CSIR COLLEGE OF SCIENCE AND TECHNOLOGY

MICROSATELLITE BASED GENETIC VARIATIONS AND

RELATIONSHIPS AMONG SOME FARMED NILE TILAPIA

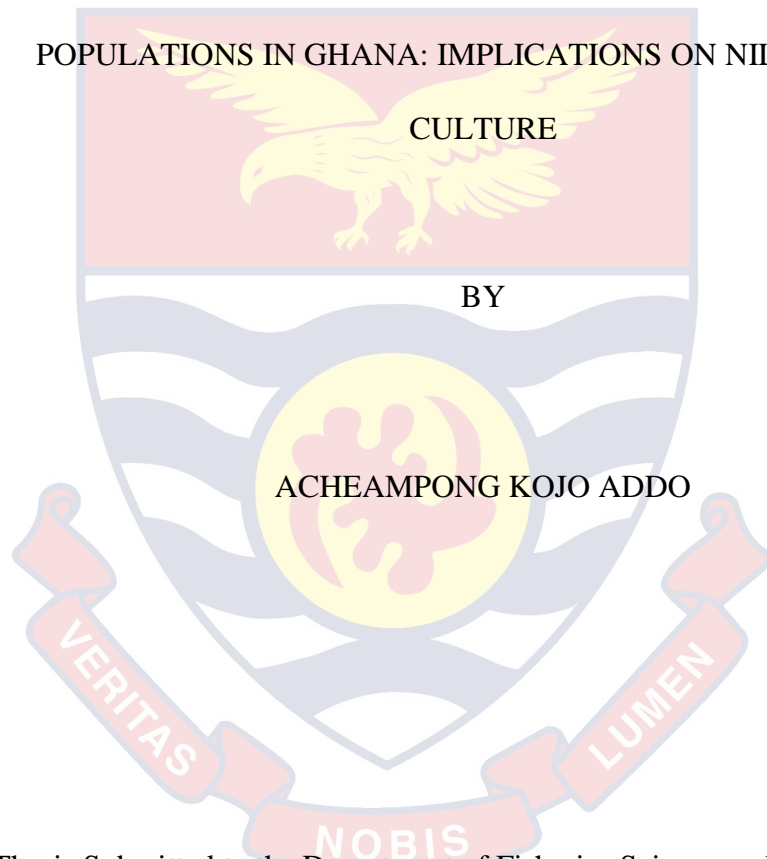POPULATIONS IN GHANA: IMPLICATIONS ON NILE TILAPIA

CULTURE

ACHEAMPONG KOJO ADDO

2021

CSIR COLLEGE OF SCIENCE AND TECHNOLOGY

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POPULATIONS IN GHANA: IMPLICATIONS ON NILE TILAPIA

CULTURE

BY

ACHEAMPONG KOJO ADDO

Thesis Submitted to the Department of Fisheries Science and Aquaculture of
the CSIR College of Science and Technology, in partial fulfilment of the
requirements for the award of Master of Philosophy degree in Aquaculture

FEBRUARY 2021
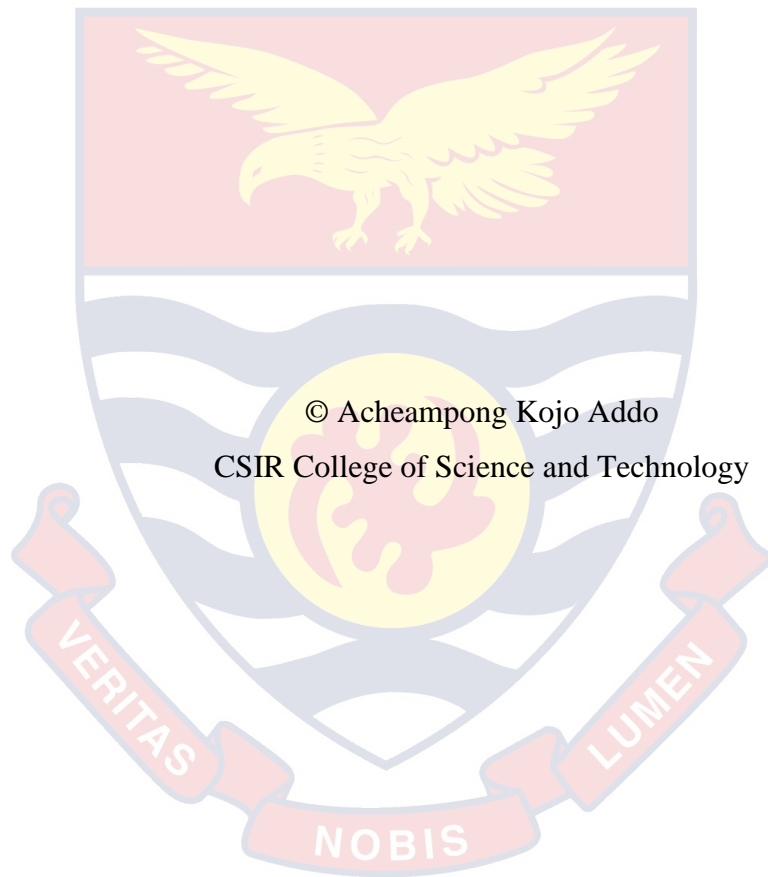
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DECLARATION

Candidate's Declaration

I hereby declare that the thesis is the result of my own original research and no part of it has been presented for another degree in this College or elsewhere.

Candidate's Signature:………………………............Date……………...............

Name: Acheampong Kojo Addo

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the CSIR College of Science and Technology.

Principal Supervisor's Signature …………………….........Date:……………........

Name: Prof. Mike Yaw Osei-Atweneboana

Co-supervisor's Signature:…………………………….Date……………............

Name: Dr. Seth Koranteng Agyakwah

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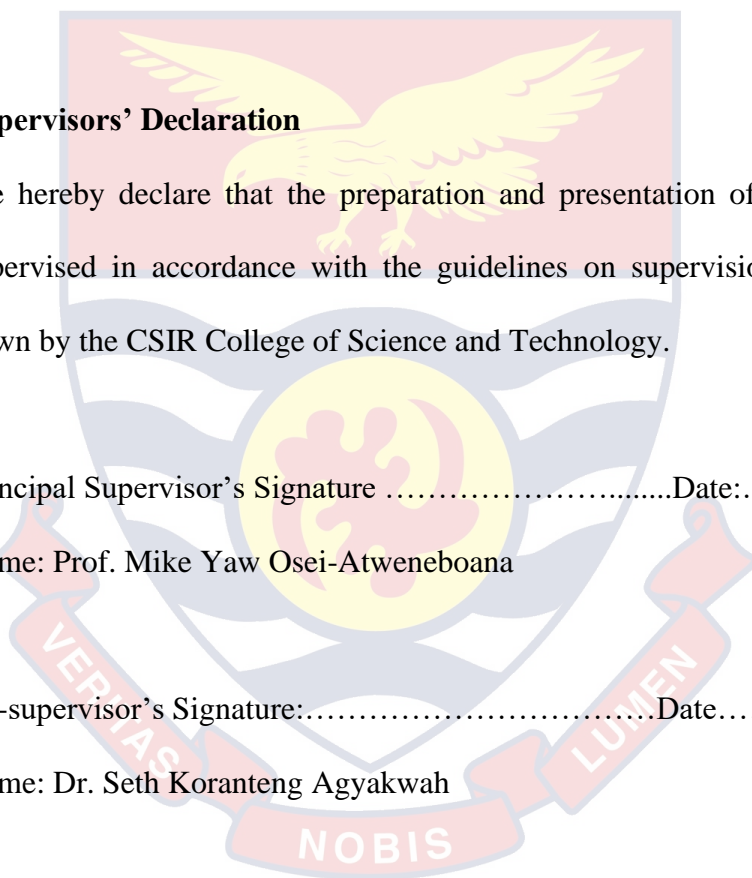
ABSTARCT

The study investigated genetic variation and relationships among populations of Nile tilapia, *Oreochromis niloticus* in pond fish farms located in six regions of Ghana. Thirty fish specimens were collected from each farm and analysed together with improved locally bred 'Akosombo strain' and imported 'GIFT-derived strain' populations. Fin clips of specimens per farm were labelled, put together, preserved in ethanol and transported to CSIR-WRI laboratory for molecular analysis. All samples were screened with five microsatellite markers using Zymo-kit DNA extraction, PCR technology and agarose gel electrophoresis. Bands visualized were scored and analysed using GenALEx, MegaX, and Genpop on the web. Two of the microsatellite markers, GM531 and GM538 showed four alleles per locus whereas UNH154, UNH222, and UNH995 showed three alleles per locus. 'GIFT- derived' was highest in heterozygosity at 0.445 whereas locally bred 'Akosombo Strain' was 0.232. Heterozygosity was also high in three populations ranging from 0.232 to 0.258 which suggest high variability among the populations. Gene diversity based on locus ranged between 0.180 to 0.430 whereas genetic differentiation between populations (F_{ST}) was 0.140 indicating moderate differentiation between the populations. Three fish population clusters were formed; four clustered closely with locally bred 'Akosombo Strain', seven clustered closely with 'GIFT- derived' and three other fish farms forming a separate cluster. This debunks common perception that *O. niloticus* farmed in Ghana is solely the Akosombo strain. Cluster of populations also suggested that farmed Nile tilapia populations are now mixed hence production from different farms would not easily be predictable or comparable.

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KEYWORDS

Nile tilapia

Populations

Microsatellites

Alleles

Heterozygosity

Genetic Variation

ACKNOWLEDGEMENT

I am grateful to my Principal Supervisor, Prof. Mike Yaw Osei-Atweneboana and Co-Supervisor, Dr. Seth Koranteng Agyakwah and Dr. Kofi Abban, for their immense support, advice, criticisms, guidance and tolerance which enabled me to commence and complete this work. I would also like to express great appreciation to Dr. Ruby Asmah, Dr. Emmanuel Tetteh - Doku Mensah, Dr. H.R. Dankwa, Dr. Kweku Amoako Atta deGraft-Johnson and all my lecturers for their great support and contributions in making this study successful. I appreciate Mr. Emmanuel Armah, Mr. Isaac Owusu - Frimpong and all the laboratory research assistants at CSIR- Biomedical and Public Health Research Unit for their selfless assistance throughout the laboratory analysis of this study.

Finally, I would like to thank all my course mates and also express profound gratitude to the Ghana Tilapia Seed Project and the Modernization of Agriculture in Ghana (MAG) Programme for providing financial support in making this study successful.

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DEDICATION

To my father, Mr. Daniel Addo, mother, Mrs. Irene Addo and brothers,
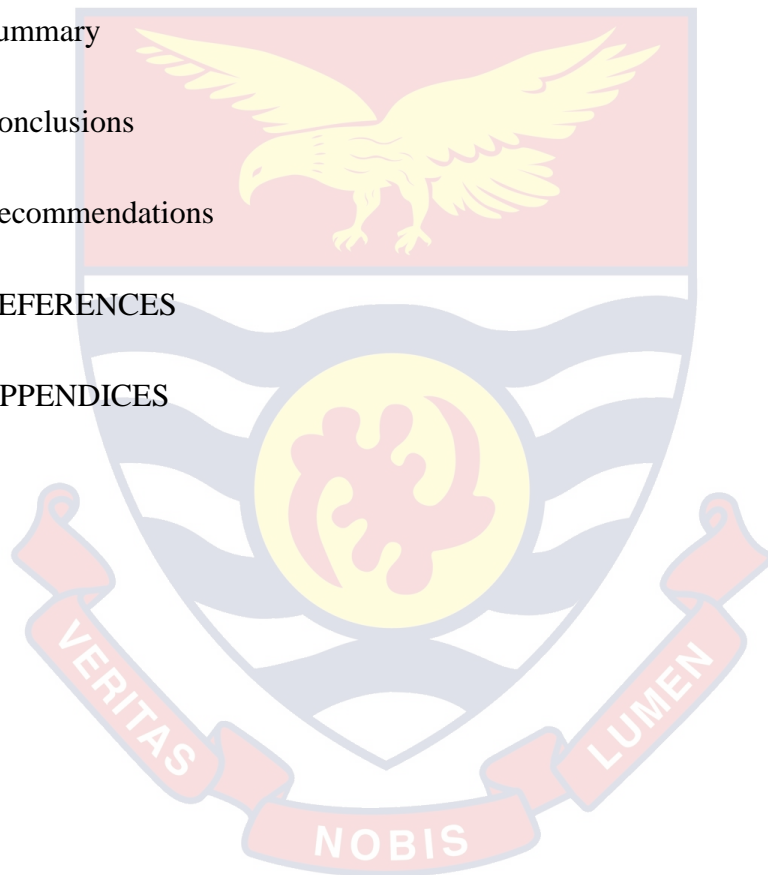
Kwaku, Kwame and Papa

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LIST OF ACRONYMS

ARDEC	Aquaculture Research and Development Centre
Bp	Base Pair
BPHRU	Biomedical and Public Health Research Unit
CSIR-WRI	Council for Scientific and Industrial Research –Water Research Institute
GDP	Gross Domestic Product
He	Expected Heterozygosity
Ho	Observed Heterozygosity
HWE	Hardy Weinberg equilibrium
I	Shannon Information Index
Na	Mean number of alleles
Ne	Number of effective alleles
PCR	Polymerase chain reaction

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CHAPTER ONE

INTRODUCTION

Nile tilapia culture has gained prominence in Ghanaian aquaculture industry and as a result, several studies are being undertaken to contribute to improving culture of the fish within the country. Some earlier studies including Attipoe, Tetteh-Doku and Agyakwah (2015); Mireku, Kassam, Changadeya, Attipoe, and Adinortey (2017), Lind *et al.* (2019) and Falk and Abban (2004), showed that genetic variation studies among fish populations provide information that can be used to improve genetic resources among fish populations.

In Ghana, continuous genetic improvement of Nile tilapia, the 'Akosombo strain', for culture enhancement and predictability of production is considered threatened or interrupted by mainly unintended introduction of unknown genetic stocks into culture stocks as well as farmers establishing their own brood stocks. The situation was probably aided by lack of published information on genetic structure and other information on the 'Akosombo strain' which was in development from Volta strain of Nile tilapia, *Oreochromis niloticus.* Anane-Tabeah, Frimpong, and Hallerman, (2019), indicated some native *O. niloticus* having genetic similarities with the non-native 'GIFT strain' in the Lower Volta Basin of Ghana.

The objective of the study was to assess genetic variations among farmed *O. niloticus* populations in six regions of Ghana and to determine how genetically related they were to the 'Akosombo strain' or 'GIFT-derived' strain. It will also provide an informed platform based on which fish farmers

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would be educated on the selection criteria for establishment of brood stock which could minimize inbreeding among fish farm populations.

