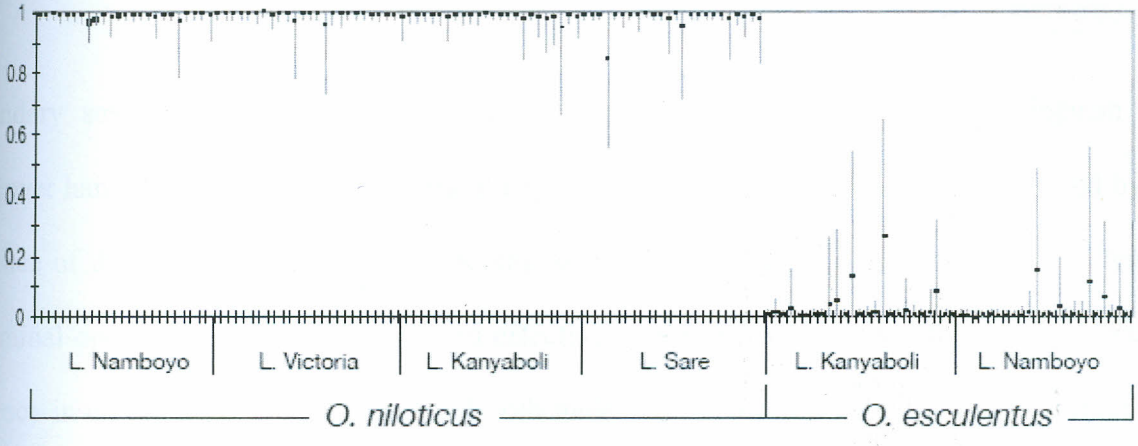


Fig. 20. The distribution of individual membership coefficients (Q ± 90% probability intervals) of *O. niloticus* and *O. esculentus* genotypes in the lakes identified through microsatellite analyses where each sample along the *x* axis represents an individual.

CHAPTER FIVE

DISCUSSION

5.1. Sexing fingerlings of *O. niloticus*

In the present study, all the Nile tilapia samples were unambiguously sexed by gonad squash technique and confirmation of the accuracy of sexing done by histological preparations of the gonads. The presence of oocytes at different stages of development were observed and identified as females, oocytes appeared as small rounded cells with very high nucleus to cytoplasm ratio whereas preparations with distinctive testicular structures including semineferous tubules and clusters of developing spermatocyes were identified as males (Fig. 3).

Secondary sex characteristics like coloration and size were inadequate to distinguish sex. Moreover hand sexing based on the genital papillae where male individuals are identified by the presence of the urinogenital and anal opening and females by an additional aperture called the abdominal opening is not an accurate and effective approach (Mair *et al.* 1997). Therefore for accuracy in determining the proportion of both male and female individuals from heat treatment experiments, a more reliable and accurate method like gonad squash and histology was necessary.

Squash technique is currently a simple laboratory technique originally developed for sexing *Oreochromis aureus* and bluegill (*Lepomis machrochirus*) by Guerero and Shelton (1974). The method can be used to sex large samples, for instance, Bruno *et al.*, (2005) used squash and histological procedures to determine the sex of 3,894 European sea bass (*Dicentrarchcus labrax*). However, histology is time consuming and labour intensive.

5.2. Effect of temperature on sex reversal in *O. niloticus*

In the current study heat treatment was performed at 10 day post yolk sac stage for 10 days. The fry survival for all temperatures were recorded 24 hours post heat treatment, and are expressed here as percentages of the total numbers of fry present at the beginning of heat treatments and each sex is expressed as a percentage of the total number of the fry (Fig. 4). Results show that rearing sexually undifferentiated *O. niloticus* fingerings at the age of 10dph at 36 ± 1.0^{0}C for 10 days has sex reversal effect in this species. These results confirm that temperature is important in gonadal sex determination in tilapias as reported by Baroiller and D'Cotta (2001). This is in agreement with the observation that sexual differentiation of gonads is triggered by temperature during the critical developmental period and that exposure to elevated temperature for 10 or more days at 9-13dph increases the proportion of male *O. niloticus* (Baroiller *et al.*, 1995). Gonadal sex is determined by temperature before the onset of gonadal differentiation in fish (Hendry *et al.*, 2002).

