GENETIC DIVERSITY IN FARMED NILE AND RED HYBRID TILAPIA STOCKS IN INDIA USING MICROSATELLITE GENETIC MARKERS

Thesis submitted in part fulfillment of the requirements for the Degree of ***Master of Fisheries Science*** *in* ***FishBiotechnology*** *to the Tamil Nadu Fisheries University, Nagapattinam*

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2014

CERTIFICATE

This is to certify that the thesis entitled **"Genetic diversity in farmed Nile and red hybrid tilapia stocks in India using microsatellite genetic markers"** submitted in part fulfillment of the requirements for the award of the degree of **Master of Fisheries Science** in **FishBiotechnology** to the Tamil Nadu Fisheries University, Nagapattinam is a record of bonafide research work carried out by **Shyamala, G. C** under my supervision and guidance and that no part of this thesis has been submitted for any other degree, diploma, fellowship or similar titles.

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Dedicated to my Parents, Teachers &Friends

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

At the outset, I place on record my heartfelt gratitude and effusive thanks to **Dr. K. Karal Marx**, Professor & Head, Department of Fisheries Biotechnology, and Chairman of the Advisory Committee for his valuable guidance, scholarly suggestions immense help rendered in carrying out research work and preparation of manuscript which helped me to shape it in its final form.

I wish to extend my profound thanks to **Dr. P. Chidambaram**, Associate Professor, Department of Fisheries Biotechnology and member of advisory committee, for his valuable help, constant inspiration and critical review of the manuscript to achieve success in this work.

I like to render my sincere thanks to **Dr. G. Jeyasekaran**, Professor &Head, Department of Fish Quality Assurance and Management and member of advisory committee, for his continuous encouragement and timely suggestions.

I sincerely thank **Dr. G. Sugumar**, Dean-in-charge, Fisheries College and Research Institute, Thoothukudi for the encouragement and facilities provided by him.

I place my sincere thank to **Dr. S.N. Sivaselvam**, Professor & Headand **Dr. S.M.K. Karthickeyan**, Professor, Department of Animal Genetics and Breeding, Madras Veterinary College, Chennai for the facility provided by them and valuable help.

I offer my profound thanks to **Dr. R. JeyaShakila**, Professor, Department of Fish Quality Assurance and Management, for her valuable help during my study.

I place my sincere thanks to **Mr. Kumaresan**,Assistant Librarian,**Mrs. Ezhil Rani,** Library Assistant and **Mrs. Indhrani,** Attendantfor their kind cooperation in giving reference materials from our college library.

I have great pleasure in expressing my gratitude to my friends **Rajan Kumar**, **Rakesh**, **GajendraGulladi** for their kind cooperation and mental support. I also extend my heartfelt thanks to **Jagadeesh**, **B. Veeranna,S. Saral, Rehana Raj, N.Thenmozhi, G. Sumati, B.R. Sona**and**BharathiRathinam**for having helped me during different phases of research work.

I express my sincere appreciation for the help rendered by**A. Hisham,G. Arunkumar**,**Rose Diana Charles, P. Hepsibha,S.Sankar Ram,Harirama Krishnan**and **Benazir Nisha.**

I have great pleasure in expressing my gratitude to **Tamilnadu Veterinary and Animal Sciences University, Chennai** for providing PG Merit Scholarship during the tenure of my study.

Above all I cannot find words to express what I really feel regarding the endless backup, moral boosting, support, love and affection showered upon me by my **family**, without whom I never could have completed the study.

SHYAMALA, G. C

ABSTRACT

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Title **:**Genetic Diversity in Farmed Nile and Red Hybrid Tilapia Stocks inIndia Using Microsatellite Genetic Markers

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Tilapia are one of the most widely introduced fish globally that has clearly emerged as a very promising group in aquaculture. The Nile tilapia,*Oreochromis niloticus* was probably introduced unofficially in West Bengal in the late 1970s or early 1980s. Nile tilapia spread all across the country within a few years due to its prolific breeding and adaptability to wide range of environmental conditions. This leads to over population and stunted growth in both aquaculture ponds and reservoirs especially in large scale aquaculture systems.In order to prevent stunted growth in culture system, aquaculturists has been turned to produce Genetically Improved Farmed Tilapia (GIFT). However, the conservation of genetic variation is an essential component of many species management programmes in fisheries and aquaculture. Scope also exists for developing further improved strains through Marker Assisted Selection (MAS). DNA markers play an important role in analysing or assessing stocks to increase the production efficiency.

Genetic diversity analysis was done to know the genetic distance between the Nile and red hybrid tilapia stocks available in different states of India. The collected fish fin-clip samples were preserved in 90% ethanol.

DNA was extracted from fin-clips by phenol-chloroform method and extracted DNA was subjected to amplification by using nine microsatellite primer pairs.

The amplified DNA fragments were then subjected to automated genotyping. Results obtained from genotyping were calculatedand input data were arranged according tosoftware's requirement and fed to POPGENE version 1.31. Data or results obtained from POPGENEwere then used for analysing genetic variation based on heterozygosity, allelic diversity, F-statistics, Hardy-Weinberg equilibrium (HWE), Polymorphism Information Content (PIC), etcin Nile and Red hybrid tilapia stocks.

All the nine microsatellite markers were polymorphic with mean allelic diversity ranging from 7.0 to 9.0 in Nileand 5.4 to 8.8 in red tilapia. Observed mean heterozygosity (H_o) (value ranged from 0.663 to 0.779 in Nile and 0.759 to 0.777 in red tilapia)was lower than that of expected mean heterozygosity (H_e) (value ranged from 0.777 to 0.856 in Nile and 0.792 to 0.873 in red tilapia) in all the nine population, which indicates the presence of low variability among all the nine populations. All nine microsatellite loci were found to have PIC value greater than 0.5 which clearly indicates that all nine markers were highly informative for studying genetic diversity analysis in Nile and red tilapia stocks. Heterozygote deficit ($F_{IS)}$) was studied in all the nine population. Four out of nine loci showed positive F_{IS}in Nile as well as in red tilapia population.

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INTRODUCTION

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 (Hulata, 2001). Fish is also the primary source of protein for some 950 million people worldwide and represent an important part of the diet of many more (UNEP, Nairobi).Globally, fish provide about 16% of the animal protein consumed by humans, and are a valuable source of minerals and essential fatty acids (FAO, 2003). Fish is the primary source of omega-3 fatty acids in the human diet (Crawford and March,1989). Fish production in least developed countries where fish protein is needed to prevent malnutrition is a key element of food security in these regions and a critical area where innovative programmes are needed to increase production. 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.

In the last three decades (1980-2010), world fish food production of aquaculture has expanded by almost 12 times, at an average annual rate of 8.8 percent. World aquaculture production attained another all-time high in 2010, at 60 million tonnes (excluding aquatic plants and non-food products) with an estimated value of US$ 119 billion. Global production of farmed food fish was 59.9 million tonnes (mt), up by 7.5% from 55.7 mt in 2009. Freshwater fishes dominate global aquaculture production (56.4%,

33.7 mt). The total farmagate value of food fish production from aquaculture is estimated at US$ 119.4 billion for 2010 (FAO, 2012).

The fisheries and aquaculture sector remains fundamental importance to the Asia-Pacific region. Production from both capture fisheries and aquaculture has grown since 2002 (3 percent for capture fisheries and 14 percent for aquaculture) (Lowe-McConnell, 2006). The region has been the world's largest producer of fish for decades and in 2008 increased its contribution to 51 percent of global capture production (48.3 million tonnes) and 89 percent of global aquaculture production (46.6 million tonnes) (FAO, 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. Aquaculture is an expanding sector in Asia-Pacific region and very important for many of its economics. The current trends and current expectations are that aquaculture will play an even more important role in future, both in terms of an important rural livelihood and an invaluable source of protein for both the poor and rich in Asia-Pacific region.

In India, fisheries sector plays an important role in the national economy and in the socioeconomic development of the country. These sectors also plays significant role in supplementing family income and generating gainful employment in the rural sector, particularly among small and marginal farmers and women, besides providing cheap nutritional food to millions of people. India is the second largest country in aquaculture production in the world next to China. Fish production has increased from 41.57 lakh tonnes (24.47 lakh tonnes for marine and 17.10 lakh tonnes for inland fisheries) in 1991-92 to 82.90 lakh tonnes (32.20 lakh tonnes for marine and 50.70 lakh tonnes for inland fisheries) in 2010-11 (Ministry of Agriculture (Government of India), 2011-12).

Unlike crops and livestock, most farmed fish have very short history of domestication and genetic improvement and many still resemble closely their wild relatives. Asia provides more than 80% of 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 husbandry 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 in farmed fish can be substantial. The need for increased investment in fish genetic resources conservation, combined with their use in genetic improvement research and fish breeding programmes, is extensive and immediate.

With increasing popularity among consumers, tilapia has become the world's second most popular farmed fish, after carps (World Bank, 2004). World tilapia production has been booming during the last decade, with output doubling from 830000 tonnes in 1990 to 1.6 million tonnes in 1999 and to 3.5 million tonnes in 2008 (FAO,

2010). Tilapia are farmed in at least 85 countries, with most production coming from the developing countries of Asia and Latin America. Thus, Asia and Latin America dominated the world's top producers of farmed tilapia (Ponzoni, 2006). The global supply of farmed tilapia surged in the 1990s and early 2000s, largely due to genetic improvement through conventional breeding methods, widespread introduction of improved tilapia breeds; feed supply availability, effective management of reproduction through sex reversal and hybridization and expansion of consumer markets. 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 programmes for aquaculture species. Four technologies are considered, namely marker assisted selection, DNA fingerprinting, *in-vitro* fertilization and cryopreservation. Marker assisted selection can results 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 programmes (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). 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).

Mozambique Tilapia *(Oreochromis mossambicus)* was introduced in India as early as 1952 with a view to filling some unoccupied ecological niches, mainly pond aquaculture and reservoir fisheries. Because of their hardy nature and adaptability to different ecological conditions the species got well established in all types of water. Due to their prolific breeding habit and inbreeding for so many generations led to stunted growth and became an aquatic pest. So the Fisheries Research Committee of India had imposed ban on Tilapia propagation in 1959 (Dept. of Animal Husbandry, Dairying and Fisheries).

The Nile tilapia, *Oreochromis niloticus* was probably introduced unofficially in West Bengal in the late 1970s or early 1980s. The fish turned out to be a prolific breeder, leading to over population and stunted growth in both aquaculture ponds and reservoirs (George, 2008).Very recently the ICAR, Government of India has permitted the farming of Nile tilapia in our country, by considering the demand for more fish and also they have been extensively used for studies on biochemical genetics and chromosome manipulations (George, 2008).

Slowly the farming of Nile tilapia has started in some of the states like West Bengal, Karnataka, Andhra Pradesh and Tamil Nadu. By considering *O. niloticus* is most widely cultured tilapia species globally, its adoption in India is worth considering. Further, in view of the immense aquaculture possibilities in India and the need for enhancing production in freshwater farming system, *O. niloticus* could emerge as a candidate species of choice (George, 2008). However, not much is known about their quality with respect to their genetic variability. Hence, the present study was undertaken, in order to find out genetic variability among the population available in different states to increase production efficiency of Nile and red hybrid tilapia stocks by using microsatellite markers.