Background to the Study

Nile tilapia is the second most extensively farmed freshwater fish worldwide (FAO, 2014). This fish is the most cultured in Ghana, making up about 80% of fish culture production with the remaining 20% being mostly *Clarias, Heterobranchus species* and *Heterotis niloticus* (FAO, 2016).

The Nile tilapia, *O. niloticus,* being a native species, has the ability to thrive in most fresh waters in Ghana. It has been introduced in many freshwaters mostly unintentionally and cultured in most parts of the country Among the freshwater species cultured, preference for culture and wide distribution of the Nile tilapia is because of its ability to spawn easily under captive conditions, consume wide variety of natural and formulated diets and grow under the temperature regime of country.

Aquaculture production, primarily *O. niloticus* culture in Ghana provides a source of livelihood and income for many individuals. It has the potential to alleviate poverty in most communities whilst contributing to their nutritional needs (Bene & Heck, 2005; Kassam & Dorward, 2017).

Nile tilapia culture was enhanced in Ghana through a natural breeding program, "Breeding Selection of the Akosombo strain of Nile tilapia" by Attipoe *et al.* (2015), based on genetic variations that exist among native *O. niloticus* populations in Ghana.

Genetic variation describes naturally occurring genetic differences among individuals of the same species co-existing within natural populations

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(Bezault *et al.,* 2011) and are assisted by Polymorphic DNA markers which are used by fisheries researchers to determine genetic variations that exist among populations including fishes (Ferguson & Danzmann, 1998).

Farmed Nile tilapia populations were studied to observe their diversity and implication of the status on aspects of its commercial venture in Ghana. Parameters of study included: Loci variability, allelic patterns, polymorphism, gene diversity, Interpopulation diversity, Heterozygosity (observed and expected), genetic distance and population clustering.

Microsatellites are one of the best suitable genetic markers for analysing population genetic structure and genome variations among populations. They are widely dispersed throughout eukaryotic genomes and consist of several repeat sequences which show high levels of variability among closely related populations (Bruford & Wayne, 1993). Single locus microsatellites are mostly used for population studies since both alleles for heterozygote genotype show codominant expression (Abdul-Muneer, Gopalakrishnan & Musammilu, 2009). Genetic data yielded by microsatellite markers make these markers one of the preferred molecular tools for many populations diversity studies (Abban, 1988). This basically involves the process of DNA extraction, polymerase chain reaction (PCR), and gel electrophoresis of PCR products to separate alleles which appear as bands on the gels with specific fragment sizes from which genetic variations are analysed (Ciofi *et al.*, 1998).

Some microsatellites markers have multiple alleles which are highly polymorphic hence very useful for applications such as parent-offspring identification in mixed populations, while others have lower numbers of

alleles and are mostly suited for fish population studies and phylogeny (Al-Atiyat *et al.,* 2012). However, different markers used for fish genetic studies vary in allele numbers. High microsatellite markers with high allele numbers are more efficient than markers with low allele numbers.

An allele is any alternative from of a gene that can exist at a single locus (Hartl & Jones, 2005). In fish populations, allelic patterns generated from allele frequencies are used to quantify genetic variation within fish populations because they describe genetic makeup of populations as well as provide information on Gene diversity and interpopulation diversity.

Gene diversity among individuals reflects different alleles present within different populations whilst genetic variations shapes and defines individuals, populations and species of organisms and also describe the tendency of genetic traits to vary within populations (Laikre *et al.,* 2010).

One key index used to determine genetic variations among fish populations is heterozygosity. This refers to the condition of having two different alleles at a locus. It can be used to describe the structure and even the history of populations. the heterozygosity for all the populations was determined in this study to know about variations within the fish populations (Ruzzante, Taggart, Cook & Goddard, 1996). High heterozygosity within populations indicates high genetic variability, low heterozygosity indicates low genetic variability within populations.

The Wright F statistics is also an important index used to measure genetic diversity among populations. It is used to measure the deficiency or excess of average heterozygotes in each population and gene differentiation

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among populations based on allele frequencies for a group of populations (de Vicente, López & Fulton, 2004).

Genetic distance and genetic identity determine relationships that exist among fish populations. This index is used to characterize *O. niloticus* populations in several genetic studies. It indicates whether or not populations are closely related or have a common ancestor (Nei, 1987). Populations with similar alleles have small genetic distances than populations with different alleles (Nei, 1987). Genetic distance in phylogenetic tree consists of branch lengths and nodes. The branches form clusters which connect two populations together based on their genetic distances. The higher the genetic distance between two populations the higher the genetic diversity hence the less genetic relationship that exist among the two populations. Two populations that cluster on the same branch are more genetically related and share a more common recent ancestor (Nei, 1987). In the study, population clustering was used to show relationship that exist among Nile tilapia populations; farms that show close relationship with the 'Akosombo strain' and farms that show close relationship with the 'GIFT - derived strain'.

The genetic variations among farmed Nile tilapia populations as well as the different strains of tilapia being cultured by fish farmers is currently a major subject of concern within the industry. This is because knowledge of genetic diversity and population structure of farmed *O. niloticus* in Ghana is inadequate. It is also unclear the type of strains cultured presently in Ghana based on findings from Anane-Tabeah *et al.* (2019). Most genetic diversity studies have looked at populations in the wild (Mireku *et al.* 2017; Lind *et al.* 2019; Falk & Abban, 2004; and Abban 1988). Some of these studies

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investigated the genetic diversity and population structure of *O. niloticus* in Lake Volta of Ghana using microsatellite markers. Lind *et al.,* also used microsatellite markers to determine the genetic diversity of *O. niloticus* throughout West Africa.

The paucity of information on genetic variations among farmed *O. niloticus* populations and information on available strains for culture, makes it difficult for farmers in most parts of the country to obtain quality seed and brood stock for culture. Success of production within most fish farms especially small-scale and semi-intensive systems is therefore not easily predictable or comparable.

The study intends to compare the genetic variations among *O. niloticus* populations being cultured in fish ponds in some regions in Ghana using microsatellite markers and develop suitable markers that can be used to identify different strains of *O. niloticus* available for culture in the country. The study would provide empirical evidence to show whether genetic variations exist among some farmed *O. niloticus* populations within the country and provide information on available strains of *O. niloticus* for culture in the country. This information is vital in predicting success of production or comparing production among different farms (Kajungiro *et al.,* 2019) since genetic makeup of fish populations has an influence on its survival and diversity status (Carlson, Cunningham & Westley, 2015). Populations with high genetic diversity would be better adapted to their environment compared to populations with low diversity (Carlson *et al.,* 2015).

Statement of the Problem

There is paucity of information on the genetic variations among farmed *O. niloticus* populations in Ghana and the type of strains available for culture. These factors among others makes it difficult to predict success of Nile tilapia production in Ghana. Many fish farmers in the country continue to struggle to make their farms productive and profitable due to various reasons, including slow and uneven growth and mortality of fingerlings (Ragasa, Agyakwah, Asmah, Tetteh-Doku & Amewu (2020), Kruijssen *et al.,* 2020).

In Ghana, continuous genetic improvement of Nile tilapia, the 'Akosombo strain', for culture enhancement and predictability of production is considered threatened or interrupted by mainly unintended introduction of unknown genetic stocks into culture stocks as well as farmers establishing their own brood stocks. The situation was probably aided by lack of published information on genetic structure and other information on the 'Akosombo strain' which was in development from Volta strain of Nile tilapia, *Oreochromis niloticus.* Anane-Tabeah, *et al.* (2019), indicated some native *O. niloticus* having genetic similarities with the non-native 'GIFT strain' in the Lower Volta Basin of Ghana.

Purpose of the Study

To determine level of uniformity of genetic structure of some farmed Nile tilapia populations and with Akosombo strain.

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Research Objectives

1. To determine Genetic variations that exist among the farmed Nile tilapia populations in some regions in Ghana.

2. To determine suitable microsatellite markers for identification of different strains of farmed Nile tilapia in Ghana.

3. To determine the phylogenetic relationship among the different populations of farmed Nile tilapia in Ghana

Research Questions

1. What genetic variations and relationships exist among farmed Nile tilapia populations studied?

2. Which microsatellite markers used will be suitable to determine variations among farmed Nile tilapia populations?

Research Hypothesis

1. H_0: Genetic variations do not exist among Nile tilapia populations cultured in Ghana

2. H_1: Genetic variations exist amongst Nile tilapia populations cultured in Ghana.

3. H_0: Microsatellite markers will not be suitable to characterize farmed *O. niloticus* populations in Ghana.

4. H_1: Microsatellite markers will be suitable to characterize farmed *O. niloticus* populations in Ghana.

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Significance of the Study

The Assessment of the genetic variations among farmed Nile tilapia populations in Ghana will provide baseline information on the genetic variations and relationships that exist among farmed *O. niloticus* populations in some regions of Ghana. This information will empower Fisheries Commission and aquaculture extension services to give technical advice to Hatchery operators. Inbreeding situations will be minimized among fish farms with knowledge of genetic differentiation among populations. Fishery scientist will rely on this information when carrying out future breeding programmes to improve farmed *O. niloticus* in Ghana.

The study will also develop molecular tools which can be used to characterize *O. niloticus* populations and distinguish between different strains of *O. niloticus* available for culture in the country.

Delimitation

O. niloticus were sampled from sixteen (16) fish farm populations located at different regions of Ghana. The study focused on six regions where pond fish culture is dominantly practiced. These included; Ashanti region, Eastern region, Volta, Bono, Bono-East and Ahafo region. Seven (7) fish populations from seven different farms were selected in Ashanti region; ASHOFM, ASHSES, ASHBOS1, ASHBOS2, ASHAAS, ASHEJJ1 and ASHEJJ2, three populations selected from Bono region; BONSUE, BONSUM and BONSUW, however, in the Eastern, Bono-East, Ahafo and Volta Regions, one population was sampled. They are EASLOM, BOETEM, AHAASU and VOLNOT populations respectively. The remaining two fish

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farm populations were the AKO and GIFT-D populations. Five microsatellite markers namely; GM531, GM538, UNH154, UNH222, UNH195 were used to assess genetic variations amongst the populations.

Limitations

All the fish populations were selected within the southern central part of Ghana excluding the northern part of Ghana. This was a major limitation to the study with regard to genetic variations amongst farmed Nile tilapia populations in Ghana. Also, five microsatellite markers were used for the study which could have been increased to further enhance development of effective markers to characterize farmed Nile tilapia populations.

Definition of terms

Microsatellites: Microsatellite is a set of short repeated DNA sequences at a particular locus on a chromosome, which vary in number in different individuals and so can be used for genetic fingerprinting.

Genetic variation: Genetic variation refers to similarities and differences that exist among individuals of the same species co-existing within natural populations.

Genetic diversity: Genetic diversity refers to the total number of genetic characteristics in the genetic makeup of a species that ranges widely from the number of species to differences within species and can be attributed to the span of survival for a species

Alleles: Alleles refers to each of two or more alternative forms of a gene that arise by mutation and are found at the same place on a chromosome

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Heterozygosity: Heterozygosity refers to the possession of two different alleles of a particular gene or genes by an individual.

Organisation of the Study

Fin clips from fish samples were collected from all designated regions and transported to the CSIR- Biomedical and Public Health Research Unit for molecular analysis. All fish samples were screened by all the five microsatellite markers to obtain allele frequencies for all fish farm populations. The results obtained (allele frequencies) were scored and subjected to GenALEx statistical software to determine parameters needed to establish genetic variations amongst the fish populations and also establish relations amongst the fish populations. Other software used included MegaX, and Genepop on the web to construct the phylogeny tree and establish the polymorphism information content (PIC) of the microsatellites markers used for the study.

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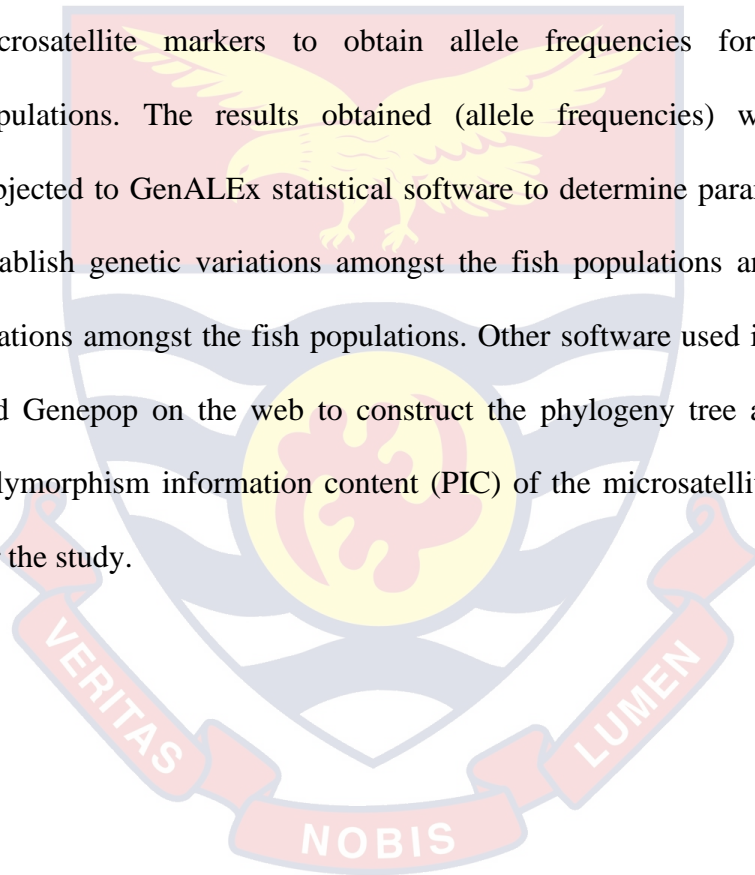
CHAPTER TWO

LITERATURE REVIEW

This section of the thesis deals with the review of related literature. The review was done on the following topics: General biology of *O. niloticus,* state of aquaculture production and more specifically state of aquaculture fish production in ponds, fish genetic variation, Microsatellite applications in fisheries and aquaculture and finally PCR (Polymerase Chain Reaction) in microsatellite application.