The results of this study show that treatment at 36°C± 1.0^{0}C was the most critical temperature condition for both sex reversal (86.31%) and fry survival (65.25) (Fig. 4). The observed temperature effect on sex reversal may suggest the existence of a critical sex reversal temperature for *O. niloticus* 36°C. Such critical conditions have been observed for other fishes (Fujioka, 2002). Previous studies have suggested the need to synchronise the ages at which the fry are heat treated and the treatment temperatures, as well as the length of treatment. For instance, *O mossambicus* juveniles that were heat treated before 5 days of age showed a higher incidence of deformities than those that were equally treated but at older ages (Wang and Tsai, 2000b). In the present study heat treatments commenced 10 days post yolk sac stage (~ 18 days post hatch) and

lasted only 10 days. No physical abnormalities were observed in the fingerlings that survived heat treatment.

5.3. Genetic mechanism of heat induced sex determination in *O. niloticus*

The present study has shown that rearing *O. niloticus* fry at 36^0C has sex reversal effect in favour of male individuals. This observation confirms that temperature plays a critical role in sex determination and gonadal differentiation in this species during the critical sex determination period (10-18dyph). Temperature has an influence in sex determination process leading to either high proportions of male individuals of *O. niloticus* (at elevated temperatures of 36 ± 1^0C) as shown in the present study or may produce high female ratios at lower temperatures as exhibited in Atlantic halibut (Van Nes and Andersen, 2006).

Unlike in medaka fish (*Oryzias latipes*) where a sex determining gene has been identified and characterised (Herpin and Scharti, 2009), no particular gene has been identified as sex determining gene on *O. niloticus* and no gene has been identified in temperature induced sex reversal. However the present study has shown that sex linked microsatellites, particularly the locus Abur36 is highly influenced by elevated temperatures during sexual differentiation. The genetic proportion of sex linked markers indicated that Abur36 was highly influenced by temperature and was associated with 95% of the sex reversed male individuals. The effect of temperature on this locus may lead to cascading events that favour development of male individuals through gene expression of male biased transcription factors (Ijiri *et al.*, 2008; Hossain *et al.*, 2008) (Fig. 14). The prediction of androgen receptor β, Hsp70 that modify steroid receptors and SRY (sex determination region on Y chromosome) transcriptional factors from the

ESTsequences generated from heat treated *O. niloticus*, indicates that heat influences this locus leading to development of male individuals.

The genetic basis of temperature dependent sex-determination in *O. niloticus* may still not be clear. However this is the first attempt to elucidate the genetic basis by investigating the effect of heat on the role of the sex linked genes in sex determination. The sex linked locus, Abur36 identified 95% of the sex reversed *O. niloticus* suggesting that this locus could be highly influenced by temperature, thereby influencing sexual determination and differentiation. Pieau, (1996) showed that temperature activates at least four genes: gene encoding for aromatase and oestrogen receptors at female producing temperatures and genes encoding for 5α-5β reductase receptors and androgen receptors at male producing temperatures. Blast results in the present study have indicated the involvement of expression factors shown in Fig 11, 12 and 13, in temperature induced sex determination in *O. niloticus.* The present study has also shown that heat treatment during ontogenic development may have 'mutagenic' effect on the expression sequence tag (EST) based sex linked genes (Fig. 6).

The EST based sex linked genes, have been shown to change DNA structure (Rich *et al.*, 1984), chromatin organization (Rich *et al.*, 1984), regulation of DNA recombination (Jeffreys *et al.*, 1998), transcription and translation (Meloni *et al.*, 1998) as well as gene expression and cell cycle dynamics (Fabregat *et al.*, 2001). Therefore, EST based sex linked microsatellites may play a pivotal role in gene expression leading to sex reversal in favour of males at elevated temperatures and could turn out to be the major genes controlling sex determination in this thermolabile species. Since microsatellites are far from being completely identified and characterised (Chistiakov *et al.*, 2006), new properties and characteristics of SSRs, will help the design of new

research fields and the practical use of these markers in unravelling the whole mechanism of sex determination in these species with temperature sex determination.