Objective of the study

i. To find out the genetic architecture of farm reared Nile tilapia and red hybrid tilapia using microsatellite genetic markers.
ii. To assess the genetic distance between populations of farm reared Nile tilapia and red hybrid tilapia.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Life history, biology of Tilapia and suitability for aquaculture

Fossil remains of tilapia that are 18 million years old have been found in Africa (Fryer and Iles, 1972). Regional interest in tilapia was stimulated by its appearance in Java early in the twentieth century, but few would have predicted the expanding importance of this group of cichlids by the beginning of the following century (Balarin and Hatton, 1979; Balarin and Haller, 1982). In the twentieth century, tilapia were introduced into some 90 countries for aquaculture and fisheries, including through Pan-African transplants (Courtenay, 1997; Pullin *et al*., 1997). Although the first introduction outside the African countries was documented early in the twentieth century, most of the remaining transfers occurred after mid-century.

Tilapia are likely to become the most important of all cultured fishes in the twenty-first century: yield has increased by 12 percent per year since 1984 (Fitzsimmons, 2000; Shelton, 2002). The suitability of tilapia for various types of culture relates to their ease of propagation; handling tolerance; good growth on natural food and with a variety of supplemental feeds (manufactured and by-products) under various degrees of intensification; tolerance of a wide range of environmental conditions, including resistance to poor water quality and disease; and being perceived as a palatable,

marketable, and nutritious product (Balarin and Haller, 1982; Teichert-Coddington *et al*., 1997).

2.2.Taxonomy and distribution of Tilapia

The name ‘tilapia’ was derived from the African Bushman word meaning ‘fish’ (Trewavas, 1982). Tilapias represent a large number of freshwater fish species within the family Cichlidae. Cichlidae 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 naming is one of confusing and constant modification (Lowe, 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* (parental or biparental mouth-brooders) 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 river 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 (*O. niloticus*), Mozambique tilapia (*O. mossambicus*) and blue tilapia (*O.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 *O. niloticus*. Red tilapias are currently produced in the USA, Philippines, Greece, Israel, Jamaica, India and other tilapia producing countries (Wohlfarth *et al*., 1990).

2.3. Need for analysing genetic diversity

All organisms are subjected to mutations because of normal cellular operation or interactions with the environment, leading to genetic variation (polymorphism). Genetic variation in a species enhances the capability of organism to adapt to changing environment and is necessary for survival of the species (Fisher, 1930). In conjugation with other evolutionary forces like selection and genetic drift, genetic variation arises between individuals leading to differentiation at the level of population, species and higher order taxonomic groups.

The conservation of genetic variation is an essential component of many species management programmes. Ultimately, it is genetic variation that allows species to adapt to changing environmental conditions and respond to selection/breeding programmes. To manage any biological resources effectively, researchers must identify the level of genetic variation within and among populations (Michael and Wright, 1997). The conclusion from genetic diversity data has varied application in research on evolution, conservation and management of natural resources and genetic improvement programmes, etc (Ferguson *et al*., 1995; Neff and Gross, 2001; Jehle and Arntzen, 2002; Wasko *et al*., 2003; Liu and Cordes, 2004).

2.4. Genetic improvement of tilapia

Production system 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).

Genetic improvement of farmed aquaculture species has the capacity to deliver cumulative and sustained improvements in production efficiency, product quality and, ultimately, financial profitability of aquaculture enterprises and industries. The potential of these gains has long been recognized as a significant impetus for domestication and controlled breeding for a range of aquaculture species. Gains in profit resulting from genetic improvement have been realized in terrestrial domesticated livestock species, agricultural, horticultural and ornamental plants, forest trees and some aquaculture species, notably salmonids (Davis and Hetzel, 2000).

Genetic improvement techniques that have delivered these gains in terrestrial animal and plant industries include formal definition of the breeding objective, estimation of genetic parameters that describe populations and their differences, evaluation of additive and non-additive genetic merit of individuals or families and defining the structure of a breeding programme in terms of mating plants. For most aquaculture species, these analytical tools are available, but the information needed to implement them successfully is lacking because of difficulty in measurement and/or a lack of domestication or controlled breeding (Davis and Hetzel, 2000).

Such programmes are particularly well suited to contribute to the fulfilment 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. 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).

One of the most recent technological developments in tilapia culture is the production of genetically improved farmed tilapia (GIFT) through a selective breeding project. The GIFT project was a multidisciplinary research and development programme implemented by the International Center for Living Aquatic Resources Management (ICLARM) (the name has recently been changed to World Fish Center) and co-partners (such as the United Nations Development Programme (UNDP)) to develop a selective breeding programme for Nile tilapia (Eknath and Acosta, 1998). It was one of the longest international research projects, since it started in 1988 and ended in 1997. The GIFT project focused on growth studies of tilapia, in addition to studying the genetic parameters of other important traits, such as size at first maturity, survival, disease resistance, skin colour, body conformation and cold tolerance.

2.5. Genetic markers

The use of molecular genetic markers to address questions related to aquaculture management has found a steadily widening application in the last two decades. These markers can provide valuable information for various aspects of aquaculture practice, such as genetic identification and discrimination of aquaculture stocks, assisting selective breeding programmes, and assessing chromosomal and gene manipulations such as induction of polyploidy and gynogenesis (Ferguson, 1994).

Utter (1991) reviewed the potential of molecular markers to fisheries management. Molecular genetic markers are powerful tools to detect genetic uniqueness of individuals, populations or species (Avise, 1994; Linda and Paul, 1995). Hillis *et al*. (1996) reported that these markers have revolutionized the analytical power, necessary to explore the genetic diversity.

In addition to protein markers, application of DNA markers is finding wide acceptance in population genetics and it is theoretically possible to observe and exploit genetic variation in the entire genome. Both genomic and mitochondrial DNA is used for varied applications. The commonly used techniques are allozyme analysis, types of restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellite typing, single nucleotide polymorphism (SNP), and expressed sequence tag (EST) markers, etc.

Molecular markers can be classified into type I and Type II markers. Type I markers are associated with genes of known function, while type II markers are associated with anonymous genomic regions (O'Brien, 1991). Under this classification, allozyme markers are type I markers because the protein they encode has known function. RAPD and microsatellite are type II markers. In general, type II markers such as RAPDs, microsatellites, and AFLPs are considered non-coding and therefore selectively neutral. Such markers have found wide spread use in population genetic studies to characterize genetic divergence within and among the population of species (Brown and Epifanio, 2003).

2.5.1. Allozyme markers

Analysis of allozyme loci remained one of the most popular approaches in examining population genetics and stock structure questions in fisheries (Suneetha, 2000). Isohyets are structurally different molecular forms of an enzyme system with qualitatively the same catalytic function encoded by one or more loci (Markert and Moller, 1959). Isohyets, which are encoded by different alleles of the same gene locus, are designated as “allozymes” or “alloenzymes” (Starck, 1998). Amino acid differences in the polypeptide chain of the different allelic forms of an enzyme reflect changes in the underlying DNA sequence. Depending on the nature of the amino acid changes, the resulting protein products may migrate at different rates (due to change and size differences) when run through a gel subjected to an electric field. Differences in the relative frequencies of alleles are used to quantify genetic variation and distinguish among genetic units at the levels of populations, species, and higher taxonomic designations (Chauhan and Rajiv, 2010).

2.5.2. Random Amplified Polymorphic DNA (RAPD) markers

RAPD markers are the amplified products of less functional part of the genome that do not strongly respond to selection on the phenotypic level. Such DNA regions may accumulate more nucleotide mutations with potential to access inter-population genetic differentiation (Mamuris *et al*, 2002). The amplification of genomic DNA by PCR with arbitrary nucleotide sequence primers, RAPD can detect high level of DNA polymorphism (Williams *et al*., 1990). The technique detects coding as well as non-coding DNA sequences, and many of the most informative polymorphic sequences are those derived from repetitive (non-coding) DNA sequences in the genome (Haymer,

1994). Because 90% of the vertebrate nuclear genome is non-coding, it is presumed that most of the amplified loci will be selectively neutral.

RAPD loci are inherited as Mendelian markers in a dominated fashion and scored as present/absent. RAPDs have all the advantages of a PCR-based marker, with the added benefit that primers are commercially available and do not require prior knowledge of the target DNA sequence or genome organization. Analysis follows the assumption that population under study follow Hardy-Weinberg expression. According to Wirgin and Waldman (1994) the presence of paralogous PCR product (different DNA region which have the same length and thus appear to be a single locus), low reproducibility due to low annealing temperature used in the PCR amplification, have limited the application of this marker in fisheries science (Chauhan and Rajiv, 2010). .

2.5.3. Restriction Fragment Length Polymorphism (RFLP)

RFLP markers were regarded as the first shot in the genome revolution (Dodgson *et al*., 1997), making the start of an entirely different era in the biological sciences. Restriction endonucleases are bacterial enzymes that recognize specific 4, 5, 6, or 8 base pair (bp) nucleotide sequences and cut DNA wherever these sequences are encountered, so that changes in the DNA sequence due to indels, base substitution, or rearrangement involving the restriction sites can result in the gain, loss, or relocation of a restriction site. Digestion of DNA with restriction enzymes results in fragments whose number and size can vary among individuals, populations, and species. Traditionally, fragments were separated using Southern blot analysis (Southern, 1976) (most recent analyses replace the tedious Southern blot method with techniques based on the PCR), in which genomic

DNA is digested, subjected to electrophoresis through an agarose gel, transferred to a membrane, and visualized by hybridization to specific probes.

The potential power of RFLP markers in revealing genetic variation is relatively low compared to more recently developed markers and technique, because of the major disadvantage of RFLP is the relatively low level of polymorphism. In addition, either sequence information (for PCR analysis) or probes (for southern blot analysis) are required, making it difficult and time-consuming to develop marker in species lacking known molecular information.

2.5.4. Single Nucleotide Polymorphism (SNP)

Single nucleotide polymorphism (SNP) describes polymorphism caused by point mutations that give rise to different alleles containing alternative bases at a given nucleotide position within a locus. SNPs are becoming a focal point in molecular marker development since they represent the most abundant polymorphism in any organism's genome (coding and non-coding regions), adaptable to automation, and reveal hidden polymorphism not detected with other markers and methods (Liu and Cordes, 2004). SNP markers are inherited as co-dominant markers. Several approaches have been used for SNP discovery including SSCP analysis (Hecker *et al*, 1999), heteroduplex analysis, and direct DNA sequencing. SNPs not without their limitations, however, might provide marginal additional, or even less, utility in some applications (e.g. relatedness) (Chauhan and Rajiv, 2010). .