General Biology of Nile tilapia (*Oreochromis niloticus*)

Nile tilapia is a tropical freshwater fish species that lives in a variety of freshwater habitats such as rivers, lakes, sewage canals and irrigation channels (Bailey, 1994). The lower and upper lethal temperatures for Nile tilapia are 11°C - 12 °C and 42 °C respectively, while the preferred temperature ranges from 31°C to 36 °C. It feeds mainly on phytoplankton or benthic algae (Phillipart & Ruwet, 1982). Additionally, insect larvae are of some importance, as are aufwuchs and detritus; juveniles tend to be more omnivorous compared to adults (Lamboj, 2004). Nile tilapia can filter feed by entrapping suspended particles, including phytoplankton and bacteria, on mucous in the buccal cavity, although its main source of nutrition is obtained by surface grazing on periphyton mats. Reproductive activities in adults may begin when water temperature reaches 24 °C. The reproductive process between a male and a female starts when a male establishes a territory, digs a craterlike spawning nest and guards the territory. The ripe female spawn eggs into the nest which is immediately after fertilization by the male, the female

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then collects the fertilised eggs into its mouth and moves off. The female incubates the eggs in her mouth and broods the fry after hatching until the yolk sac is absorbed. Incubating and brooding are accomplished in 1 to 2 weeks, depending on temperature. After incubation, fry is released, but they may swim back into the mouth if danger threatens. Being a maternal mouth brooder, the number of eggs per spawn is small in comparison to stratum incubating tilapias, such as *Sarotherodon* and *Tilapia* species. Egg number is proportional to the body weight of the female and its 'situation'.

Nile tilapia has a number of important characteristics which makes it a key species for freshwater aquaculture. It has a relatively short generation time (approximately 6 months) relative to other species such as carp and trout, which ensures that the production cycle is completed within a single year. They also survive in water conditions that normally would not support the most of the other aquaculture species (Fitzsimmons, 2000).

In Ghana Nile tilapia are mostly cultured in floating cages, earthen ponds, and concrete tanks. Majority of farmed fish in Ghana, about 90 percent are from cage culture system with the remaining percentage from ponds (FAO, 2012). Most of the farms do not have hatcheries thus buy fingerlings from hatcheries for their production. Some others import fingerlings from other countries which is illegal with the objective to obtain quality fast growing fingerlings, however, most fish farmers have incurred adverse results as a result of low-quality fingerings (Kassam, 2014).

State of Aquaculture in Ghana

Population increases coupled with growing taste and preference for fish and fish products due to their nutritional value and other health benefits makes supply of capture fisheries inadequate (Rurangwa, Agyakwah, Boon, & Bolman, 2015) thereby making increased aquaculture production necessary in Ghana (FAO, 2012). *O. niloticus* is the dominant and preferred cultured fish species in Ghana accounting for over 80% of the farmed fish harvest in Ghana and also a source of income for many farmers countrywide. Some fish farmers have failed due to a number of reasons which include: use of low-quality fish feed, poor extension services, low funding, lack of comprehensive policy, lack of investment by private sector and unfocused promotion of aquaculture through many institutions (Munguti, Kim & Ogello, 2014).

Farmers are therefore challenged to produce their own brood stock with the aim of enhancing quality fingerlings for production since this is a vital component for successful aquaculture production. Commercial fish farmers who use intensive culture systems though in the minority produce about seventy-five percent (75%) of Ghana's total aquaculture production (MoFAD, 2016). Pond culture system is the prevailing production system in the southern and central part of the country, which covers about 98 percent of farms, which is also primarily small scale and semi-intensive in status. The prevailing culture system for tilapia production has however changed, and the immense bulk of cultured tilapia is now cultured intensively in cages, especially in Lake Volta (Kassam, 2014).

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Aquaculture production systems

Pond fish culture was adopted in Ghana in 1953, when there was no apparent decline in capture fishery (Kassam, 2014). This involved the growing of fish under controlled or semi-controlled aquatic environments usually in earthen and concrete tanks either for subsistence or commercial purposes. Pond aquaculture in Ghana has a huge potential for growth through sustainable intensification. Although mostly small in scale, pond fish farms are fairly uniformly distributed throughout the southern regions of the country. Majority of community farmers also employed the extensive culture system by the use of dams, dugout, ponds, and reservoirs for fish culture. Commercial fish farmers who use intensive culture systems though in the minority produce about 75 percent of Ghana's total aquaculture production (MoFAD, 2016).

Fish production from aquaculture has been estimated at 950 tonnes for 2004 and this continues to increase in demand at dwindling catch levels of capture fisheries. This is also due to intensification in production systems to meet increase demand. The aquaculture sub sector comprises largely small-scale subsistence farmers who practice extensive aquaculture in earthen ponds in contrast to the intensive practices of commercial farmers. The sector therefore lacks the organization to take up the challenges of providing inputs such as fish seed and feed as viable commercial activities to support the development of the industry.

Fish Genetic Variation

Genetic variation is one of the fundamental subjects of investigation in population genetics and this is of major importance in studies such as

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evolutionary biology. It describes naturally occurring genetic differences among individuals of the same species co-existing within natural populations (Bezault *et al.,* 2011). The variations that exist among individual organisms within a population increases adaptation of organisms to their environment which enhances survival in the face of changing environmental circumstances (Carlson *et al.,* 2015). The addition of a new allele to a population makes it more able to survive, less able to survive or has no effect on the survival of the organism.

Genetic variations among populations are caused due to Non-random mating resulting in inbreeding or outbreeding among populations, genetic drift, and migration of species from one population to the other (Zhao *et al*., 2011). Inbreeding amongst populations occurs when individuals with similar genotypes mate with each other rather than with individuals with different genotypes which results in reduction of genetic variation within populations whereas outbreeding occurs when individuals with a particular genotype mate with individuals of another particular genotype (Zhao *et al.,* 2011).

To improve culture of Nile tilapia in Ghana, Akosombo strain was developed by reciprocal crosses of four populations *viz* Nawuni, Yeji, Kpando and a farmed stock from Nsawam, through a selective breeding programme in Ghana (Attipoe *et al.,* 2015). Fish genetic resources have economic, ecological and social value and need to be characterized. The proper identification of breeding stock has potential benefits regarding the characterization, conservation and sustainable use of resources (Carvalho & Pitcher, 1994). In Conservation programs whereby genetic variations are determined, the loss of genetic diversity of fish resources are minimized in order to increase the

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chances of successful population restoration and long-term viability (Carlson *et al.,* 2015). Translocation of fish to supplement suppressed populations may have in fact harmful effects if the recipient population is genetically different (Allendorf & Luikart, 2007).

Available knowledge regarding genetic variations of farmed Nile tilapia populations in Ghana will provide information on the genetic variations among populations which will be used to improve *O. niloticus* culture through future breeding programmes (Angienda *et al*. 2011; Oldenbroek, 2017).

Microsatellite Application in Fisheries and Aquaculture

Microsatellites are tracts of repetitive DNA (Dioxyribonucleicacid) in which certain DNA motifs are repeated, typically 5–50 times). They occur at thousands of locations within an organism's genome (Phumichai, Phumichai & Wongkaew, 2015) and have a higher mutation compared to other areas of DNA within an organism hence high diversity in its application in genetic diversity studies among organisms (Brinkmann, 1998). They are co-dominant markers that present high numbers of alleles compared to allozyme markers (Slatskin, 1995) hence are mostly used in forensic genetics and genetic geology thereby making their application a very important molecular tool in genetic diversity studies. They are also used for DNA profiling in cancer diagnosis, in kinship analysis (especially paternity testing) and in forensic identification. They are also used in genetic linkage analysis to locate a gene or a mutation responsible for a given trait or disease.

Recent advances in aquaculture have adopted the use of microsatellites and other molecular markers in characterizing fish species. Studies carried out

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by Falk and Abban (2004), Bardakci and Skibinski (1994), Hassanien and Gibey (2005), Bezault *et al*. (2011), Chi, Huang, Wu, and Hu, (2014), Mireku *et al*. (2017) and Anane-tabeah *et al*. (2019), used microsatellites and other molecular markers such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphisms (AFLPs) to assess the genetic diversity of *O. niloticus* in the Volta Lake and tributaries of the Volta system. Mireku *et al*. (2017), in particular used 15 microsatellite markers with the advent of PCR technology to investigate genetic diversity and population structure of *O. niloticus* in the Volta Lake of Ghana and concluded that Nile tilapia populations exhibit high within population variability and low among population variability. This gives further insight in the genetic diversity of Nile tilapia populations in the Volta Lake.

Lind *et al.* (2019), conducted a genetic survey of Nile tilapia throughout West Africa, sampling 23 wild populations across eight countries (Benin, Burkina Faso, Côte d'Ivoire, Ghana, Togo, Mali, Gambia and Senegal), representing the major catchments of the Volta, Niger, Senegal and Gambia River basins. In the study microsatellite markers (192 Single Nucleotide Polymorphisms) were used in differentiating tilapia populations throughout West Africa. Findings showed that Nile tilapia populations in the Volta Lake of Ghana have lower heterozygosity. The genetic structure and diversity among seven Nile tilapia populations in Tanzania was investigated by Kajungiro *et al.* (2019), using SNP (Single Nucleotide Polymorphism) markers. This was intended to provide valuable genetic diversity information for the future management of Nile tilapia (*Oreochromis niloticus*) in Tanzania. Study showed that genetic diversity that exist within and among Nile tilapia

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populations cultured in Tanzania which will inform fishery scientist on selection of base populations in breeding programmes. Anane-tabeah *et al.,* (2019) also used Microsatellite markers to investigate the invasion of Genetically Improved Farmed Tilapia (GIFT) strain in the lower Volta basin of Ghana. Findings showed that some selected farms of the study were growing non-native *O. niloticus* strains genetically distant from the Akosombo Strain.

In addition, Basiita, Zenger, Mwanja and Jerry (2018), investigated the population genetic structure of the Nile perch in six populations from Western and Eastern Africa using 19 polymorphic microsatellite loci using ten (10) species specific markers.

Polymerase Chain Reaction (PCR) in Microsatellite application

Polymerase chain reaction (PCR) is reaction which consist of nuclease-free water, forward and reverse primers, DNA material and PCR reagents which may include taq polymerase, PCR buffer, magnesium chloride, deoxynucleotide triphosphates (dNTP), or Sybr mix depending on the PCR protocol being used. For instance, reagent such as Sybr mix in the PCR reaction mix will exclude reagents such as PCR Buffer, MgCl2, dNTP and Target DNA Polymerase. Some reaction components such as Water (nuclease-free water, Forward primers, reverse primers and DNA) are constant for all PCR protocols. Conventional PCR is done at relatively low cost, and includes a unique combination of specificity and sensitivity coupled with great flexibility.

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The advent of PCR technology has led to a revolution in genetic studies worldwide. PCR has opened doors to areas hidden to all but a few for most of the history of genetics. The purpose of a PCR primer or microsatellite marker is to specify a unique address in the background of the target DNA. In order to do this, two aspects must be considered. First is the fragment length of the molecular marker and second is the actual sequence of the primer. Most studies on population genetics have employed conventional PCR in determining genetic diversity among species.

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CHAPTER THREE

RESEARCH METHODS

Microsatellite markers were used to determine the genetic variations and relationships among farmed *O. niloticus* populations studied in six regions of Ghana. Fish samples were collected from these six regions; Ashanti, Bono, Bono East, Ahafo, Eastern and Volta region and sent to the CSIR- WRI, Biomedical and Public Health Research Unit laboratory for molecular analysis. Conventional PCR technology was used to amplify fish DNA samples using Primers GM531, GM538, UNH154, UNH222 and UNH995 to obtain allele frequencies for all fish populations. Co-dominant data obtained were scored and analysed using GenALEx, MegaX software and Genpop on the web.

Study Area

A total of 16 farmed populations of *O. niloticus* (grow-outs) with average size of 250 - 500 grams were sampled from fish farms in Ashanti, Bono, Eastern, Bono East, Ahafo and Volta Regions (Figure 1). Population names as used in this study, were derived from a combination of the regions and districts from which the samples were collected, with the first three letters representing the regions and the remaining letters representing the district names as shown in Table 1. Fifty (50) percent of the total populations sampled were from the Ashanti Region; ASHOFM, ASHSES, ASHBOS1, ASHBOS2, ASHAAS, ASHEJJ1 and ASHEJJ2; In the Bono region, three populations, BONSUE, BONSUM and BONSUW were sampled; however, in the Eastern, Bono-East, Ahafo and Volta Regions, one population was sampled which

were; EASLOM, BOETEM, AHAASU and VOLNOT populations respectively. Global positioning system coordinates of the sampling sites are provided in Table 1.
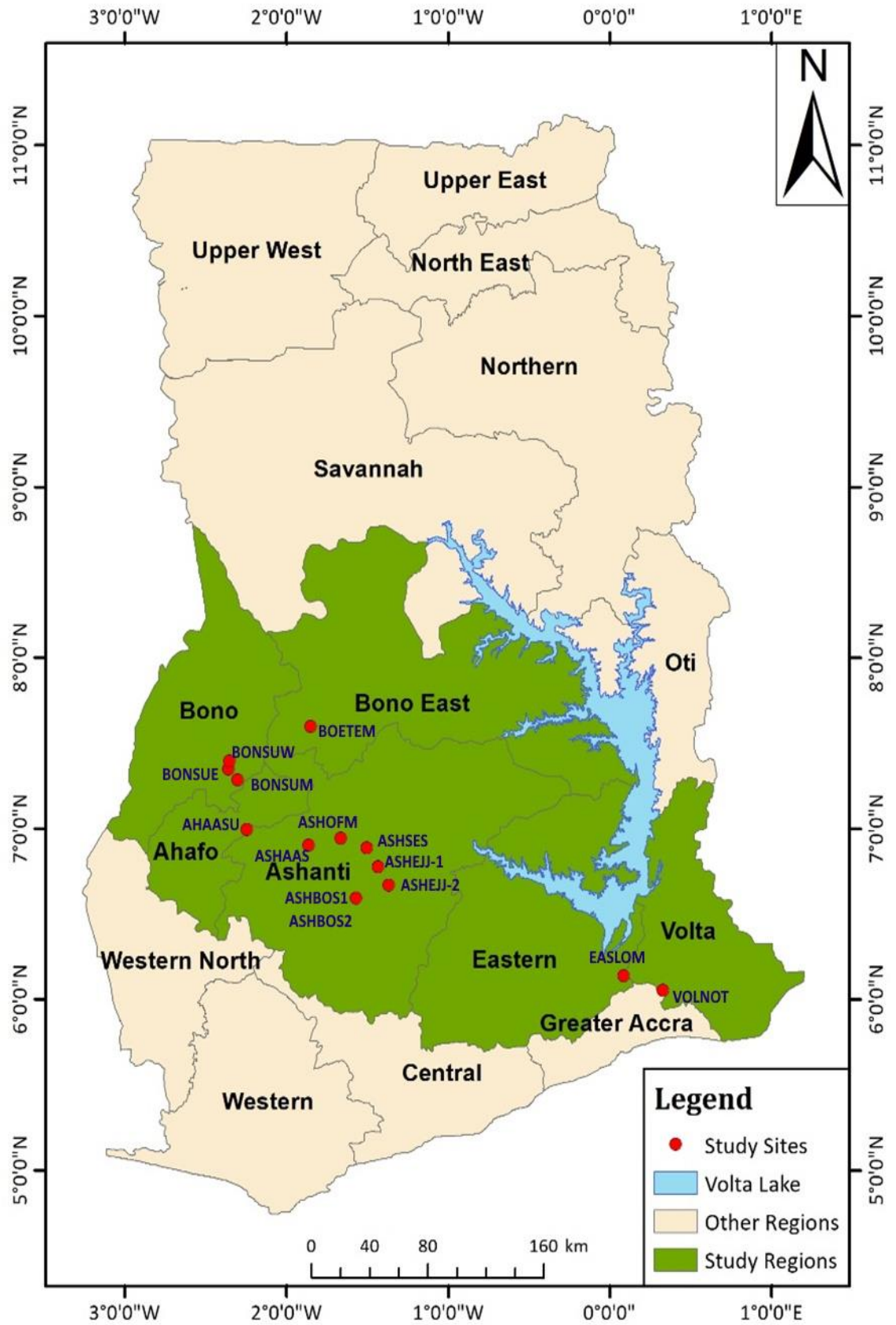
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Figure 1 : Map of Ghana showing fish sample collection sites.