SSR variation produce drastic and quantitative variations in gene expression producing male biased transcription factors summarised in Fig. 14. The variations in the SSR sequences are presented in Fig. 7, Fig. 8 and Fig. 9. Because of genomic overabundance and high mutability of SSRs, changes in SSR array size may serve as a rich source of variation in fitness-related traits in natural populations (Streelman and Kocher, 2002). Its role may be especially important for adaptation to varying environmental conditions (Blankenship *et al.*, 2002).

SSRs located in promoter regions can influence gene expression level. Loci containing tandem repeats within either a coding sequence or a promoter can be hypermutable. Altered numbers of repeats thus cause switches in the reading frame of translation or changes in the level of promoter activity, while SSR situated in the 3′-UTR could affect gene expression through their influence on the stability of transcribed products (Chistiakov *et al.*, 2006).

In the present study the putative functions of the sex linked genes were predicted by comparing the sequences to the amino acid public database sequence (Fig. 11 – 13). The predicted proteins and transcription factors indicated the involvement of gonadotropin receptors (follicle stimulating hormone and leutenising hormone receptor). Recent studies on the expression of gonadotropin and gonadotropin receptor genes during early sexual maturation in male Atlantic salmon (Maugars and Schmitz, 2008) showed that both gonadotropin receptor genes are expressed in immature testis. The androgen receptor β predicted from the public database of amino acid

sequences (Fig. 11) plays a key role in vertebrate sex differentiation. Action of androgen is mediated through androgen receptor during sex determination (Zhang *et al.*, 2006).

The present study predicted the involvement of DM-related transcription factor (DMRT2b) in the heat induced sex determination (Fig. 12). A related transcription factor expressed by DMRT1bY gene has also been predicted to be involved in sex determination (Zhang *et al.*, 2006). The authors predict that the gene could be the master of sex-determination but concludes that the relationship between environmental factors and sex-related genes in sex-determination remains largely unknown.

The present study has also indicated the involvement of heat shock protein 70 in sex determination. This concurs with the work of Khuo *et al.*, (2009) that showed that hsp70 have the ability to modify steroid receptors and that heat shock protein binding protein (HSPBP) mRNA is expressed in the gonadal-adrenal complex in male individuals. Cytochrom P450 aromatase is also critical during sex-differentiation. It converts oestrogens into androgens and the brain aromatase act as a synchroniser in the sex differentiation initiated by gonadal cues in *O. niloticus* (Sudhakumari *et al.*, 2005).

Therefore the heat induced sex-determination mechanism in *O. niloticus* can be modelled from this genetic approach. It is therefore clear that heat has an effect on the sex-linked genes with a resultant effect of a male biased cascade of genetic events. Temperature dependent sex reversal requires obtaining recently hatched fry and rearing them in aquaria with high quality water. It remains the most effective and environmentally friendly approach in terms of production and

adoptability to local farmers for seed production in facilitating mono sex culture of all-male *O. niloticus*.

5.3.1. Genetic variability of *O. niloticus* populations compared to *O. esculentus*

The present study attempts to elucidate the genetic population structure of *O. niloticus* and to assess whether it has undergone introgression hybridization with *O. esculentus* within the Lake Victoria region (Yala swamp) based on both mtDNA and microsatellite analyses. All lines of evidence suggest that there is only a limited amount of gene flow among the different populations of both species of tilapia.