2.6. Microsatellite Markers

Microsatellites consist of multiple copies of tandemly arranged simple sequence repeats (SSRs) that range in size from 1 to 6 base pairs (Tautz, 1989; Litt and Luty,

1989). Abundant in all species studied to date, microsatellite motifs have been estimated to occur as often as once every 10 kb in fishes (Wright, 1993). Microsatellites tend to be evenly distributed in the genome on all chromosomes and all regions of the chromosome. They have been found inside gene coding regions (Liu *et al*, 2001), introns, and in the non-gene sequences. Most microsatellite loci are relatively small, ranging from a few to a few hundred repeats. Regardless of specific mechanisms, changes in numbers of repeat units can results in a large number of alleles at each microsatellite locus in a population. Microsatellites have been inherited in a Mendelian fashion as codominant markers (Chauhan and Rajiv, 2010).

Microsatellites were found to be informative in several species, which showed almost no variation at other markers (Taylor *et al.*, 1994). Each microsatellite locus has to be identified and its flanking region sequenced to design PCR primers. Due to polymerase slippage during replication, small size differences between alleles of a given microsatellite locus (as little as 2 bp in a locus comprised of di-nucleotide repeats) are possible. Microsatellites recently have become an extremely popular marker type in a wide variety of genetic investigations (Chauhan and Rajiv, 2010).

2.6.1. Attributes of microsatellites as genetic markers in aquaculture and fisheries research

Microsatellites exhibit attributes that make them particularly suitable as genetic markers for numerous applications in aquaculture and fisheries research. They are very abundant, so sufficient markers can be rapidly developed for any research objective using the single strategy (Jonathan and Paul, 1994).

Some microsatellites exhibit extremely high level of allelic variation. This attribute makes microsatellites especially attractive in a variety of research contexts, including the following: (1) species that show low overall levels of variation with conventional markers such as allozyme or mtDNA, for example Atlantic salmon (*Salmo salar*, Salmonidae) and Atlantic cod (Bentzen *et al*., 1991; Wright, 1993); (2) populations that are inbred (such as might occur in aquacultural situations), or have experienced severe bottlenecks; (3) recently derived or geographically proximate populations where genetic differentiation may be limited; (4) pedigree analysis, such as might be employed in aquacultural husbandry or studies of variation in reproductive success among individual. Although some microsatellites display extremely high levels of allelic variation, this offers the opportunity to select for microsatellite markers that exhibit the appropriate level of variation for any given research problem. For example, microsatellite loci that exhibit only three to five alleles may be preferred for population studies, whereas hypervariable loci exhibit dozen of alleles are likely to be more suitable for aquacultural genetic studies (Jonathan and Paul, 1994).

Microsatellite alleles are codominant markers inherited in a Mendelian fashion, as is not the case with dominant/recessive RAPD (Wright, 1993) or haploid mtDNA markers. Codominant Mendelian inheritance makes microsatellites more informative in pedigree studies, as well as in population studies, where the extent to which genotypes conform to Hardy-Weinberg expectations confers added information about population structure (Jonathan and Paul, 1994).

Microsatellites are assayed using PCR, only minute amount of tissue are required for analysis. This feature allows use of logically convenient tissue sources such as fin-

clips and scales preserved by drying or storage in alcohol at ambient temperatures. This property can confer significant saving in sample acquisition and transport costs (Jonathan and Paul, 1994).

Although the initial development of a suite of microsatellite markers may be more costly and time consuming then the development efforts needed with other markers, this effort is repaid with gains in efficiency at a later stage. Microsatellites can be assayed rapidly compared with many other DNA markers. Sample sizes in the hundreds or even thousands are readily assayed. Moreover, the highly allelic nature of microsatellites means that they confer more information per unit assay than most other marker systems. In addition, microsatellite assays promise to become even more rapid with the introduction of automation and fluorometric detection methods (Ziegle *et al*., 1992). The development efforts required for microsatellites are not unduly onerous given their long-term utility.

2.6.2. Microsatellite studies in Indian Major Carps

Microsatellite analysis research was also carried out in Indian major carps and common carp, in India and Bangladesh.

McConnel *et al*. (2001) used microsatellite markers in *Catla catla* genetic diversity analysis. Bartfai *et al*. (2003) used four microsatellite and ten polymorphic RAPD markers to find out the genetic analysis of two common carp broodstocks. Lal *et al*. (2004) identified five polymorphic microsatellite markers for *Cirrhinus mrigala* using heterologous primers.

2.7. DNA-level polymorphisms in tilapia genetic studies and stock management

The application of DNA-based genetic analysis in tilapia research and stock development and management is still not fully maximised. Only limited research is available or carried out separately and independently. They have revealed the potentials of DNA-level polymorphisms as tools for tilapia genetics and management.One of the fundamental concerns of Tilapia genetics is the identification and assessment of available stocks, particularly hybrids from parental species (Omitogun, 2005). Data obtained so far from this type of molecular analysis is able to: (1) provide information regarding the discreteness of stocks; (2) quantify introgression within populations; (3) establish genetic variation or relatedness of different stocks; (4) elucidate evolutionary trends within the Tilapiine genera; and should (5) identify loci controlling production traits (or QTLs: quantitative trait loci) and (6) serve as models for studies in other fish systems. Such information is indeed valuable to the overall scientific study of Tilapia and to the management programmes for its genetic resources necessary for its farming and breeding and development of superior stains and breeds through marker-assisted selection (MAS) (Omitogun, 2005).

Stock identification and assessment in tilapia has been rapidly improved. Morphological description and morphometric analyses were the first tools used to define tilapiine species (Galman and Avtalion, 1983). But these techniques are rather arbitrary, and biochemical means (i.e. electrophoresis of expressed isozymes) soon found a more reliable use in such studies (Macaranas *et al*., 1986). Biochemical investigations, however, are still limited in that most of the isozymes are affected by environmental and/or developmental conditions (Galman and Carino, 1979).

DNA-level investigations were thus developed for fish genetic studies. Such approach provides direct investigations of the genetic make-up of several fish species, thus eliminating the effects of extraneous factors. Furthermore, polymorphisms in the DNA are highly numerous as compared to that of isozymes. This equips DNA-level analysis with a lot of genetic markers from which accurate stock identification and assessment can be deduced (Omitogun, 2005). Some of these DNA markers are described below giving examples of the application of the use of this type of molecular analysis to tilapia research.

2.7.1. Mitochondrial DNA RFLP

DNA-level analysis of genetic make-up proves advantageous against protein analyses. Between mtDNA and genomic DNA, the former would also have certain advantages over the latter, although such advantages are not strictly empirical. To begin with, mtDNA is smaller, making it easier to handle than genomic DNA. Also, it is highly uniform in size, at least among the vertebrates and the invertebrates. Due to the maternal inheritance, sequences within a population are highly conserved, so that it is possible to tell the relatedness of the populations of interest (Ferris and Berg, 1982).

Several investigations on species and subspecies variation of tilapias have been successfully shown to generate RFLPs. Some enzymes are only able to distinguish between tilapia species, but as for *Apa*I, differentiation has been shown down to subspecies level of *O.niloticus* (Seyoum and Kornfield, 1992).

2.7.2. Genomic DNA RFLPs

Methods employed in the analysis of genomic DNA include RE digestion, hybridization and amplification via PCR to reveal polymorphic fragments. Several DNA sequences however are more practical amplified randomly and thus AP-PCR is used instead to produce RAPDs as was done simultaneously in three different fish species such as *Barbus terazona, Poecilla reticulate and O. niloticus* (Harris *et al*., 1991). However, this technique requires rigidly controlled set-ups that, for instance, a slight sample contamination may cause sizeable errors.

2.7.3. Different molecular techniques used in tilapia for genetic diversity analysis

Majumdar *et al*. (1997) used DNA fingerprinting technology for analysing genetic diversity in Indian major carps and tilapia by Bkm 2(8) and M13 probes.

Ahmed *et al*. (2004) used 20 random 10 or 20-mer primers to assay RAPD polymorphism among three genera of Tilapia: *Tilapia zillii*, *Sarotherodon galilaeus* and *Oreochromis niloticus* in Alexandra, Egypt. Similarly, Hassanien *et al*. (2004) found genetic diversity among different populations of *O. niloticus* using RAPDs in Egypt.

Another powerful tool developed for plants, AFLP is now being applied by an Isaeli-US research project on the creation of synthetic strain with cold and low salinity tolerance from crosses of 4 species: *O. mossambicus*, *O. noliticus, O. aureus*, red *O. niloticus* and *S. galilaeus.* Out of the 162 AFLP bands scored from 17 primer combinations, about 25% can differentiate parental species and their F_1 progeny (Agresti *et al*., 2000).

Rashed *et al*. (2008) used dominant DNA markers to evaluate genetic structure of natural Egyptian *Oreochromis niloticus*. Saad *et al*. (2012b) analysed the genetic diversity among some tilapia species based on ISSR markers.

2.7.4. Satellite DNA

The entire genome is too large and excessively polymorphic that genetic comparisons using RE digestion hardly facilitates RFLP analysis. However certain noncoding and repetitive segments of the genome known as *satellite* DNA, thought to be only structurally functional, have now found use in genetic analysis. It has the advantage of ease in analysis in that it is rather small (less than 1 kb) and thus enables even sequence comparisons. Due to these characteristics, the resolution becomes more defined and detailed studies such as linkage analysis and DNA fingerprinting can be done (Omitogun, 2005).

A general protocol involved in satellite DNA analysis involves basic molecular biology techniques. Initially, isolated genomic DNA is digested with an appropriate RE. Satellite DNA, after gel electrophoresis, is seen as heavily stained bands in the DNA smear. Southern blotting is done for a more permanent record of the fragment patterns. Furthermore, the generated fragments may be cloned for further analysis, such as PCR and/or sequencing studies, or labelled to be used as a probe in genotyping unknown stocks, mixed or introgressed populations and offspring from parental stocks. This type of analysis was used in differentiating different tilapia species (Franck *et al*., 1992). This showed the utility of microsatellite genetic markers as tool in distinguishing tilapia species particularly those that look morphologically similar, e.g. *O. aureus* and *O. niloticus* or *O. mossambicus* and *O.hornorus*.

Lee and Kocher (1996) used the microsatellite DNA markers to describe the isolation of seven tri- and 133 di-nucleotide microsatellite markers from a tilapia, *O. niloticus*. They also discussed regarding an efficient methodology for isolating large

numbers of such markers and their potential applications in applied aquaculture and evolutionary genetics.

Bhassu *et al*. (1998) used microsatellite markers to find out variation between five populations of *Oreochromis* spp before progressing into breeding work. Bezault *et al*. (2011) studied population genetic structuring of *O. niloticus* in Africa using nine microsatellite markers and 350 samples from ten natural populations and they found the highest genetic differentiation among the three ichthyofaunal provinces and regions (Ethiopian, Nilotic and Sudano-Sahelian).

2.8. Testing the basic assumptions for population genetic analysis

2.8.1. Microsatellite Alleles

Rutten *et al*. (2004) detected genetic variation in four strains of nile tilapia using microsatellite markers and identified the total average number of alleles per marker varied from 5 to 20.

Rowena *et al*. (2004) analysed the genetic diversity in Asian Nile and Red tilapia stocks by using five microsatellite as well as mt-DNA RFLP markers. They identified number of alleles per locus ranged from 11 to 22 in microsatellite markers.

