CHAPTER I

INTRODUCTION

1.1 Introduction

Aquaculture has been growing more rapidly than any other animal food producing sector in the world. Most of the world's recent increases in per capita food fish supply have been obtained from aquaculture. Worldwide, more than 1 billion people rely on fish as an important source of animal protein, healthy lipids and essential micronutrients (FAO, 2010). Fish is particularly important in the diet of people in the world's poorest countries, supplying more than 50% of their animal protein intake (World Bank, 2009). Attention to fish production and consumption is vital to achieving one of the Millennium Development Goals- that of eradicating hunger. The target is to halve, between 1990 and 2015, the number of undernourished people from 800 million to 400 million (Pullin, 1982). Enhancing access of the poor to the food they need and creating livelihood opportunities to hasten their exit from poverty are part of the current fight against global hunger and extreme poverty.

During 1970-2000, global aquaculture production grew at an average annual rate of 9.2%, compared with only 1.4% for capture fisheries and 2.8% for terrestrial farmed meat production (FAO, 2006). In 2000, global aquaculture production was 45.7 million metric tons (t), valued at $56.5 billion. Finfish accounted for 23 million tons, or about half of total aquaculture production. In the past three decades, aquaculture has expanded intensified and made major technological advances. Most of the world's recent increase in per capita food fish supply has been obtained from aquaculture (Hulata, 2001).

The fisheries and aquaculture sector remains of fundamental importance to the Asia-Pacific region. Production from both capture fisheries and aquaculture has grown since 2002 (3 % for capture fisheries and 14 % for aquaculture). In 2010, the region contributed 49 % of the global production of captured fish (46.7 million tonnes) and 91 % of global aquaculture (54.3 million tonnes) (Lowe-McConnell, 2010). Both capture fisheries and aquaculture sectors continue to be of fundamental importance to the Asia-Pacific region in terms of food security, revenue generation and employment. In many of the countries, catching or farming aquatic resources forms a vital part of rural people's livelihoods. Fisheries and aquaculture also have deep cultural significance and are more than just a source of income or food supply; traditional fishery products such as fish sauce and fish-based condiments have always been important ingredients of people's daily diet which are not easily substituted. All sizes and types of fish are utilized in a wide variety of ways and very little is discarded or wasted. The role that fish play in both the food security and nutritional security of many rural and coastal populations was often nderestimated in the past (Lowe-McConnell, 2010)

In Malaysia, aquaculture plays an important role in the economy besides providing fish as a source of food and protein. Aquaculture contributes 1.73% in 2011 compared to 1.37% in 2003 to GDP. This sector also provided direct employment to 89,453 fishermen and 21,507 fish culturists. The aquaculture sector recorded a production of 202,225 tonnes, which constituted about 13.2% of the total fish production, increasing by 2.72% from the production in 2009. However, the value from the aquaculture sector increased by 7.86% from RM1, 172.3 million in 2003 to RM 1.264.5 million in 2009 (FAO, 2010)

Freshwater aquaculture contributed 27.5% of the total aquaculture production in 2010, increasing by 11.23% from 49,947 tonnes to 55,556 tonnes in 2010. Freshwater pond culture spearheaded production from culture systems with 70.6% (39,233.43 tonnes) of the total freshwater production. The major freshwater species cultured were Red Tilapia (12,709 tonnes), Black Tilapia (2,664 tonnes) and River Catfish (2,558 tonnes) in year 2010. Production from ex-mining pools increased by 33.11% from 6,495 tonnes in 2010 to 8,646 tonnes in 2011. Major species produced were Red Tilapia (4,082.26 tonnes) followed by Big Head Carp (1,817.75 tonnes) and Black Tilapia (1,206.54 tonnes) (FAO, 2006)

Unlike crops and livestock, most farmed fish have very short histories of domestication and genetic improvement and many still resemble closely their wild relatives. Asia provides more than 80% of the world's farmed fish. Until relatively recently, most were grown from wild fish seed (fry and fingerlings) or from the progeny of captive spawners (called broodstock) that were managed with little or no application of genetics (Ponzoni, 2006) Production of fish seed in hatcheries and the ability to grow successive generations of broodstock to sexual maturity began in the 1970s for most Chinese and Indian carps and in the 1980s and 1990s, respectively, for farmed shrimp (*Penaeidae*) and milkfish (*Chanos chanos*). The world's first International Symposium on Genetics in Aquaculture was convened in 1982. Up to the mid-1980s, most aquaculture research and development (R&D) was targeted at seed production technology and improved fish husbandary rather than at genetic improvement. (Pullin, 1982)

The application of genetics in aquaculture still lags behind its application in terrestrial crops and livestock. As evidenced through the development of Genetically Improved Farmed Tilapia (GIFT), the rewards for aquaculture from public and private investment in genetic improvement of farmed fish can be substantial (Khaw et al,.2010). The need for increased investment in fish genetic resources conservation, combined with their use in genetic improvement research and fish breeding programs, is extensive and immediate.

With increasing popularity among consumers, tilapia has become the world's second most popular farmed fish, after carps (World Bank, 2010). Global production of farmed tilapia exceeded 1.5 million tons (t) in 2010, valued at about $2.0 billion (World Bank, 2010). Tilapias are farmed in at least 85 countries, with most production coming from the developing countries of Asia and Latin America. The global supply of farmed tilapia surged in the 1990s and early 2000s, largely due to genetic improvements through conventional breeding methods, widespread introductions of improved tilapia breeds; feed supply availability, effective management of reproduction through sex reversal and hybridization and expansion of consumer markets. Asia and Latin America dominated the world's top producers of farmed tilapia (Ponzoni, 2006). There is scope for further genetic improvement of farmed tilapia for improved feed conversion and growth using plant – based feeds, as well as for dressing weight and other performance traits, including cold tolerance and saltwater tolerance. GIFT and GIFT derived strains are currently a good basis for the pursuit of further genetic improvement of farmed tilapia.

The use of reproductive and genetic technologies can increase the efficiency of selective breeding programs for aquaculture species. Four technologies have been widely practised, namely marker assisted selection, DNA fingerprinting, in-vitro fertilization and cryopreservation. Marker assisted selection can result in greater genetic gain, particularly for traits difficult or expensive to measure, than conventional selection methods but its application is currently limited by lack of high density linkage maps and by the high cost of genotyping. DNA fingerprinting is most useful for genetic tagging and parentage verification. Both in- vitro and cryopreservation techniques can increase the accuracy of selection while controlling accumulation of inbreeding in long-term selection programs (Nguyen et al, 2006).

Selective breeding in aquaculture species has been very successful, averaging a genetic gain of 10 to 20 % per generation (Ponzoni, 2006 & Dey et al,.2010). Such progress has been achieved through the application of quantitative genetics and statistical methods, whereby genetically superior animals are identified based on their own performance or that of their relatives. Recently the advent of molecular genetics has opened possibilities for direct selection of animals on genotype or alternatively, selection based on linkage associations between markers and quantitative trait loci (QTL) (Nguyen *et al*, 2006).

1.1.1 Objectives of the study

The main objective of this study was to evaluate the four stocks of red tilapia (*Orechromis sp*) in a diallel cross with regard to growth rate assessment and determining the genetic variability of the four populations stocks by microsatellite analysis.

The objectives were:

i) To estimate the mean observed effective allele numbers and heterozygosity levels in Tilapia populations.

ii) To determine whether the sampled populations conformed to Hardy-Weinberg equilibrium.

iii) To measure genetic distance and evaluate the relationship among the four sampled populations.

iv) To evaluate the growth and survival rate of the four tilapia.

1.1.2 Justification of this Study

Justification of this study is to increase production efficiency and to estimate genetic parameters for growth and survival of the four stocks.

CHAPTER II

LITERATURE REVIEW

2.1 Life History, Biology of Tilapia and suitability for aquaculture.

Worldwide harvest of farmed tilapia has now surpassed 800,000 metric tons and tilapia is second only to carps as the most widely farmed freshwater fish in the world (Frank, 2000). Positive aquaculture characteristics of tilapia are their tolerance to poor water quality and the fact that they consume a wide range of natural food organisms (Lowe-McConnell, 2006).

2.1.1 Taxonomy and distribution Of Tilapia

Tilapia is a generic term used to designate a group of commercially important food fish belonging to the family Cichlidae; the expression derived from the African native Bechuana word “thiape”, meaning fish. Cichlids are classified in the large order Perciformes, and inhabit the fresh and brackish waters of Africa, the Middle East, coastal India, Central and South America (Frank, 2000)

Cichlids are well known as colourful aquarium fish and for their ability to adapt to new environments. Cichlids also display highly organized breeding activities. Because of their complex evolutionary biology, cichlid classification and naming is one of confusion and constant modification (Lowe-McConnell, 2006). Consequently, the tilapias have recently been classified into three genera. The most commercially important tilapia genera are: *Oreochromis*, *Tilapia* and *Sarotherodon*. In addition to anatomical characteristics, criteria for genetic distinction include the following differences in their reproductive biology: *Tilapia* (sub-strate spawners), *Sarotherodon* (paternal or biparental mouthbrooders) and *Oreochromis* (maternal mouth-brooders) (Frank, 2000) The genus *Oreochromis* is the largest, with

approximately 79 species, followed by *Tilapia* with approximately 41 species and the genus *Sarotherodon* with approximately 10 species. *Oreochromis* is typical of the rivers and lakes of East and Central Africa and the Jordan valley. *Tilapia* distribution coincides with that of *Sarotherodon* and in addition with *Oreochromis* in the Zambezi basin and southwards. Nile tilapia (*Oreochromis niloticus*), Mozambique tilapia (*Oreochromis mossambicus*) and blue tilapia (*Oreochromis aureus*) are the most commercially important species found in the genus *Oreochromis*. Red tilapia was first isolated in Taiwan by crossing a red *O. mossambicus* with an *O. niloticus*. They are currently produced in the USA, Philippines, Greece, Israel, Jamaica, India and other tilapia producing countries (Wohlfarth et al., 1990).

2.1.2 Physical Characteristics

Tilapia are shaped much like sunfish or crappie but can be easily identified by an interrupted lateral line characteristic of the Cichlid family of fishes. They are laterally compressed and deep bodied with long dorsal fins. The forward portion of the dorsal fin is heavily spined. Spines are also found in the pelvis and anal fins. There are usually wide vertical bars down the sides of fry, fingerlings and sometimes adults (Popma & Masser, 2000).

2.1.3 Banding Patterns and Colouration

The main cultured species of tilapia usually can be distinguished by different banding patterns on the caudal fin. Nile tilapia has strong vertical bands, Blue tilapia has interrupted bands and Mozambique tilapia has weak or no bands on the caudal fin. Male Mozambique tilapia also has upturned snouts. Colour patterns on

the body and fins also may distinguish species. Mature male Nile has gray or pink pigmentation in the throat region, while Mozambique tilapia has more yellow colouration. However, colouration is often an unreliable method of distinguishing tilapia species because environment, state of sexual maturity and food source greatly influence colour intensity. The “red” tilapia has become increasingly popular because its similar appearance to the marine red snapper gives it a higher market value (Wohlfarth et al.,1990).

2.1.4 Reproduction

A distinguishing characteristic between the genera is the type of care the parents provide to their young. In the species of genera *Sarotherodon* and *Oreochromis* the parents will incubate and protect the young in their mouths (mouth brooding); in particular the *Oreochromis* species are distinguished by maternal mouth- brooding where parental care of the young is performed primarily by the female. A female lays her eggs in a simple nest, the male fertilizes the eggs and then the female picks the eggs up and incubates them in her mouth. Even after the eggs hatch, the fry will remain in the mouth. Once the fry are free swimming they will still return to her mouth for protection (Popma & Masser, 1990).

In contrast, incubation of eggs in lake or pond bottom built-in “nest” is exhibited by those species belonging to the genus *Tilapia*. They also invest in the care of the young by producing large eggs and then protecting the embryos and fry in the nest. Females can produce several hundred to several thousand young fries per spawn (Frank, 2000).

Tilapia are known for their ability to sexually mature at a small size, around 8-10 cm (3-4 in) in body length and at a young age (2-3 months old). Adult fish are known to live six to eight years, but some fish eleven to twelve years of age have been reported. In temperate regions, the spawning season of tilapia usually begins during the spring months when water temperatures rise and spawning continues throughout the year as long as water temperature are above 22 ^{0}C (Popma & Masser, 1990)

Depending on age, body size and mode of egg incubation, female tilapias have a large variation in the number of eggs they produced. Blue female tilapia are reported to lay around 9-10 eggs per gram of body weight (around 4,500 eggs/pound). The eggs of red tilapia are yellow-brown in colour, egg shaped and will sink to the bottom when spawned. The eggs vary in size from an average of 2 to 4 mm (0.08-0.16 in) in diameter, depending on the species and number of spawns. After fertilization, eggs hatch in 2 to 4 days, depending on water temperature. Newly hatched embryos absorb their yolks for 3 to 4 days. After yolk absorption, young tilapias actively feed on varied diet, such as plankton and detritus (Wohlfarth et al., 1990).

2.1.5 Feeding behavior and nutrition requirements

Tilapia ingests a wide variety of natural food organisms, including plankton, some aquatic macrophytes, planktonic and benthic aquatic invertebrates, larval fish, and detritus decomposing organic matter. With heavy supplemental feeding, natural food organisms typically account for 30 to 50 % of tilapia growth (Popma & Masser, 1990).

Hybrid tilapia has high efficiency to convert feed into meat. With the use of high quality formulated diets, the feed conversion should range between 0.33 to 0.67 g of fish weight gain per gram of practical diet consumed. Young hybrid tilapias are easily weaned and grow fast to market size when fed formulated diets. Fast growth rates are common when fish are fed foodstuffs containing levels of 35-50% protein for fish of < 1g (0.04 oz); 30 -40 % for 1-5 g (0.04-0.18 oz) fish and 25-30 % for 5-25 g (0.18-0.88 oz) animals. For larger fish, recommended dietary levels vary from 25% protein for fish raised in ponds, 28-32% when reared in cages, to 35-40% when fish are grown in tanks (Popma & Masser, 1990).

2.1.6 Environmental Requirements

Tilapias are more tolerant than most commonly farmed freshwater fish to high salinity, high water temperature, low dissolved oxygen and high ammonia concentrations (Popma & Masser, 1990).

2.1.6 (a) Salinity

All tilapia are tolerant to brackish water. The Nile tilapia is the least saline tolerant of the commercially important species, and grows well at salinities up to 15 ppt. The Blue tilapia grows well in brackish water up to 20 ppt salinity and the Mozambique tilapia grows well at salinities near or at full strength seawater. Therefore, the Mozambique tilapia and some mossambicus derived “red” tilapia are preferred for saltwater culture (Popma & Masser, 1990).

2.1.6 (b) Water temperature

The tolerance of tilapia to low temperatures is a serious constraint for commercial culture in temperate regions. The lower lethal temperature for most species is 50 ^{0}C to 52 ^{0}C for few days but the Blue tilapia tolerates temperatures to about 48 ^{0}C. (Frank, 2000)

Tilapias generally stop feeding when water temperature falls below 63 ^{0}C. Disease-induced mortality after handling seriously constrains sampling, harvest and transport below 65 ^{0}C. Reproduction is best at water temperatures higher than 80 ^{0}C and does not occur below 68 ^{0}C. In subtropical regions with a cool season, the number of fry produced will decrease when daily water temperature averages less than 75 ^{0}C. (Frank, 2000)

Optimal water temperature for tilapia growth is about 85 ^{0}C to 88 ^{0}C. Growth at this optimal temperature is typically three times greater than at 72 ^{0}C (Popma & Masser, 1990).

2.1.6 (c) Dissolved oxygen concentration

Tilapia survive routine dawn dissolved oxygen (DO) concentrations of less than 0.3 mg/L, considerably below tolerance limits for most other cultured fish. In research studies, Nile Tilapia grew better when aerators were used to prevent morning DO concentrations from falling below 0.7 to 0.8 mg/L (compared with unaerated control ponds). Growth was not further improved if additional aeration kept DO concentrations above 2.0 to 2.5 mg/L. Although tilapia can survive acute low DO concentrations for several hours, tilapia ponds or tanks should maintain DO concentrations above 1mg/L. Metabolism, growth and disease resistance are

depressed when DO falls below this level for prolonged periods (Popma & Masser, 1990).

2.1.6 (d) pH

In general, tilapia can survive in pH ranging from 5 to 10 but do best in a pH range of 6 to 9 (Popma & Masser, 1990).

2.1.6 (e) Ammonia

Massive mortality of tilapia occurs within a few days when fish are suddenly transferred to water with unionized ammonia concentrations greater than 2mg/L. However, when gradually acclimated to sub-lethal levels, approximately half the fish will survive 3 or 4 days at unionized ammonia concentration as high as 3mg/L. Prolonged exposure (several weeks) to un-ionized ammonia concentration greater than 1 mg/L causes losses, especially among fry and juveniles in water with low DO concentration. The first mortalities from prolonged exposure may begin at concentrations as low as 0.2mg/L. Un-ionized ammonia begins to depress food consumption at concentrations as low as 0.08mg/L (Popma & Masser, 1990).

2.1.7 Diseases

Tilapia is more resistant to viral, bacterial and parasitic diseases than other commonly cultured fish, especially at optimum temperatures for growth. Lymphocystis, columnaris, whirling disease and hemorrhagic septicemia may cause high mortality, but these problems occur most frequently at water temperatures below 68 ^{0}C. “Ich”, caused by the protozoan *Ichthyopthirius multifiliis*, can cause serious losses of fry and juveniles in intensive recirculation systems (Popma &Masser, 1990).

External protozoan such as *Trichodina* and *Epistylis* also may reach epidemic densities on stressed fry in intensive culture. In recent years the bacterial infection *Steptococcus inae* has caused heavy losses, primarily in recirculating and intensive flow-through systems (Popma & Masser, 1990).

2.1.8 Stocking Density

Recommended stocking densities for table-size tilapia production are extremely variable and vary according to fish size and system of production. (Agresti et al., 2000; Barman & Little,2011) In fed and aerated production ponds, young (50g) tilapia usually stocked at 9,500 to 19,500 fish per hectare. In tanks, tilapia of 25-50 g in body size is stocked at densities between 140 and 248 fish/m^3 of container space. In final grow-out production cages, stoking densities for 60-100 g tilapia range from 250 to 400 fish per cubic meter of cage (Frank, 2000).

2.1.9 Yields in aquaculture

Tilapia is a good fish for warm water aquaculture. They are easily spawned, use a variety of natural foods as well as artificial feeds, tolerate poor water quality and grow rapidly under warm temperatures. These attributes, along with relatively low input costs, have made tilapia the most widely cultured freshwater fish in tropical and subtropical countries (Popma & Masser, 1990).

Live tilapia is marketed in the 450 to 680 grams range and yield between 30 to 39 percent whole fish to boneless fillets. Fish are most often traded as whole (dressed or undressed), fresh and frozen. Nutritive value of tilapia is considered around: 96 kcal/100grams of raw meat, 19.2 % protein and 2.3% fat by weight.

A serious problem when marketing tilapia is “off flavor”, the flesh or fish having a musty / muddy odor and flavor. Holding the fish in clean and continually flowing water for 7 to 10 days will usually reduce the problem (Frank, 2000).

2.2 Genetic Improvement of Tilapia

Production systems in developing countries are largely based on the use of unimproved species and strains. As knowledge and experience are accumulated in the management, feeding and animal health issues of such production systems, the availability of genetically more productive stock becomes imperative in order to use the resources more effectively (Ponzoni, 2006 & Dey et al,.2010 .

In terrestrial animal species like dairy cattle, pigs and poultry, genetic improvement programs have made a substantial contribution to industry productivity and viability . The gains achieved among plants species have been even more spectacular (Weeratunge et al,.2010). There appears to be great potential for improvement in aquatic animal species because comparatively little application of genetic improvement technology has taken place to date (Agresti et al., 2000).