Table 1 - GPS Locations of Aquaculture Farms Where Fish Samples Were
Collected

Name of Farm	Type of Farm	Region	District/ Municipal	Population Name	GPS
Romilla farms	Hatchery	Ashanti	Offinso Municipal	ASHOFM	Elev. 260m N 06°56.444' W 001°39.932'
Pilot Aquaculture Centre	Hatchery	Ashanti	Sekyere South	ASHSES	Elev. 292m N 06°53.136' W 001°30.222'
Bososmtwi Integrated Aqualife-Village (Grow-Out)	Grow-out	Ashanti	Bosomtwi	ASHBOS-1	Elev. 237m N 06°35.441' W 001°34.316'
Bososmtwi Integrated Aqualife Village (Hatchery)	Hatchery	Ashanti	Bosomtwi	ASHBOS-2	Elev. 237m N 06°35.441' W 001°34.316'
Jodaps farms	Grow-out	Ashanti	Ahafo Ano South	ASHAAS	Elev. 232m N 06°54.003' W 001°51.815'
Asomdwe farms	Grow-out	Ashanti	Ejisu-Juabeng	ASHEJJ-1	Elev. 271m N 06°46.429' W 001°26.201'
Oserbey Unique Ventures	Grow-out	Ashanti	Ejisu-Juabeng	ASHEJJ-2	Elev. 16.9km N 06°40'09° W 1°22'.13°
University of Energy and Natural Resources Demonstartion Farm	Hatchery	Bono	Sunyani East	BONSUE	Elev. 290m N 07°20.714' W 002°21.658'
Brit Addo farms	Hatchery	Bono	Sunyani Municipal	BONSUM	Elev. 259m N 07°16.924' W 002°18.210'
Dartah farms	Grow-out	Bono	Sunyani West	BONSUW	Elev. 263m N 07°23.667' W 002°21.247'
Alphonse farms	Grow-out	Bono East	Techiman Municipal	BOETEM	Elev. 316m N 07°35.830' W 001°51.169'
Frankoboam Fishery	Grow-out	Ahafo	Asutifi	AHAASU	Elev. 191m N 06°59.510' W 002°14.860'
University Cage Fish Farm	Grow-out	Eastern	Lower Manya	EASLOM	Elev. 10m N 06°08.167' E 000°05.056'
Xu Zhou Industrial Ltd	Grow-out	Volta	North Tongu	VOLNOT	Elev. 12m N 06°03.053'E 000°19.661'

Source: Field Data (2020)

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Sampling Procedure

Thirty (30) *O. niloticus* grow-outs were collected from each farm with average weights ranging between (250-500) grams were collected from the farms. Fish were sampled in the morning between 9:00GMT to 10:00 GMT using scoop net and placed in sampling bowls half filled with water to avoid stressing fish.

Fin clips were taken for each fish by cutting a portion of the caudal fin using a pair of pre-cleaned scissors and sterilized after each use with 70% ethanol to prevent cross-contamination of samples. After collection of fin clips, the fish were placed back into the ponds. Fin clip specimens of each fish was kept in a separate1.5ml Eppendorf tube containing 95% ethyl alcohol, labelled and transported to the CSIR- WRI Biomedical and Public Health Research Unit) for molecular analysis. This was carried in all farms where the samples were taken and analysed together with native genetically improved 'Akosombo strain' and non-native 'GIFT-D strain'.

DNA Extraction

Genomic DNA extracts were obtained from the fin-clips of *O. niloticus* using the *Quick*-DNA™ Miniprep Plus Kit D4068 by Zymo Research with modifications of the manufacturer's protocol. The volume of lysis buffer was increased from 150µl to 200µl in order to enhance the breakdown of fish tissues thereby making enough genetic material (DNA) available from the fin clips. Overnight incubation at 50 °C instead of 3hrs incubation was also adopted to enhance chemical breakdown of the tissue cells to increase the concentration of genetic material (DNA).

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1. 200µl of genomic lysis buffer and 5µl of proteinase K was measured into each sample.

2. Samples were vortexed for one (1) minute and incubated at 56°C for 12 hrs. (Overnight).

3. Samples were centrifuged at 10,000 rpm for Five (5) minutes

4. Samples (supernatants) were transferred into zymo-spin columns in collection tubes and centrifuged at 10,000rpm for 1 minute.

5. Flow through was discarded and 200µl of DNA pre-wash buffer was added and centrifuged at 10,000rpm for one (1) minute.

6. Flow through was discarded again and 500µl of Genomic DNA (g-DNA) wash buffer was added to the spin column and centrifuged for 1 minute at 14,000 rpm.

7. The spin column was then transferred into 1.5ml Eppendorf tube and 100µl of elution buffer was added.

8. After addition of elution buffer, it was incubated for five (5) minutes at room temperature.

9. The sample was then centrifuged at top speed (14,000 rpm) for one (1) minute to elute the DNA.

10. DNA elute was labelled as '1st elution' and kept in a refrigerator.

11. The spin column was removed and transferred into a different Eppendorf tube and the procedure in step nine was repeated to obtain the second DNA elute which was labelled as '2nd elution' and kept in refrigerator.

PCR Amplification

Five Microsatellite Markers (UNH541, UNH 222 and UNH 995, GM 531 and GM 538) previously used by (Ukenye, Taiwo, Oguntade, Oketoki, & Usman, 2015) and (Mireku *et al.,* 2017) were used in this study. A single locus PCR amplification was performed for each of the five microsatellite primers. The PCR 'Master Mix' or solution contained 5X Sybr Mix, 10µM forward primer, 10µM reverse primer Nuclease free water and 2.0µl DNA template.

Table 2 - *Master Mix with Concentrations of PCR Amplification*

Reagent	Conc. Initial	x1/µl	Conc. Final
Sybr Mix	5X	5	1X
Forward primer	10µM	0.2	0.2 µM
Reverse primer	10µM	0.2	0.2 µM
ddH$_2$O		2.6	
DNA Template		2	
	Total	10µl	

Source: *Bio- RAD* Laboratories. iQ ™ SYBR ® Green Supermix.

Polymerase Chain Reaction with few modifications to manufacturers protocol was carried out on all fish samples using the conventional PCR machine. Each microsatellite loci used had specific cycling conditions, notable amongst them was the annealing temperature which differed amongst the microsatellites that were used for the study. The annealing temperatures were 48.1°C, 49.3°C, 50.1°C, 53.4°C and 52.1°C for loci GM531, GM538, UNH154, UNH995, and UNH222 respectively. Conditions that were optimized also included the following:

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1. Adjustment of annealing temperatures. A gradient PCR was performed for the microsatellite loci that did not show any amplification. PCR analysis was done for a few selected numbers of fish samples at varying annealing temperatures (Tm) holding all other PCR conditions constant. The annealing temperature which gave the best amplification after the reaction was then adopted and used;

2. Increasing DNA volum0es in the Reaction Mix: For some of the farms, the volume of DNA was increased from 2µl to 3µl in order to increase the DNA concentration in the reaction mix since low DNA concentrations can result in no amplifications and

3. Increasing the cycling conditions: the cycling conditions for some microsatellite loci was increased from 30 cycles to 35 cycles in order to enhance DNA amplifications for some of the fish specimen that could not amplify.

Gel electrophoresis and Band visualisation.

At the end of the PCR reaction, two microliters of loading dye (6X) were added to ten microliters of the final PCR product thus homogenized PCR 'reaction mix' and centrifuged at 8000rpm for about 30 seconds. The addition of the loading dye renders the PCR product denser than the running buffer in order for the products to sink to the bottom of the well when loaded. The essence of the addition of bromophenol blue in the dye also assists in tracking the loaded PCR product when being run on the gel. All PCR products were separated on 3% agarose gel.

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Three percent (3%) agarose gel was prepared using 3grams of agarose powder dissolved in 100ml TBE (Tris-borate EDTA) and heated using the conventional microwave for four minutes. The mixture was allowed to cool for 20minutes and then stained with 4µl ethidium bromide. The mixture was swirled to ensure complete dissolution of the ethidium bromide because it intercalates with the DNA molecules in the PCR product to make it visible under UV radiation. The prepared gel was casted in a gel tray aligned with gel combs to create wells within the gel. After 25 minutes, the gel was removed from the tray and placed in the gel tank containing TBE buffer. DNA is negatively charged and therefore the PCR product is negatively charged. The PCR products mixed with loading dye was loaded into the gel wells and allowed to run on electric fields moving from the positively charged electrodes to the negatively charged electrodes. The Gel was allowed to run for one hour at 100V to separate the alleles of different fragment sizes and the resulting amplifications visualized as bands using a UV Trans illuminator (BioDoc-it imaging system, Upland, USA).

Data Processing and Analysis

Allele patterns (bands) visualized were scored as 150bp, 200bp, 300bp and 500bp and expressed as codominant data in GenAIEx 6.502 software (Peakall & Smouse, 2012). This was used to determine inter-populations differentiation (Gst) and Shannon's information index (I). It was also used to estimate the genetic variations amongst the populations by estimating mean number of alleles (Na) per locus, number of effective alleles (Ne), observed

heterozygosity (Ho), and expected heterozygosity (He). These parameters were further used to estimate Wrights F statistics (F_{ST}, F_{IT}, F_{IS}).

The pairwise F_{ST} values were used to generate a matrix on the number of migrants exchanged per generation (Nm). The genepop on the web (Raymond & Rousset, 1995) was used to estimate the Polymorphism information content (PIC) value of the microsatellite loci used for the study. Nei's genetic distance (D) and Nei's genetic identity (I) were calculated by the GenAIEx 6.502 software and exported to Mega X (Tamura, Dudley, Nei, & Kumar, 2007) from which a phylogenic tree was constructed using Neighbour-joining method generated by Mega X of phylogeny tree construction to determine the relationships or closeness that exist among the farm populations. The following are the formulae of the indices that were used to determine genetic variations among the populations;

Mean number of alleles (Na) per locus: <u>Total number of alleles at all loci</u>

Number of loci

Allele frequency: <u>Number of individual genotypes</u>

Number of alleles

Heterozygosity index: $p^2+2pq+q^2=1$ and $1-\sum_{i=1}^{K} pi^2$

where the heterozygosity is given by $2pq$. The rest of the expression $(p^2 + q^2)$ is the homozygosity also in the second formulae p_i is the frequency of the ith of k alleles.

Shannon diversity: $H = \Sigma pi \log_2 pi = 3.3219 (\log_{10}N - N1s\Sigma n1 \log_{10} ni) i = 1$

Where, N = total number of alleles ni = number of copies of the ith allele

s = number of alleles.

Standard error of the mean (SE): Standard error of the mean is the standard deviation divided by the square root of the number of individuals.

$SE = \dfrac{s}{\sqrt{n}}$

Wrights F statistics (F_{ST}, F_{IT}, F_{IS}): Variation among subpopulations and total populations. The equation for the genetic structure of populations is;

$(1 - F_{IT}) = (1 – F_{IS}) (1 – F_{ST})$.

F_{IT}: $F_{IT} = 1 – (H_I/H_T)$

F_{IS}: $F_{IS} = 1 – (H_I/H_S)$

Where, H_T= total gene diversity or expected heterozygosity in the total population as estimated from the pooled allele frequencies

H_I= intrapopulation gene diversity or average observed heterozygosity in a group of populations

H_S= average expected heterozygosity estimated from each subpopulation $_{ST}$:

$F_{ST} = 1 – (H_S/H_T)$

Genetic distance also provides a way of measuring the probability of encounter between equal alleles. The statistical indices include;

F_{IS}= the deficiency or excess of average heterozygotes in each population

F_{ST}= the degree of gene differentiation among populations in terms of allele frequencies

F_{IT}= the deficiency or excess of average heterozygotes in a group of populations

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Chapter Summary

The study focused on six administrative regions in Ghana as mentioned in the sampling procedure. Genetic variations of Nile tilapia populations were determined in fish ponds which were sampled. The criteria for selection of fish farm populations were based on the prevalence of Nile tilapia pond culture in the selected administrative regions in Ghana. The limitation of the study was that ponds sampled did not cover all sixteen administrative regions of the country.

The core activities that were carried out involved the cutting of fin clips for each fish on the field, preserving the fin clip specimens in 1.5 Eppendorf tube filled with 95% ethanol and transportation of the specimens to Biomedical and Public Health Research Unit laboratory of the CSIR-WRI for molecular analysis. All the samples were screened with the five microsatellite markers involving DNA extraction, Conventional PCR technology, Gel electrophoresis and visualization of Gel images. Allele frequencies were obtained for each population using the GenAlEx statistical software package to compute genetic parameters to determine variations in heterozygosity, Number of alleles, number of effective alleles, and Shannon index amongst the fish populations. Pairwise genetic distance and genetic identity were computed in GenAlEx which was used to draw a dendrogram showing genetic relationships amongst the fish populations using Mega X software.

CHAPTER FOUR

RESULTS

This Chapter presents the results of the study based on the objectives of the study. Results for five microsatellite markers used in the study are presented to determine genetic variations amongst some farmed *O. niloticus* populations in Ghana. Two of the markers (GM531, and GM538) were more polymorphic compared to the other three; UNH154, UNH222, UNH995 which were less polymorphic. Genetic parameters which included mean number of alleles per locus, number of effective and private alleles, Heterozygosity index and Shannon diversity were also analysed using GenALEx statistical package. MegaX and genepop on the web were used to determine variations among sixteen (16) populations. Relationships among the sixteen (16) populations studied were established using pairwise genetic distance and pairwise genetic identity.