Hesham and John (2005) determined the genetic variability in nile tilapia using six microsatellite loci. The mean number of alleles per locus ranged from 11.5 to 13.16. Boris *et al*. (2011) analysed the genetic variability in six populations of red hybrid tilapia using 5 microsatellites. Microsatellite alleles varied from 4 to 16 alleles per locus.

Li-hao *et al*. (2012) used nine microsatellites for comparison of genetic diversity among stocks of *O. niloticus, O. aureus* and red tilapia. They detected total 60 alleles

from 109 individuals. The mean number of alleles was 4.11, 1.33 and 3.44 for *O. niloticus, O. aureus* and red tilapia stocks respectively.

Karuppannan *et al*. (2013) analysed the genetic diversity in five red tilapia stocks using 20 microsatellite markers. They identified the mean number of alleles ranged between 2.7 to 3.4. Saad *et al*. (2013) characterized the genetic diversity analysis in some tilapia fish species (*O. niloticus, O. aureus, S. galilaeus and T. zillii*) using ten microsatellite markers. They detected that the allele numbers ranged from seven (for GM538 locus in *O. niloticus* and *O. aureus*, UNH106 locus in *O. niloticus*, UNH123 locus in *O. aureus* and UNH995 locus in *O. aureus*) to zero (for UNH104, UNH185 and UNH995 loci in *T. zillii*).

Cuevas-Rodriguez *et al*. (2014) studied genetic variability of commercial species of the tilapia genus *Oreochromis* in Mexico by using eight microsatellite markers. They obtained average number of allele 6.42 in *O. mossambicus*, 11.71 alleles in *O. aureus* and 11.25 alleles in *O. niloticus.*

2.8.2. Polymorphism Information Content (PIC)

The Polymorphism Information Content (PIC) is a parameter, originally introduced by Botstein *et al*. (1980). The PIC values usually ranged from 0 to 1. More number of alleles at a given locus and PIC value of 1 are most desirable. PIC refers to the value of a marker for detecting polymorphism within a population, depending on the number of detectable alleles and the distribution of their frequency, and has been proved to be a general measure of how informative a marker is (Guo and Elston, 1999).

Li-hao *et al*. (2012) studied the PIC values in nine loci (UNH985, UNH871, UNH995, UNH855, UNH1007, UNH735, UNH846, UNH849 and UNH954) in three

different tilapia species and they obtained PIC values 0.580 in *niloticus*, 0.077 in *O. aureus* and 0.466 in red tilapia

Cuevas-Rodriguez *et al*. (2014) studied the PIC values of eight loci in three different tilapia species belonging to the genus *Oreochromis* and the observed value ranged from 0.66 to 0.87 (UNH145), 0.46 to 0.81 (UNH155), 0.60 to 0.75 (UNH160), 0.63 to 0.91 (UNH166), 0.21 to 0.89 (UNH190), 0.25 to 0.87 (UNH207) and 0.2 to 0.8 (UNH211).

2.8.3. Heterozygosity

Heterozygosity is one of the indices used to assay the genetic variation of each population. The amount of actual or potential heterozygosity measures the gene diversity in the population. The observed heterozygosity (H_o) is a measure of actual percentage of heterozygotes occurring in the sample population. Whereas the expected heterozygosity (H_e) which measures gene diversity is considered to be a better estimator of the genetic variability in the population. The high mean heterozygosity value indicates the low level of inbreeding, low selection pressure and larger number of alleles in the population.

Rutten *et al*. (2004) studied 14 microsatellites in four strains of nile tilapia and reported that the average H_o values ranged from 0.61 to 0.69 and H_e values ranged between 0.62 to 0.71.Rowena *et al*. (2004) reported that pooled mean observed and expected heterozygosity values for Asian Nile tilapia and red hybrid tilapia were 0.680 and 0.422, and 0.791 and 0.697 respectively.

Hesham and John (2005) reported that mean observed heterozygosity ranged from 0.493 to 0.900, whereas expected heterozygosity varied between 0.843 and 0.886 in

different Nile tilapia stocks. Boris *et al*. (2011) assessed the microsatellite polymorphism in six populations of red hybrid tilapia and reported that the mean expected heterozygosity ranged from 0.586 to 0.837.

Karuppannan *et al*. (2013) reported that the mean observed heterozygosity ranged from 0.70 to 0.78 and expected heterozygosity ranged between 0.56 to 0.57. Saad *et al*. (2013) obtained the observed heterozygosity values were higher than the expected values in all *O. niloticus* microsatellite loci except three of them (GM211, GM531 and UNH207)

2.8.4. Hardy-Weinberg Equilibrium (HWE)

If analyses are to be made using allele frequencies, rather than genotypic frequencies, it is necessary to ensure the populations are in HWE. This law states that in a large random mating population with no selection, mutation or migration, the allele frequencies and the genotype frequencies are constant from generation to generation and that, furthermore, there is a simple relationship between the gene frequencies and the genotype frequencies (Wright, 1969).

Rowena *et al*. (2004) studied microsatellite variability in Asian Nile and red tilapia stocks. A significant deviation from Hardy-Weinberg Equilibrium was observed at five loci in both Nile and red tilapia populations.

Boris *et al*. (2011) observed highly significant deviation from HWE, in all five loci while assessing the population structure of five stocks of red hybrid tilapia with microsatellite markers.

2.8.5.Genetic Distance

Genetic distance is the difference between populations as expressed by a function of gene differences. Knowledge about genetic distance is important in two aspects of evolutionary study. First, it provides information on phylogeny. Second, measurement of genetic distance is useful in the study of genetic structure of populations within one species (Nei, 1973).

Rutten *et al*. (2004) studied genetic distance in four strains of Nile tilapia. Genetic distance in five populations of Nile and five populations of red tilapia was studied by Rowena *et al*. (2004).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Materials

3.1.1 Samples

Farmed Nile (Plate 1a) and Red hybrid (Plate 1b) tilapia fin-clip samples were collected from different states of India. Nile tilapia samples were collected from Tamil Nadu (Dharmapuri, Thanjavur, Sathanoor and Krishnagiri), Kerala (Trivandrum), Karnataka (Mangalore) and Andhra Pradesh (Vijayawada). Red hybrid tilapia samples were collected from Tamil Nadu (Thanjavur) and Andhra Pradesh (Vijayawada) during December, 2013 to March, 2014.

3.1.2. Ethanol (99.9%)

3.1.3. Reagents for DNA extraction

3.1.3.1. Lysis buffer

200mM Tris HCl	24.228 g
100mM EDTA	37.224 g
250mM NaCl	146.1 g

Distilled water	1000 ml

The mixture was vigorously stirred and the pH was adjusted to 8.0.

3.1.3.2. 20% SDS

SDS	20 g
Distilled water	100 ml

The mixture was gently mixed until SDS gets completely dissolved.

Plate 1 a. Nile Tilapia

Plate 1 b. Red Tilapia

3.1.3.3. Proteinase K

Proteinase K	10 mg
Distilled water	1000 µl

The mixture was gently mixed until Proteinase K gets dissolved and stored at -20ºC.

3.1.3.4. Phenol-Chloroform-Isoamyl alcohol mixture

Saturated Phenol (Tris-HCl)	25 ml
Chloroform	24 ml
Isoamyl alcohol	1 ml

3.1.3.5. 10 M Ammonium acetate

Ammonium acetate	77.08 g
Distilled water	100 ml

3.1.3.6. 70% ethanol

Ethanol	70 ml
Distilled water	30 ml

3.1.3.7. TE buffer

Tris (1M)	5 ml
EDTA (0.5 M)	1 ml
Molecular grade water	496 ml

The mixture was gently mixed and pH was adjusted to 8.0

3.1.4. Reagents for Polymerase chain reaction (PCR)

Taq 2X Master Mix manufactured by Ampliqon, Denmark was used. Nuclease free water supplied by Qiagen, India Pvt. Ltd. was used. DNA marker 50bp (0.5 µg/µl) and gel loading buffer (6X) supplied by Merck Specialities Pvt. Ltd., Mumbai, India were used.

Note: Taq 2X Master Mix contained

150 mM Tris-HCL pH 8.5, 40 mM $(NH_4)_2 SO_4$, 3.0 mM $MgCl_2$, 0.2% Tween 20®

0.4 mM dNTPs

0.05 mM units/µl Ampliquon Taq DNA polymerase

Inert red dye and a stabilizer

3.1.5. Primers selected for amplification

Fluorescent tagged oligonucleotide primers supplied by M/s. Eurofins Genomics, Bangalore were used for amplification. The primers and base pairs were chosen based on the earlier reports of Rowena *et al.* (2004) and Boris Brinez *et al.* (2011) and the selected sequence are given in the Table 1.

Note: The primers were supplied by M/s. Eurofins Genomics, Bangalore in lyophilised form with the OD values ranging from 2.2 to 7.8. This was diluted as per the instructions in the primer synthesis report to obtain a stock primer solution having a final

concentration of 100 pmol/µl. Then, the primers were further diluted (1 in 10 dilution) to a working solution of 10 picomole/µl concentration with Millipore water (45 µl Millipore water + 5 µl primer stock solution).

Table 1.Primers used for the amplification based on earlier literature

Primers	Sequences	Product size(bp)	Accessi-on	Modifi cation
UNH106	F-CCTTCAGCATCCGTATAT R-GTCTCTTTCTCTCTGTCACAAG	130-240	G12259	FAM
UNH222	F-CTCTAGCACACGTGCAT R-TAACAGGTGGGAACTCA	122-209	G12373	HEX
UNH172	F-AATGCCTTTAAATGCCTTCA R-CTTTTATAGTCGCCCTTTGTTA	119-194	G12324	TET
UNH123	F-CATCATCACAGACAGATTAGA R-GATTGAGATTTCATTCAAG	145-208	G12276	FAM
UNH216	F-GGGAAACTAAAGCTGAAATA R-TGCAAGGAATATCAGCA	116-154	G12367	HEX
UNH104	F:GCA GTT ATT TGT GGT CAC TA R:GGT ATA TGT CTA ACT GAA ATC C	138-152	G12257	TET
UNH211	F:GGG AGG TGC TAG TCA TA R:CAA GGA AAA CAA TGG TGA TA	112-135	G12362	FAM
UNH213	F:ACT GCT CCT CTT GTT TT R:TGT GAT AAG GTT AAT TAA AGT TAG G	194-240	G12364	HEX
UNH147	F: ATAACTTGCACATTGGC R: GACTTTACATATGCATATTTCTG	125-168	G12299	TET

3.1.6. Reagents for agarose gel electrophoresis

3.1.6.1. Tris acetate buffer (TAE buffer) (50X)

Tris base (Genetix Pvt. Ltd., New Delhi, India)	48.4 g
Glacial acetate acid (Ranbaxy, New Delhi, India)	11.42 ml
Disodium Ethylenediamine-tetraacetate.$2H_2O$ (0.5M)	20 ml
Molecular grade water	1000 ml

The mixture was vigorously stirred on a magnetic stirrer and the pH was adjusted to 8.5.

3.1.6.2. TAE Buffer (1X)

20 ml of 50X TAE Buffer was diluted to 1000 ml with 980 ml of molecular grade water.