Such programs are particularly well suited to contribute to the fulfillment of noble aims, such as increasing the amount of animal protein available to a greater number of the population of developing countries, thus assisting in achieving greater food security (Bentsen et al.,2012). Three factors have resulted in a greater demand for fish in the world; namely, an ever-increasing human population, improved economic situation in some sectors and greater awareness of the health aspects of food. Since capture fisheries have stagnated, fish farming has become a burgeoning food production system (Ponzoni, 2006).

Genetic improvement program for fish can contribute to the production system output, both in quantitative and qualitative terms by enhancing traits of major importance such as growth rate to harvest weight, survival, stress or disease resistance, cold water tolerance, sexual maturation, product quality and feed efficiency (Hulata, 2001).

Application of genetic improvement to develop better breeds of tropical finfish, which make up 90 percent of all global aquaculture production, is a relatively new phenomenon. It was only introduced in the last three decades. It was started by World Fish Center, which managed to modify the Nile Tilapia strain or better known as GIFT (Genetically Improved Farmed Tilapia). The GIFT project demonstrated the potential of using selective breeding to genetically enhance the production performance of Tilapias. After five generations of selection, the growth performance of the GIFT strain has improved to 80 % of the base population (Ponzoni, 2006).

2.3 DNA marker technologies and their application in aquaculture genetics

DNA marker technologies have revolutionized the way aquaculture genetics research is conducted. The dramatic development of molecular genetics since the first widespread use of allozymes in the 1970s and currently exemplified by the Human Genome Project, has laid the groundwork for genomics. Broadly defined as the study of genes and their functions, genomics is rapidly impacting many facts of life, from health care and food safety to reproduction and law enforcement. Keys to the emergence of genomics were advances in DNA marker technology. These advances have resulted in a wealth of genetic markers including allozymes, mitochondrial DNA (mtDNA), restriction fragment length polymorphic (RFLP),

random amplified polymorphic DNA (RAPD), amplified fragment length polymorphic (AFLP), microsatellites or simple sequence repeat (SSR), polymorphic expressed sequence tag (EST) with potentially widespread utility in a variety of aquaculture endeavors. (Liu and Cordes, 2004)

All organisms are subject to mutations as a result of normal cellular operations or interactions with the environment, leading to genetic variation (polymorphism). In conjunction with selection and genetic drift, there arises genetic variation within and among individuals, species and higher order taxonomic groups. At the DNA level, types of genetic variation include: base substitutions, commonly referred to as single nucleotide polymorphism (SNPs), insertions or deletions of nucleotide sequence within a locus, inversion of a segment of DNA within a locus and rearrangement of DNA segments around a locus of interest (Liu & Cordes, 2004).

Several marker types are highly popular in aquaculture genetics. In the past, allozyme and mtDNA markers have been popular in aquaculture genetics research. More recent marker types that are finding service in this field include microsatellite, restriction fragment length polymorphic (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphic (AFLP), and polymorphic expressed sequence tag (EST). Molecular markers are classified into two categories: type I are markers associated with genes of known function, while type II markers are associated with anonymous genomic segments (O'Brien, 1991) Table 2.1 summarizes the basic properties of these marker types and each is discussed in detail below.

Table 2.1

Types of DNA markers, their characteristics and potential applications (Liu and Cordes,2004).

Marker Type	Acronym or alies	Requires prior molecular information?	Mode of inheritance	Type	Locus under investigation	Likely allele numbers	Polymorphism or power	Major applications
Allozyme		Yes	Mendelian, Codominant	Type I	Single	2-6	Low	Linkage mapping, population studies
Mitochondrial DNA	mtDNA	No	Maternal inheritance	-		Multiple haplotypes		Maternal lineage
Restriction fragment length polymorphism	RFLP	Yes	Mendelian, Codominant	Type I or Type II	Single	2	Low	Linkage mapping
Random amplified polymorphic DNA	RAPD	No	Mendelian, Dominant	Type II	Multiple	2	Intermediate	Fingerprinting for population studies, hybrid identification
Amplified fragment length polymorphism	AFLP	No	Mendelian, Dominant	Type II	Multiple	2	High	Linkage mapping, population studies
Microsatellites	SSR	Yes	Mendelian, codominant	Mostly Type II	Single	Multiple	High	Linkage mapping, population studies, paternity analysis
Expressed sequence tags	EST	Yes	Mendelian, codominant	Type I	Single	2	Low	Linkage mapping, physical mapping, comparative mapping
Single nucleotide polymorphism	SNP	Yes	Mendelian, codominant	Type I or Type II	Single	2-4	High	Linkage mapping

2.4 Microsatellites – Genomic distribution, evolution, function and applications

Microsatellites or simple sequence repeats (SSRs) represent a unique type of tandemly repeated genomic sequences which are abundantly distributed across genomes and demonstrate high levels of allele polymorphism. They are codominant markers of relatively small size, which can be easily amplified with the polymerase chain reaction. These features provide the foundation for their successful application in a wide range of fundamental and applied fields of biology and medicine including forensics, molecular epidemiology, parasitology, population and conservation genetics, genetic mapping and genetic dissection of complex traits. (Li et al., 2004)

In the field of fisheries and aquaculture, microsatellites are useful for the characterization of genetic stocks, broodstock selection, constructing dense linkage maps, mapping economically important quantitative traits and identifying genes responsible for these traits and application in marker assisted breeding programmes (Liu & Cordes et al., 2004).

Although microsatellites are considered selectively neutral markers, they often represent functionally relevant polymorphisms. Microsatellites contribute to DNA structure, chromatin organization, regulation of DNA recombination, transcription and translation, gene expression and cell cycle dynamics.

2.4.1 Genomic Distribution

Microsatellites are stretches of DNA consisting of tandemly repeated short units of 1-6 base pairs (bp) in length. They typically span between twenty and a few hundred bases (Beckmann and Weber, 1992). They are ubiquitous in prokaryotes and eukaryotes, present even in the smallest bacterial genomes (Gur-Arie et al., 2000). The existence of microsatellites in eukaryotic genomes has been known since the 1970s (Bruford et al.,1996), demonstrated a large number and wide occurrence of these sequences from yeast through to vertebrates (Tautz & Renz, ,1984), hybridized different microsatellite sequence to genomic DNA from a variety of organisms and reported many types of simple sequences. Figure 2.1 shows anatomy of microsatellites (Beckman & Weber, 1992).

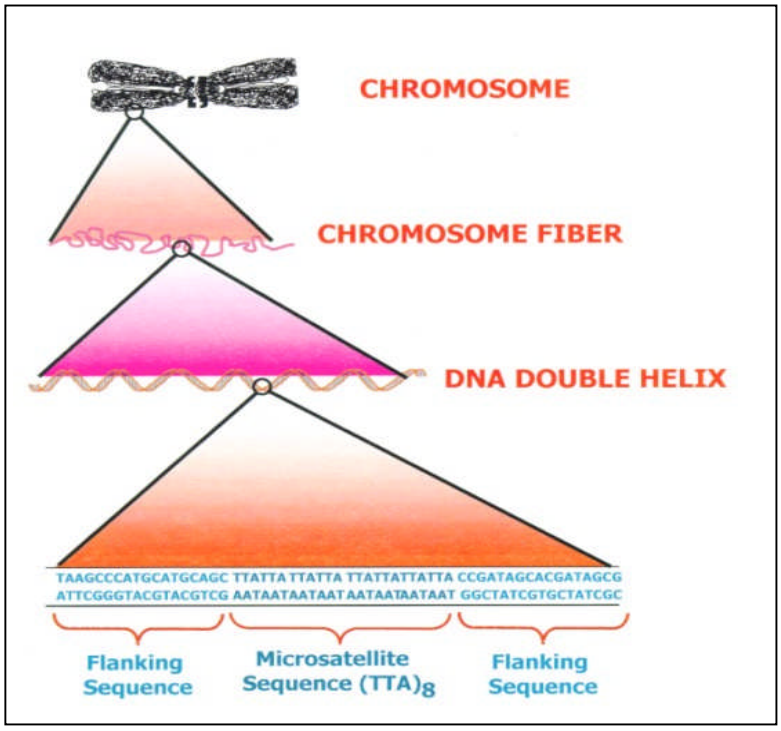


Figure 2.1 Anatomy of microsatellite (Beckman & Weber, 1992).

The majority of microsatellites (30-67%) found are dinucleotides. In the genome of vertebrates, $(AC)_n$ is the most common dinucleotide motif. It is 2.3- fold more frequent than $(AT)_{n,}$ the second most general type of dinucleotides (Toth et al., 2000). Interestingly, in primates mononucleotide repeats are mostly represented by poly (A/T) tracts, which are most frequent classes of SSRs (Bruford et al,.1996). In total, higher order of microsatellite classes (tri-, tetra-, penta- and hexanucleotides) are about 1.5- fold less common in genomic DNA of vertebrates than dinucleotides (Toth et al., 2000).

Microsatellites can be found anywhere in the genome, both in protein-encoding and noncoding DNA (Toth et al., 2000). In eukaryotic organisms, microsatellites have been shown to be in excess in noncoding regions where compared to a random distribution pattern (Metzgar et al., 2000). They are relatively rare in coding DNA, ranging between 7-10 % in higher plants and between 9-15 % in vertebrates. Only 11.6 % of a total of 6042 microsatellites were found in protein- coding regions in the genome of Japanese pufferfish (Wang et al., 1994).

The most common way to detect microsatellites is to design PCR primers that are unique to one locus in the genome and that base pair on either side of the repeated portion (Figure 2.2). Therefore, a single pair of PCR primers will work for every individual in the species and produce different sized products for each of the different length microsatellites (Beckman & Weber, 1992).

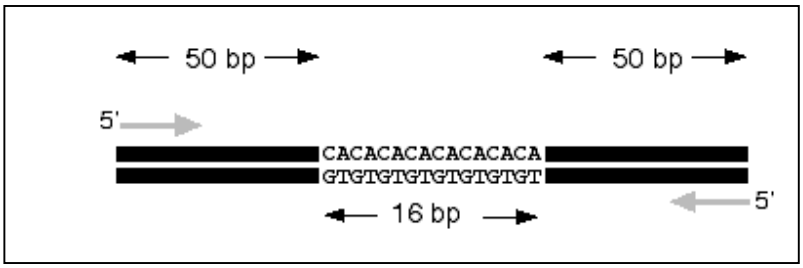


Figure 2.2: Detecting microsatellites from genomic DNA (Beckman & Weber, 1992).

Two PCR primers (forward and reverse gray arrows) are designed to flank the microsatellite region. If there were zero repeats, the PCR product would be 100 bp in length. Therefore, by determining the size of each PCR product (in this case 16 bp), we can calculate number of CA repeats present in each microsatellite (8 CA repeats in this example)

2.4.2 Evolution of microsatellites

The key features of mirosatellites as molecular marker is their hypermutability and hence, their hypervariability in species and populations. The microsatellites mutation rate is estimated at 10^{-2} - 10^{-6} per locus per generation (Ellegren, 2000; Norton & Ashley,2004), which is several orders of magnitude greater than that of regular nonrepetitave DNA (10^{-9}) (Wan et al., 2004). Analysis of $(AC)_n$ microsatellites in five vertebrate classes (mammals, birds, reptiles, amphibians and fishes) showed that length is a major factor influencing mutation rate.

Two models (DNA polymerase slippage and unequal recombination) have been suggested to explain microsatellite generation and evolution. The first model involves transient dissociation of the replicating DNA strands with subsequent reassociation (Schlötterer & Tautz, 1992). The structure can be stabilized by

hairpin, triplex or quadruplex arrangement of DNA strands. Since DNA repeat regions represent preferred target sites for mutations during DNA replication, microsatellite stability is controlled at multiple steps *in vivo* through the DNA mismatch repair (MMR) system, as shown for *Escherichia coli*, yeast and humans (Sia et al., 1997)

Nonreciprocal recombination (gene conversion) may also play a role in genetic instability of some microsatellites. Replication slippage and recombination could interact, affecting stability of microsatellite loci. For example, such "repair-slippage" mechanisms are probably involved in the instability of tri-and di nucleotides in yeast, polarity of substitutions within repeats in $(CA)_n$ stretches in bovine genomes and evolution if intronic microsatellites and some haplotypes within orthologous Rhesus (RH) genes in vertebrates (Okuda & Kajii, 2002).

2.4.3 Function of Microsatellites

Microsatellites typically represent selectively neutral DNA markers. However, multiple studies proved the functional relevance of a significant number of microsatellites (Beckman & Weber, 1992).

2.4.3.1 DNA Structure

Microsatellites are involved in forming a wide variety of unusual DNA structures with simple and complex loop- folding patterns. Telomeric and centromeric chromosome regions have been shown to be rich in long arrays of a variety of mono-, di-, tri-, tetra- and hexanucleotide motifs. Satellite sequences enriched by AT- dinucleotides have been found in the centromeric DNA of various gobiid species (Canapa et al., 2002).They are considered important for the control

of centromeric chromatin compactness in these fishes. The expanded stretches of a simple repeat sequence (TTAGGG) oriented in the 5' to 3' direction towards the end of the eukaryotic chromosomes constitute a substantial portion of the repetitive DNA in telomeric regions (Henderson, 1995). For example, the telomeric repeat in the Nile tilapia varies in size from 4 to 10 kb (Chew et al., 2002).

The (TTAGGG) hexamer sequence is recognized by ribonucleoprotein polymerase, a telomerase, which synthesizes telomere repeats onto the chromosome ends to overcome the loss of sequences during DNA replication, whereas other proteins prevent nucleolytic degradation and confer stability of chromosomes. Therefore, microsatellites play an important role in the organization of the chromosome structure (Fang & Sech,1995).

2.4.3.2 DNA recombination

Microsatellites are considered hot spots for recombination. Dinucleotide motifs are preferential sites for recombination events due to their high affinity for recombination enzymes (Biet et al., 1999). Some microsatellite sequences, such as GT, CA, CT, GA and others, may influence recombination directly through their effects on DNA structure (Okuda et al., 2000).

2.4.3.3 DNA replication

Microsatellites may influence DNA replication. For example in rat cells, DNA amplification is terminated within a specific fragment which consists of a $d(GA)_{27}$ X $d(TC)_{27}$ tract. This sequence is situated at the end of an amplicon and forms a loop, which serves as a stop signal for DNA polymerase (Li et al.,

2004).These observations suggest that microsatellites can affect enzymes controlling mutation rate and cell cycles (Chang et al., 2001).

2.4.3.4 Gene expression

Numerous data show that microsatellites located in promoter regions can influence gene expression. The 5' upstream region of the insulin gene of Nile tilapia contains a microsatellite close to the same position of a unique minisatellite found only in humans and primates (Mansour et al., 1998). The human insulin minisatellite is highly polymorphic and some of its alleles were shown to regulate the expression of the insulin gene (Melloul et al., 2002). An analogous function might be predicted for the insulin microsatellite in Nile tilapia.

In many cases, microsatellite repeat number could significantly influence gene expression level. Such an effect was described for a dinucleotide $(CA/GT)_n$ microsatellite in the Nile tilapia prolactin 1 (*PRL1*) promoter (Streelman and Kocher, 2002). Individuals homozygous for long microsatellite alleles express less (*PRL1*) in freshwater, but more in half- seawater than fish with other genotypes. Interestingly, a similar activity was previously reported by

(Naylor & Clark, 1990) for TG/CA repeat sequences in the promoter of the rat prolactin gene. It suggests the conservation of the regulatory function for CA/GT microsatellites in the *PRL1* promoter over 300 million years of vertebrate evolution.

2.4.4 Application of microsatellites

Microsatellites are often highly polymorphic due to variation in the number of repeats. They can be simply and rapidly detected by the polymerase chain reaction (PCR) using two unique oligonucleotide primers that flank the

microsatellite locus. Because of their multiallelic nature, codominant inheritance, small length, extensive genome coverage and relative abundance, microsatellites have been successfully applied in a wide variety of research fields and practical disciplines (Powel et al., 1996).

2.4.4.1 Genetic mapping

Genetic mapping represents one of the major research fields in which microsatellite markers have been applied. SSRs remain the marker choice for the construction of linkage maps, because they are highly polymorphic, highly informative and require small amount of DNA for each test. Methods for microsatellite detection can be readily automated. A disadvantage of microsatellite is that they are mostly anonymous DNA fragments (Cullis, 2002). However, type II (noncoding) microsatellites are very helpful for building a dense linkage map framework into which type I (coding) markers can then be incorporated.

2.4.4.2 Individual DNA identification and parentage assignment

Microsatellites represent codominant single- locus DNA markers. For each microsatellite, a progeny inherits one allele from the male and another from female parent. This simple inheritance pattern can explain the extreme popularity of polymorphic microsatellite loci in paternity testing. Using a panel of several microsatellite loci, a unique combined microsatellite genotype profile can be produced for each individual tested. The genotype profile is highly discriminating, which suggests that a random individual would have a low probability of matching a given genotype (Beckman & Weber, 1992).

Microsatellites are extensively exploited for paternity and relatedness analysis of natural populations, hatchery broodstocks and trade control of fish products, including those from aquaculture (Liu & Cordes, 2004). An example of successful application of microsatellite markers in relatedness testing was described by (Herbinger et al.,1995), who analyzed a rainbow trout broodstock in a small hatchery in Canada. Using only four of five microsatellite markers, they were able to match 91% of offspring to one or two parental couples of 100 % possible parental pairs (ten sires X ten dams) and in addition, to estimate parental effects on progeny growth and survival.

Due to the small size of microsatellites, they are relatively stable in degraded DNA. This is one reason why polymorphic microsatellites are widely used in forensic science for individual DNA identification. In addition, they show a high degree of allelic variability and hence uniqueness (Schneider et al., 2004).

2.4.4.3 Phylogeny, population and conservation genetics

The molecular structure and genetic variability of microsatellites is extensively exploited in evolutionary studies of a wide variety of fish species. The vast majority of these studies attempt to infer phylogenetic relationships from microsatellite data at levels below the species level or for recently diverged species (McCartney et al., 2003), using variability within stretches of tandem repeats which evolve significantly more rapidly than flanking regions.

Flanking regions of microsatellites have proven their value in establishing phylogenetic relationships between species and families, because they evolve much more slowly than tandem repeats. For example, a phylogeny of cichlid fishes was studied based on information from DNA sequences of the flanking region of a

(CA)n microsatellite locus TmoM27, which showed particular conservation in several lineages of cichlids diverged more than 80–100 million years ago (Zardoya et al., 1996). Analysis revealed that the repeat region was nearly lost in the ancestor to cichlids and then amplified extensively in African taxa (Streelman et al., 2002). Indian and Malagasy cichlids formed a basal, paraphyletic group, while African and Neotropical cichlids were both monophyletic and sister groups (Zardoya et al., 1996; Streelman et al., 2002). The authors suggested that marker TmoM27 could be widely applied in phylogenetic studies in other perciform fishes.

2.4.4.4 Quantitative trait loci mapping

A quantitative trait loci (QTL) is one that has measurable phenotypic variation owing to genetic and environmental influences. The variation can be measured numerically by height, size or age and quantified. Generally, quantitative traits are complex and influenced by several polymorphic genes and by environmental conditions. A QTL is a genetic locus (gene), the alleles of which affect phenotypic variation. One or many QTLs can contribute to a trait or phenotype. (Howe & Kocher, 2003)

Microsatellite based strategies (scans across individual chromosomes and a whole genome) represent appropriate techniques to identify QTLs, particularly those that are associated with medically, economically and evolutionarily important complex traits. Due to the genome-wide distribution and high levels of allelic polymorphism, microsatellite loci are very helpful in coarse and fine linkage mapping approaches. Coarse mapping resolves detection of a putative QTL in a chromosomal region, usually within a range of 10 -30 cM. For a given QTL, the likelihood of success and mapping resolution depends on the number of loci screened and the magnitude of their effect on the trait of interest. Also important are

recombination events in the mapping population, the mode of expression of the trait (dominant, recessive or additive), size of the mapping population and number of genes that define the quantitative trait (Glazier et al., 2002).