Locus Variability

Two of the markers (GM531, and GM538) were more polymorphic as represented in Figure 5 and Figure 6 compared to the other three (UNH154, UNH222, UNH995) which were less polymorphic as represented in Figure 2, Figure 3, and Figure 4. Loci GM531 expressed four different allele patterns which were similarly observed in loci GM538 whereas loci UNH154, UNH222, and UNH 995 had three alleles. Alleles 150,200,300 and 500 were shared by loci GM531 and GM 538 whereas alleles 150,200 and 300 were shared by loci UNH 154 and UNH 222. Loci UNH995 had allele 150, 200, and

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500. Alleles 150 and 200 were shared by all the microsatellite loci when screened against all the sixteen (16) populations.

Table 3 showed gene diversity at the various loci. GM538 and GM531 recorded a high gene diversity of 0.444 and 0.430 respectively whereas UNH154, UNH222 and UNH995 recorded low gene diversity of 0.181, 0.137 and 0.058 respectively. The Polymorphism Information Content (PIC) values showed similar trends with GM538 and GM531 being the highest at 0.7173 and 0.6411 respectively and lowest at 0.2771, 0.2272 and 0.0882 for UNH154, UNH222 and UNH995 respectively. Heterozygosity was also high in GM538 and GM531 at 0.406 and 0.316 and lowest at 0.052 in UNH995.
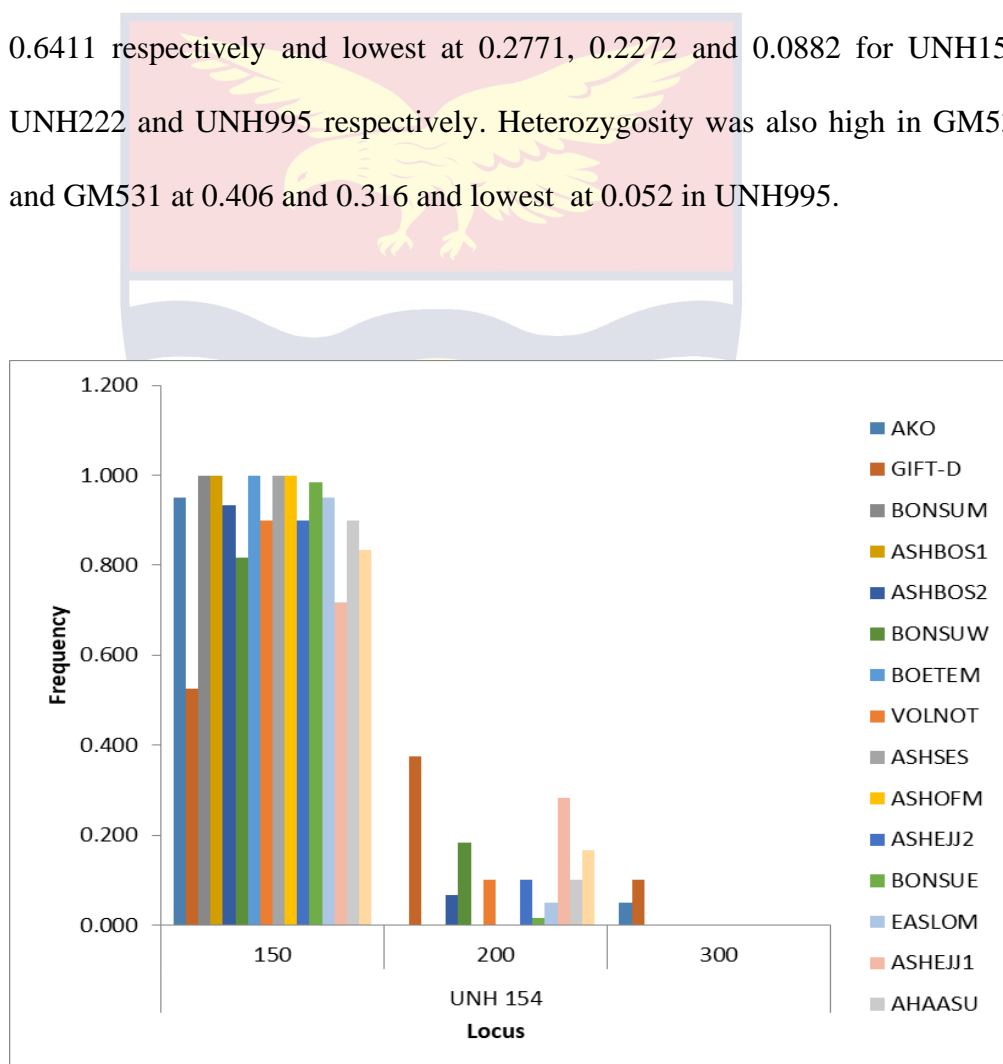

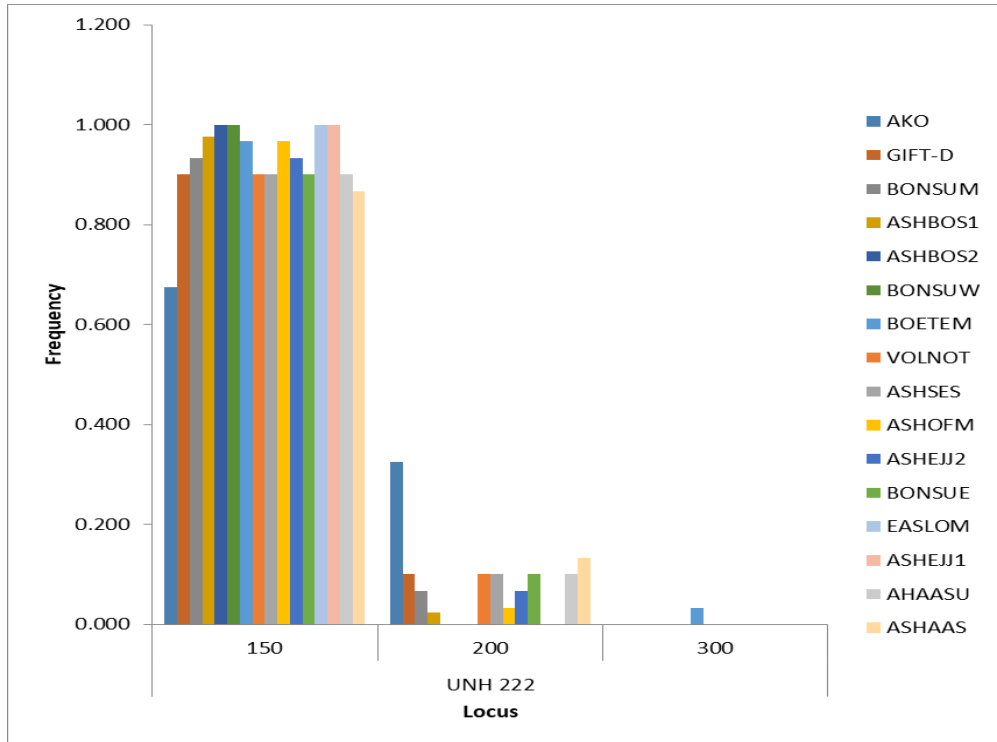*Figure 2* : Allele frequency of 16 farmed fish populations for Loci UNH154.

Figure 3 : Allele frequency of 16 farmed fish populations for loci UNH222.
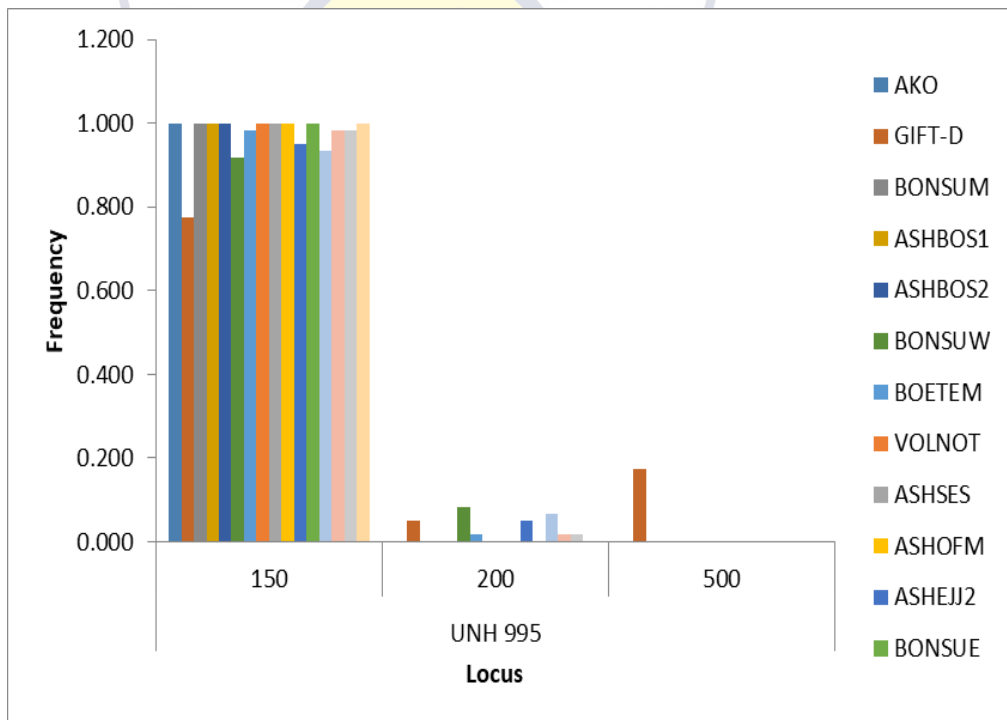

Figure 4 : Allele frequency of 16 farmed Nile tilapia populations for loci UNH995.

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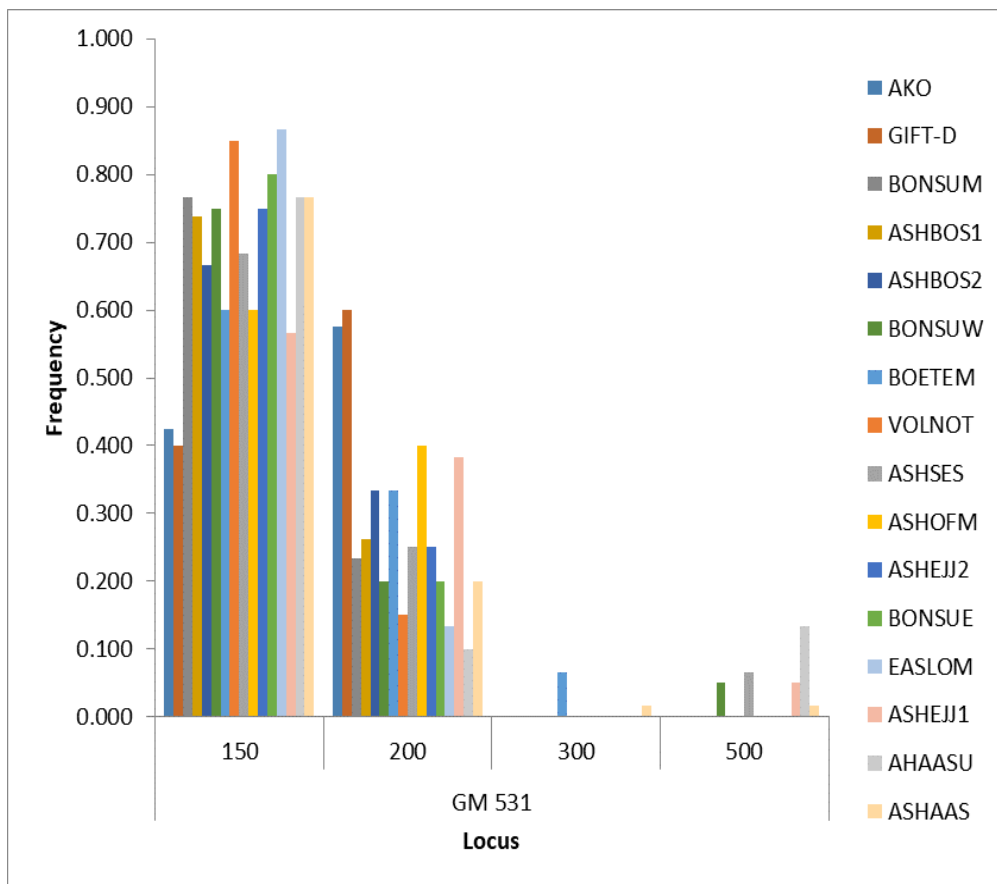
Figure 5 : Allele frequency of 16 farmed Nile tilapia populations for Loci GM 531.