For the native critically endangered species *O. esculentus*, population genetic analysis revealed two discrete populations based on microsatellite data, indicative of low gene flow between the two satellite lakes Kanyaboli and Namboyo. This lack of gene flow is most likely due to physical isolation between the two populations of *O. esculentus*. This eco-physiological barrier could also limit gene flow between the two populations of *O. esculentus* (Crispo and Chapman, 2008). This result of population structure was further supported by mtDNA analysis that revealed unique mtDNA haplotypes in each population of *O. esculentus*. *Oreochromis esculentus* from Lake Namboyo exhibited higher haplotype diversity and more private alleles than the population from Lake Kanyaboli. This higher genetic diversity in Lake Namboyo may be due to higher effective population size as a result of lower fishing pressure compared to Lake Kanyaboli. Higher fishing pressure has been shown to reduce genetic diversity in other fish species (Hauser *et al.*, 2002).

The present study revealed four genetically distinct populations of the introduced species *O. niloticus* following exact tests defined by the four lakes based on the mtDNA and microsatellite markers. The genetic structuring of the introduced *O. niloticus* is most likely caused by the fact that the four lakes are geographically isolated from one another, thus preventing movement of individuals between populations, thereby diminishing gene flow. Introduction of *O. niloticus* into the Lake Victoria basin began as early as 1924 (Trewavas, 1983). However, widespread introduction of this species have been in the last half century (EAFFRO, 1964) since it was re-introduced by human for developing local fishery during 1951 – 1954 (Beauchamp, 1958). Based on this historical information on the local introductions, data suggest that duration of time of ~ 60 or 90 years is sufficiently long enough for the subsequent generations of the originally introduced individuals into different lakes to have become genetically diverged from one another via genetic drift (Crispo and Chapman, 2008). Both mitochondrial DNA and microsatellite markers revealed distinct private haplotypes and alleles that were restricted to a single of the four lakes. Satellite lakes tended to have slightly lower genetic diversity probably due to population bottlenecks, and/or smaller effective population sizes (Nei *et al.*, 1975). Nonetheless, the difference in genetic diversity between Lake Victoria and the satellite lakes was not substantial. This observation concurs with previous similar studies on haplochromine cichlids (Abila *et al.* 2004, 2008) that there are similar levels of genetic diversity in satellite lakes as in Lake Victoria, although larger population sizes for Lake Victoria would have suggested that it might have a higher genetic diversity as well (Lee and Boulding, 2009). *O. niloticus* was also introduced in these satellite lakes but unlike Lake Victoria where the native *O. esculentus* has been extirpated, remnants of this species still exist in sympatry with the introduced *O. niloticus*, perhaps because of the absence of Nile perch (Aloo, 2003).

5.3.2. Admixture between *O. niloticus* and *O. esculentus*

Both mitochondrial and nuclear markers indicate that the two tilapia species have remained genetically distinct. There is no evidence for introgression of mtDNA between species, as might have occurred via occasional hybridization (Redenbach and Taylor 2003; Barton and Hewitt 1995). However, there are very low levels of individual-level genetic admixture at microsatellites. The levels of admixture were slightly higher in *O. esculentus* than *O. niloticus* (Fig. 20), which suggests that there may be some very low levels of introgression from *O. niloticus* into *O. esculentus*.

Previous attempts to study hybridization in Lake Victoria tilapias led to conflicting results. Based largely on allozymes, Agnèse *et al.* (1999) suggested that *O. niloticus* from Lake Kanyaboli are genetically 'pure'. A study conducted by Mwanja (2004) on characterization of Lake Victoria tilapia fishes using RAPD markers revealed 6.72 % of bands of *O. Niloticus* that appeared in populations of *O. esculentus* and 0.91% of the *O. esculentus* bands present in *O. niloticus*. Our data support this finding of some low levels of introgression from *O. Niloticus* into *O. esculentus*. However, the overall low-levels of shared bands between *O. Niloticus* and *O. esculentus* indicated a limited level of hybridization between the two species. Since both studies were based on relatively small sample sizes and genetic markers that might be considered to suffer from inherent limitations, a re-evaluation of the question of hybridization between invasive and native tilapia species in the Lake Victoria basin would be timely. A combination of microsatellite genotyping and mtDNA sequence analyses is more sensitive in detecting potential hybridization events (Frankham *et al.*, 2004) and therefore the results presented in this study support the hypothesis that hybridization between the two species is not entirely absent, but the two species might be introgressed with each other as revealed by microsatellite analysis.