For farmed fishes, the first mapping of an economically important QTL was reported in 1998 (Jackson et al., 1998). No QTL gene has been defined, but several microsatellite based QTL screenings have been performed. Most of these mapping experiments have targeted three salmonid species (Atlantic salmon, rainbow trout and Arctic charr). These screenings include searches for QTLs related to temperature tolerance, body weight, body length, spawning date, embryonic development rate and condition factor (Nichols et al., 2000 , Nichols et al,2003 and Cnaani et al., 2004)

An interspecific hybrid tilapia stock (four-way cross between *Oreochromis mossambicus, O. aureus, O. mossambicus* and *Sarotherodon galilaeus*) has been successfully developed for linkage mapping and QTL analysis (Agresti et al., 2000; Cnaani et al., 2003). A search for association with deleterious alleles and sex ratio distortions revealed three microsatellite loci linked to sex distortion genes in tilapia, and one of the markers was likely to be related to a modifier of these genes (Shirak et al., 2002). A recent complex search for genetic linkage between DNA markers and QTLs for innate immunity, response to stress, biochemical parameters of blood, and fish size in the tilapia hybrid revealed 35 significant marker-trait associations involving 26 microsatellite markers in 16 linkage groups (Cnaani et al., 2004). A different hybrid line was used for the detection of QTLs controlling body color (Howe & Kocher, 2003) and sex determination (Lee et al., 2003; Lee et al., 2004).

2.4.4.5 Marker - assisted selection

Marker- assisted selection (MAS) is based on the concept that it is possible to infer the presence of a gene from the presence of a marker tightly linked to the gene. For this purpose, it is important to have high- density and high – resolution genetic maps, which are saturated by markers in the vicinity of a target locus (gene) that will be selected (Dixon et al., 1995).

Breeders select animals or plants carrying beneficial genotypes and alleles of markers that associate with or contribute to a trait of interest. Successful implementation of MAS requires well-developed genomic tools, including optional information on genetic variations relevant to the QTL phenotype, mode of inheritance, interactions with other contributing QTLs and economical magnitude of the QTL studied (Poompuang & Hallerman, 1997). To plan MAS, breeders also should take into account possible interactions between QTLs, which could relate to each other and have overlapping genetic backgrounds. In that case, MAS should preferably represent a complex selection index and take into consideration all economically significant traits that interact. (Howe & Kocher, 2003)

Production (such as growth rate, stress response and disease resistance) and reproductive (such as sex determination and development rate) traits are extremely important to breeders. Discovery of genes that control these features could greatly benefit MAS in breeding programs. Some marker-assisted breeding approaches have been implemented in agricultural plants, for which significant genetic and genomic knowledge is available (Koebner & Summers, 2002; Dale & Bradshaw, 2003). Microsatellites markers are useful in early stages of MAS for the primary selection of parents for further crossing and subsequent genetic characterization of progeny (Hulata, 2001)

2.5 Microsatellite in Aquaculture

With special emphasis on fishes, microsatellites are considered as structural genomic components and as genetic markers which have specific evolutionary mechanisms, functions and applications. Microsatellite loci have a high utility for constructing a genetic framework onto which other markers and genes are incorporated using various mapping strategies (linkage mapping, physical mapping and comparative genetic and genomic tools). Using microsatellite markers will greatly benefit the genetic dissection of complex and quantitative traits in order to map identify and eventually clone and characterize the candidate genes controlling economically important traits. (Liu & Cordes,2004)

In aquaculture, microatellite represent the markers of choice for genetic monitoring of farmed stocks in view of breeding programs through the analysis of genetic variability and pedigree structure to design beneficial crosses, select genetically improved stocks, minimize inbreeding and increase selection response (Davis & DeNise, 1998).

CHAPTER III

MATERIALS AND METHODS

3.1 Experimental Studies

Two experimental studies were conducted. The first study was on population genetic study of four populations. The second study was quantitative genetic study of four populations.

3.2 Experimental stocks

Four populations of *Oreochromis sp* were used in this study. The four populations were: PKPS, Bentong, Enggor and Negeri Sembilan. All these families were obtained from Freshwater Fisheries Research Center, Jelebu (Department of Fisheries, Malaysia).

3.2.1 Origin / History of the four populations

In 2005, twelve stocks were collected from twelve different locations from Peninsular Malaysia in Sungai Buloh, PKPS, Bentong, Lorong Pandan, Pekan, MADA, Rawang, Tanah Merah, PPPAT, PPA Tapah, Tapah Chenderiang and Simpang Pertang. All tilapia populations were collected from various farmers. Growth Assesment Study was done in Freshwater Fisheries Research Center, Jelebu, Negeri Sembilan (Department of Fisheries, Malaysia) until stocks gained weight around 250g.

In 2006, four stocks were chosen based on their best specific growth rate (SGR) for diallel cross. The four stocks were from PKPS, Bentong, Enggor and Negeri Sembilan. Basic measurements such as Total length (TL), Standard length (SL), Head length (HL) and Body depth (BD) were taken from all the stocks.

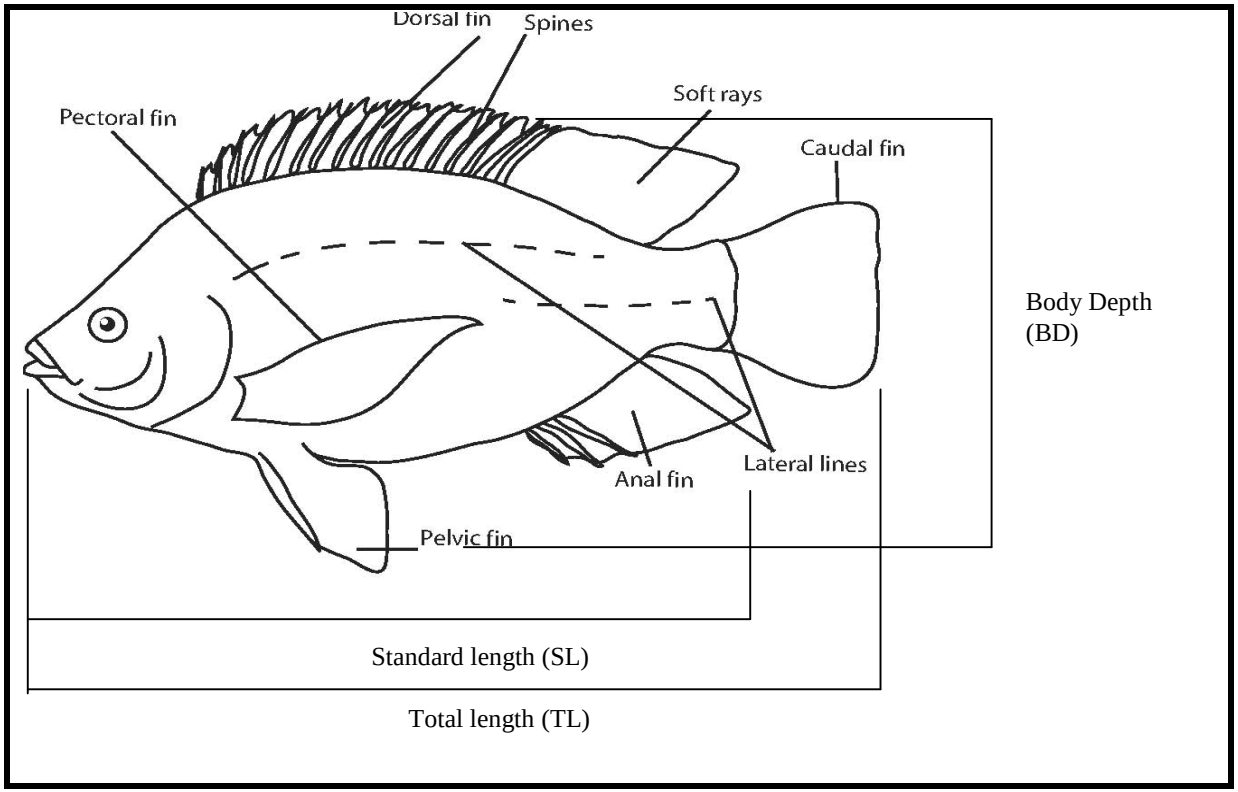


Figure 3.1 Basic measurements in tilapia specimen (Nguyen *et al*., 2006)

3.2.1.1 PKPS

Fries were collected on 15 June 2005 from a farm named PKPS, Sg Buloh, Selangor. The average fry weight was 7.68 g and the length was 8.18 cm. Fries were transported from Selangor to FFRC in plastic bags. The temperature and pH of the water in the plastic bag was 28.6 ^{0}C and 5.5 respectively. The fries were placed in a quarantine tank in FFRC. The temperature and pH of the tank were maintained at 27.9 ^{0}C and 6.67 respectively. After two weeks of quarantine period, these fries were transferred to stocking tank for growth assessment studies. Figure 3.2 shows a fry from PKPS.

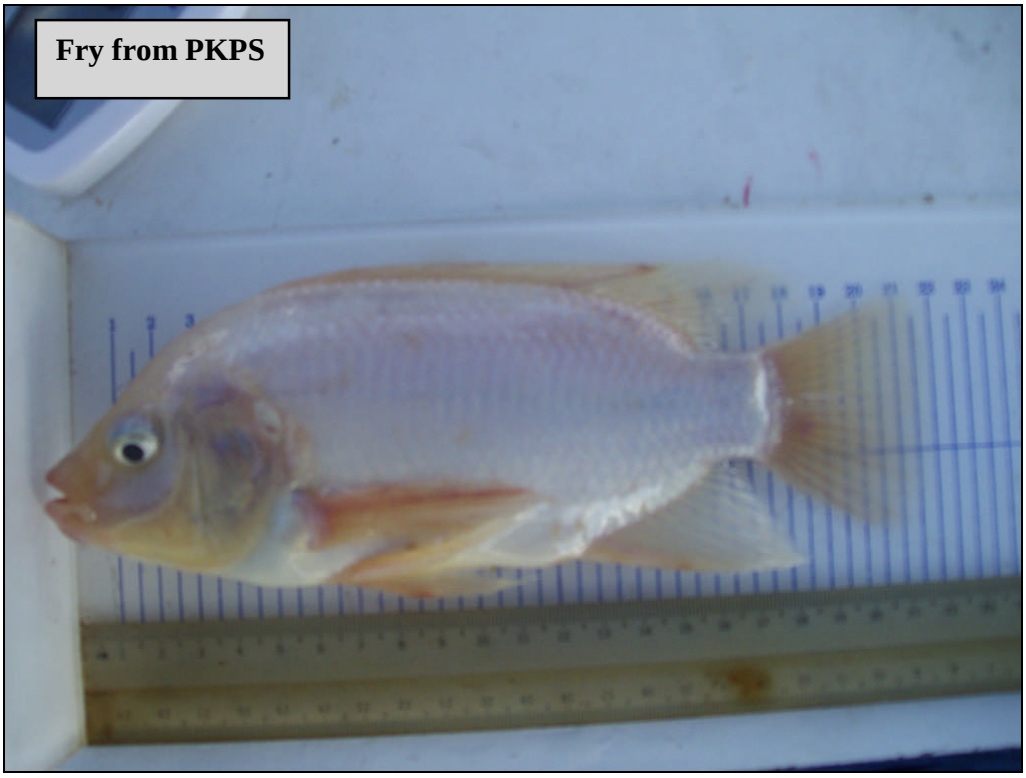


Figure 3.2: Specimen from PKPS

3.2.1.2 Bentong

Fries were collected on 6 July 2005 from Bentong, Pahang. The average of the fries weight was 2.30 g and the length was 5.26 cm. Fries were transported from Pahang to FFRC in plastic bags. The temperature and pH of the water in the plastic bag was 28.4 ^{0}C and 5.98 respectively. The fries were placed in a quarantine tank in FFRC. The temperature and pH of the tank were maintained at 27.8 ^{0}C and 7.05 respectively. After two weeks of quarantine period, these fries were transferred to a stocking tank for growth assessment studies. Figure 3.3 shows a fry from Bentong.

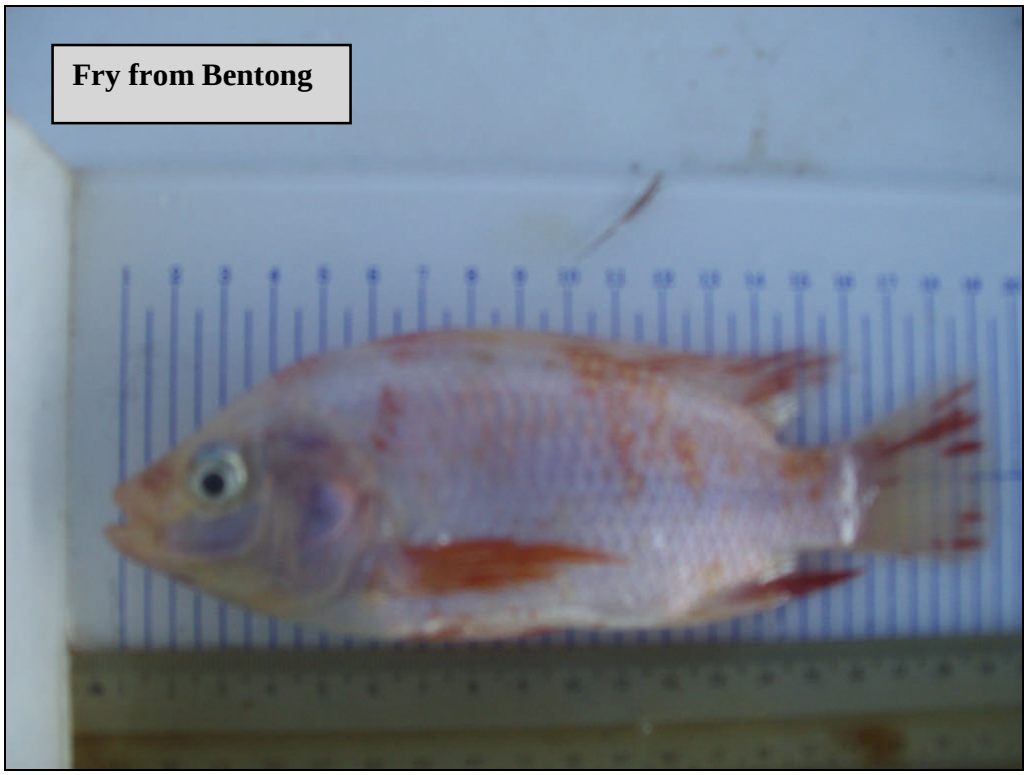


Figure 3.3: Specimen from Bentong

3.2.1.3 Enggor

Fries were collected on 15 June 2005 from Enggor, Pahang. The average weight of the fries was 1.70 g and the length was 4.76 cm. Fries were transported from Pahang to FFRC in plastic bags. The temperature and pH of the water in the plastic bag was 27.5 ^{0}C and 6.34 respectively. The fries were placed in quarantine tanks in FFRC. The temperature and pH of the tank were maintained as 27.5 ^{0}C and 6.27 respectively. After two weeks of quarantine period, these fries were transferred to a stocking tank for growth assessment studies. Figure 3.4 shows a fry from Enggor.

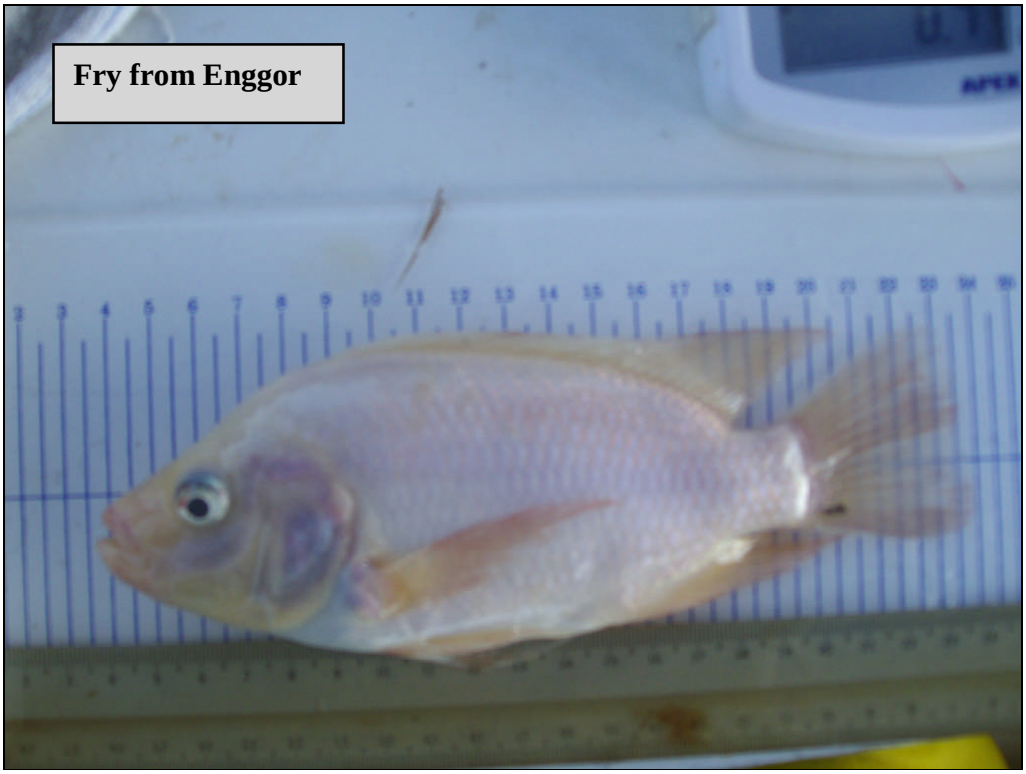


Figure 3.4: Specimen from Enggor

3.2.1.4 Negeri Sembilan

Fries were collected on 15 June 2005 from Simpang Pertang, Negeri Sembilan. The average weight of the fries was 2.18 g and the length was 5.04 cm. Fries were transported from Negeri Sembilan to FFRC in plastic bags. The temperature and pH of the water in the plastic bag was 27.8 ^{0}C and 6.31 respectively. The fries were placed in a quarantine tank in FFRC. The temperature and pH of the tank were maintained as 27.8 ^{0}C and 6.75 respectively. After two weeks of quarantine period, these fries were transferred to a stocking tank for growth assessment studies. Figure 3.5 shows a fry from Negeri Sembilan.

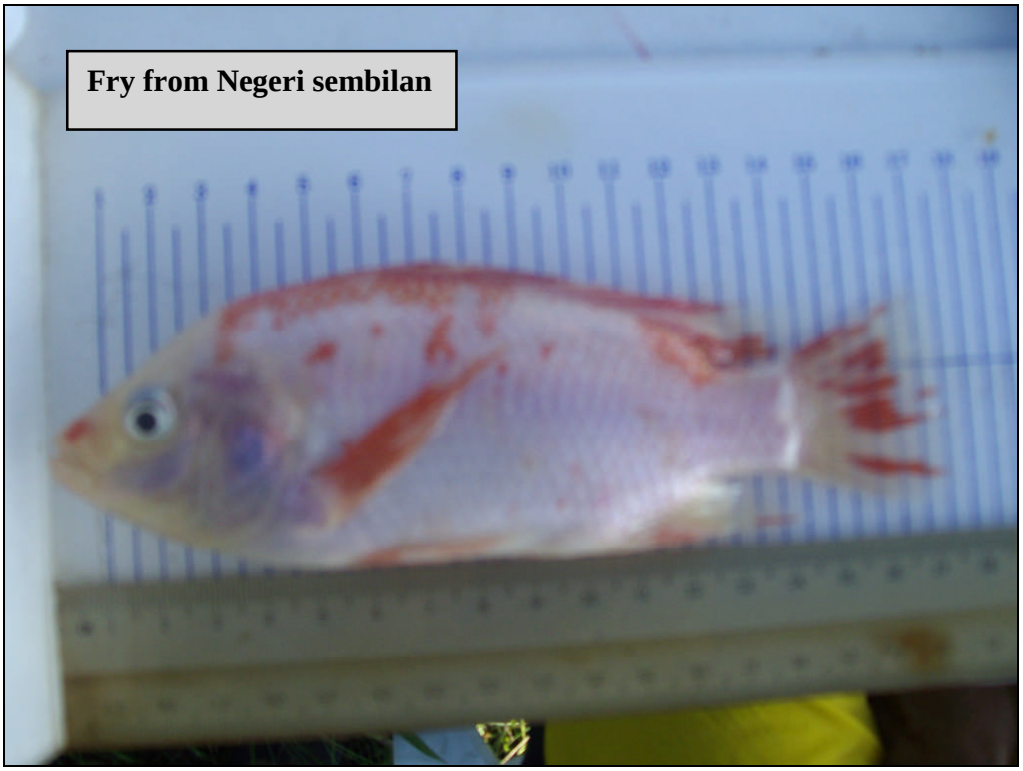


Figure 3.5: Specimen from Negeri Sembilan

3.2.2 Quarantine Procedure

Quarantine is a compulsory procedure which has to be done when receiving new families of fries or adult fish from different farms. Quarantine preparation starts two days before receiving the new families. Three quarantine tanks with a capacity of 25 tan each were cleaned and fill up with water. Good aeration was fixed to each tank.