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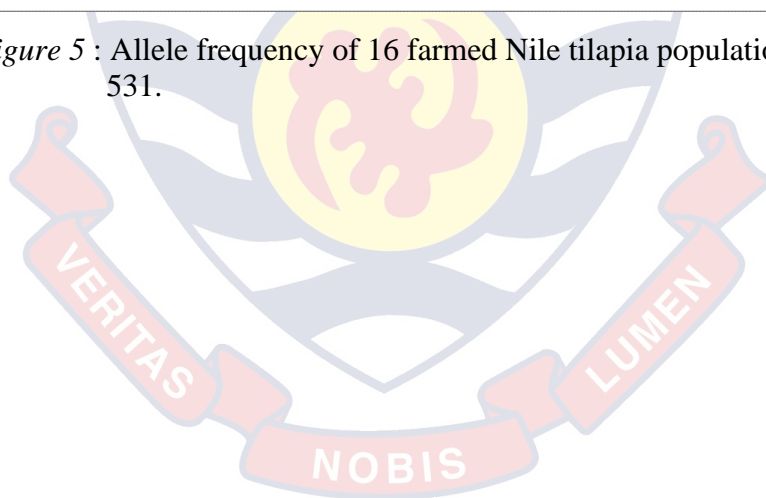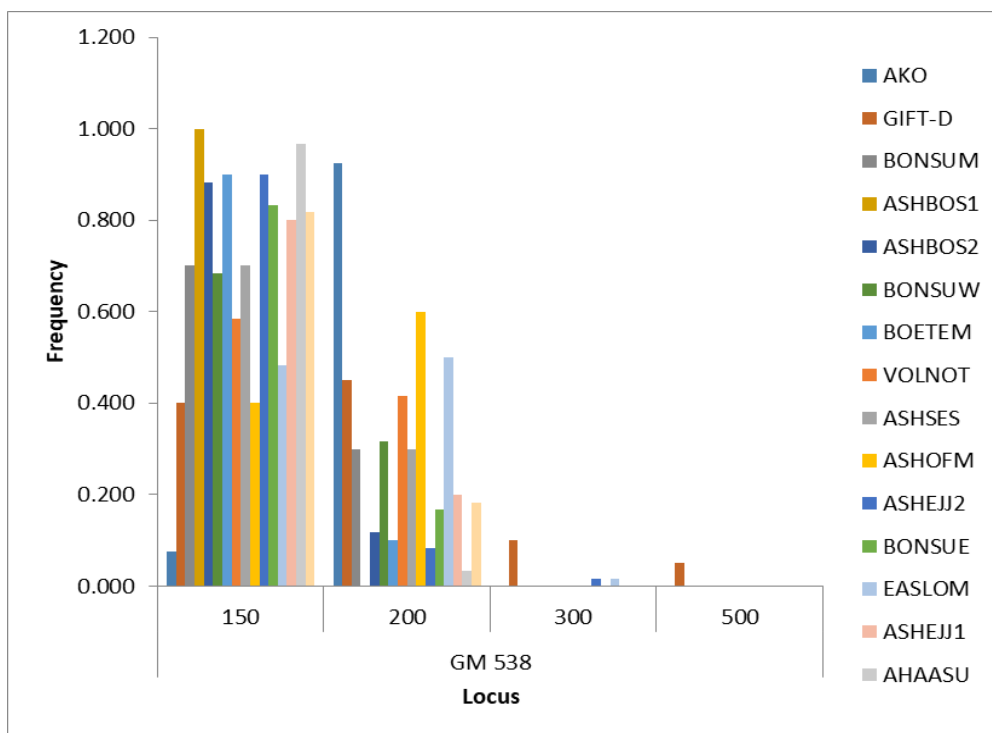
Figure 6 : Allele frequency of 16 farmed Nile populations for Loci GM 538.

Table 3 - *Total Gene diversity, Observed Heterozygosity (Ho), Expected Heterozygosity (He) and Polymorphism Information Content (PIC) for all Loci.*

Locus	Gene Diversity	Ho	He	PIC
UNH 154	0.181	0.117	0.153	0.2771
UNH 222	0.137	0.044	0.125	0.2272
UNH 995	0.058	0.055	0.052	0.0882
GM 538	0.444	0.374	0.406	0.7173
GM 531	0.430	0.153	0.316	0.6411
Mean	0.250	0.148	0.210	0.3902

Source: Field Data (2020)

Intra-population Diversity

The mean number of alleles per locus (Na) and number of effective alleles (Ne), number of private alleles (Np), and Fixation index were estimated for all sixteen (16) populations as presented in Figure 7. ASHBOS1

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populations (BIA-B farms) recorded the least Number of alleles (Na) of 1.600 whiles the highest, 2.800 was recorded in 'GIFT-derived' Populations. BONSUW (DA), BOETEM (ALP), ASHEJJ2 (OSE), EASLOM (UGK), ASHEJJ1 (ASO), AHAASU (FRA) and ASHAAS(JOD) populations also recorded higher Na values when compared to ASHBOS1(BIA-B) populations. The number of effective alleles (Ne) was lowest in ASHBOS1 (BIA-B) population at 1.136 and highest at 1.947 in the 'GIFT - derived' population. Most of the fish populations however recorded (Ne) values that ranged from 1.200 to 1.300.

The high number of alleles (Na) and effective alleles (Ne) recorded in 'GIFT- derived' population were evident in the heterozygosity values recorded in the GIFT populations (Figure. 8). The observed heterozygosity in the 'GIFT- derived' population was 0.550 which was highest amongst all the *O. niloticus* populations followed by 'Akosombo strain' populations with observed heterozygosity of 0.250. BONSUW (DA) population had the next highest heterozygosity of 0.213 whilst the other populations recorded observed heterozygosity within the range of 0.100 to 0.200. Similar trend was recorded for the expected heterozygosity amongst the sixteen populations. BONSUW (DA) populations recorded an expected heterozygosity of 0.256 and therefore succeeded 'GIFT derived' populations which had the highest expected heterozygosity of 0.445. 'The expected heterozygosity for Akosombo Populations was 0.232 whiles VOLNOT (XZ), ASHSES (PAC) and ASHOFM (ROM) populations were 0.220, 0.213 and 0.205 respectively. All the other populations recorded expected heterozygosity below 0.200. GIFT-D, AKO and ASHBOS1 (BIA-B) populations recorded negative FIS values of -

0.228, -0.071, -0.066 respectively indicating excess heterozygosity in these three Nile tilapia populations. However, 'GIFT derived' and Akosombo populations were the only populations which had a greater observed heterozygosity than expected heterozygosity
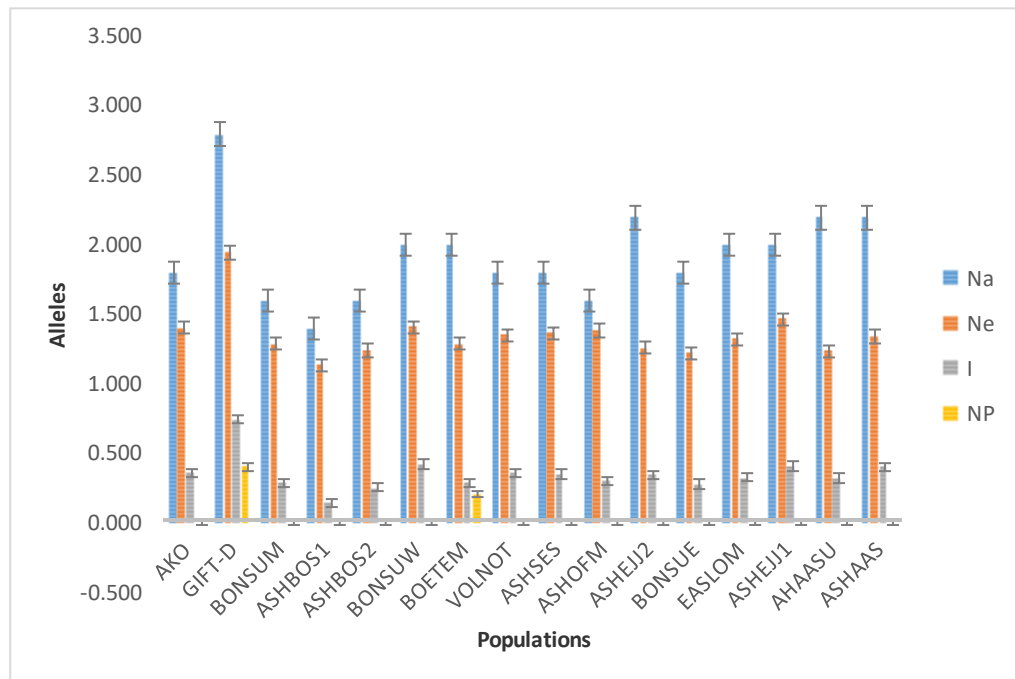
Figure 7: Allelic patterns showing genetic variations amongst 16 Nile tilapia populations studied

Table 4 : *Shannon Diversity Index Recorded for Each Farmed Population of O. niloticus Studied*

Populations	Mean (I) Value
AKO	0.355
GIFT	0.739
BOETEM	0.280
ASHBOS1	0.138
ASHBOS2	0.248

Source: Field Data (2020)

Table 4: *Continued*

Populations	Mean (I) Value
BONSUW	0.415
BOETEM	0.282
VOLNOT	0.350
ASHSES	0.345
ASHOFM	0.298
BONSUM	0.340
BONSUE	0.272
EASLOM	0.320
ASHAAS	0.404
AHAASU	0.317
ASHEJJ1	0.396

Source: Field Data (2020)

The mean Shannon index was determined for all the populations. GIFT-D had the highest Shannon diversity of 0.739. BONSUW (DA) had the high Shannon diversity of 0.415. ASHAAS (JOD), ASHEJJ1(ASO) and AKO populations recorded relatively high Shannon diversity of 0.404,0.396 and 0.355 respectively. Shannon diversity recorded in the other populations were low when compared to AKO populations.

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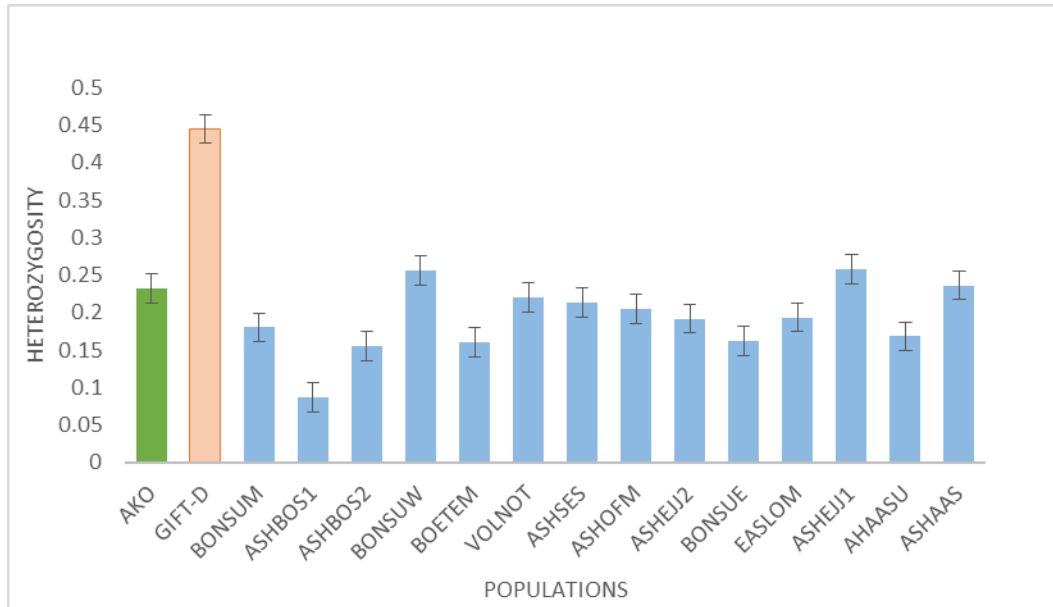
Figure 8 : Graph showing expected heterozygosity in all 16 Nile tilapia
populations studied.
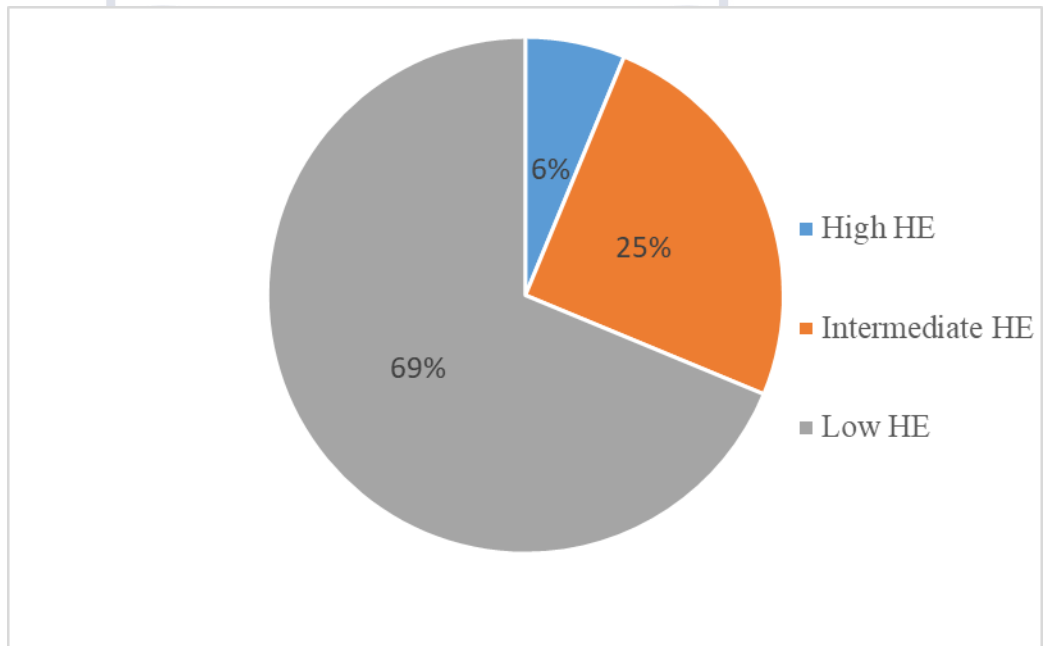
Figure 9*: Pie chart showing categories of heterozygosity levels among 16*
populations studied.

About 70% of the *O. niloticus* populations had low and heterozygosity as shown in figure 9. Low Shannon diversity values were also recorded in about 70% of the populations as presented in table 4. The results indicated that low heterozygosity and low Shannon diversity exist among the 16 populations studied.

Inter-Population Diversity

Inbreeding co-efficient of the five markers shown in table (4) were calculated as inbreeding co-efficient of individual relative to the total sub-population (F_{IS}), individual relative to total population (F_{IT}) and sub-population relative to the total population (F_{ST}) as well as the number of effective migration (Nm). These parameters measured the amount of subdivision in populations. The mean F_{ST} values of all five markers was 0.140.

Table 5: *Inbreeding Coefficient Values of the Five Microsatellite Loci*

Locus	F_{IS}	F_{IT}	F_{ST}	Nm
UNH 154	0.236	0.355	0.155	1.360
UNH 222	0.650	0.682	0.091	2.489
UNH 995	-0.056	0.049	0.099	2.285
GM 531	0.079	0.159	0.087	2.611
GM 538	0.515	0.644	0.267	0.687
Mean	0.285	0.378	0.140	1.886

Source: Field Data (2020)

Genetic distance and population clustering

The phylogenetic tree for the sixteen populations generated from Nei genetic distance in Figure (5) clearly shows three clusters. the first cluster included ASHBOS1, ASHEJJ2, BOETEM, ASHEJJ1, ASHSES, GIFT-D, ASHBOS2 and BONSUW populations. The second cluster included ASHOFM, BONSUE, EASLOM, AKO and ASHAAS populations whiles the third populations comprised of VOLNOT, BONSUM and AHAASU populations. ASHBOS2 populations paired closely with 'GIFT-D' populations in the first cluster whereas EASLOM and ASHAAS populations paired closely with AKO populations in the second cluster.