3.1.6.3. TAE Buffer (0.5X)

10 ml of 50X TAE buffer was diluted to 1000 ml with 990 ml of molecular grade water.

3.1.6.4. Agarose solution (2%)

Agarose (Medox, Chennai, India)	2 g
1X TAE buffer	100 ml

Agarose was dissolved in buffer by microwave heating for 2 min.

3.1.6.5. Agarose solution (1%)

Agarose (Medox, Chennai, India)	1 g
1X TAE buffer	100 ml

Agarose was dissolved in buffer by microwave heating for 2 min.

3.1.6.6. Ethidium bromide solution (10mg/ml)

Ethidium bromide (Merck Specialities Pvt. Ltd., Mumbai, India)	10 mg
Molecular grade water	1000 µl

In the working solution, the ethidium bromide was used at the rate of 2.5 to 5 µg/ml.

3.1.6.7. Gel loading buffer (6X)

Bromophenol blue (10%)	200 µl
Xylene Cyanol (10%)	200 µl
EDTA Disodium salt (0.5 M)	400 µl
Formamide to make up to	10 ml

Gel loading buffer was aliquoted into 1 ml and stored under refrigeration (4ºC)

3.1.7. Equipments

3.1.7.1. Biosafety cabinet

The extraction of DNA from fish and mixing of PCR products were done in the Biosafety cabinet (Clean Air System, Chennai, India).

3.1.7.2. Water purification system

Distilled water obtained from water purification system (Riviera, Mumbai, India) was used for the reagent preparation and other laboratory uses like washing glass wares, etc.

3.1.7.3. Refrigerated Microfuge

Refrigerated Microfuge (Micromax, USA) was used for the extraction of DNA from the fish.

3.1.7.4. Gradient Master Cycler

Gradient Master Cycler (model 7F06, Eppendorf, Germany) was used for the standardization of PCR conditions, particularly annealing temperature.

3.1.7.5. Spectrophotometer

Spectrophotometer (Perkin Elmer, Lambda 25) was used to quantify the DNA.

3.1.7.6. Hot Air Oven

A hot air oven (EverflowScientific Instruments, Chennai, India) set at 100ºC was used for sterilization of glass wares used during carrying out research work.

3.1.7.7. Serological Water Bath

Serological water bath (Labnet Scientific Services, Chennai, India) set at 48ºC was used for desolation of fish tissue for DNA extraction.

3.1.7.8. Submarine Electrophoresis System

Submarine electrophoresis system (Genei Pvt. Ltd., Bangalore, India) was used for running the PCR products on agarose gel at 110V.

3.1.7.9. Gel Documentation System

Gel Documentation System having UV Transilluminator (Bio-Rad, USA) was used for capturing and documenting the gel images.

3.1.7.10. pH meter

pH meter (Eutech Instruments, Singapore) was used to check and adjust the pH of buffer used in DNA extraction.

3.1.7.11. Other instruments

Other instruments used in this study included deep freezer (Sanyo, Japan), electronic balance (model CPA224S, Sartorius Pvt. Ltd., Germany), microwave oven (LG electronics, Seoul, South Korea) and autoclave (Everflow Scientific Service, Chennai, India).

3.2. Methods

Tilapia fin-clip samples were collected from different states of India. Two species of tilapia were collected viz. Nile tilapia (*Oreochromis niloticus*) and Red hybrid tilapia (*O. mossambicus* × *O. niloticus*) (10-20 samples in each place). Samples were placed in micro-centrifuge tubes containing 99.9% ethanol and transported to the laboratory using insulated box. The samples, on reaching the laboratory, ethanol was replaced by adding fresh ethanol and stored in deep freezer until DNA extraction.

3.2.1. DNA extraction

The DNA extraction protocol was followed according to Ravindrakumar *et al*. (2007).

Approximately, 50 mg of fish fin tissue were cut into small pieces and placed in a 2 ml eppendorf tube containing 940 µl lysis buffer, 30 µl Proteinase K and 30 µl 20% SDS. The contents in the tubes were incubated at 48ºC for 45-50 min in a water bath. After incubation, an equal volume of phenol: chloroform: isoamyl alcohol mixed in the ratio of (25:24:1) was added. The contents were then mixed properly by gently inverting

the tube for 10 min to precipitate the proteins and other part of the nucleic acids. The tube was then centrifuged for 10 min at 9200 rpm. The top aqueous layer was transferred to a new 1.5 ml-eppendorf tube, leaving inter phase and lower phase. The DNA was then precipitated by adding equal volume of isopropanol and 0.2 volumes of 10 M ammonium acetate and inverting the tubes gently several times. The precipitated DNA was then pelleted by centrifugation at 13200 rpm for 10 min. The supernatant was removed by pouring out gently, taking care to avoid loss of DNA pellet. The pellet was then washed briefly in 500 µl chilled 70% ethanol, air-dried and resuspended in 200 µl of TE buffer.

3.2.2. Determination of quality and quantity of genomic DNA

3.2.2.1. Spectrophotometric measurement for DNA purity and concentration

Perkin Elmer Spectrophotometer was used to estimate the purity of the extracted DNA. 1.5 µl of DNA was diluted in 1500 µl with deionized water and read at A_{230}, 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 standard DNA in a 1cm quartz cuvette. Nucleic acid concentration is calculated as follows:

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

3.2.2.2. Agarose gel electrophoresis

Quality of the DNA was also checked by agarose gel electrophoresis.

Edges of a clean, dry gel plate was sealed with cellophane tape so as to form a mould and kept over a horizontal plane. The comb was positioned 0.5-1 mm above the plate so that a complete well was formed when the agarose solution was added. One

percent agarose solution was prepared using agarose powder and TAE buffer and boiled in a microwave oven. Ethidium bromide (4 µl) was added to the prepared agarose solution and poured into the mould when the temperature of the gel was around 60ºC and allowed to set completely. After the gel was completely solidified, the comb and the tape were removed carefully and the gel was mounted in an electrophoresis tank. 2 µl of DNA stock solution was mixed with 1 µl of loading dye and loaded in the wells. An electromotive force of 110V and current of 500A was applied for 20 minutes for migration of DNA towards the anode. The DNA in the gel was visualized by UV transilluminator and the quality was assessed based on thickness or density of the bands developed.

3.2.3. Template DNA preparation for PCR

Based on the bands observed in the agarose gel and concentration determined by spectrophotometer measurement (purity and concentration), the DNA was diluted using Tris EDTA buffer in 1 in 25 or 50 or 100 dilutions to obtain the template DNA (working DNA) concentration of approximately 50 to 100 ng per µl and stored at -20ºC till further processing.

3.2.4. Standardization of PCR conditions

Standardization of PCR condition was done by performing PCR in a Gradient Master Cycler. Several thermal cycling programmes were performed to standardize the reaction conditions. Different annealing temperatures ranged from 40-60ºC were tested to standardize the PCR condition for UNH106, UNH222, UNH172, UNH123, UNH216, UNH104, UNH211, UNH213, UNH147 primers.

3.2.5. Polymerase Chain Reaction (PCR)

Nine polymorphic pairs of primers were used for amplification and identification of polymorphic products in nine populations. The PCR reactions were carried out in a total volume of 10 µl containing 5 µl of 2X PCR master mix (Ampliquon), 0.3 µl each of forward and reverse primer, 3.7 µl of nuclease free water and 0.7 µl crude DNA extract (template DNA). The components were mixed thoroughly and the PCR amplification was performed in an Eppendorf Thermal Cycler with following temperatures given in the Table 2.

Table 2: PCR protocol used for amplifying specific areas in the genomic DNA with varying annealing temperatures.

Step	Process	Temperature	Duration
1	Initial denaturation	94ºC	5 min
2	Denaturation	94 ºC	35 sec
3	Annealing	Varies for each locus (51.5 ºC to 55.4 ºC	30 sec
4	Extension	72 ºC	35 sec
5	Repeat steps 2 to 4	35 cycles	
6	Final extension	72 ºC	4 min
7	Hold	4 ºC	Until the samples are removed

3.2.6. Checking of PCR products

Amplified PCR products were checked on 2% gel and the bands developed were observed in a GelDoc (Bio-Rad, USA) system and the images were stored. The samples, upon confirmation of the size of the amplified products, were used for further study.

3.2.7.Automated Genotyping

Automated genotyping was outsourced to M/s. Eurofin Genomics, Bangalore. The Automated Genetic Analyzer has the ability to determine the size of DNA fragments by

using a fluorescence-based detection system, thus dispensing with the need for radioactivity or laborious manual polyacrylamide gel techniques. Each 1µl of PCR product were mixed with 0.50 µl of size standard fluorescent dyeGene Scan Liz 500 (Applied Biosystems, USA) and made up the volume to 10 µl with Hi-Diformamide. Samples were denatured for 5 minutes at 95ºC and snap chilled on ice for 5 minbefore being run on ABI 3730XL Genetic Analyzer for genotyping. As the DNA migrated through the detection cell, the 96 capillaries were simultaneously illuminated from both sides of the array by an argon-ion laser. To accomplish this, the beam from a single laser source was split using a series of mirrors to form a dual pathway. The fluorescent emission were spectrally separated by a spectrograph and focussed on to a charged couple device, which were then converted to digital information that is processed by the collection software. A fluorescent size standard in each capillary eliminated variability.

3.2.8. Analysis of microsatellite data

Microsatellite allele frequencies, effective number of alleles, test for Hardy-Weinberg equilibrium, Shannon's index, observed heterozygosity (H_o), expected heterozygosity (H_e) and F-statistics were estimated by using POPGENE version 1.31 (Yeh *et al*., 1999).

3.2.8.1. Estimation of polymorphism information content

The polymorphism information content (PIC) was calculated using the individual frequencies of the alleles occurred at each locus (Botstein *et al*., 1980) using the following formula:

$$PIC = 1 - \sum_{j=1}^{k} 2X_i^2 X_j^2$$

where, k = number of alleles

X_i = allele frequency at homozygous loci
X_j = allele frequency at heterozygous loci

3.2.8.2. Estimation of heterozygosity

The observed heterozygosity (H_o) was calculated as the actual percentage of heterozygosity occurring in the sample population.

$$H_o = \frac{\text{Number of heterozygotes}}{\text{Total number of samples}} \times 100$$

The expected heterozygosity (H_e) of each microsatellite locus was measured as per Nei (1978)by usingtheformula:

$$H_e = 1 - \Sigma P_i^2$$ where, P_i is the frequency of i^{th} allele

3.2.8.3. Testing for Hardy-Weinberg equilibrium

The chi-square (χ^2) test of goodness of fit was carried out with observed and the expected numbers to check whether the population was in Hardy-Weinberg equilibrium or not at all loci under study (Falconer and Mackay, 1996).

$$\chi^2 = \sum \frac{(O-E)^2}{E}$$

where, O = observed number

E = expected number

3.2.8.4. Testing for Analytical Molecular variance (AMOVA)

Genetic differentiation among stocks are quantified through ananalysis of molecular variance (AMOVA) (Excoffier *et al*., 1992) using Arlequin, ver. 3.0 (Excoffier *et al*., 2005). The hierarchical AMOVA isused to partition the total genetic variance into components due to differences between groups and among individuals in Nile tilapia.