During transferring of fries or adult fish to the quarantine tanks, the un-opened plastic bags which contain the fries was kept in the quarantine tank for 5 minutes. Temperature and pH of the water in the plastic bag were recorded. The plastic bag was then opened and the fries slowly released into the quarantine tank.

Fifty fries were randomly collected to examine parasite and bacterial infection. Salt treatment at 1000 parts per million (ppm) was given after 3 hours of transferring fries into the quarantine tank for 24 hours. Fries which shows abnormality was separated from the rest of the fries. Feeding was done after 48 to 72 hours of stocking in quarantine tank. All the fries were transferred to a stocking tank after 14 days of quarantine period.

3.3 Study 1: Populations Genetic Study

In the first phase studies were conducted to estimate effective allele numbers and heterozygosity levels among these four Tilapia stocks, to determine if the sampled populations conformed to Hardy-Weinberg equilibrium and to measure genetic distance among all these four stocks. In this study, sample from Nile Tilapia (*Oreochromis niloticus*) or Genetically Improved Farmed Tilapia (GIFT) was used as control populations. Nile tilapia was chosen as control populations because Red Tilapia is a hybrid of *Oreochromis niloticus* and *Oreochromis mossambicus* .

3.3.1 Tissue Collection

Tissue collections were done on the four testing populations and one control population. The four testing populations were PKPS, Bentong, Enggor and Negeri Sembilan. The control population is GIFT. Thirty individuals were chosen from each population for tissue collection. Fin-clip samples were taken from each of these populations. All the samples were labeled with details of date, family and sex. Samples were kept at -80 0^C for long term preservation.

3.3.2 DNA Extraction

In this studies DNA extraction was done using the Genipin Tissue DNA kit (1^{st} BASE). Extraction starts with adding 200 µl Buffer TL for cell lysis and 25 µl OB Protease to 30 mg of tissue. Tissue sample was kept for incubation at 55 ^{0} C for 3 hours tissue lysis. After incubation, 220 µl BL buffer was added to the tissue sample and incubated at 70 ^{0} C for 10 minutes. Absolute ethanol (220 µl) was added to the sample for precipitation and mixed well. Samples were transferred to a spin column and spun at 8000 rpm for one minute. Flow through was discarded and 750 µl wash buffer were added and spun at 8000rpm for one minute. To elute the DNA, 200 µl of pre heated Elution Buffer was added to the sample and spun at 8000 rpm for one minute. The entire DNA samples were stored at – 20 ^{0}C for long term preservation purposes.

3.3.3 DNA Purity

Bio-Rad SmartSpec Plus Spectrophotometer was used to estimate the purity of the DNA. Diluted 1.5 µl of DNA in 1500 with deionized water was use to read at A_{260} and A_{280}. In a pure sample, ratio of A_{260} / A_{280} will approximately 1.8. Lower values indicate protein contamination. An A_{260} of 1 corresponds to approximately 50 µl/ml of double stranded DNA in a 1cm quartz cuvette. Nucleic acid concentration is calculated as follows:

A_{260} X 50 mg/ µl X 0.001 µl/ml X dilution factor (1500 µl/1.5 µl)

3.3.4 Polymerase Chain Reaction (PCR)

Twenty polymorphic pairs of primers obtained from gene bank were used for amplification and identification of polymorphic products in five populations. Details of primers are given in Appendix 1. The PCR reactions were carried out in a total volume of 10 µl containing 30 ng of genomic DNA, 2.5 mM $MgCl_2$, 10mM Tris-HCl, 50 mM KCl, 0.1% Triton-X 100, 0.5mM each of dATP, dCTP, dGTP and dTTP, 10 pmol of primer and 2.5 U *Taq* DNA polymerase (GoTaq® Flexi DNA Polymerase, Promega, USA). PCR amplifications were performed in a Perkin Elmer Thermal Cycler, Bio-Rad My cycler and Corbett palm cycler with the following temperatures: a predenaturation at 95 ^{0}C, followed by 35 cycles of denaturation at 94°C, annealing at 55°C, extension at 72°C and concluded with a final extension at 72°C .The PCR product were separated according to size on a 4% metaphore gel and polyacrylamide gel and visualized over ultraviolet light after ethidium bromide staining.

3.3.5 Electrophoresis and Polyacrylamide Gel Electrophoresis

Seven µl of PCR product was mixed with 4 µl 6X loading dye (Promega, USA). Blue/Orange Loading Dye, 6X, is a convenient marker dye containing 0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10mM Tris-HCl (pH 7.5) and 50mM EDTA (pH 8.0). One µl of a 20bp molecular marker was used as ladder. PCR products were run on agarose methapore (4%) gel using Tris-Borate-EDTA buffer (TBE). AlphaImager, Alpha Innotech gel documentation machine was used to view the gel. The same PCR product was run on polyacrylamide gel electrophoresis (PAGE) using Gel Scan 3000 (Corbett, Life Science). Gel Acquisition and Analysis Software were used to score bands

3.3.6 Statistical Analysis

Prior to the analysis of data for population structure, it is essential to test the genetic variation found at the different microsatellite loci. Selective neutrality of each locus is very important and subsequent analysis is based on the interactions of forces such as genetic drift, mutation and migration.

Genetic drift and mutation will cause divergence of allele frequencies among the subpopulations and migration will cause homogenization of allele frequencies. Selection may lead to an underestimation of genetic distance. However, differences in the selective pressures among regions may cause fixation of alternative alleles in different sub populations and cause an overestimation of these parameters. It is important to eliminate loci that are under selective pressure. This can be done by comparing observed and expected genotype frequencies from predictions under Hardy-Weinberg equilibrium. A selection of tests is explained below:

An unbiased estimation of heterozygosity, He (Edwards *et al*, 1992) is determined as,

$$H_e = \frac{n\,(1-\sum p_i^{\,2})}{n-1}$$

n is sample size
$\mathbf{p_i}$ is frequency of the $\mathbf{i^{th}}$ allele

Hardy Weinberg equilibrium is an important factor before doing genetic analysis of natural populations. In the absence of forces such as selection, migration, mutation and small sample size that change the gene frequencies, the allelic and genotypic frequencies will remain constant from generation to generation.

In Hardy Weinberg principle, if the alleles **A** and **a** at this locus were frequencies **p** and **q,** such that **p+q = 1**, then the frequencies of **AA**, **Aa** and **aa** for this would be constant with frequencies of $\mathbf{p^2}$, $\mathbf{q^2}$ and **2pq**, where $\mathbf{p^2 + 2pq + q^2 = 1.}$

The chi-square analysis was carried out to test the above. If the observed chi-square value (χ^2) was larger than the expected chi-square value for the degree of freedom at the P = 5% level of significance, then it was concluded that the population was not in Hardy Weinberg equilibrium. Otherwise the null hypothesis that the population is in Hardy Weinberg equilibrium was accepted.

$$\chi^2 = \frac{\sum (\textbf{Observed number-expected number})^2}{\textbf{Expected number}}$$

Degree of Freedom (df) = no of genotypes – no of genes

Heterozygosity per locus was estimated as follows:

$$H_m = \frac{\text{Observed number of heterozygotes}}{\text{Total number of individuals}}$$

Expected heterozygosity per locus, H_L:

$$H_L = 1 - \sum X_i^2$$

X_i = Frequency of the allele at the locus

The mean heterozygosity per locus, including monomorphic loci, H was determined by:

$$H = \frac{\sum H_L}{\sum L \text{ loci}}$$

L = mean the number of loci

The allele frequencies were estimated by the following equations:

$$F(\text{allele}) = \frac{P + \frac{1}{2}(H)}{N}$$

P= number of homozygotes for that allele

H= number of heterozygotes for that allele

N= total number of individuals examined

Chi square test, allele frequency estimation, genetic variation estimation, heterozygosity analysis and Hardy Weinberg expectations were done by PopGene 2.11 (Yeh et al., 1997) and GenePop 4.0 (Raymond & Rousset, 2007).

Genetic distance between two populations may be used as a relative estimate of the time that has passed since the populations existed as a single cohesive unit. When two populations are genetically isolated, mutations and genetic drift can lead to differentiation in allele frequencies at selectively neutral loci. As time passes, the difference in allele frequencies will increase until each population is sometimes fixed for separate alleles. A measure of genetic distance was done by PopGene 2.11 (Yeh et al., 1997) and GenePop 4.0 (Raymond & Rousset, 2007).

When the measurements of genetic distance has been compiled for all possible pairs of populations, there are presented in the form of a matrix. The method used here was unweighted pair group with arithmetric average (UPGMA) clustering. A test of bootstrapping was performed using Phylogeny Inference Package (PHYLIP; 3.5c version) (Felsenstein, 1993). Estimation of null alleles and scoring errors was also carried out using Micro-Checker (Oosterhout et al., 2005)

Small effective population size can result in a high occurrence of inbreeding, or mating between close relatives. One of the effects of inbreeding is a decrease in the heterozygosity (increase in homozygosity) of the population as a whole, which means a decrease in the number of heterozygous genes in the individuals. This effect places individuals and the population at a greater risk from homozygous recessive diseases that result from inheriting a copy of the same recessive allele from both parents. The impact of accumulating deleterious homozygous traits is called inbreeding depression - the loss in population vigor due to loss in genetic variability or genetic options.

In the 1950's, Sewell Wright developed a set of parameters called F statistics. The simplest of these is the inbreeding coefficient defined as the probability that two homologous (same) alleles present in the same individual are identical by descent. The inbreeding coefficient (F) is calculated by comparing the expected heterozygosity with observed heterozygosity (H_o), and ranges from -1 (no inbreeding) to +1 (complete identity). PopGene 2.11 (Yeh et al., 1997) and GenePop 4.0 (Raymond & Rousset, 2007). As such it describes the amount of inbreeding like effects:

F_{IS} : within sub populations

F_{ST} : among subpopulations

F_{IT} : entire population

F statistics within sub population can be estimated from this ratio.

$$F_{IS} = \frac{H_s - H_i}{H_s}$$

H_s = Expected average Heterozygosity
H_i = Observed Heterozygosity

Population substructure will also lead to inbreeding like effects, such as a reduction in observed heterozygosity when compared to expected values. This relationship shows that as the allele frequencies in two subpopulations deviate, the average expected heterozygosity in those populations will always be less than that expected from pooled allele frequencies, H_T. An among subpopulations F-statistics can be estimated from this ratio.

$$F_{ST} = \frac{H_T - H_S}{H_T}$$

H_T = Total heterozygosity

The measure of the correlation of alleles for the entire population is thus a combination of both the within and among subpopulation effects and can be estimated by this ratio.

$$F_{IT} = \frac{H_T - H_I}{H_T}$$

3.4 Study 2: Quantitative Genetic Study

The second studies were conducted to evaluate the four populations (PKPS, Bentong, Enggor and Negeri Sembilan) in a diallel cross with special reference to growth. Attention was given to survival too.

3.4.1 Broodstock and Synchronization of Spawning

Equal number of males and females were used from all the four populations. Fifty males and females were chosen from each population. Synchronization of spawning was done to produce progeny groups as uniform in size and age as possible. Therefore, the strategy for synchronizing spawning were by maintaining broodstock separated by sex in a holding tank, conditioning by proper feeding and evaluation of sexual maturity condition of females.

3.4.2 Conditioning of Breeders

Breeders were conditioned for two weeks separately prior to stocking in breeding tanks. During conditioning, breeders were fed with balanced feed. The feed was given twice daily, in the morning and in the evening. Figure 3.6 shows stocking tanks which were labeled by family name and sex.

Figure 3.6 - Stocking tanks

3.4.3 Evaluation of Sexual Maturity Condition

After conditioning, the female breeders were checked for their readiness to spawn by visually examining their morphological characteristics. Female breeders were categorized into the following maturity conditions: ‘Ready to Spawn’ (RS), ‘Swollen’ (S), ‘Not Ready to Spawn’ (NR) and ‘Has spawned’ (HS). Descriptions of these four categories are given in Table 3.1. Female breeders categorized as ‘Ready to Spawn’ were first selected for pairing with a male in breeding tanks. Figure 3.7 shows a ‘Ready to spawn (RS) female as observed by the pink to red and protruding genital papilla, fully opened genital pore and distended abdomen.

Table 3.1: Different categories of sexual maturity condition in female breeder and the expected number of days until spawning (Nguyen et al., 2006).

Category	Code	Morphological Characteristics	Days until spawning
Ready to spawn	RS	Pink to red protruding genital papilla, fully opened genital pore & distended abdomen	3 to 7
Swollen	S	Pink to yellow genital papilla, slightly opened genital pore & slightly distended abdomen.	5 to 10
Not ready to Spawn	NS	White to clear & flat genital papilla & normal abdomen.	21 to 30
Has spawned	HS	Red genital papilla & shrunken to compressed abdomen	15 to 30

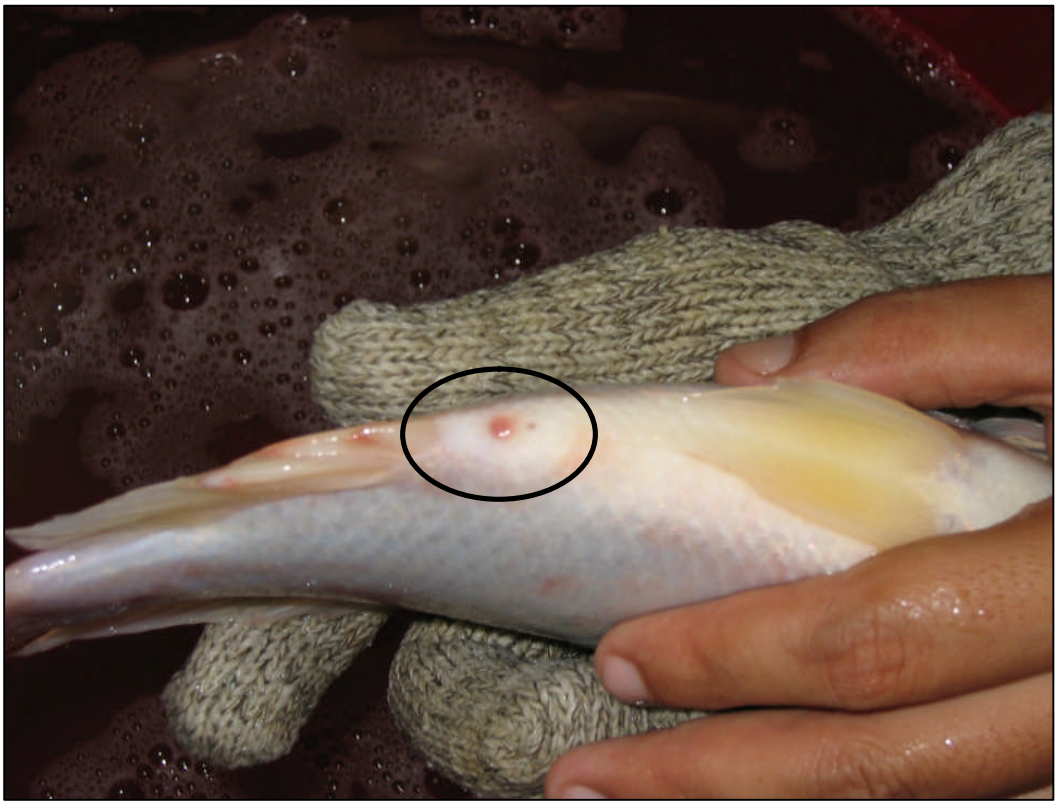


Figure 3.7: Ready to spawn (RS) female

3.4.4 Mouth Clipping for males

To avoid or at least to reduce mortalities, body weights of female and male breeders should be as close as possible to each other. If the male breeders are much larger than their female counterparts, it is necessary to carry out a mouth clipping of male breeders before transfer to breeding tanks. The male breeders were anesthetized before removing their upper lips. Wounds will be disinfected using an antiseptic. Figure 3.8 shows mouth clipping of male breeder (Nguyen et al., 2006).

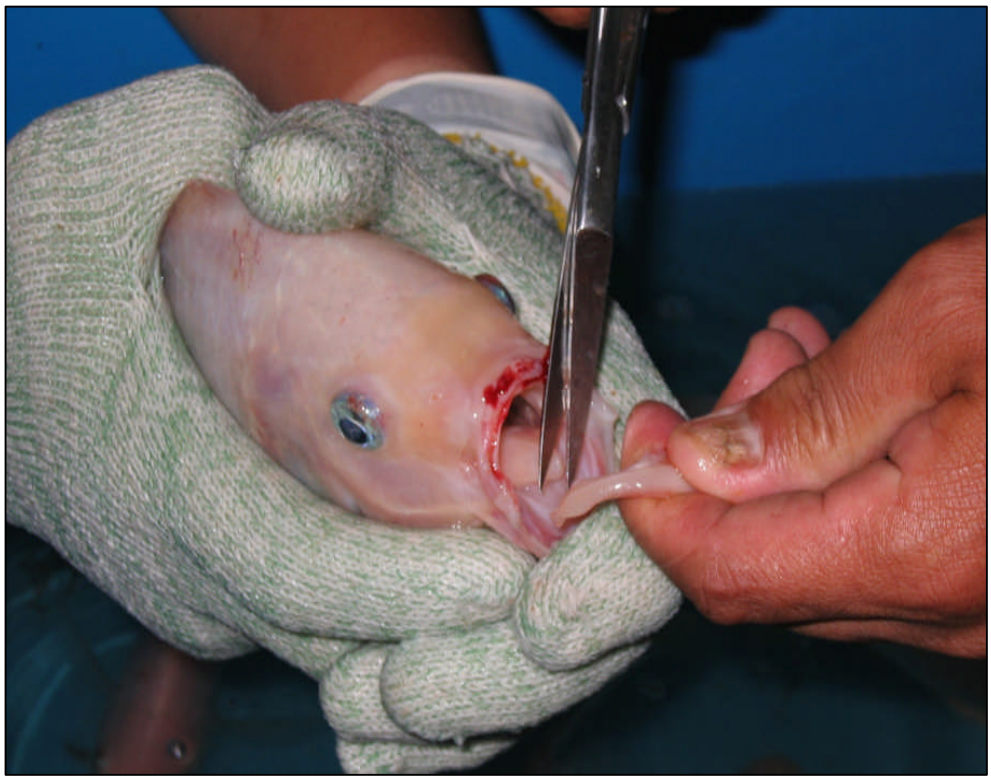


Figure 3.8: Mouth clipping of male breeder

3.4.5 Diallel Cross

In a full diallel cross, all strains were used as female parents, as well as male parents. Table 3.2 shows a pattern of diallel cross for these four populations. The cells shaded green represent purebred progeny, whereas those shaded yellow are crossbreds. There were three replicates for each treatment.