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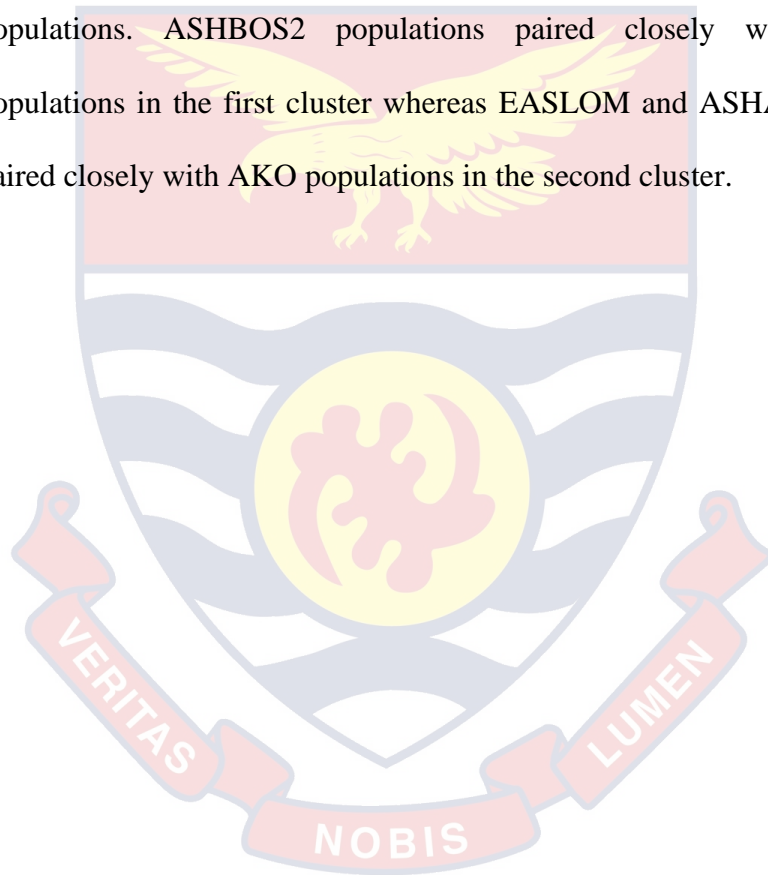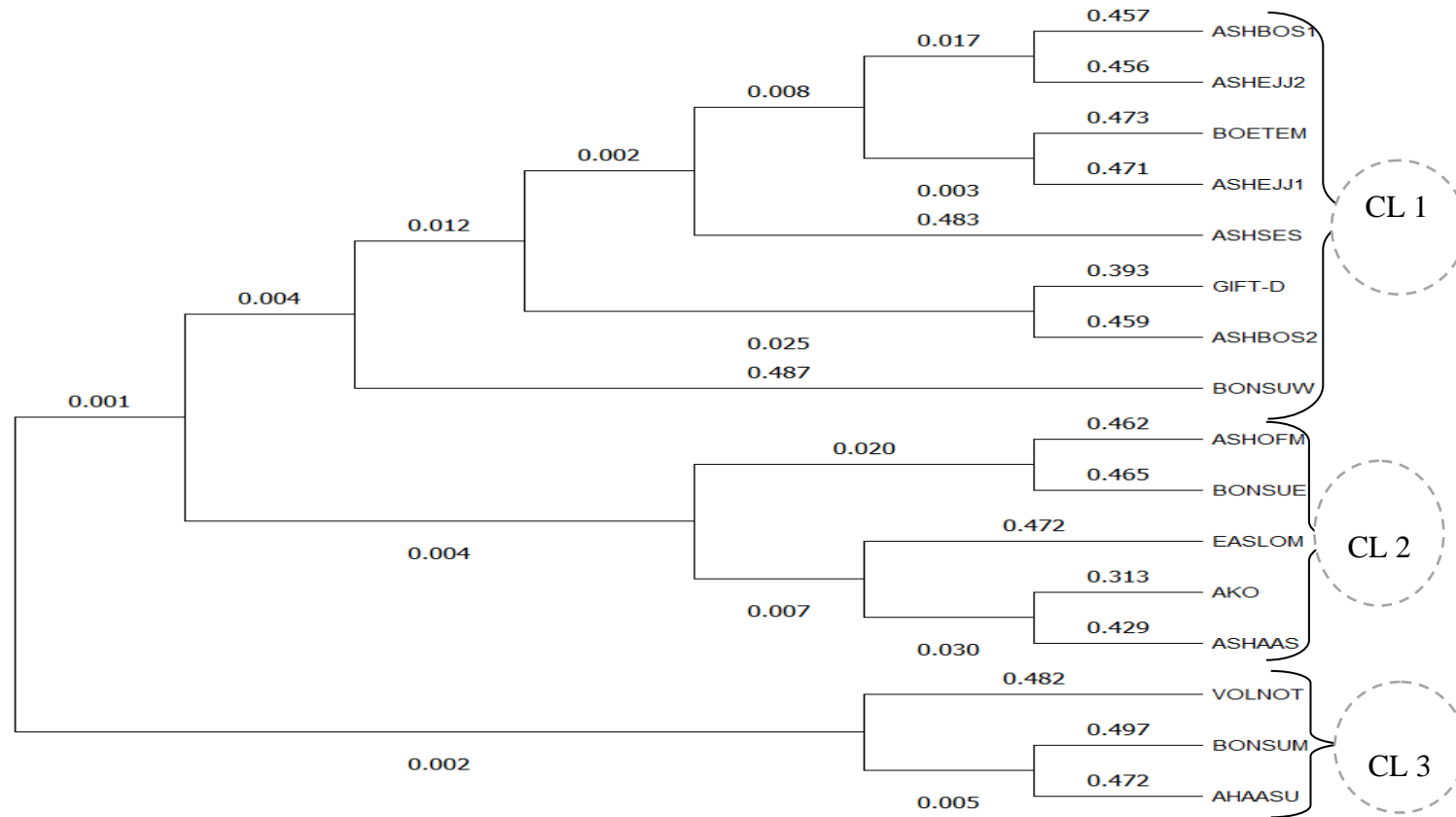
Figure 10 : Phylogenetic tree and branch lengths using Neighbour-joining method generated by Mega X (Tamura *et al.,*2007) showing clusters and relationships among 16 farmed Nile tilapia populations studied.

CHAPTER FIVE

Discussion

Genetic variation studies in individuals of a population, among populations, and sub-populations is one of fundamental subjects of population genetics (Bezault *et al.,* 2011). Such studies help to identify genetic differences that exist between individuals and sub-populations. These studies help to describe genetic differences in and among populations using markers which may be: morphological, meristic, biochemical and in recent times, molecular (Abban, 1988) with microsatellite markers amongst most recent (Gao *et al*., 2013).

Locus variability

The study showed genetic differences or variations among the sixteen populations when screened with five (5) microsatellite markers. GM 531 and GM538 as shown in Figure 5 and Figure 6 were more efficient in determining genetic variations among the populations because these markers showed variable levels of polymorphism, compared to UNH154, UNH222, and UNH995 which were monomorphic (Mireku *et al.,* 2017), as shown in Figure 2, Figure 3 and Figure 4.

High Polymorphism information content (PIC) of 0.7173 and 0.6411 and high gene diversity of 0.406 and 0.316 were recorded for GM531 and GM538 respectively whereas low PIC values of 0.2771, 0.2272 and 0.0882 were recorded in UNH154, UNH222 and UNH995 respectively as presented in Table 3. Low gene diversity of 0.181, 0.137, and 0.058 were respectively recorded in these microsatellite markers. Polymorphism Information Content

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(PIC) value of a marker refers to the ability of a marker to express different forms of alleles at a particular gene locus of an organism within a population. This describes the ability of a marker to distinguish between closely related populations. Microsatellite markers with high PIC values are therefore employed in genetic variation studies than microsatellite markers with low PIC values. Mireku *et al.* (2017), also compared variable levels of loci using their PIC values and gene diversity. Microsatellite markers with high PIC values were used to assess genetic variations among *O. niloticus* populations compared to microsatellite markers with low PIC values.

Inter-population diversity

Heterozygosity is an important measure of population diversity at the genetic level (Mu *et al*. 2011), because it helps to determine genetic variations among populations based on allele patterns expressed among the populations (Gu *et al.,* 2014).

High Shannon information Index and high expected heterozygosity were observed in GIFT-D, AKO, BONSUW, ASHEJJ1 and ASHAAS populations. Allele numbers obtained for each locus were used to estimate Shannon diversity and heterozygosity for each population. GIFT-D populations recorded the highest Shannon diversity of 0.739 and heterozygosity of 0.445 among the sixteen (16) populations studied. High heterozygosity and Shannon diversity of GIFT-D populations can be attributed to genetic modifications of the GIFT strain in selective breeding programs for the past thirty (30) years (Falk & Abban, 2004). High Shannon diversity within a population suggests high genetic variability within a population (Lind

et al., 2019). GIFT-D populations were more genetically diverse than all the populations studied.

Four other populations i.e., AKO, BONSUW, ASHEJJ1 and ASHAAS populations recorded high Shannon diversity of 0.355, 0.415, 0.396 and 0.404 respectively. Shannon diversity recorded in these four populations indicated high genetic variability among these populations (Koseman *et al.,* 2020). The remaining populations; BOETEM, ASHBOS1, ASHBOS2, BOETEM, VOLNOT, ASHSES, ASHOFM, BONSUM, BONSUE, EASLOM, and AHAASU recorded low Shannon diversity when compared to AKO populations. Low Shannon diversity indicated low genetic variability among these populations according to Koseman *et al.,* and therefore it is possible these populations share similar genetic materials. Majority of the populations studied had low genetic variability which is possibly due to inbreeding among *O. niloticus* pond fish farms where the populations were sampled. Farmers within these farms are likely to experience slow growth of fishes which will lead to low production since growth performance of fish is influenced by its genetic makeup and environment. AKO, BONSUW, ASHEJJ1, and ASHAAS populations had high heterozygosity of 0.232, 0.256, 0.258 and 0.236 respectively. This is an indication of how effective the genetic characteristics of these populations have been managed (Holsinger & Weir, 2009; Whitlock 2011). This further shows that selection pressure for characters such as growth has not affected the gene pool of the strain suggesting that a strain has not lost alleles through random genetic drift.

Heterozygosity and Shannon diversity recorded in ASHAAS, ASHEJJ1 and BONSUW populations similar to AKO population is an

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indication that these three farms obtain their fingerlings from farms growing 'Akosombo strain' and are practicing effective brood stock farm management (Holsinger & Weir, 2009; Whitlock, 2011). Majority of the populations recorded low heterozygosity and low Shannon diversity values which could be possibly due to continuous use of same brood stock by farmers for long periods which has reduced genetic variation among the populations due to inbreeding among the populations.

Other deductions could be that, populations with low heterozygosity and Shannon diversity with reference to the third cluster where populations did not group with AKO and GIFT-D populations have genetic similarities with wild *O. niloticus* from the Volta Lake. *O. niloticus* in the Volta Lake of Ghana are not improved strains for culture as in the case of 'Akosombo strain' and 'GIFT strain'. Studies by Lind *et al.* (2019), showed that *O. niloticus* from the Volta Lake have naturally low heterozygosity. The Akosombo strain however, has a high heterozygosity because of the base population assemblage which was obtained by selecting *O. niloticus* populations from different geographic locations within the country. This genetic improvement of *O. niloticus* for culture was initiated by Attipoe *et al.,* (2015) through a natural selective breeding program which was aimed at enhancing the growth performance of the fish for culture.

High heterozygosity in the GIFT-D populations compared to AKO populations was confirmed by the fixation indices values (F_{ST} value), and negative FIS values is an indication of excess heterozygosity. This suggests that sexual selection, mutation or migration, the allele frequencies and the genotype frequencies are not constant from generation to generation as

expected under Hardy-Weinberg equilibrium (HWE) hence high genetic diversity amongst these two populations.

The observed and expected heterozygosity revealed that the GIFT-D populations are more genetically diverse compared to Akosombo populations, this is further confirmed by Shannon diversity being high in GIFT-D populations compared to AKO populations.

The results showed an opposite trend in ASHAAS, ASHEJJ1 and BONSUW populations though high heterozygosity was recorded, Fis values were positive an indication of reduced genetic diversity when compared to AKO and GIFT-D populations. ASHBOS1 farms on the contrary recorded a negative Fis value of -0.066 with a low heterozygosity of -0.066 which implied excess of heterozygosity especially when there are alleles with low frequencies (Crow & Kimura, 1970).

Furthermore, Locus UNH995 recorded a negative FIS value. This suggested that there is no inbreeding amongst some fish farm populations which possibly accounted for the high heterozygosity values recorded in the AKO, GIFT-D, BONSUW, ASHEJJ1 and ASHAAS populations. Most of the loci, UNH 154, UNH 222, UNH 995, and GM 538 recorded a positive FIS values, indicating presence of inbreeding amongst most of the populations under study hence the reason for the low heterozygosity values recorded amongst most of the fish farms (Crow & Kimura, 1970).

Genetic distance and clustering

Diagrammatic representation of groupings and linkages among the 16 populations studied is shown in figure 10. The groupings were generated by

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Mega X using Neighbour - joining method (Tamura *et al.,* 2007) program based on genetic distances which ranged from 0.001 to 0.497 between and among the populations studied. The Genetic similarity among different populations were determined using both genetic distance and genetic identity. These two parameters are inversely proportional. Two genetically similar populations have a high genetic identity and a low genetic distance.

Each branch length in the phylogenetic tree represents the number of genetic changes that occurred in the sequences prior to the next level of separation. Two populations at the end of the phylogeny tree are more closely genetically related if they share a recent common linkage because they have a more recent common ancestor, and less closely genetically related if they do not share a recent common linkage because they have a less recent common ancestor.

The results in Figure 10 showed that there were very closely genetically related groups. These consisted of the following populations: ASHBOS1 and ASHEJJ2; BOETEM and ASHEJJ1; GIFT-D and ASHBOS2; ASHOFM and BONSUE; AKO and ASHAAS; and BONSUM and AHAASU. In the first grouping both populations were sampled from farms located within the Ashanti region of Ghana as represented in Figure 1 hence most expected these populations are genetically closely related. This observation was however not so for the other closely genetically related groups. In the second group, BOETEM population were sampled from Bono East whereas ASHEJJ1 were sampled from Ashanti region, Similarly, 'GIFT-D' populations obtained from ARDEC in Eastern region paired closely with ASHBOS2 population located in Ashanti region. ASHOFM population from

Ashanti region also paired closely with BONSUE in the Bono region, 'AKO' populations also from ARDEC also paired closely with ASHAAS population from Ashanti region. BONSUM populations from Bono region also paired closely with AHAASU population from Ahafo region.

This is a clear indication that *O. niloticus* populations studied do not follow logical groupings per the regions from where the populations were sampled. It was expected that genetically close linkages will be between populations sampled from farms located within the same region which was not the case. Most of the close linkages were observed between populations sampled from farms located in different regions.