RESULTS

4. RESULTS

4.1. Isolation of genomic DNA

The mean yield and purity of genomic DNA isolated from Nile and red hybrid tilapia by phenol-chloroform method ranged from 10 to 200ng per µl and mean purity of DNA was 1.76 ± 0.027 with range of 1.49 to 21.9.

4.2. Primer screening and banding pattern

A total of nine primers were used in the present study. Nine primers, their base pair range and annealing temperature are furnished in Table 3. The size of the band is estimated by unweighted linear regression relative to the position of the 50bp DNA ladder. Figure 1a shows the different bands obtained for two different primers (UNH216 and UNH106) tested on ten individuals of Karnataka stocks and Figure 1b shows the different bands obtained for seven different primers (UNH104, UNH172, UNH222, UNH211, UNH123, UNH147 and UNH213) tested on two individuals of Kerala stocks after running in agarose gel electrophoresis (2%).

Table 3.Annealing temperature and basepair ranges of primers used in the present study.

Primers	Product size or allele size (bp)	Annealing temperature (^{o}c)

UNH106	130-240	51.5
UNH104	130-230	55
UNH213	170-260	54.1
UNH211	80-195	55.4
UNH147	230-281	51.7
UNH216	116-200	51.7
UNH123	145-240	55
UNH172	119-240	52.8
UNH222	122-240	55

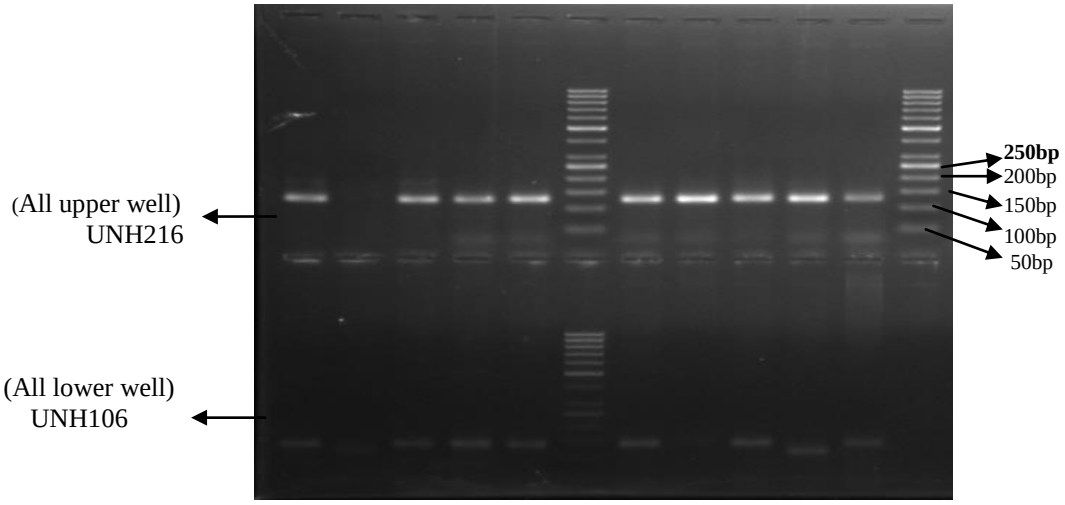


Fig. 1(a). Gel image showing bands obtained by two primers tested on ten individuals of Karnataka stocks using 2% agarose gel

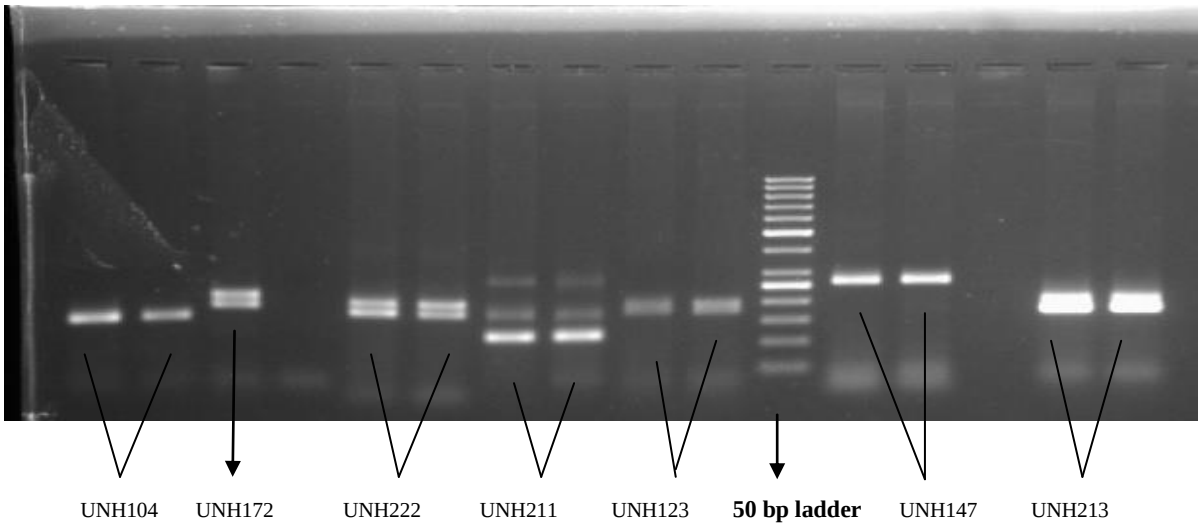


Fig. 1(b). Gel image showing bands obtained by seven primers tested on two individuals of Kerala stocks using 2% agarose gel

4.3. Genetic architecture of Nile tilapia

4.3.1. Allelic Diversity

Microsatellite alleles and allele sizes at different loci in Nile tilapia are presented in Table 3and allele numbers, mean number of alleles and effective number of alleles in each population of Nile tilapia are furnished inTable 5.All the nine microsatellite markers were polymorphic with UNH211 and UNH222 as the most variable locus with 14 alleles, followed by UNH172 (13 alleles), UNH123 (12 alleles), UNH 104 (11 alleles), UNH106 and UNH216 (10 alleles), UNH213 (8 alleles) and UNH147 (7 alleles). The Krishnagiri (Tamil Nadu) and Vijayawada (Andhra Pradesh) stocks had the highest mean number of alleles per locus with 9.44 alleles, followed by, Thanjavur (TN) selected stocks (9 alleles). Trivandrum (Kerala) stocks showed the least mean number of alleles with 7 alleles. The effective number of alleles ranged from 5.39 (UNH216) to 10.6 (UNH222)

with mean 8.26. The Vijayawada (AP) stocks had the highest effective numbers of alleles with 6.24 alleles, followed by Thanjavur (TN) with 6.02 and Trivandrum(Kerala) had the least number of alleles with 4.8 alleles.

4.3.2. Polymorphism Information Content (PIC)

The polymorphism information content (PIC) values for the 9 microsatellite loci in Nile tilapia are furnished in Table 4. The PIC values for Nile tilapia ranged from 0.7735(UNH147) to 0.8981(UNH222) with mean value of 0.855. All nine microsatellite loci were found to have PIC value greater than 0.5 which clearly indicates that all nine markers were highly informative for studying genetic diversity analysis in Nile tilapia.

4.3.3. Heterozygosity

The heterozygosity measures are presented in the Table 5. The average observed and expected heterozygosities pooled over different loci in Nile tilapia were 0.74 and 0.87 respectively. The observed heterozygosity (Ho) ranged from 0.3803 (UNH147) to 0.9014 (UNH222) and the expected heterozygosity (He) ranged between 0.8203 (UNH216) and 0.9121 (UNH222). Mean expected heterozygosity at the nine loci was highest in Krishnagiri (TN) stocks with 0.856 and lowest in Vijayawada (AP) stocks with 0.777.

4.3.4. Hardy-Weinberg Equilibrium (HWE)

The results of Chi-square (χ^2)test for goodness of fit revealed that among nine microsatellite loci studied across all populations, two were in HWE proportions, and the

remaining seven loci departed from HWE and they showed highly significant deviation with probability of <0.01.

4.3.5. Heterozygote Deficit (F_{IS})

The overall mean F_{IS} value across all the loci in Nile tilapia was 0.0472. Totally, four out of nine microsatellite loci showed positive F_{IS} values; while remaining five loci had negative F_{IS} values. The F_{IS} calculated in the present study ranged from -0.0143 (UNH104) to 0.3671 (UNH213).

4.3.6. Analytical Molecular Variance (AMOVA)

Genetic differentiation among stocks were quantified through an analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992) which showed the percentage of variation among the seven studied groups was 0.16, among population within group was -0.18 and among individuals within population was 0.00 in Nile tilapia stocks.

4.4. Genetic architecture of red hybrid tilapia

4.4.1. Allelic Diversity

Microsatellite alleles and allele sizes at different loci in red tilapia are presented in Table 3and allele numbers, mean number of alleles and effective number of alleles in each population of red tilapia are furnished in Table 5.All the nine microsatellite markers were polymorphic with UNH211 as the most variable locus with 13 alleles, followed by UNH172 and UNH222 (11 alleles), UNH104 and UNH123 (10 alleles), UNH106 (9 alleles), UNH216 (8 alleles) and UNH213 and UNH147 (7alleles). The Vijayawada (AP) stocks had the highest mean number of alleles per locus with 8.88 alleles and Thanjavur (TN) had the lowest mean number with 5.44 .The effective numbers of alleles ranged from 2.58 (UNH147) to 9.8 (UNH222) with mean of 6.53.

4.4.2. Polymorphism Information Content (PIC)

The PIC values for the nine microsatellite loci in red hybrid tilapia are furnished in Table 4. The PIC values for red hybrid tilapia ranged from 0.5883(UNH147) to 0.8898(UNH222) with mean value of 0.79174. All nine microsatellite loci were found to have PIC value greater than 0.5. The PIC value greater than 0.5 indicates that the loci used for this study were highly informative.

4.3.3. Heterozygosity

The heterozygosity measures are presented in the Table 5. The average observed and expected heterozygosities pooled over different loci in red hybrid tilapia were 0.76 and 0.83 respectively. The observed heterozygosity (Ho) ranged from 0.3125 (UNH213) to 1.0000 (UNH172 and UNH222) and the expected heterozygosity (He) ranged between 0.6331 (UNH147) and 0.9274 (UNH222). Mean expected heterozygosity (He) at the nine loci was highest in Thanjavur (TN) stocks with 0.873 and lowest in Vijayawada (AP) stocks with 0.792.

4.4.4. Hardy-Weinberg Equilibrium (HWE)

The results of Chi-square (χ^2)test for goodness of fit revealed that among nine microsatellite loci studied across all populations, seven were in HWE proportions, and the remaining two loci departed from HWE and they showed highly significant deviation with probability of <0.01.

4.4.5. Heterozygote Deficit (F_{IS})

The overall mean F_{IS} value across all the loci in red hybrid tilapia was -0.0125.Totally, four out of nine microsatellite loci showed positive F_{IS} values; while

remaining five loci had negative F_{IS} values. The F_{IS} calculated in the present study ranged from -0.0566 (UNH104) to 0.5333 (UNH213).