Since *Oreochromis esculentus* and *O. Niloticus* are phylogenetically closely related (although they are not a sister taxa) (Klett and Meyer 2002), both species might share certain alleles and/or allele size classes naturally at microsatellite loci examined. However, this hypothesis seems highly unlikely because (1) no mtDNA haplotypes were found to be shared by these two species (see Fig. 17 and 20) and (2) the sequence divergence in the mtDNA control region between the species was considerably large [uncorrected *p*-distance (i.e. average number of nucleotide differences per site) between haplotypes of the species: 13.9 %, suggesting that these two species are clearly "genetically unique". Therefore, it is hard to think of how *O. esculentus* innately shares alleles with *O. Niloticus* at only microsatellite loci, but not at a mitochondrial marker.

A better understanding of historical and contemporary levels of hybridization between *O. niloticus* and *O. esculentus* would be aided by additional temporal and/or geographical samples, particularly for *O. esculentus*. Comparing population genetic structure of native *O. esculentus* before and after an introduction of *O. Niloticus* will provide insights into a direct connection of admixture between these two species. It would also be interesting to see how stable the population structure of these two species is at a contemporary time-scale. In addition, contrasting population structure of *O. esculentus* in Lake Victoria region, where there is potential for admixture with *O. niloticus*, to that from a native and genetically "pure" source population without *O. Niloticus* will gain insights into how much the genome of *O. esculentus* has been "polluted" by *O. Niloticus* or vise versa in Lake Victoria region.

5.3.3. Satellite lakes as refugia for endemic fishes and their potential use in aquaculture development

Introduction of exotic organisms including fishes have been one of the major threats to global biodiversity. The Lake Victoria cichlid fish species flock experienced one of the worst mass extinctions of the twentieth century (Barel *et al.*, 1985). Lake Victoria alone originally contained over 500 indigenous species of cichlid fishes, but mainly due to anthropogenic influences, particularly introduction of exotic species such as the Nile Perch, *Lates niloticus*, hundreds of endemic species (including *O. variabilis* and *O. esculentus*) became extinct (Balirwa *et al.* 2003). Invasive *O. niloticus* is also believed to contribute to the disappearance of the native tilapia species from Lake Victoria through its competitive superiority and its capability to hybridize with them (Ogutu-Ohwayo, 1990; Goudswaard *et al.*, 2002).

However, recent studies on fish species genetic diversity in satellite lakes within the Lake Victoria region have lead to the discovery of genetic diversity that have either previously not been sampled from Lake Victoria or arisen In situ (Mwanja, 2004; Abila *et al.*, 2004, 2008). Such findings support the hypothesis that satellite lakes and other small water reservoirs surrounding Lake Victoria could have played an important role in the evolution and conservation of the genetic diversity of the ichthyofauna in the Lake Victoria region now and as refugia during the late Pleistocene desiccation (Abila *et al.*, 2008; Elmer *et al.*, 2009). Chapman *et al.*, (1996) also suggested that wetlands in Lake Nabugabo near Lake Victoria, Uganda protect endemic haplochromine cichlid fishes – *Haplochromis velifer*, *H. simpsoni*, *H. beadlei*, *H. annectidens* and *Prognathochromis venator* – from predation of Nile perch, serving as refugia for these now endangered species. Similarly, *O. esculentus* is now extirpated from Lake Victoria (Balirwa *et*

al., 2003), yet its existence in the minor water bodies provides an opportunity to unravel its genetic diversity as well as population structure in relation to the introduced *O. niloticus*.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1. Summary

The current study was set out to determine the genetic basis of heat induced sex determination in *O. niloticus* population from the Gulf of L. Victoria and to determine the genetic structure of the species within the Lake Victoria and the associated satellite lakes. The genetic composition of invasive *O. niloticus* was compared to that of the native *O. esculentus* to determine whether the species has undergone introgressive hybidization especially where they co-exist. To determine the effect of temperature on sexual determination and differentiation, fingerlings where obtained and reared under constant temperature conditions (ranging from 28 to 38^0C) and 26^0C being control for 10day. Treatment of the fry commenced at the age of 10dph. Sexing of the fry was done at 4 and 6 months post heat treatment by gonadal squash technique and confirmed by histology. Male individuals where identified by the presence of clusters of spermagocytes and developing semineterous tubules. While females where identified by the presence of developing oogonia and oocytes.