Table 3.2: Diallel cross pattern

Male / Female	PKPS (PS)	Enggor (EG)	N.Sembilan (NS)	Bentong (BN)
PKPS (PS)	PSPS	EGPS	NSPS	BNPS
Enggor (EG)	PSEG	EGEG	NSEG	BNWG
N.Sembilan (NS)	PSNS	EGNS	NSNS	BNNS
Bentong (BN)	PSBN	EGBN	NSBN	BNBN

3.4.6 Stocking of Breeders

The female breeders should be stocked into the breeding tanks before the males. Ratio of female: male is 2:1 in each treatment tank. The males were then transferred to the females that were most ready to spawn. Only ready to spawn and swollen females were chosen as breeders. Mouth clipped males were chosen as breeder. After fries had been produced and collected, the males would be separated from the females and immediately transferred to the stocking tank. The tilapias being mated should not be fed when the female breeders are expected to spawn since this might cause the females to swallow the eggs. Figure 3.9 shows breeders in treatment tanks.

Figure 3.9: Breeders in breeding tank

3.4.7 Eggs collection and artificial incubation

The first egg collection was done 10 days after stocking of breeders. The egg/fry collection will be done in the morning to avoid stress and mortalities. Eggs were collected from the female mouth. Figure 3.10 and 3.11 shows the method of collecting eggs from a female's mouth. The eggs will then be rinsed with salt water (3ppt for 2 minutes). Figure 3.12 shows rinsing of eggs in salt water. The eggs will be transfered to artificial incubators (Figure 3.13 and Figure 3.14). Photos were taken to count the initial number of the eggs. Figure 3.15 shows collected eggs on 1st day. The eggs usually hatch after 6 to 7 days (Figure 3.16). The fries were incubated for 21 days before being transferred to nursery tanks. The fries were fed with powdered pellets after complete yolk absorption. Figure 3.17 shows fries after completion of yolk absorption in artificial incubators. This particular female was kept separately to be tagged.

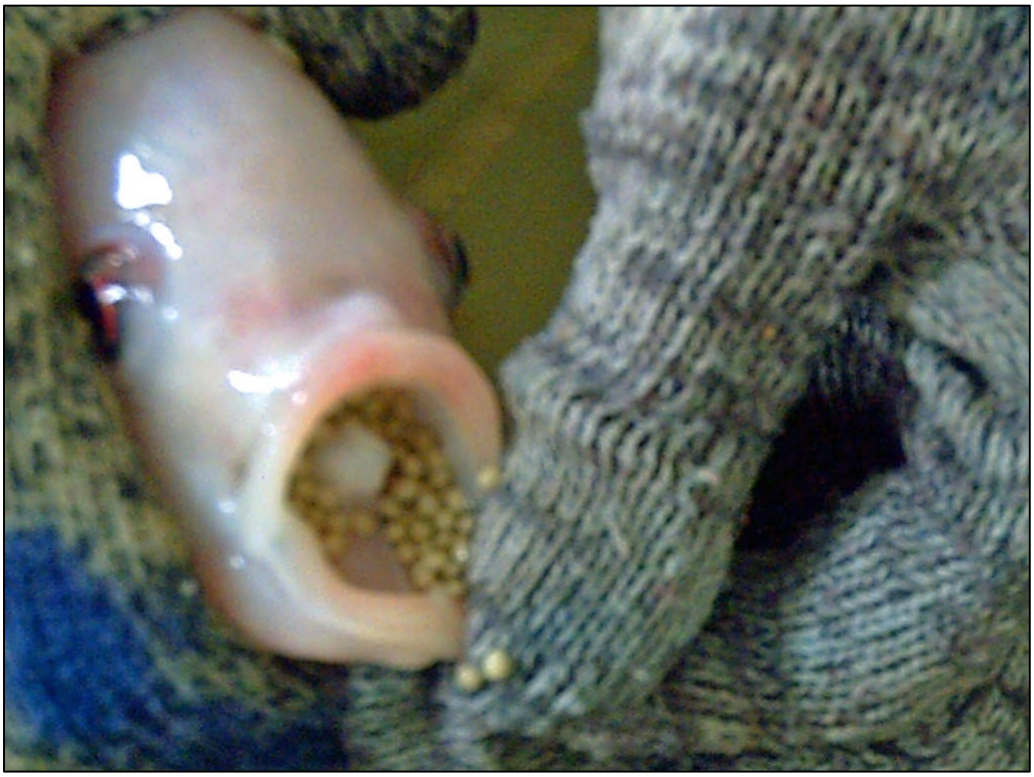


Figure 3.10 Method of collecting eggs from female mouth

Figure 3.11 Method of collecting eggs from female mouth

Figure 3.12 Rinsing of eggs in salt water

Figure 3.13: Artificial incubators

Figure 3.14: Artificial incubators

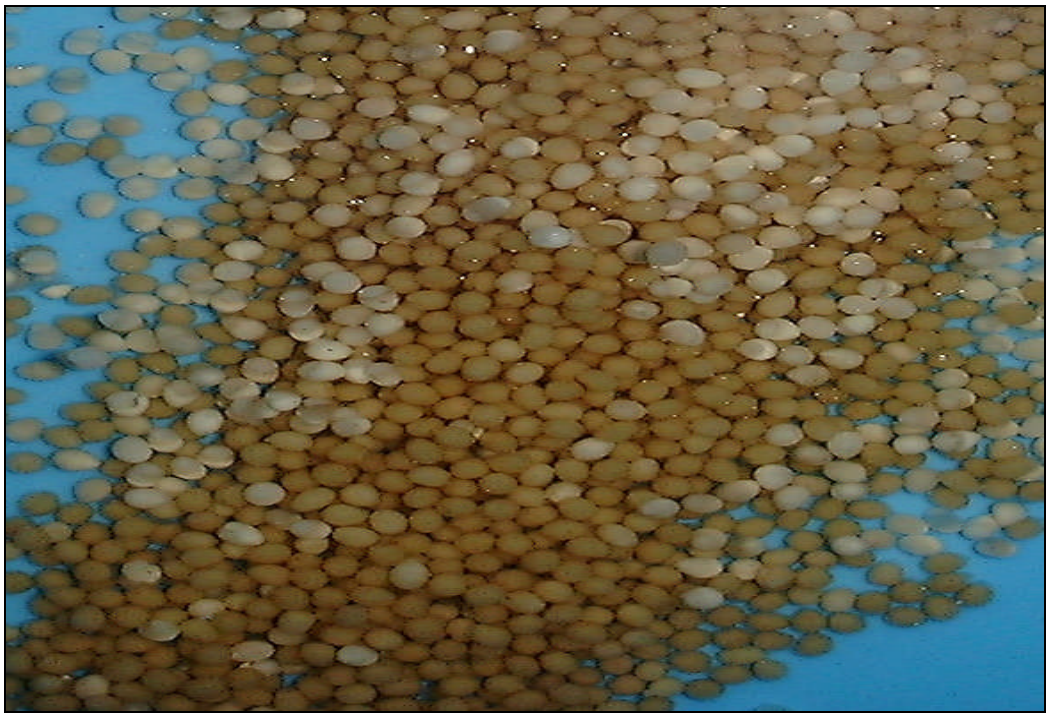


Figure 3.15: Eggs on 1st day

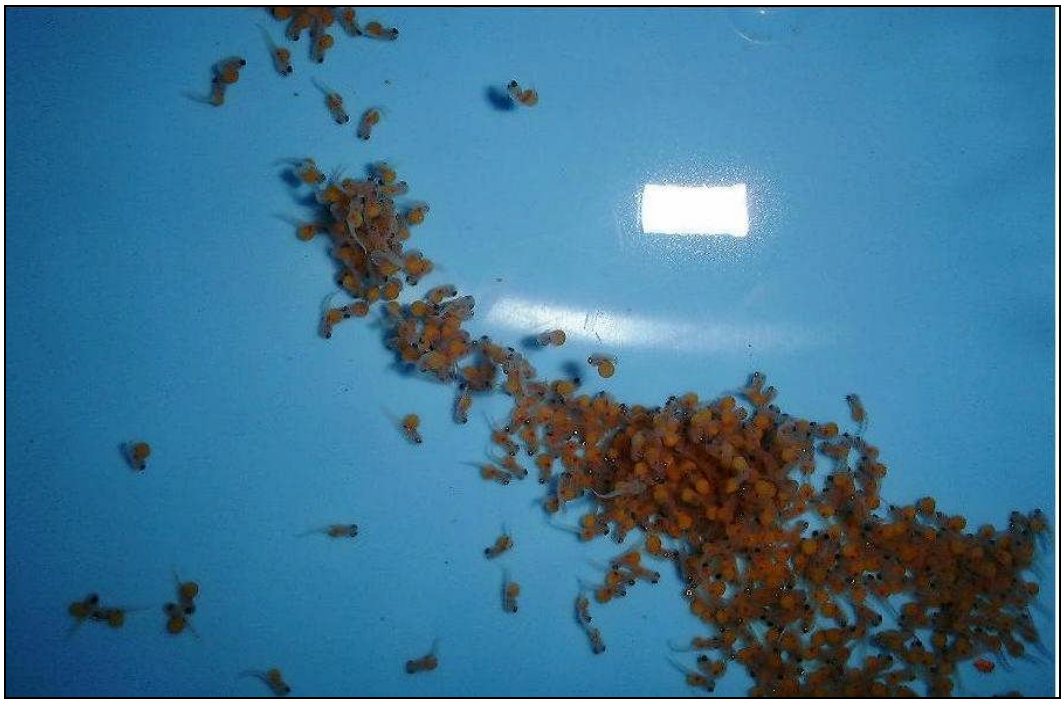


Figure 3.16: Fries after 1 week

Figure 3.17: Fries after completion of yolk absorption

3.4.8 Parental tagging

Each female was tagged after producing eggs. Pit-Tag was used to tag the breeders. The fish were anaesthetized to reduce or eliminate the stress experienced during handling in connection with tagging. Tricaine methanesulfate was used at a concentration of 0.33 gl^{-1} as an anesthetization agent. However, the breeders were removed immediately from the anesthetic solution after tagging and transferred into aerated freshwater. Body weight and total length measurements of the female will be measured prior to tagging. Figure 3.18 shows tagging of the female breeder. Figure 3.9 shows the way to scan for the tag number after tagging.

Figure 3.18: Parental Tagging

Figure 3.19: Scan of tag number after tagging

3.4.9 Fry Nursing

Fry nursing was done in nursing tanks. We used hapa (small cages) were utilized to nurse the fries in the tanks. After 21 days in artificial incubator, fries will be transferred to nursing tanks each of which were partitioned into three by using hapa . Fries will first be counted before transfer to nursing tanks. The fries were fed with powdered pellets twice a day. Figure 3.20 shows a nursing tank with three hapa in one nursing tank. Each hapa will be labelled by details of parents, egg collection date, and number of fry and data of transferring date to nursing hapa.

Figure 3.20: Nursing tank with hapa

3.4.10 Nursing in pond

Fries were transferred to “Pusat Perkembangan Akuakultur , Jitra Kedah” after two months of nursing in nursing tanks in Freshwater Fisheries Research Center, Jelebu, Negeri Sembilan. In Kedah, all the fries were stocked in rearing ponds. Fries were transferred to nursery hapas in pond at density of 150 to 200 fry/m^3. Figure 3.21 shows the rearing ponds with hapas in Kedah. Figure 3.22 shows fries for stocking. Parameters such as body weight, standard length, total length, body depth and body width were recorded as shown in Figure 3.23. Tagging (Figure 3.24) was done to all fries before being transferred to pond. Pit Tag was used for tagging the fries. Five parameters were taken during stocking. The parameters were tag number, standard length, weight, body width and body depth.

Figure 3.21: Rearing pond with labelled hapas.

Figure 3.22: Fries before tagging

Figure 3.23: Measuring all parameters before stocking

Figure 3.24: Tagging using Pit-Tag

3.4.11 Water Quality

Water quality tests were taken to monitor the quality of the water through out this research. Four parameters such as temperature, dissolved oxygen, pH and ammonia were taken to monitor the quality of the water.. Temperature, dissolved oxygen and pH for stocking tanks, breeding tanks, artificial incubators and nursing tanks were taken every morning while tests for ammonia were done once in a week.

Low levels of dissolved oxygen limit active breeding and ability of females to re-condition. Extended periods of low oxygen will also affect courtship having a negative effect on the ability to mouth brood eggs and fry (Popma & Masser, 2000).

Tilapias can tolerate high temperature, however: too much handling at high temperature could result in high mortality. The range of lethal temperature is 10^0C to 11^0C. Reproduction of tilapias will be inhibited at a temperature of 20^0C. Frequent reproduction will occur in the range of temperature 25^0C to 35^0C. The ranges of stress and mortality temperature are 37^0C to 38^0C (Popma & Masser, 2000).

Tilapias seem to grow best in water that is near neutral or slightly alkaline. pH 7-9.5 mg/l is desirable for fish culture. Ammonia is more toxic at higher temperatures of 24 to 32^0C. Unionized ammonia low as 0.08 mg/l will depress appetite. Prolonged exposure (weeks) to unionized ammonia concentration > 1 mg/l causes losses in fry and juveniles; massive mortality occurs when unionized ammonia concentrations are > 2 mg/l (Popma & Masser, 2000).

3.4.12 Final Harvesting

Final harvesting of all the stocked families was done after four months stocking in the pond. Six measurements were taken during final harvesting. The measurements were tag number, standard length, weight, body width, body depth and sex.

This was done early in the morning to minimize heat stress. All harvested families were transferred to different hapa for 1 to 2 days conditioning without feeding, before recording all the six measurements.

3.4.13 Analysis

Growth rate and Survival were the two interested traits in this study. Progress in crossbreeding is virtually synonymous with increment in size per unit time. Therefore it is important that relative growth performance of the four stocks be evaluated. While survival is an important aspect of production with respect to both reproductive efficiency and yield.

3.4.13(a) Growth rate

Growth rate is very important because it speeds up the turnover production. Frequently larger animals attain greater prices. It also easy to estimate through measurements of body weight (BW), body length (BL), body width (BW) and body depth (BD). All these parameters were also considered as quality traits.

For each measured characteristic, descriptive statistics were calculated per population per stock. The analysis mentioned above was done using SPSS software.

3.4.13(b) Survival

Three traits were studied under factor of survival. Those traits were percentage of egg hatchability, percentage of fry survival and percentage of adult fish survival.

Percentage of egg hatchability.

= (the number of swim up fry / number of eggs at spawning) x 100

Percentage of fry survival.

= (number of two months old fry / number of swim up fry) x 100

Percentage of adult fish survival.

= (number of 6 months old fish harvested / number of fish stocked) x 100

CHAPTER IV

RESULTS

4.1 Population Genetic Study of four populations

4.1.1 Template DNA Concentrations

Concentrations of template DNA tested ranged from 5ng to 200ng per µl. It was observed that the template DNA concentration influenced the number of fragments generated. Higher DNA concentrations lead to loss of the bands.

4.1.2 Primer Screening

Twenty pairs of polymorphic primers were selected and amplified at various annealing temperature using Perkin Elmer Thermal Cycler, Bio-Rad My cycler and Corbett palm cycler. Once the annealing temperatures were optimized, the primers were tested on ten individuals from the four testing populations and one control population (Appendix 1).

4.1.3 Banding Pattern and scoring polymorphism

DNA polymorphism is one of two or more alternate forms (alleles) of a chromosomal locus that differ in nucleotide sequence or have variable numbers of repeated nucleotide units. The size of the band is estimated by unweighted linear regression relative to the position of the 20 bp DNA ladder. The bands were scored based on the molecular weight. Figure 4.1 shows an example screening of one of the microsatellite markers (UNH 173) in populations of this study using 4% MetaPhore agarose gel. Figure 4.2 shows schematic representation of figure 4.1. Figure 4.3 shows the interpretation of polymorphic banding patterns into genotypes. The same samples were screened using 6% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS- PAGE) to visualize the allelic bands more clearly (Figure 4.4).

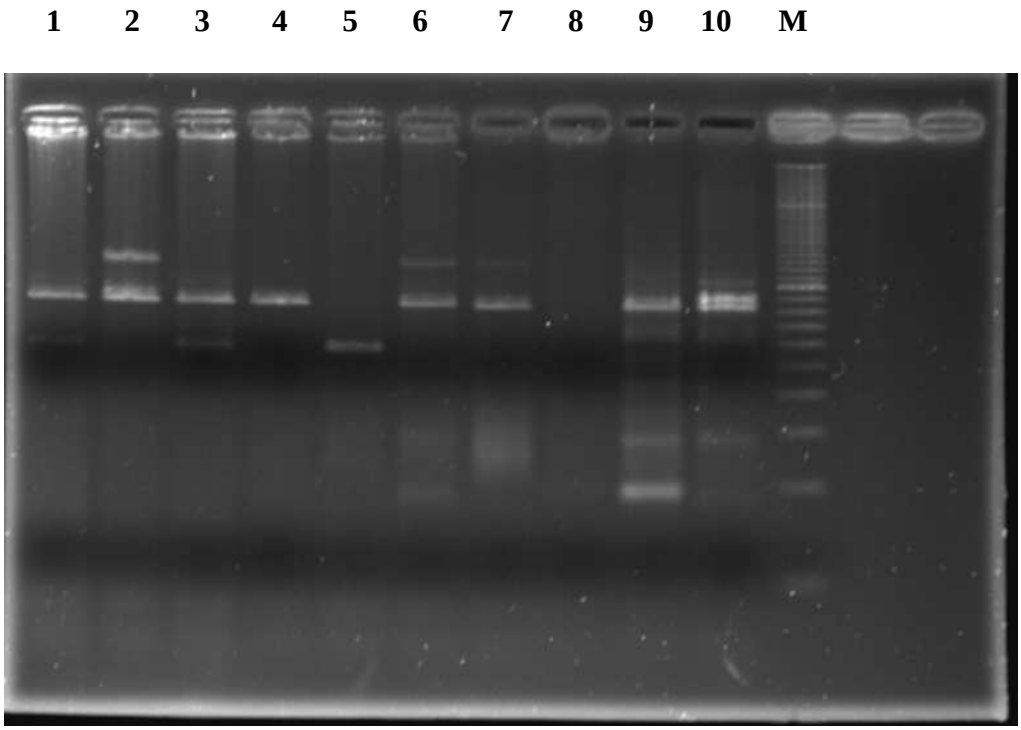


Figure 4.1 Banding pattern generated by primer UNH 173, tested on 10 individuals of Bentong using 4% MetaPhore agarose gel.