This could be attributed to the transportation of fingerlings from one farm to the other which has led to low genetic differentiation of farmed *O. niloticus* populations in the regions where the study was conducted (Basiita *et al.,* 2018). According to Wang (2013), geographic distance affects the level of genetic differentiation among populations but close linkages in figure 10 showed an opposite trend.

This confirms mix-up of *O. niloticus* populations in Ghana and the extent to which it has affected genetic differentiation of Nile tilapia populations in most parts of the country. The implication is that fish farmers located within the study region will have challenges in acquiring quality seed and brood stock for culture. There is a possibility that most of these farms have inbred lines based on study findings and therefore may not exhibit the desired growth performance.

GIFT-D and AKO populations were formed on different clusters on the phylogenetic tree as presented in Figure 10. Three clusters were formed on

the phylogenetic tree with most of the populations clustered around GIFT-D populations. ASHBOS1, ASHEJJ2, BOETEM, ASHEJJ1, ASHSES, ASHBOS2 and BONSUW clustered with 'GIFT-D' Populations whereas ASHAAS, EASLOM, BONSUE and ASHOFM were clustered with AKO populations. Three populations namely, VOLNOT, BONSUM and AHAASU however formed a separate cluster in the phylogenetic tree.

ASHBOS2 population which paired closely with GIFT-D suggest that GIFT-D populations share similar genetic materials with ASHBOS2 population because clustering of populations confirms the presence of same alleles (Avise, 1994). Populations that share a more recent common ancestor are also very genetically close than populations that do not share a more recent common ancestor. (Nei, 1987). Fish populations clustered with GIFT-D populations is possibly an indication that the farmers are growing the 'GIFT strain' or its derivatives. Initial studies by Osei-Atweneboana *et al.* (2019), revealed some Chinese farms in Ghana growing derivatives of the 'GIFT strain'. Findings showed two *O. niloticus* populations sampled from Chinese farms closely linked to the 'GIFT strain' in the phylogenetic tree. It is therefore possible that *O. niloticus* populations that clustered with GIFT-D populations in this study are farms that obtain *O. niloticus* fingerlings or brood stocks from these Chinese farms located in the Eastern region for culture (Osei-Atweneboana *et al.*, 2019). ASHAAS also paired closely with AKO populations which confirmed genetic similarities between these two *O. niloticus* populations (Avise, 1994). It is possible fish farmers were growing the 'Akosombo Strain' or derivatives of the 'Akosombo strain' in farms that clustered with AKO populations.

The last cluster however comprised of three populations; VOLNOT, BONSUM and AHAASU. These three populations have diverged from the phylogenetic tree and therefore may be closely related to wild *O. niloticus* populations in the Volta Lake which are not improved strains for culture. Farmers located within farms where these populations were selected could be culturing *O. niloticus* that are genetically similar to wild *O. niloticus* populations in the Volta Lake (Lind *et al*., 2019). Farmers within this category may therefore experience low production in their farms.

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

This chapter consist of the summary, conclusions and recommendations based on the findings of the study in consistency with the objectives of the study as well as the research questions and research hypothesis that guided the study.

Summary

Results on genetic differentiation among the populations sampled and clusters from the phylogenetic tree indicated genetic variation among the populations which was confirmed by the clustering patterns in the study. It was evident that high genetic variation does not exist among the farmed *O. niloticus* populations cultured in ponds within the six regions studied. Majority of the populations had low genetic variations existing among.

Microsatellite markers were suitable tools to characterize *O. niloticus* populations in Ghana. These markers were also efficient in determining *O. niloticus* populations that had genetic similarities with indigenous 'Akosombo strain' and non-native 'GIFT' strain.

Findings from the study suggested that *O. niloticus* farmed in Ghana are not only derived from 'Akosombo strain', some farmers are culturing the GIFT or derivatives of the GIFT strain which are not approved strain for culture. Clustering pattern further indicated that *O. niloticus* populations in Ghana are mixed; low genetic variability exists among different populations found in different geographic locations. This will make quality brood stock acquisition difficult for farmers located within the six regions studied as

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inbreeding is likely to occur within most of the farms. Success of production cannot be easily predicted.

Conclusions

Results obtained from the study showed that *O. niloticus* sampled genetically were made up of three clusters. The First cluster involved 'GIFT-D' populations i.e., *O. niloticus* populations which originated from a Chinese farm in the Eastern region of Ghana. The second cluster involved populations close to AKO while the third cluster involved *O. niloticus* populations which were neither close to AKO nor GIFT-D populations.

The result from the clustering also indicated that *O.niloticus* populations are genetically mixed. Population from Bono-East region will be genetically similar to population from Ashanti region. the implication is that inbreeding is likely to occur among *O. niloticus* farms located among different regions. Farmers producing fish within these regions will experience low growth and survival rates which will affect production. Fishery scientist will also find it difficult to carryout breeding programmes because of low genetic differentiation among populations.

In conclusion, low genetic variations exist among most of the farmed Nile tilapia studied, and the genetic linkage or relationship among the populations implies that general perception by most farmers that *O. niloticus* farmed in Ghana is solely from Akosombo strain genetically is erroneous; which also suggest that success of production from different farms will not be easily predictable.

Recommendations

1. Future studies should be extended to cover other regions not covered by the current study. This would further advance knowledge in the field and give a very clear picture with respect to genetic variations among farmed *O. niloticus* populations in Ghana.

2. The sign of inbreeding among the Nile tilapia populations studied could slow down growth and reduce overall production and fitness within population. Nile tilapia farmers should be educated (through workshops and seminars) and advised to desist from producing their own brood stock and rely in certified brood stocks from recognized institutions such as Aquaculture Research and Development Centre (ARDEC) Akosombo.

3. Assessment of genetic variations among other important commercially farmed species cultured in Ghana such the African Catfish- *Clarias gariepinus* should be undertaken as a matter of urgency, this would inform scientists and fish farmers to know the status and adapt good culture practices to ensure high production levels.

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APPENDICES

Appendix 1 - *Allele Frequencies of the Sixteen (16) Fish Populations per Microsatellite Marker*

Locus	Allele	AKO	GIFT	BA	BIA-B	BIA-F	DA	ALP	XZ	PAC	ROM	OSE	UEN	UGK	ASO	FRA	JOD
UNH 154	150	0.950	0.525	1.000	1.000	0.933	0.817	1.000	0.900	1.000	1.000	0.900	0.983	0.950	0.717	0.900	0.833
	200	0.000	0.375	0.000	0.000	0.067	0.183	0.000	0.100	0.000	0.000	0.100	0.017	0.050	0.283	0.100	0.167
	300	0.050	0.100	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
UNH 222	150	0.675	0.900	0.933	0.976	1.000	1.000	0.967	0.900	0.900	0.967	0.933	0.900	1.000	1.000	0.900	0.867
	200	0.325	0.100	0.067	0.024	0.000	0.000	0.000	0.100	0.100	0.033	0.067	0.100	0.000	0.000	0.100	0.133
	300	0.000	0.000	0.000	0.000	0.000	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
UNH 995	150	1.000	0.775	1.000	1.000	1.000	0.917	0.983	1.000	1.000	1.000	0.950	1.000	0.933	0.983	0.983	1.000
	200	0.000	0.050	0.000	0.000	0.000	0.083	0.017	0.000	0.000	0.000	0.050	0.000	0.067	0.017	0.017	0.000
	500	0.000	0.175	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GM 531	150	0.425	0.400	0.767	0.738	0.667	0.750	0.600	0.850	0.683	0.600	0.750	0.800	0.867	0.567	0.767	0.767
	200	0.575	0.600	0.233	0.262	0.333	0.200	0.333	0.150	0.250	0.400	0.250	0.200	0.133	0.383	0.100	0.200
	300	0.000	0.000	0.000	0.000	0.000	0.000	0.067	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017
	500	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000	0.067	0.000	0.000	0.000	0.000	0.050	0.133	0.017
GM 538	150	0.075	0.400	0.700	1.000	0.883	0.683	0.900	0.583	0.700	0.400	0.900	0.833	0.483	0.800	0.967	0.817
	200	0.925	0.450	0.300	0.000	0.117	0.317	0.100	0.417	0.300	0.600	0.083	0.167	0.500	0.200	0.033	0.183
	300	0.000	0.100	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.017	0.000	0.000	0.000
	500	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Source: Field Data (2020)

Appendix 2- *Some Genetic Parameters Recorded Amongst the Sixteen Populations*

PARAMETER		AKO	GIFT	BA	BIA-B	BIA-F	DA	ALP	XZ	PAC	ROM	OSE	UEN	UGK	ASO	FRA	JOD	TOTAL
N	MEAN	20.000	20.000	30.000	21.000	30.000	30.000	30.000	30.000	30.000	30.000	30.000	30.000	30.000	30.000	30.000	30.000	28.188
	SE	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.425
NA	MEAN	1.800	2.800	1.600	1.400	1.600	2.000	2.000	1.800	1.800	1.600	2.200	1.800	2.000	2.000	2.200	2.200	1.925
	SE	0.200	0.374	0.245	0.245	0.245	0.316	0.316	0.200	0.374	0.245	0.200	0.200	0.316	0.316	0.200	0.490	0.075
NE	MEAN	1.401	1.947	1.285	1.136	1.240	1.405	1.285	1.345	1.363	1.383	1.258	1.222	1.323	1.463	1.233	1.341	1.352
	SE	0.195	0.259	0.150	0.124	0.148	0.142	0.208	0.160	0.184	0.221	0.088	0.093	0.192	0.210	0.105	0.097	0.043
NP	MEAN	0.000	0.400	0.000	0.000	0.000	0.000	0.200	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	SE	0.000	0.245	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
I	MEAN	0.355	0.739	0.280	0.138	0.248	0.415	0.282	0.350	0.345	0.298	0.340	0.272	0.320	0.404	0.317	0.396	0.344
	SE	0.131	0.134	0.130	0.112	0.120	0.125	0.152	0.109	0.159	0.155	0.063	0.099	0.128	0.158	0.108	0.109	0.032
HO	MEAN	0.250	0.550	0.067	0.095	0.113	0.213	0.087	0.080	0.140	0.067	0.127	0.087	0.107	0.187	0.113	0.093	0.148
	SE	0.106	0.100	0.042	0.084	0.061	0.081	0.051	0.034	0.098	0.042	0.078	0.051	0.036	0.138	0.089	0.046	0.022
HE	MEAN	0.232	0.445	0.180	0.087	0.155	0.256	0.160	0.220	0.213	0.205	0.191	0.162	0.193	0.258	0.168	0.236	0.210
	SE	0.097	0.080	0.089	0.076	0.082	0.080	0.096	0.079	0.100	0.113	0.049	0.064	0.089	0.104	0.062	0.063	0.021
FIS	MEAN	-0.071	-0.228	0.717	-0.066	0.231	0.089	0.433	0.631	0.484	0.769	0.448	0.417	0.306	0.286	0.480	0.644	0.343
	SE	0.019	0.063	0.112	0.027	0.131	0.205	0.193	0.136	0.240	0.095	0.233	0.200	0.146	0.260	0.254	0.130	0.050

Na = number of different alleles; Ne = number of effective alleles; I = Shannon's Information Index; He = expected heterozygosity or gene diversity; Ho = observed heterozygosity; Fis = fixation index
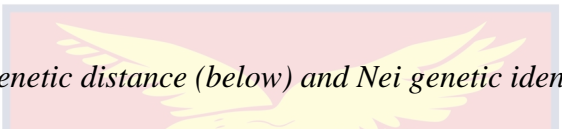
Appendix 3. *A matrix of pairwise Nei genetic distance (below) and Nei genetic identity (above) for all Sixteen Populations.*

	AKO	GIFT-D	BONSUM	ASHBOS1	ASHBOS2	BONSUW	BOETEM	VOLNOT	ASHSES	ASHOFM	ASHEJJ2	BONSUE	EASLOM	ASHEJJ1	AHAASU	ASHAAS
AKO		0.882	0.855	0.754	0.797	0.832	0.795	0.872	0.863	0.943	0.778	0.809	0.878	0.807	0.742	0.807
GIFT-D	0.125		0.892	0.852	0.894	0.918	0.880	0.899	0.895	0.928	0.879	0.870	0.894	0.939	0.842	0.892
BONSUM	0.156	0.114		0.980	0.987	0.989	0.984	0.992	0.998	0.971	0.986	0.995	0.984	0.969	0.976	0.989
ASHBOS1	0.283	0.160	0.020		0.995	0.970	0.995	0.955	0.979	0.913	0.996	0.992	0.936	0.970	0.992	0.986
ASHBOS2	0.227	0.112	0.013	0.005		0.983	0.997	0.968	0.987	0.941	0.996	0.992	0.952	0.987	0.986	0.990
BONSUW	0.183	0.086	0.011	0.030	0.017		0.974	0.989	0.986	0.960	0.984	0.984	0.984	0.983	0.974	0.989
BOETEM	0.229	0.128	0.016	0.005	0.003	0.026		0.959	0.986	0.938	0.992	0.990	0.944	0.977	0.982	0.983
VOLNOT	0.138	0.107	0.008	0.046	0.033	0.011	0.042		0.989	0.972	0.970	0.983	0.994	0.958	0.961	0.983
ASHSES	0.147	0.111	0.002	0.021	0.013	0.014	0.014	0.011		0.972	0.985	0.994	0.978	0.970	0.976	0.988
ASHOFM	0.059	0.074	0.030	0.091	0.061	0.040	0.064	0.028	0.029		0.927	0.943	0.978	0.937	0.901	0.937
ASHEJJ2	0.251	0.129	0.014	0.004	0.004	0.016	0.008	0.030	0.015	0.076		0.996	0.952	0.981	0.994	0.995
BONSUE	0.211	0.139	0.005	0.008	0.008	0.016	0.011	0.017	0.007	0.058	0.004		0.967	0.969	0.991	0.995
EASLOM	0.130	0.112	0.016	0.066	0.049	0.016	0.058	0.006	0.022	0.022	0.049	0.034		0.941	0.938	0.962
ASHEJJ1	0.215	0.063	0.032	0.030	0.014	0.017	0.023	0.043	0.030	0.065	0.019	0.031	0.060		0.967	0.982
AHAASU	0.298	0.172	0.025	0.008	0.014	0.027	0.018	0.040	0.024	0.104	0.006	0.009	0.064	0.033		0.991
ASHAAS	0.214	0.114	0.011	0.014	0.010	0.012	0.017	0.017	0.013	0.065	0.005	0.005	0.038	0.019	0.009	