Three sex linked microsatellite locus (Abur36, Abur100 and UNH846) were employed to determine the effect of temperature at the gene level. The loci Aur36 identified 95% of the sex reversed individuals suggesting that the locus is highly influence by temperature. Determination of the putative function of the microsatellite sex linked gene was determined following sequencing of the gene. Male biased transcription factors were identified as being transcribed by this gene suggesting that temperature could either trigger or alter gene expression in favour of male individuals.

To determine the genetic structure of *O. niloticus* populations, eight microsatellite DNA markers obtained from eight independent chromosomes were amplified and genotyped. Mitochondria DNA sequences where also obtained from each sample amplified and sequenced. The genetic structure up of *O. niloticus* was compared to that of *O. esculentus* and results revealed the existence of four populations of *O. niloticus* defined by the four lakes (Lake Victoria, Sare, Namboyo and Kanyaboli) and two populations of *O. esculentus*. There was very low genetic admixture between the two species indicating lack of gene flow or very low levels of introgressive hybridization suggesting purity of the two species. This suggested the existence of genetically distinct populations of Nile tilapia within the region, which could potentially be used as broodstock populations in the temperature treatment technology for sex reversal in all-male tilapia culture.

6.2. Conclusion

1. This study has demonstrated the effect of temperature on sexual determination and differentiation in *O. niloticus* at 36^0C, being the optimal temperature for sex reversal and survival.
2. The present study has also adduced evidence that temperature as an environmental factor interacts with the genome altering gene expressions leading to male biased individuals at specific temperature sensitive periods during ontogenic development of the embryos. The effect of elevated temperature is mainly at the sex-linked locus affecting gene expression thereby leading to sex reversal in favour of male individuals.
3. Studies on genetic variability of *O. niloticus* compared to *O. esculentus* have shown the existence of four distinct populations of *O. niloticus* within the region defined by the individual

lakes, and two distinct populations of *O. esculentus*. This offers an opportunity to explore the potential genetic variability of these distinct populations into aquaculture systems.

6.2. Recommendations

6.2.1. Application of the present work

1. Unlike genetic approaches, heat treatment technology for sex reversal is a consumer friendly method that does not require specialised facilities and high tech expertise knowledge. It can therefore be easily adapted by local fish farmers to produce all-male fingerlings in facilitating all-male (mono-sex) culture practices to overcome the inherent problems of mixed sex cultures. The use of hormones for sex reversal is being discouraged due to public resentment and the potential health risk to the consumers, thus heat treatment technology remains as the only cheap and reliable method with no known inherent health risks for production of viable mono sex tilapia fish.
2. The identified genetically diverse and unique populations of *O. niloticus* provide a unique opportunity to explore their potential as broodstock genetic resource that can be used for production of all-male fingerlings of high quality.

6.2.2. Recommendations for future studies

1. Further studies on gene expressions should be carried out to identify the main gene(s) responsible for sex determination, for example, the role of Abur36 and other sex-linked microsatellites that play a role in sex determination in *O. niloticus* by isolating and genetically characterising these genes to identify their putative functions. Invitro studies on the functions of these genes should also be carried out based on gene-knock out technology.

2. The genetic distinctiveness of the two species should be conserved as management units and further genetic characterisation of the *O. niloticus* within the Lake Victoria region based on microsatellite set of the entire genome should be carried out to identify the most genetically robust and diverse population with suitable characteristics such as diseases resistance and faster growth in marker assisted selection (MAS) programmes.

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