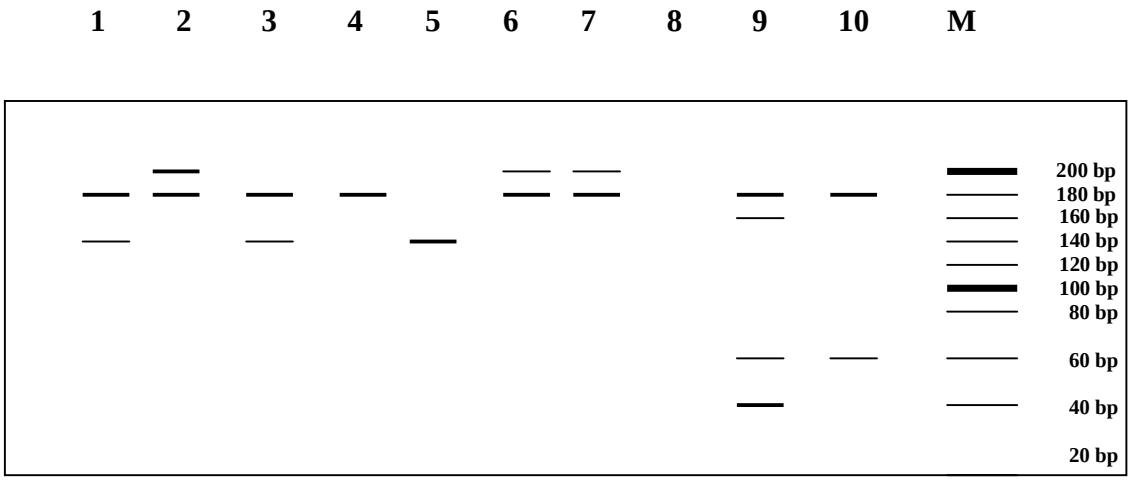
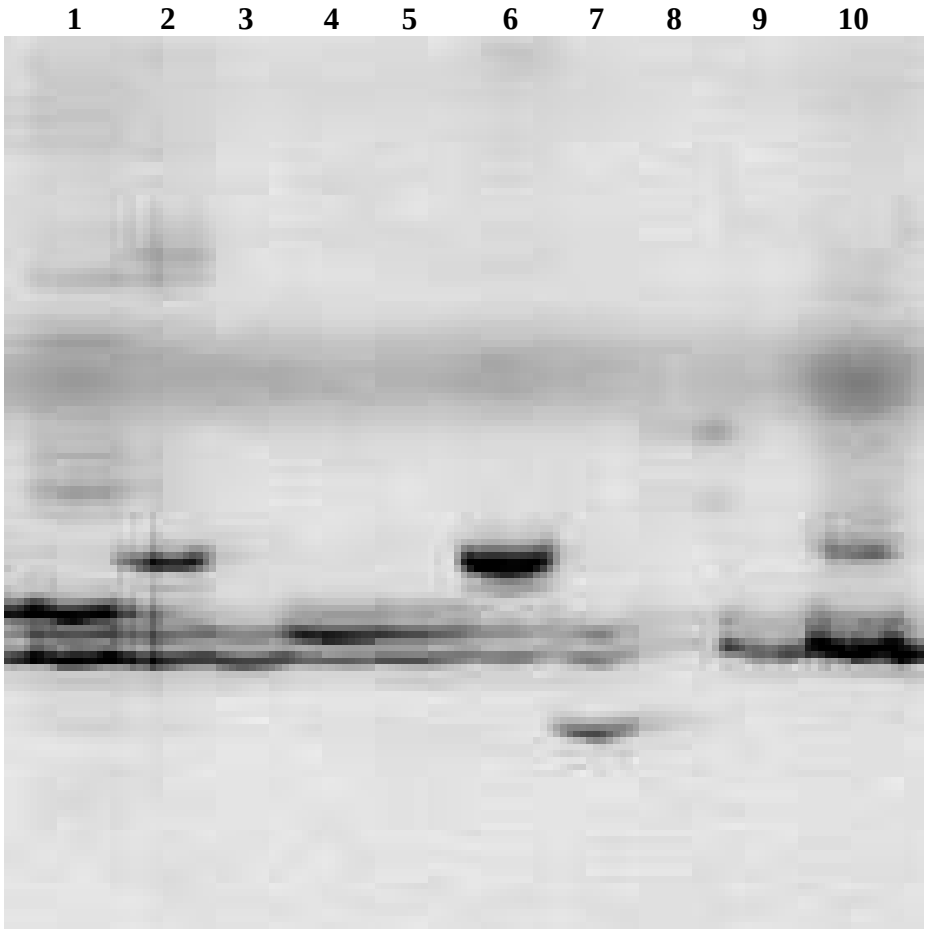


Figure 4.2 shows schematic representation of the same figure of Figure 4.1.

Figure 4.3 shows the interpretation of polymorphic banding patterns into genotypes.

Lane	Genotype	Phenotype
1	180/140	Hetrozygote
2	200/180	Hetrozygote
3	180/140	Hetrozygote
4	180/180	Homozygote
5	140/140	Homozygote
6	200/180	Hetrozygote
7	200/180	Hetrozygote
8	PCR Failed (No product was formed)	
9	180/40	Hetrozygote
10	180/60	Hetrozygote

Figure 4.4 shows banding patterns generated by primer UNH 173, tested on 10 individuals of Bentong using 6% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS- PAGE).



4.1.4 Allele number and Effective Allele number

For the 20 pairs of primer used in the population studies, the total number of observed alleles ranged from 2 to 6 alleles per locus for all the five populations and the effective alleles ranged from 1 to 5 alleles. The mean value for observed alleles was 4.4 and mean value for expected alleles was 3.1.

4.1.5 Level of Genetic Variation among Five Populations

The allele frequencies at informative microsatellite loci among the populations are given in Appendix 2.

4.1.6 Population Differentiation

F-statistics and gene flow for all loci were estimated and are shown in Table 4.1. Inbreeding coefficient (F_{IS}) estimates range from -0.0236 to -0.4427. The mean value of F_{IS} is -0.3149. The results show all the individuals tested were heterozygous.

Fixation index (F_{ST}) estimates ranging from 0.0857 to 0.2841. In other word 8% and 28% of the total genetic variation were distributed among subpopulations, with 92% and 72% of the variation within subpopulations.

Table 4.1: The summary of the genetic structure for the five populations with respect to the 20 loci

Primer	**Na**	**Ne**	**Exp Het**	**F_{is}**	**F_{it}**	**F_{st}**	**Nm**
UNH 104	5.0000	4.0409	0.7554	-0.1197	-0.0238	0.0857	2.6806
UNH 172	6.0000	3.7560	0.7366	-0.3821	-0.1187	0.1906	1.1607
UNH 146	6.0000	4.5544	0.7832	-0.1838	-0.2650	0.3791	0.4876
UNH 207	5.0000	3.3616	0.7051	-0.1442	0.2977	0.1793	1.2314
UNH 166	5.0000	2.6934	0.6312	-0.4224	-0.2242	0.1393	1.8762
UNH 190	5.0000	3.3736	0.7062	-0.0940	-0.0314	0.0572	3.4581
UNH 111	5.0000	3.0462	0.6744	-0.4108	-0.2168	0.1375	1.6551
UNH 173	6.0000	3.2863	0.6984	-0.0236	0.1555	0.1351	1.6063
UNH 149	3.0000	2.6768	0.6287	-0.3090	0.0628	0.2841	0.7539
UNH 189	4.0000	3.0782	0.6775	-0.4173	-0.1869	0.1626	1.5628
UNH 214	4.0000	2.7782	0.6423	-0.4427	-0.3217	0.0839	3.0108
UNH 216	4.0000	2.6400	0.6235	-0.3324	-0.1664	0.1246	1.9540
UNH 231	4.0000	2.9044	0.6580	-0.4539	-0.0801	0.2571	0.8736
UNH 233	4.0000	2.9147	0.6592	-0.3362	-0.2135	0.0919	2.7544
UNH 213	4.0000	2.2558	0.5587	-0.4377	-0.3485	0.0620	4.0680
UNH 145	4.0000	2.9632	0.6648	-0.3747	-0.2327	0.1033	2.4635
UNH 162	4.0000	2.6443	0.6240	-0.3655	-0.1993	0.1218	2.0630
UNH 194	4.0000	3.1583	0.6858	-0.3724	-0.0356	0.2454	0.9603
UNH 211	2.0	1.9459	0.4880	-0.2458	-0.2355	0.0083	13.1624
UNH 120	4.0000	3.4775	0.7150	-0.4310	-0.1368	0.2056	1.1816
Mean	4.4000	3.0775	0.6658	-0.3149	-0.0995	0.1527	1.5098
Std Dev	0.9947	0.5961	0.0665	-	-	-	-

Notes: Nm : Gene flow estimated from Fst = 0.25(1 - Fst)/Fst.
Exp Het: Expected Heterozygosity
Na: Observed allele number
Ne: Effective allele number

4.1.7 Hardy Weinberg equilibrium

Genetic variability is a measure of the tendency of individual genotypes in a population to vary from one another. Variability is different from genetic diversity, which is the amount of variation seen in a particular population. While, high heterozygosity means lots of genetic variability. Low heterozygosity means little genetic variability. Often, we will compare the observed level of heterozygosity to what we expect under Hardy-Weinberg equilibrium (HWE).

If the observed heterozygosity is lower than expected, we seek to attribute the discrepancy to forces such as inbreeding. If heterozygosity is higher than expected, we might suspect an isolate-breaking effect (the mixing of two previously isolated populations) (Beckman & Weber, 1992). Estimates of observed and expected heterozygosity of all the five populations and tests for conformity to Hardy-Weinberg and genotypic equilibrium expectations were performed with PopGene 2.11 (Yeh et al., 1997) and GenePop 4.0 (Raymond & Rousset, 2007)

All twenty microsatellite loci were polymorphic. Heterozygosity value was estimated according to Nei (1973). The mean levels of average observed heterozygosity ranged from 0.7039 to 0.7834 and the mean levels of average expected heterozygosity ranged from 0.5608 to 0.5852 for all the populations based on the 20 microsatellite loci.

Significant deviations from Hardy-Weinberg equilibrium (HWE) were observed for all twenty loci analyzed in the five populations. The mean probability value showed that loci from PKPS, Enggor, Negeri Sembilan and Bentong stocks conform to Hardy Weinberg (HW) equilibrium. Loci from Gift did not conform to HW expectations. Table 4.2 shows an analysis of variability at twenty microsatellite loci

Table 4.2: An analysis of variability at twenty microsatellite loci

		UNH104	UNH172	UNH146	UNH207	UNH166	UNH190	UNH111	UNH173	UNH149	UNH189	UNH214
GT	*N*	30	30	30	30	30	30	30	30	30	30	30
	A	5(4.2)	2(1.9)	4(2.7)	5(3.4)	3(2.9)	4(3.0)	2(1.9)	2(1.9)	2(1.8)	2(1.9)	2(2.0)
	Ho	0.9000	0.7333	0.5667	0.6333	0.9000	0.5667	0.7667	0.7667	0.7333	0.7667	0.8000
	He	0.7600	0.4911	0.6294	0.7039	0.6594	0.6639	0.4950	0.4851	0.4644	0.4861	0.5000
	P	0.0296	0.0090	0.0267	0.1740	0.0305	0.0038	0.0036	0.0021	0.0020	0.0021	0.0014
PS	*N*	30	30	30	30	30	30	30	30	30	30	30
	A	5(4.6)	3(2.9)	3(2.8)	4(2.6)	3(2.1)	3(2.8)	4(2.9)	5(4.1)	3(2.4)	2(1.9)	3(2.5)
	Ho	0.7857	0.7826	0.9259	0.3333	0.6522	0.6087	0.8400	0.6250	0.7667	0.6667	0.8148
	He	0.7812	0.6626	0.6399	0.6172	0.5132	0.6474	0.6560	0.7578	0.5894	0.4938	0.5919
	P	0.0000	0.0207	0.0000	0.0003	0.4375	0.8438	0.0688	0.0000	0.0171	0.0851	0.0078
BN	*N*	30	30	30	30	30	30	30	30	30	30	30
	A	3(2.7)	5(2.6)	2(1.7)	4(2.2)	3(2.2)	5(3.10	4(3.0)	5(3.6)	2(1.6)	3(2.9)	4(2.5)
	Ho	0.4762	0.8636	0.5769	0.4167	0.8636	0.6087	0.9000	0.6818	0.5385	0.9667	0.8667
	He	0.6315	0.6147	0.4105	0.5521	0.5331	0.6759	0.6663	0.7190	0.3935	0.6594	0.5994
	P	0.0104	0.0058	0.0471	0.3381	0.0165	0.8269	0.0000	0.1135	0.0720	0.0066	0.0100
EG	*N*	30	30	30	30	30	30	30	30	30	30	30
	A	3(2.9)	3(2.9)	2(1.7)	3(2.0)	2(1.6)	3(2.7)	3(2.3)	2(1.9)	2(2.0)	3(2.8)	3(2.5)
	Ho	0.7857	0.9643	0.5926	0.6897	0.5357	0.9000	0.8148	0.5185	0.5556	0.9643	0.9286
	He	0.6607	0.6524	0.4170	0.5018	0.3922	0.6328	0.5658	0.4938	0.5000	0.6429	0.6046
	P	0.2150	0.0065	0.0351	0.0558	0.0630	0.0189	0.0070	0.8714	0.6317	0.0014	0.0002
NS	*N*	30	30	30	30	30	30	30	30	30	30	30
	A	3(2.5)	3(2.9)	2(1.9)	3(2.3)	3(2.9)	3(2.9)	3(2.4)	3(2.4)	2(1.6)	3(2.6)	3(2.9)
	Ho	0.9231	1.0000	0.4667	0.5357	1.0000	0.9286	0.8462	0.5517	0.5200	0.7407	0.9286
	He	0.5969	0.6578	0.4801	0.5695	0.6005	0.6524	0.5747	0.5850	0.3848	0.6166	0.6658
	P	0.0004	0.0021	0.8062	0.1503	0.0031	0.0262	0.0041	0.0645	0.0936*	0.0294	0.0214

		UNH216	UNH231	UNH233	UNH213	UNH145	UNH162	UNH194	UNH211	UNH120	MEAN
GT	*N*	30	30	30	30	30	30	30	30	30	30
	A	3(2.6)	2(1.9)	2(1.8)	2(1.8)	3(2.7)	3(2.5)	3(2.5)	2(1.9)	4(2.9)	2.9(2.4)
	Ho	0.9667	0.8000	0.5333	0.6667	0.6667	1.0000	1.0000	0.6000	0.9333	0.7650
	He	0.6294	0.4800	0.4911	0.4444	0.5578	0.5978	0.5578	0.4800	0.6617	0.5620
	P	0.0000	0.0004	0.7051	0.0079	0.5488	0.0000	0.0000	0.2010	0.0040	**0.120**
PS	*N*	30	30	30	30	30	30	30	30	30	30
	A	2(1.9)	2(1.8)	3(2.4)	3(2.0)	3(2.9)	2(1.9)	2(1.9)	2(2.0)	4(2.5)	3.1(2.6)
	Ho	0.5714	0.6296	0.8621	0.6786	0.9667	0.5862	0.6333	0.6154	0.8571	0.7101
	He	0.4974	0.4314	0.5797	0.5057	0.6550	0.4905	0.4994	0.5000	0.5950	0.5852
	P	0.4896	0.0212	0.0012	0.0741	0.0013	0.3384	0.1689	0.2815	0.0201	**0.019**
BN	*N*	30	30	30	30	30	30	30	30	30	30
	A	3(2.6)	3(1.8)	4(3.1)	3(2.7)	4(2.1)	3(1.9)	2(1.8)	2(1.9)	3(2.0)	3.4(2.4)
	Ho	0.8571	0.6000	0.9333	0.9655	0.7586	0.4815	0.5000	0.5556	0.6667	0.7039
	He	0.6224	0.4294	0.6811	0.6249	0.5238	0.4561	0.4362	0.4890	0.5000	0.5608
	P	0.0013	0.1609	0.0008	0.0001	0.1139	0.5200	0.4957	0.5422	0.0699	**0.015**
EG	*N*	30	30	30	30	30	30	30	30	30	30
	A	2(1.9)	3(2.5)	3(2.7)	3(2.0)	3(2.8)	3(2.7)	3(2.6)	2(1.9)	3(2.4)	2.7(2.4)
	Ho	0.5926	0.8929	0.8929	0.7500	0.9259	0.8621	0.8077	0.6667	0.8846	0.7762
	He	0.4829	0.6078	0.6244	0.5121	0.6495	0.6332	0.6191	0.4965	0.5865	0.5638
	P	0.2781	0.0083	0.0202	0.0230	0.0278	0.0158	0.1357	0.1152	0.0014	**0.026**
NS	*N*	30	30	30	30	30	30	30	30	30	30
	A	3(2.0)	3(2.5)	3(2.8)	3(2.2)	3(2.7)	3(2.4)	3(2.4)	2(1.7)	3(2.5)	2.9(2.4)
	Ho	0.7200	0.8333	0.8571	0.7500	0.8667	0.8846	0.7857	0.6000	0.9286	0.7834
	He	0.5056	0.6044	0.6371	0.5651	0.6228	0.5910	0.5899	0.4200	0.6046	0.5777
	P	0.0601	0.0536	0.0002	0.0230	0.0006	0.0014	0.1522	0.0396	0.0002	**0.015**

N=Number of samples
A=Number of alleles
( )=Effective number of alleles
Ho=Observed Heterozygosity
He=Expected Heterozygosity
P=Probability value estimates conformation to Hardy-Weinberg expectations.
* Significant conformation to HW expectations at P<0.05

4.1.8 Detection of null alleles and scoring error

Point mutation in the primer annealing sites in such species may lead to the occurrence of ‘null alleles’, where microsatellites fail to amplify in PCR assays. PCR failure may result when particular loci fail to amplify, whereas others amplify more efficiently and may appear homozygous on a gel assay, when they are in reality heterozygous in the genome (Raymond & Rousset, 2007).

We used Micro-Checker (Oosterhout et al., 2005), which uses an iterative EM (expectation and maximization) approach to find the maximum likelihood estimate of null allele frequency. Analyses were done on all five populations and no loci show evidence for a null allele in all the populations. All the four tested populations were in Hardy Weinberg equilibrium.

4.1.9 Genetic Distance between five populations

Genetic distant measures can be grouped or clustered together to form dendogram construction. The five populations were clustered using the unweighted pair group with arithmetic averaging method (UPGMA), Figure 4.5. In UPGMA method, the first two populations to be clustered are those with the highest genetic identity values or lowest distance between them. Population from PKPS (P) and GIFT (G) were joined by vertical line. The next most similar pair joined in the dendogram was population from Negeri Sembilan (N) and population from Enggor (E).

Bentong populations clustered the furthest with highest genetic distance from other populations. Their dendogram relationship showed presence of high genetic variability.

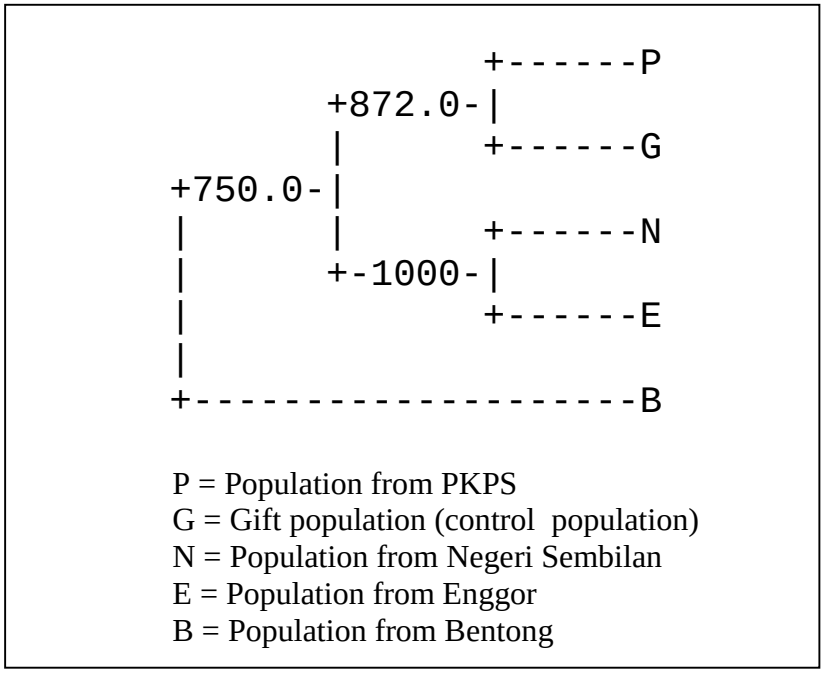


Figure 4.5: Dendogram relationship between four tested population and one control population

4.2 Quantitative Genetic Studies

4.2.1 Growth pattern of *Oreochromis spp*

The growth rate of tilapia is determined by several factors and it is important to take all these factors into consideration. The growth rate will for instance be affected by water quality, temperature, oxygen levels and the general health of fish. The type of food provides them with and in which quantities will naturally also be of imperative importance.