Table 4.The summary of the genetic structure for the nine populations with respect to the nine loci.

Nile Tilapia							
Primer	**Na**	**Ne**	**Fis**	**Fit**	**Fst**	**Nm**	**PIC**
UNH104	11.0000	9.4271	-0.0143	0.0515	0.0649	3.6018	0.8842
UNH106	10.0000	6.7900	-0.1065	-0.0466	0.0541	4.3689	0.8364
UNH213	8.0000	6.5311	0.3671	0.5024	0.2139	0.9189	0.8287
UNH147	7.0000	5.0334	0.3353	0.5221	0.2810	0.6397	0.7737
UNH211	14.0000	10.5571	0.0642	0.1017	0.0400	5.9989	0.8971
UNH216	10.0000	5.3914	0.1160	0.1986	0.0934	2.4262	0.7961
UNH123	12.0000	10.3618	-0.0245	0.0176	0.0411	5.8290	0.8950
UNH172	13.0000	9.7129	-0.0706	0.0085	0.0739	3.1330	0.8884
UNH222	14.0000	10.6015	-0.0835	0.0013	0.0782	2.9467	0.8981
Mean	11.0000	8.2674	0.0472	0.1443	0.1019	2.0230	0.8552
St. Dev	2.5000	2.3032	-	-	-	-	0.0478
Red Hybrid Tilapia							
Primer	**Na**	**Ne**	**Fis**	**Fit**	**Fst**	**Nm**	**PIC**
UNH104	10.0000	7.8769	-0.0566	-0.0213	0.0334	7.2273	0.8602
UNH106	9.0000	5.5652	-0.2052	-0.1405	0.0537	4.4038	0.7992

UNH213	7.0000	3.9690	0.5333	0.6203	0.1864	1.0909	0.7146
UNH147	7.0000	2.5859	0.0972	0.3077	0.2332	0.8222	0.5883
UNH211	13.0000	9.3091	-0.1500	-0.0920	0.0504	4.7059	0.8829
UNH216	8.0000	3.1605	0.0955	0.1325	0.0410	5.8529	0.6634
UNH123	10.0000	7.0137	0.1570	0.1815	0.0291	8.3448	0.8422
UNH172	11.0000	9.4815	-0.1852	-0.1228	0.0526	4.5000	0.8851
UNH222	11.0000	9.8462	-0.1852	-0.1163	0.0581	4.0500	0.8898
Mean	9.5556	6.5342	-0.0125	0.0644	0.0759	3.0417	0.7917
St. Dev	2.0069	2.8257	-	-	-	-	0.1105

Na = Observed allele number

Ne = Effective allele number

Nm = Gene flow estimated from Fst = 0.25(1- Fst)/Fst

PIC = Polymorphism Information Content (based on frequencies obtained from POPGENE)

F_{IS} = Heterozygote Deficit

Table 5.Genetic variability indices obtained from analysis of variability at nine microsatellite loci for Nile and red hybrid Tilapia.

locus		**Nile tilapia**							**Red tilapia**	
		KT	**KE**	**KG**	**TH**	**DH**	**AP**	**ST**	**AP**	**TH**
UNH104	N	10	10	10	10	10	12	09	12	13
	A	10 (6)	11 (8.5)	10 (6.8)	9 (5.7)	10 (7.4)	8 (6.4)	8 (4)	10 (8)	5 (4.5)
	H_o	0.888	0.875	0.800	0.800	0.800	1.000	0.777	1.000	0.750
	H_e	0.882	0.941	0.900	0.868	0.910	0.880	0.797	0.913	0.892
	P	0.827	0.240	0.870	0.274	0.904	0.605	0.334	0.459	0.074
UNH106	N	10	10	10	10	10	12	09	12	13
	A	8 (5.4)	8 (6)	8 (6.2)	9 (5.4)	10 (6.5)	8 (4.4)	6 (3.5)	6 (4.5)	6 (5.33)
	H_o	0.888	0.875	1.000	0.800	0.900	1.000	0.777	0.916	1.000
	H_e	0.862	0.891	0.884	0.857	0.889	0.808	0.758	0.811	0.928
	P	0.043	0.086	0.888	0.133	0.227	0.010	0.076	0.013	0.318
UNH213	N	10	10	10	10	10	12	09	12	13
	A	4 (2)	5 (2.9)	7 (5.1)	8 (5.5)	5 (3.5)	3 (1.8)	7 (3.3)	7 (2.9)	3 (2.4)
	H_o	0.333	0.125	0.600	0.500	0.600	0.250	0.555	0.333	0.250
	H_e	0.542	0.708	0.847	0.863	0.752	0.489	0.738	0.684	0.678
	P	0.046	0.000	0.193	0.053	0.125	0.117	0.094	0.000	0.148
UNH147	N	10	10	10	10	10	12	09	12	13

	A	6 (3.1)	5 (2.3)	7 (3.3)	6 (2.1)	7 (2.4)	4 (1.5)	5 (2.7)	5 (1.7)	5 (3.2)
	H_o	0.600	0.200	0.500	0.500	0.500	0.166	0.222	0.250	0.750
	H_e	0.715	0.600	0.742	0.573	0.584	0.369	0.673	0.438	0.785
	P	0.005	0.000	0.175	0.052	0.133	0.000	0.000	0.000	0.661
UNH211	N	10	10	10	10	10	12	09	12	13
	A	9 (5)	10 (7.4)	13 (8.3)	11 (7.6)	10 (7.1)	14(10)	13 (10.8)	13 (8.7)	6 (4.5)
	H_o	0.800	0.800	0.800	0.800	0.800	0.916	0.777	0.916	1.000
	H_e	0.842	0.910	0.926	0.915	0.905	0.942	0.960	0.923	0.892
	P	0.461	0.521	0.639	0.261	0.471	0.104	0.219	0.500	0.819
UNH216	N	10	10	10	10	10	12	09	12	13
	A	7 (3)	7 (3.3)	9 (4.4)	10 (6.2)	8 (5.5)	9 (3.0)	6 (3.2)	8 (2.7)	5 (4)
	H_o	0.500	0.600	0.500	0.900	0.800	0.500	0.777	0.750	0.500
	H_e	0.710	0.736	0.815	0.884	0.863	0.706	0.732	0.659	0.857
	P	0.153	0.061	0.007	0.732	0.167	0.000	0.116	0.986	0.144
UNH123	N	10	10	10	10	10	12	09	12	13
	A	9 (7.6)	7 (5.4)	11 (8)	12 (10)	10 (8)	12 (9)	9 (6)	9 (6.1)	7 (6.4)
	H_o	1.000	0.800	0.900	0.900	0.800	0.916	0.888	0.666	0.750
	H_e	0.915	0.857	0.921	0.947	0.921	0.927	0.882	0.873	0.964
	P	0.147	0.049	0.198	0.371	0.042	0.749	0.376	0.637	0.293
UNH172	N	10	10	10	10	10	12	09	12	13
	A	13 (8.6)	5 (3.5)	10 (4.8)	8 (5.7)	10 (6.2)	13 (9)	9 (6.4)	11 (8)	6 (5.33)
	H_o	1.000	0.800	0.900	0.800	0.800	0.916	1.000	1.000	1.000
	H_e	0.931	0.757	0.836	0.868	0.884	0.934	0.895	0.913	0.9286
	P	0.619	0.120	0.997	0.186	0.026	0.133	0.044	0.654	0.819
UNH222	N	10	10	10	10	10	12	09	12	13
	A	13 (8.6)	5 (3)	10 (4.8)	8 (5.7)	10 (6.2)	14 (9)	9 (6.4)	11 (8)	6 (5.33)
	H_o	1.000	0.900	0.900	0.800	0.800	0.916	1.000	1.000	1.000
	H_e	0.931	0.778	0.836	0. 868	0.884	0.938	0.895	0.913	0. 928
	P	0.619	0.265	0.997	0.186	0.026	0.045	0.288	0.654	0.819
Mean	A	8.7 (5.5)	7 (4.83)	9.44 (5.7)	9 (6.0)	8.8 (5.8)	9.4(6.2)	8 (5.18)	8.8(5.6)	5.4(4.57)
	H_o	0.779	0.663	0.766	0.755	0.755	0.731	0.753	0.759	0.777
	H_e	0.815	0.798	0.856	0.849	0.843	0.777	0.814	0.792	0.873

KT = Karnataka KE = Kerala

KG = Krishnagiri TH = Thanjavur

DH = Dharmapuri AP = Andhra Pradesh

ST = Sathanoor

N = Number of samples Ho = Observed heterozygosity

A= Number of alleles He = Expected heterozygosity

() = Effective number of alleles P = Probability value estimates for test of Hardy- Weinberg equilibrium

4.5. Genetic Distance between seven populations

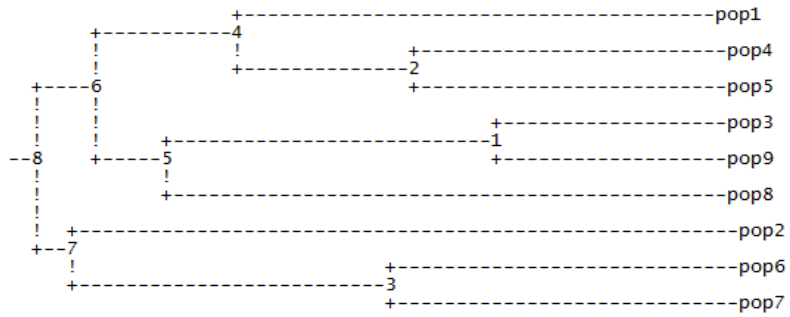
Genetic distance measures were found out and it was used to construct the dendogram. The nine populations were clustered using the unweighted pair group with arithmetic averaging method (UPGMA). Figure 2 shows the dendrogram in which individual populations are clustered based on their genetic distance mentioned in the Table 6.

Table 6. Distance between each Nile and Red tilapia populations based on their genetic identity values.

Pop. ID	1	2	3	4	5	6	7	8	9
1	****	0.5852	0.5878	0.5686	0.7327	0.4742	0.5743	0.5125	0.5956
2	0.5358	****	0.4597	0.4887	0.5305	0.4871	0.6147	0.5845	0.5547
3	0.5314	0.7771	****	0.6029	0.7001	0.5915	0.6219	0.6825	0.8098
4	0.5646	0.7161	0.5060	****	0.7528	0.5100	0.6893	0.4898	0.5018

5	0.3111	0.6339	0.3565	0.2840	****	0.5220	0.5066	0.6047	0.5473
6	0.7461	0.7192	0.5251	0.6733	0.6501	****	0.7314	0.4453	0.5500
7	0.5546	0.4866	0.4749	0.3721	0.6800	0.3127	****	0.5979	0.5223
8	0. 668	0.5370	0.3820	0.7137	0.5030	0.8090	0.5143	****	0.5335
9	0. 518	0.5892	0.2109	0.6895	0.6027	0.5979	0.6495	0.6283	****

Genetic identity (above diagonal) and genetic distance (below diagonal).