Basic statistics for body traits pooled across strains are given in Table 4.3 The fish reached a harvest weight of 330 g over 120 days of culture from stocking at around 10 g, with average daily weight gain of 2.67 g. The coefficient of variation in harvesting body weight was large (62.7%), consistent with other studies for Nile tilapia (Ponzoni et al., 2005; Nguyen et al., 2007). In a review, Gjedrem (1983) also showed a very wide range of coefficient of variation for body weight at various ages in different fish species (13 to 78%).

Table 4.3: The number of family (N), mean, standard deviation (S.D) and coefficient of variance (%).

Parameter	**N**	**Mean**	**S.D**	**CV (%)**
Stocking weight	48	147.5	84.7	57.4
Harvesting weight	48	166.2	104.2	62.7
Stocking length	48	8.6	0.8	9.3
Harvesting length	48	15.1	3.6	23.8
Stocking depth	48	1.6	0.5	31.3
Harvesting depth	48	6.2	1.9	30.6
Stocking width	48	2.7	0.4	14.8
Harvesting width	48	2.3	0.9	39.1

Relationship between weight and length during stocking and harvesting are shown in Figure 4.6 and Figure 4.7. Relationship between depth and width during stocking and harvesting are shown in Figure 4.8 and Figure 4.9. The coefficients of variation were significant.

Figure 4.6: Relationship between Weight and Length during stocking

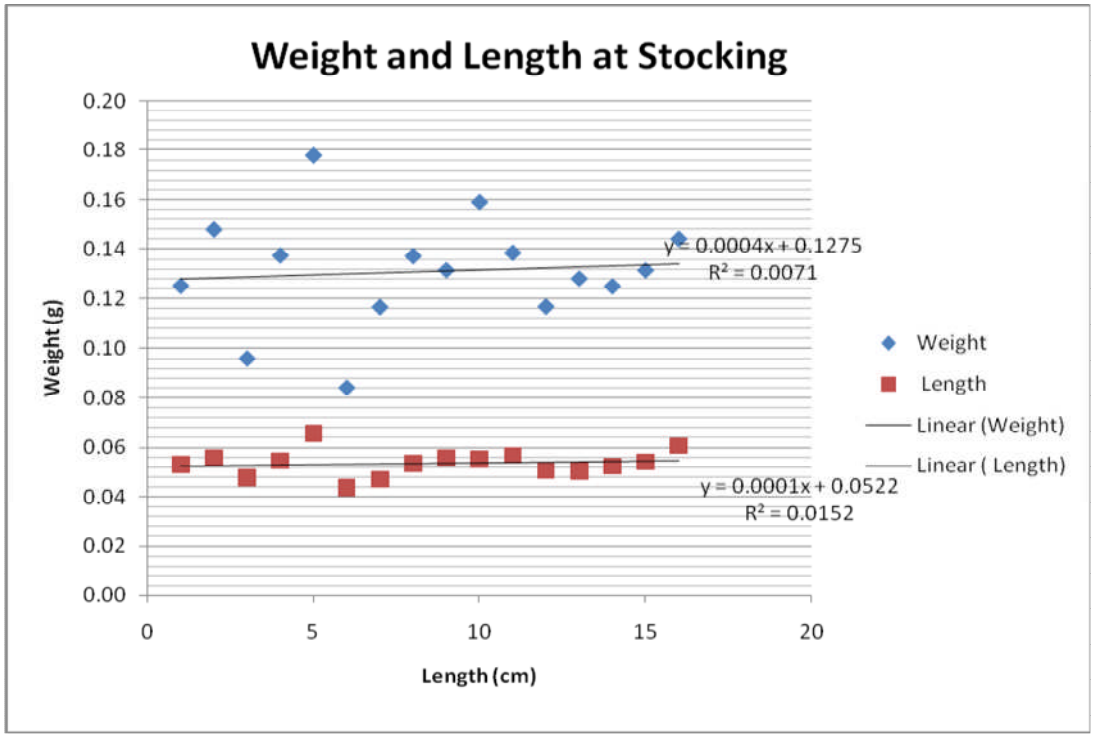


Figure 4.7: Relationship between Weight and Length during harvesting

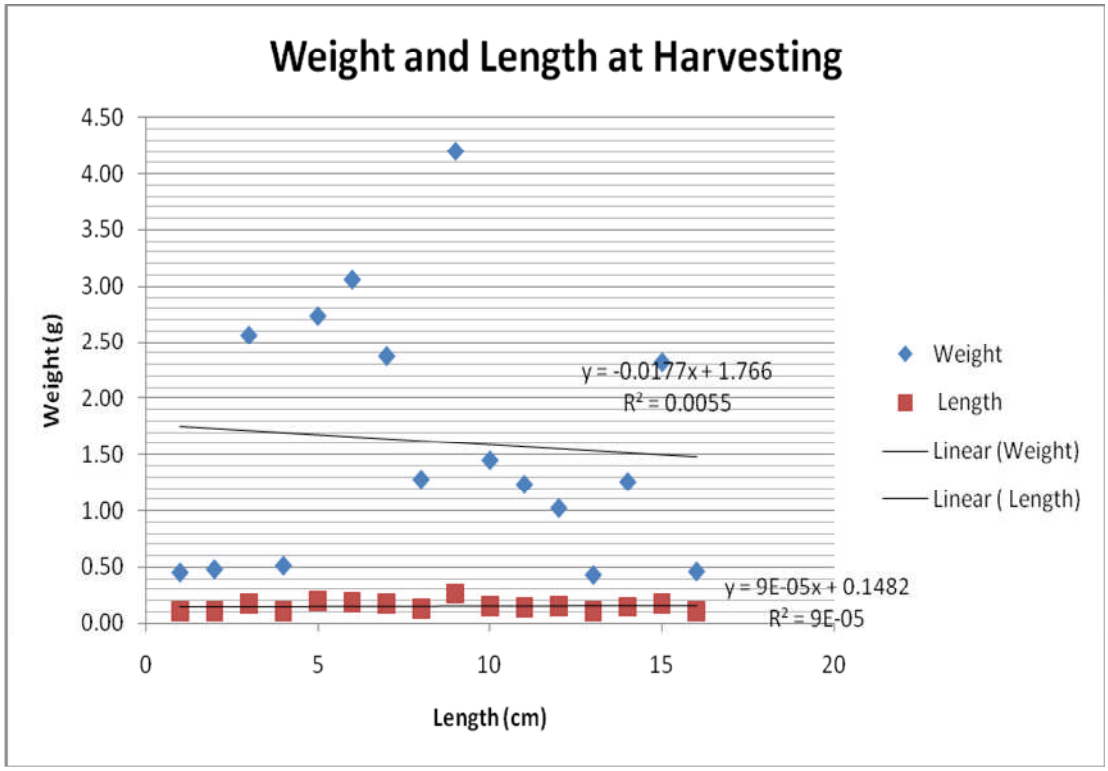


Figure 4.8: Relationship between Depth and Width during stocking

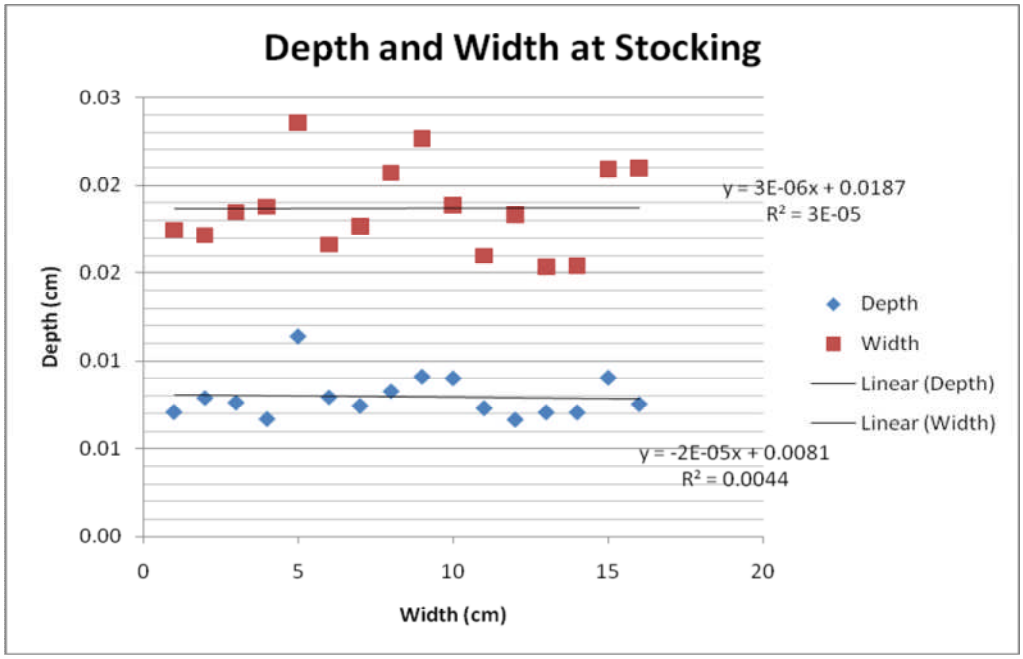
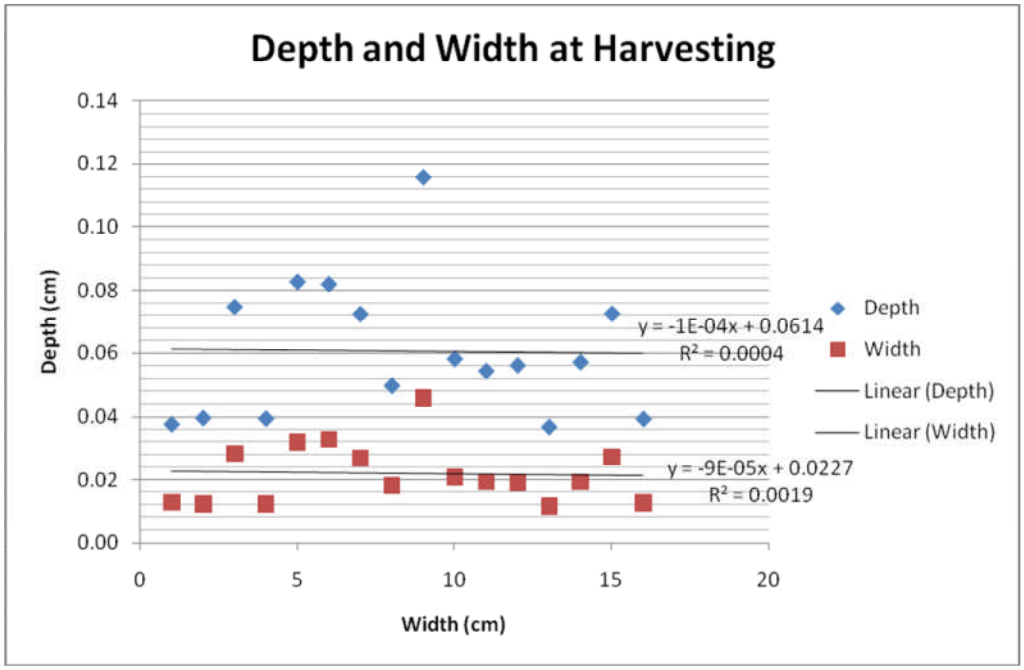


Figure 4.9: Relationship between Depth and Width during harvesting



4.2.2 Survival

Percentage of egg hatchability, percentage of fry survival and percentage of adult fish survival were the three traits measured under survival. Figure 4.10 shows percentage of the three traits from three replicates. There were sixteen families in each replicate.

In egg hatchability category, fingerlings from replicate 2 showed the highest survival percentage (87.24%). The second highest survival rate shows by fingerlings from replicate 3 (84.78%). In fry survival category, fries from the third replicate shows a highest survival rate (80.17%) and followed by fries from second replicate (80.08%).

Finally for adult fry survival category fingerling, the first replicate showed the highest survival rate (72.91%) compared to other two replicates. Fingerlings from the first replicate showed the lowest survival rate in the egg hatchability and fry survival category.

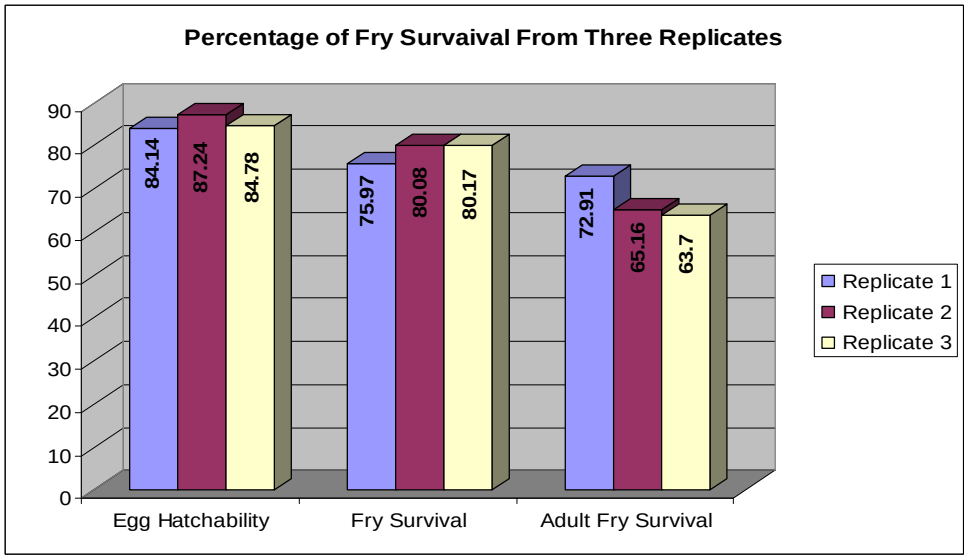


Figure 4.10 shows percentage of fry survival from three replicates.

4.2.2 Water Quality

The main factors that influence growth of tilapia under farming conditions are the water quality factors like dissolved oxygen, temperature, ammonia and pH. . Four parameters of water quality test were taken to monitor the quality of water. There were temperature, dissolved oxygen, pH and ammonia. The following charts show average readings of water quality for Stocking Tanks, Treatment Tanks, Artificial Incubators and Nursery Tanks. Figure 4.11 shows an average reading for stocking tanks. The average temperature was 23.9 ^{0} C, which is optimum temperature for stocking breeders. An average dissolved oxygen and a pH reading shows 6.40 mg/l and 6.90 mg/l. These readings were desirable for fish culture. Average of ammonia level was 0.13 mg/l. The level of ammonia is still acceptable because in fish culture, ammonia readings more than 2mg/l will cause massive mortality in breeders.

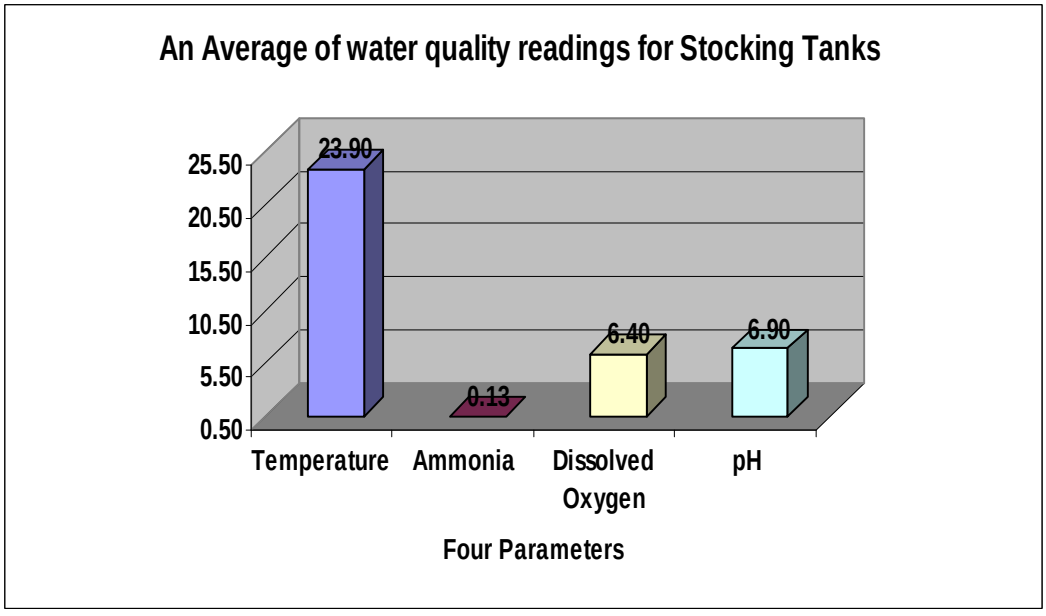


Figure 4.11 Average water quality readings for Stocking Tanks

Water quality readings also were taken from treatment tanks. Figure 4.12 shows average reading for treatment tanks. Average temperature in treatment tanks was 25.20 ^{0} C, which was very good temperature for frequent reproduction. Average readings for dissolved oxygen, pH and ammonia were 6.20 mg/l, 5.59 mg/l and 0.17 mg/l which were optimum readings for reproduction.

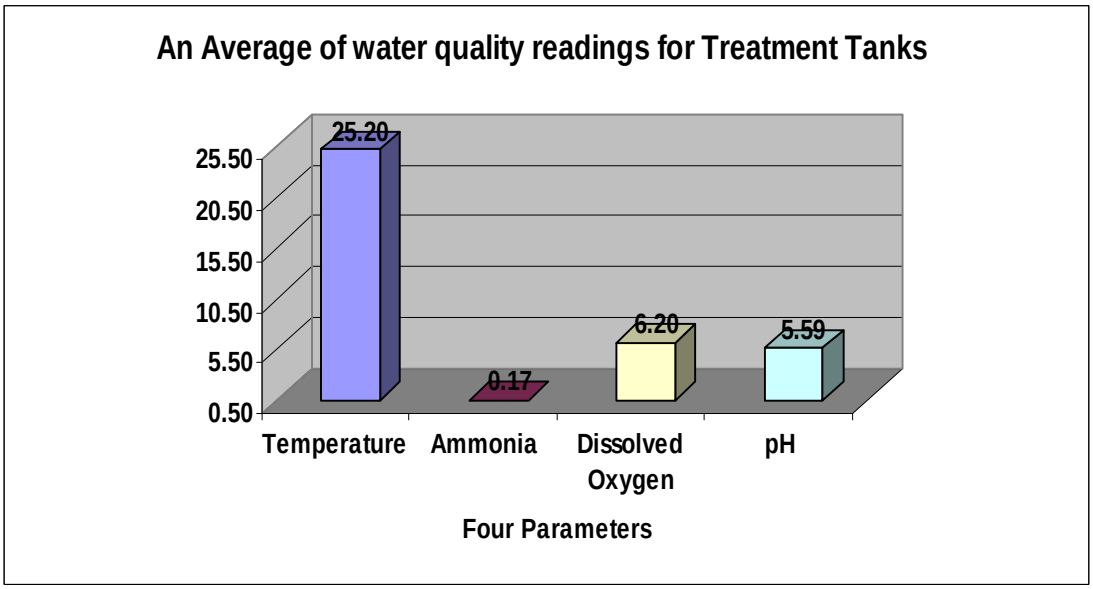


Figure 4.12 Average water quality readings in Treatment Tanks.

Water quality management in artificial incubation tanks and nursery tanks were very important to produce high number of fingerlings. All the water quality parameters were also taken in these two tanks. Figure 4.13 and Figure 4.14 shows an average water quality reading for artificial incubation and nursery tanks. All the readings were optimum for egg incubation and fry nursery.