Pop 1- Karnataka (nile) Pop 2- Kerala (nile)
Pop 3- Krishnagiri (nile) Pop 4- Thanjavur (nile)
Pop 5- Dharmapuri (nile) Pop 6-Andhra Pradesh (nile)
Pop 7 - Sathnoor (nile)
Pop 8- Andhra Pradesh (red) Pop 9- Thanjavur (red)

Fig. 2.UPGMA dendrogram showing the phylogenic relationship among Nile and Red tilapia.

DISCUSSION

5. DISCUSSION

5.1. Amplification of microsatellite markers in different stocks of Nile and Red Hybrid tilapia

The DNA extracted from the fish fin-clip samples of different stocks of red hybrid and Nile tilapia was subjected to PCR amplification by using nine microsatellite markers. The Nile and red tilapia gene was amplified between 130-240 bp in UNH106, 130-230 bp (UNH104), 170-260 bp (UNH213), 80-195 bp (UNH211), 230-281 bp (UNH147), 116-200 bp (UNH216), 145-240 bp (UNH123), 119-240 bp (UNH172) and 122-240 bp (UNH222) from different places of red and Nile tilapia stocks (Fig. 1a,b). Romana-Eguia *et al.* (2004) and Boris Brinez *et al.* (2011) had earlier successfully identified the differentiation in both red and Nile tilapia stocks by using the same primers for

amplification. Many authors have also studied the genetic diversity analysis using same as well as different microsatellite primers in tilapia (Rutten *et al*; 2004; Hesham and John, 2005; Karuppannan *et al*., 2013; Breidy *et al*., 2014). In this study, the use of microsatellite markers in population study appears to be advantageous because they can produce reproducible results by characterizing stock differences. Romana-Eguia *et al.* (2004) also reported that microsatellite variation at different loci was more informative in characterizing stock differences than mtDNA-RFLP markers.

5.2. Genetic differentiation between Nile and red tilapia

The Nile and red tilapia are phenotypically distinguishable. The observation made on Nile tilapia has a distinct silver gray colour with dark grey to black markings that appears as vertical bands along the entire body and is similar to the report of Macintosh and Little (1995), while the red tilapia hybrids are coloured either albino, pink, red-orange and dark-blotched. In most cases, the characteristics of red tilapia were morphometrically intermediate (in terms of snout shape, mouth width) between the species used in the hybrid crosses (Galman and Avtalion, 1983). However, Pante*et al*. (1998) found no significant differences between Nile and red hybrid tilapia based on canonical discriminant analysis of some morphometric and meristic characters (e.g., body depth, head length, standard length, dorsal spine count, gill raker count). They further noted that the presence or absence of caudal fin bars is the only character that can be used to distinguish between Nile and red tilapias. In terms of growth and reproductive behaviour, Nile and red tilapias are generally similar; however, the red tilapia hybrids are more salt-tolerant, and grow and survive better in saline environments (Romana-Eguia and Eguia, 1999).

The screened Nile tilapia stocks were observed to be slightly divergent from the red tilapias based on microsatellite markers but Nile tilapia samples from Krishnagiri (TN) was clustered with red tilapia from Thanjavur (TN),this may be because of breeding management scheme practiced by some commercial farms. The genetic uniqueness of each of these stocks, shown diagrammatically in the dendrograms (Fig. 2), was such that red tilapia from Vijayawada(AP) formed a separate cluster from all other Nile tilapia populations.Contradictory to the present study Romana-Eguia *et al.* (2004) obtained clear divergence of the Asian Nile tilapias from the red tilapia stocks.

Microsatellite marker data showed mean expected heterozygosities in the red tilapias (value ranged from 0.792 to 0.873) were higher than in the Nile tilapia stocks (value ranged from 0.777 to 0.856) in the present study (Table 5).These results may be counterintuitive in that interspecific hybrids such as the red tilapia might be presumed to be more variable and divergent from each other than the Nile tilapias, having been produced by crossing entirely different species. Romana-Eguia *et al.* (2004) obtained mean expected heterozygosities in red tilapias (value ranged from 0.567 to 0,715) were lower than those in Nile tilapia stocks (from 0.666 to 0.813).

In the present study, the numbers of alleles through estimated microsatellite loci ranged from 14 (for UNH211 and UNH222 locus) to 7 alleles (for UNH147 locus) in Nile tilapia and 13 (for UNH211 locus) to 7 alleles (for UNH147 locus) in red tilapia. All the nine microsatellite loci were polymorphic in both red and Nile tilapia (Table 4). Thus,DNA microsatellites are valuable markers for fish stock discrimination (Rashed *et al*. 2009) due to the high polymorphism levels compared with other genetic markers such

as RAPD (Rashed *et al*., 2008: Saad *et al*., 2011) and ISSR (Saad *et al*., 2012a and Saad *et al*., 2012b).

5.3. Genetic diversity in Farmed Nile tilapia

According to Xu *et al*. (2001) heterozygosity is an important measurement of population diversity at the genetic level and has drawn much attention from ecologists and aquaculturists.

Although varying among populations, the observed mean heterozygosity was lower than the expected mean heterozygosity for all populations studied in the present study (Table 5). There are number of possible explanations for this observation. The presence of null alleles and/or the inability to separate closely sized alleles due to the presence of stutter bands in the electropherograms of the dinucleotide microsatellites used will both lead to reduced measures of heterozygosity. A further possible mechanism for low levels of observed heterozygosity could be the Wahlund (1928) effect. This causes reduced heterozygosity in populations due to subpopulation structuring. If the populations sampled actually contained two or more subpopulations, each in Hardy-Weinberg equilibrium and these have different allele frequencies, then the overall the heterozygosity for each population is reduced as reported by Hartl and Clark(1989).

The phylogenetic tree obtained from the analysis of nine microsatellite loci screened in the seven nile tilapia populations showed a clustering of populations (Fig. 2). Nile tilapia population of Dharmapuri (TN) and Thanajavur (TN) formed one cluster, Vijayawada (AP) and Sathanoor (TN) populations formed one cluster, whereas Mangalore(Karnataka), Trivandrum (Kerala) and Krishnagiri (TN) were observed to be not clustered with other populations.

The findings from the current study help our understanding of the broad-scale population structuring of the Nile and red tilapia in India. Extending the knowledge of tilapia phylogeography, and the nature and extend of biodiversity in this species, will aid the development of management strategies, which have a better chance of conserving such diversity and ensuring the continued existence of the various sub-populations. These sub-populations can then act as a source of genetic variation which could be included in future Marker-Assisted-Selection programmes as suggested by Davis and Hetzel (2000) with this economically important aquaculture species.

5.4. Variability in red tilapia stocks

The original red tilapias were bred in separate instance in Taiwan, Israel, and Florida by crossing mutant-coloured *O. mossambicus* with either the Nile (*O. niloticus*), blue (*O. aureus*) or Zanzibar tilapia (*O. urolepishornorum*) (Lovshin, 2000).

Hence, because of (a) the use of relatively few and possibly bottlenecked mutant coloured female founder stocks in the original hybrid crosses, and; (b) the propagation of stocks through several generations using the red-orange hybrid progenies as parents for subsequent crosses, genetic variability of the red tilapia hybrids is not as high as expected and the stocks have become less divergent (Rowena*et al*., 2004).

The results obtained in the present study, for mean observed heterozygosity ranging from 0.792 to 0.873 were lower than that of mean expected heterozygosity ranging from 0.759 to 0.777. Similarly Rowena *et al.* (2004) found expected heterozygosity values ranging from 0.567 to 0.715 and Boris *et al.* (2011) found 0.587 to 0.837 which indicated that variability of the red tilapia is low. These results may be counterintuitive in that interspecific hybrids such as the red tilapias might be presumed to

be more variable and divergent from each other than the Nile tilapias, having been produced by crossing entirely different species.

The phylogenetic tree obtained from the analysis of nine microsatellite loci screened in the two red tilapia populations showed a clustering of populations (Fig. 2). Both the population of Vijayawada (AP) and Thanajavur (TN) formed different cluster. But red population from Thanajavur (TN), clustered with Nile population from Krishnagiri (TN).

Inbreeding caused by poor broodstock management could be detected as low generic variability or as an excess of homozygotes in the clustered stocks. Hence, genetic variation in both the farmed Nile and red tilapias should be monitored, and specific matings including outcrosses, should be used to avoid the negative consequences associated with inbreeding depression (e.g., morphological abnormalities, poor seed stock survival) as suggested by Lovshin(2000).For getting exact details about genetic diversity of Nile and red tilapia population more number of samples may be analysed in the future study.

SUMMARY

6. SUMMARY

The present study was carried out to analyse genetic diversity among farmed Nile and red hybrid tilapia stocks in India. The salient findings of the present study are as follows.

Samples of Nile and red hybrid tilapia were collected from different states of India. A genetic diversity analysis was done to know the genetic distance between and within Nile and red tilapia populations by using nine polymorphic pairs of primers.

DNA was extracted from all the collected samples by phenol-chloroform method and amplified by using nine microsatellite markers. The amplified DNA product was then subjected to automated genotyping.

The results obtained from genotyping were calculated according to the requirements of software used in the present study and based on data obtained from software were then used to differentiate the Nile and red tilapia populations.

All the nine microsatellite markers used in the present study was highly informative in characterizing the different stocks of Nile and red tilapia populations by obtaining their Polymorphism Information Content (PIC) value more than 0.5.

Heterozygote deficit (F_{IS}) was checked for all the nine population. Four out of nine loci showed positive F_{IS}in red as well as in Nile tilapia populations. The positive F_{IS}value indicates or shows stocks are fairly inbred.

The observed heterozygosity (H_o) values were lower than that of expected heterozygosity (H_e) for all nine populations, which indicates that all the populations are genetically less variable.

The results of Chi-square (χ^2)test for goodness of fit revealed that among nine microsatellite loci studied across all nine populations, two locus in Nile tilapia and seven locus in red tilapia were in HWE proportions, and the remaining seven locus in Nile and two locus in red were departed from HWE and they showed highly significant deviation with probability of <0.01. If locus is in HWE indicates no genetic forces acting on them.

Analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992) was calculated to know the genetic differentiation among Nile tilapia stocks. It has shown that the percentage of variation among groups was 0.16, among population within group was -0.18 and among individuals within population was 0.00 in Nile tilapia stocks.

Genetic distance measures for all nine populations were found out and dendrogram were constructed based on their genetic identity and distance were such that

red tilapia from Vijayawada (AP) formed separate cluster with other Nile and red populations but red tilapia stocks from Thanjavur (TN) was clustered with Nile populations from Krishnagiri, Nile populations of Dharmapuri (TN) and Thanjavur formed one cluster, Vijayawada (AP) and Sathanoor (TN) populations formed one cluster, whereas Mangalore(Karnataka), Trivandrum (Kerala) and Krishnagiri (TN) were observed to be not clustered with other populations.

The findings of the present study indicates that, the breeding management practice in tilapia populations in India still lags behind and necessary steps to be taken for further genetic improvement in order to obtain good quality seeds for tilapia farming.

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7. REFERENCES

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