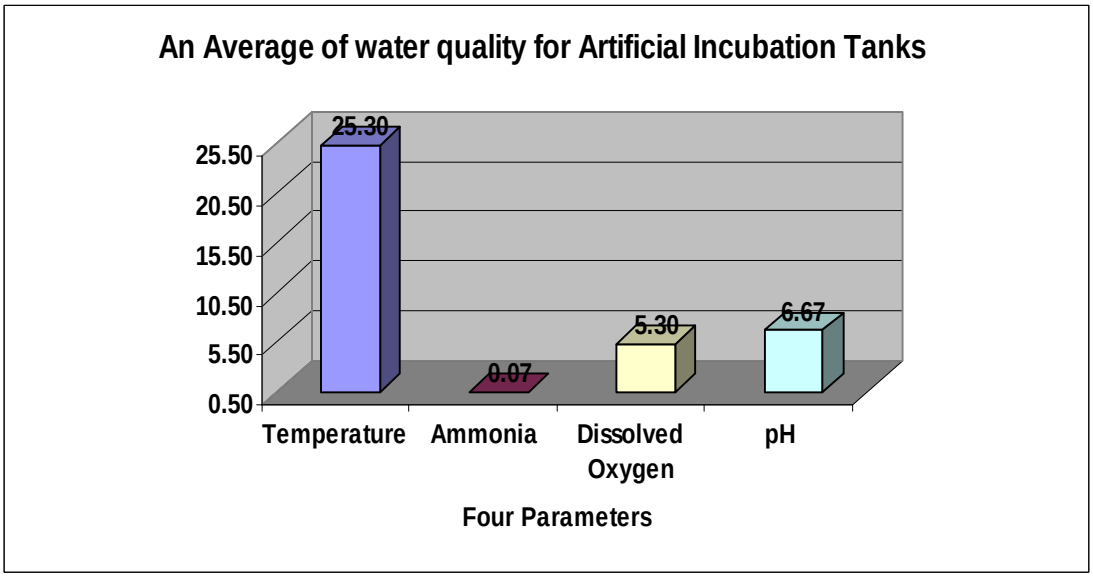


Figure 4.13 Average water quality readings in Artificial Incubation Tanks.

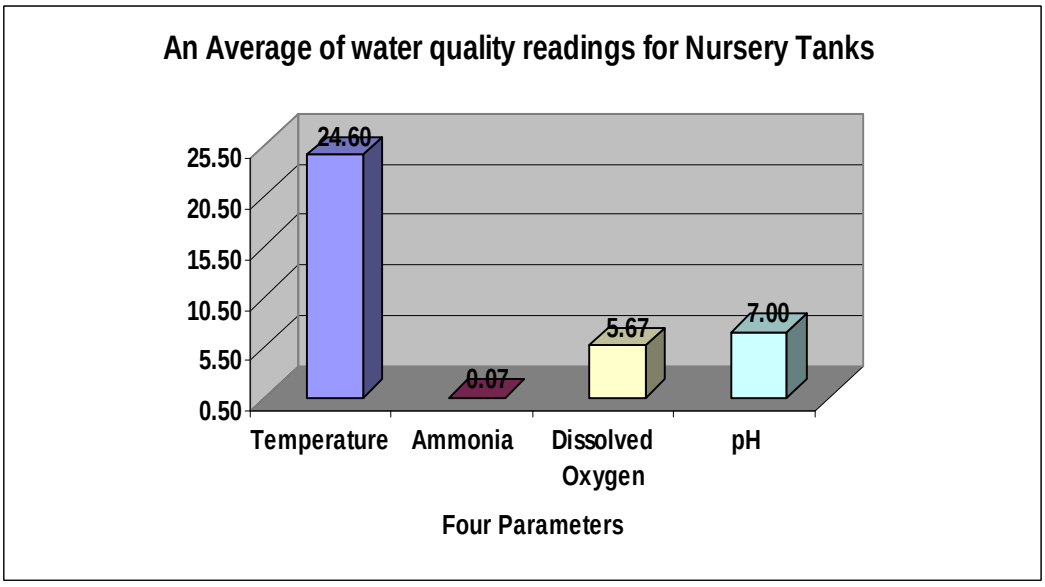


Figure 4.14 Average water quality readings in Nursery Tanks.

CHAPTER V

DISCUSSION

5.1 Population Genetic Study of four test and control populations

5.1.1 The Test of Reproducibility and Reliability of Microsatellites.

The use of microsatellite markers in population studies appear to be advantageous because they can produce reproducible results (Schierwater & Endler, 1993). In recent years, microsatellite markers have been widely used in revealing genetic variation and population structure (Broders et al., 1999; Polziehn et al., 2000). This is because of its advantages of being condominant, highly polymorphic, abundant across the genome, and easy genotyping (Røed, 1998).

In this study, the fragments generated were the same over a range of DNA concentrations (5ng to 200 ng). Only 5 ng was required for microsatellite analysis. Banding patterns were also not affected by slight variations in DNA concentrations. Different *Taq* polymerases were able to produce the same banding patterns and this shows that this technique is highly viable. The same banding patterns were observed, resulting from using the minimum amount of polymerase which was sufficient for producing the required bands (Chong et al., 1998).

The results obtained in this study showed that microsatellite marker results are reproducible. Even when different thermal cyclers were used, the same amplification banding patterns were always generated. Similarly, Subha (2002) showed that results from microsatellite analysis were reproducible even when template DNAs were extracted using the same protocol and experimental conditions but amplifications was done using different thermal cyclers. Thus, microsatellites can be considered to be a reliable marker.

Clear banding patterns were also obtained even when the annealing temperatures varied from 50^0C to 60^0C. Primers that were optimized in one laboratory can be used in others, provided all other experimental parameters are maintained.

Polymorphic microsatellite loci were resolved using 4% MetaPhore agarose gels and 6% SDS PAGE gels which produced clear bands. Heterozygous individuals were characterized by the presence of extra bands that were slower than the allelic bands. Presumably these bands resulted from heteroduplex molecules formed as combinations of complementary strands which reannealed after denaturation without amplification. For most loci, only one heteroduplex slower band was observed, but occasionally two bands were observed, referred to as stutter bands. Stutter bands migrate more slowly than the actual bands (May et al.,1997).

In this study, the method of sampling also modified from invasive sampling to nonlethal sampling. Fins from fish were collected for DNA extraction without causing death to the fish. Therefore, this gives opportunities for research to be conducted on a large scale and monitoring of fish stocks with minimal disturbance of the system.

Microsatellite markers have been widely used in many types of research such as parentage studies, population studies and mapping (Liu & Cordes, 2004). Microsatellites are particularly useful because they are codominant in their phenotypic expression and commonly exhibit more alleles segregating per locus. Microsatellites genotypes are useful in estimating the relationship and relatedness between individuals of unknown ancestry. This information is very important to increase the efficiency of selective breeding programs for

aquaculture species. Analysis using microsatellite markers in general has a very high degree of accuracy.

Hence, microsatellite markers were further tested in population studies to assess the integrity of the Malaysian tilapia stocks.

5.1.2 Genetic Structure among Five Populations

All the 20 microsatellite loci surveyed were highly polymorphic genetic markers. This could be due to the likelihood that microsatellites are situated at the non coding region thereby being unaffected by functional constraints. Microsatellites offer great advantages when compared to the other markers, although they may become suboptimal due to large sampling errors. Large number of alleles may impose problems with sample size and in subsequent genetic studies sample sizes should be increased from 30 to 50 (Carvalho & Hauser,1994).

The number of observed alleles (na) varied among the 20 loci with an average of 4.4. The effective number of alleles (ne) was less than the observed values ranging from 1.95 to 4.55 with mean of 3.48. The level of variation depicted by number of alleles at each locus in the present study serves as a measure of genetic variability having direct impact on differentiation of individuals within a species.

Wright's *F*-statistics, provide important insights into the evolutionary processes that influence the structure of genetic variation within and among populations, and they are among the most widely used descriptive statistics in population and evolutionary genetics. If the values for both observed and expected heterozygosity are the same, F will be zero (Dorak, 2007).

A positive value indicates that there is an increased number of homozygotes, and population may be inbred - the larger the number, the greater the extent of inbreeding. A negative value indicates that there are more heterozygous individuals than would be expected; this might happen for the first few generations after two previously isolated populations become one (Dorak, 2007).

The high mean heterozygosity values could be attributed to low level of inbreeding, low selection pressure and large number of alleles present in a population. Within population inbreeding estimates (F_{IS}) for investigated population (-0.3149) further showed a very low rate of inbreeding, perhaps due to absence of any directional selection.

Mean value of fixation index F_{ST} 0.1527 shows genetic differentiation among subpopulations. Putting this in other words, 15% of the total genetic variation is distributed among subpopulations, with 85% of the variation within subpopulations (Kent & Bruce, 2009).

Although F_{ST} has a theoretical range of 0 to 1.0, the observed maximum is usually much less than 1.0. Wright (1978) suggests the following qualitative guidelines for the interpretation of FST (based on allozyme loci): (Hedrick, 2000).

- The range 0.0 to 0.05 may be considered as indicating little genetic differentiation
- The range 0.05 to 0.15 indicates moderate genetic differentiation
- The range 0.15 to 0.25 indicates great genetic differentiation

F_{ST} value (0.1527) from current studies shows there is great genetic differentiation among subpopulations.

5.1.3 Degree of Heterozygosity

Heterozygosity is a measure of genetic difference within a population, and to some degree is a measure of the population's ability to withstand disaster. Breeding closely related animals reduces the level of heterozygosity in the offspring. When inbreeding occurs within population rare genes can be lost and the frequency of deleterious genes can increase or even become fixed, and overall genetic variability is reduced (Nguyen et al., 2006).

Inbreeding depression is where deleterious alleles increase in frequency in the population, and variability decreases. The effects manifest as decreased fitness. Less offspring are born, and these have a lower chance of survival than previous generations, generally due to birth abnormalities. Inbreeding depression can, and generally does, lead to and cause extinction. The opposite of inbreeding depression is out breeding depression. This is where the animals involved aren't closely related enough (Balloux & Lugon, 2002).

Significant differences in the mean observed heterozygosities ($p<0.05$) for all 20 loci among five populations were observed. All the populations show high heterozygosities and the highest (0.9286) were observed for Negeri Sembilan (NS) and Enggor (EG) population.

High heterozygosity among all the tested population shows a great genetic variability. Often, we compare the observed level of heterozygosity to what we expect under Hardy-Weinberg equilibrium (HWE). In these studies the observed heterozygosity is higher than expected and we might suspect an isolate-breaking effect or the mixing of two previously isolated populations had occurred.

5.1.4 Hardy–Weinberg Equilibrium

In a large, randomly breeding (diploid) population, allelic frequencies will remain the same from generation to generation; assuming no unbalanced mutation, gene migration, selection or genetic drift. When a population meets all of the Hardy- Weinberg conditions it is said to be in Hardy-Weinberg equilibrium**.** Populations in their natural environment can never meet all of the conditions required to achieve Hardy-Weinberg equilibrium, thus their allele frequencies will change from one generation to the next and the population will evolve (Gregorius,1978).

Just how far the population deviates from Hardy-Weinberg is an indication of the intensity of external factors, and can be determined by a statistical formula (chi-squared), which is used to compare observed versus expected outcomes: is used to determine the probability that the observed number differs from the expected number due to chance alone. This high probability indicates that some external factor (i.e., migration, selection, inbreeding, or drift) is influencing the frequencies of alleles (Steven et al., 2006).

In these studies three populations were found to have significant conformations to Hardy-Weinberg Equilibrium expectations (Table 4.2). Their probability value, $P < 0.05$ which indicates these populations conforms to HWE expectations and there is different between populations. Populations from PKPS showed probability value (P) 0.019. While populations from Bentong and Enggor showed 0.015 and 0.026. While population from Negeri Sembilan showed 0.015. All these probability value showed that migration, selection, inbreeding or drift doesn't occur in these studies.

The control population, GIFT showed significant deviation from Hardy-Weinberg Equilibrium. The probability (P) value for this population was 0.120 indicates deviation from HWE expectations. These deviations from HW equilibrium could indicate the presence of mutation, migration, natural selection or small sample size (Nguyen et al., 2006). Generally, for loci which did not conform to HW equilibrium, a significant lack of heterozygotes was observed.

In general, this problem is due to the small sample size. Large number of allelic variants occurring mostly in low frequencies, more samples (50 instead of 30) would have been ideal for microsatellite analysis (Bhassu et al., 2004 and Ruzzante, 1998). A larger sample size would reflect genotype frequencies more accurately and minimize bias and sampling variance for variability and genetic distance estimates (Carvalho & Hauser., 1994 and Simianer., 2005). Because of the constraints of sampling, the maximum sample size obtained for the study was 30 for each population.

5.1.5 Genetic Distance

A way of measuring the amount of evolutionary divergence in two separated populations of a species is by counting the number of allelic substitutions per locus that have cropped up in each population. The most popular and frequently used method of tree building can be classified as phenetic methods based on distances. The measures of pair-wise distance/dissimilarity between two genes, the actual size of which depend on different definitions, and construct the tree totally from the resultant distance matrix. The latter evaluate all possible trees and seek for the one that optimizes the evolution. The most popular distance-based methods are the unweighted pair group method with arithmetic mean (UPGMA) (Higgs & Manchester, 2001).

In this study, a phenogram was constructed based on 20 microsatellite loci. Unweighted pair group method with arithmetic mean (UPGMA) was used. If there was mixture of these populations, the presence of gene flow is minimal. PKPS population and control population, GIFT grouped together. Populations from Negeri Sembilan and Enggor clustered together and were therefore genetically similar. Bentong population was clustered the furthest with the highest genetic distance from the other populations. Thus GIFT is a control population used to compare the closeness of populations.

This study indicated that the five populations are suitable for selective breeding program. Characterization of genetic variation among populations in this way aims to determine strains to be included in strain evaluation trials. This will control inbreeding, elimination of deleterious recessive genes and prediction of heterosis.

5.2 Quantitative genetic studies

5.2.1 Growth performance

Genetically selected stocks will play an important role in aquaculture development and ensure the best use of the environment and at the same time improve production cost-efficiency. The main objectives of selective breeding programmes today are faster growth, late sexual maturity, higher resistance to diseases (higher survival) and better flesh quality (lower fat-content, colour, texture etc.) (George & Ronald, 2005).

Across two generations, all the four tested populations displayed better growth rates. The significant difference between these generations was rearing factor. The generations were reared in tanks and the second generations were reared in ponds. It has been reported that rearing in ponds and tanks can affect the growth cycle of the tilapia stocks (Subha, 2002).

Basic statistics for body traits showed that growth performance of red tilapia strains from PKPS, Bentong, Enggor and Negeri Sembilan were good. Strains from Enggor (male) and Bentong (female) crosses possessed better growth performance compared to the other crosses. This strain had the fastest growth rate. This cross has shown a highest mean in weight, length, depth and width. The existence of a positive correlation in the two stocks could be due to genetic explanations. Heterozygous individuals may have higher fitness than homozygotes (Wohlfarth et al, 1990). However, this would unlikely be the reason as the correlations could have just happened. The positive correlations that were observed in this study clearly suggest that some of the microsatellite loci have associative over dominance effect on the fitness. Even though, microsatelites are at the non coding regions, they may be situated in close

proximity to the coding region that are responsible for size. In most studies, observed heterozygosity and growth correlations considered microsatellites as markers in linkage association with the genetic conditions that were responsible for differences in growth among individuals (Toth et al,.2000).

In a same descriptive analysis, the worst growth performance were showed by strains from Bentong (male) – Enggor (female) and Bentong (male)-Bentong (female) crosses. The lowest mean level in weight, length, depth and width were observed. This situation may be due to the favorable additive gene effect and lack of dominance in the alleles concerned. The growth performance of the other strains was intermediate. The results show that there is genetic difference in performance among the strains studied.

In this tilapia breeding program, the first generation progeny were from random mating of parents. Selection across a few generations would have caused the allele frequencies to be more fixed. The progeny were a mixture from four families, thus contributing to some extent to the variability and high allele number. If the allele number is high, then the number of the individuals tested should be high too (Himadri & Ashish, 2003).

However, in this study this was not possible as the number of fish was limited. Across the two generations, there were still variations observed in the loci, which made it possible to detect associations between heterozygosity and growth rate (Toth et al,. 2000).

5.2.2 Survival

Survival rate of all the crosses in egg hatchability, pre swim fry level and adult fry level were more than 90%. This may due to optimum level of environmental factors. The main environmental factors were temperature, ammonia level, dissolved oxygen and pH. All the parameters were in optimum level for stocking tanks, treatment tanks, artificial incubation tank and fry nursery tanks.

However, there were high numbers of mortalities during early stage of grow out. We suspect that this may be attributed to a higher proportion of small fish at stocking hapas. The small fish from all the strain may have been disadvantaged from food competition in early stage of grow-out, and they could be more vulnerable to environmental conditions and thus having a higher risk of deaths (Subha, 2002).

For survival, data recording and analysis methods for this trait reported in the literature have raised concerns. In almost all studies where full pedigree or measurement of individual fish is not available, survival is often expressed as average percentage of the difference between stocking and harvest fish. In this way, there is a very limited number of observations per treatment group. In summary, since survival rate differed among strains, there may be a within strain genetic component in this trait that could be taken into account in breeding programs for red tilapia (Subha, 2002).

CHAPTER VI

CONCLUSIONS

Microsatellites have been used increasingly in studies of tilapia in recent years. A number of issues have been examined which include genetic structure of populations, effective populations sizes and temporal changes in allele frequencies (Li et al,. 2004). The results obtained from these studies have been economically important for aquaculture and fisheries. So microsatellite markers are considered the most appropriate approach for documenting and monitoring the characteristics of the cultured tilapia stocks. Hence the development of these markers will be of great importance for use in the future breeding programs (Hulata, 2001).

Microsatellite markers have proven to be reliable and reproducible. It also allows non lethal sampling of the fishes. In this study, time and cost limited the number of variable microsatellites loci examined. Population genetic parameters based on microsatellite markers were considered stronger than those generated from the allozyme markers. This was due to the variability of the number of alleles and higher heterozygosity levels. Microsatellite markers were able to detect a greater number of variable loci.

In the current study, the major aim was to study a molecular genetic evaluation in diallel crosses among four stocks of red tilapia (*Oreochromis sp*) in Malaysia. Microsatellite markers were used in this study to determine the population structure and monitor the levels of genetic variation within and among tested populations.

It was not necessary to sacrifice the sample in order to obtain the tissue sample since non invasive sampling were successfully tested found to be sufficient to do the analysis. Therefore with the quick, reliable and cost effective approach developed and without the necessity to sacrifice individuals, microsatellite markers are considered to be suitable for aquaculture and also other field studies in Malaysia.

Use of molecular markers has been suggested as an aid in the improvement of stocks to be used for selective breeding. Cultured tilapia stocks which showed poor growth rates and poor performance are likely to have lost genetic variation and are experiencing inbreeding depression and the negative effects of gene introgression and poor broodstock management (Zardoya et al,.1996).

In the current study, the four stocks that we obtained from different states in Peninsular Malaysia initially showed better growth performance and survival as well. Crosses from PKPS and Bentong really showed a good growth performance and survival. While stocks from Negeri Sembilan and Enggor was inferior compared to other two stocks. It could be due to previous hatchery practices that may have contributed to the restriction of gene pools. Loss of genetic variation may have adverse effect on the gene pools of the cultured tilapia stocks. This could lead to reduced tilapia productivity under culture and make future growth improvement via selection mode difficult. Good breeding practices with large effective breeding populations and good management will ensure that the observed levels of genetic variation and stock integrity are maintained at the hatchery over the long term.

There were few steps in tilapia strain evaluation suggested by Nguyen in Genetic improvement programs for red tilapia Oreochromis spp in Asia (2011). The steps involve choice of strains, population sampling, preparation of testing environments, determination of sample sizes, and implementation of the experiment, collection of data, statistical analysis and interpretation of the results. All the steps were followed as suggested but some shortcomings could not be avoided in this experiment. However, most of possible systematic effects associated with the observed traits were generally accounted for in statistical models.

However in future, uncertainty could develop in the genetic quality of the fries provided to the farmers. Therefore the status of the tilapia stocks need to be reevaluated among the strains originating from the different places. They are needed to be characterized by using microsatellite markers. If there is need to do cross breeding, then the actual status of the stocks first needs to be determined, in order to improve the stocks. Genetic markers are considered the most appropriate approach for documenting and monitoring the characteristics of the cultured Malaysian stocks. This will ensure that the goal of improving the tilapia stocks used in Malaysian aquaculture can be achieved in the future.