A GENETIC LINKAGE MAP OF ASIAN SEABASS, *Lates calcarifer* USING DNA MICROSATELLITE MARKERS IN APPLICATION TO QUANTITATIVE TRAIT LOCI (QTL)

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by

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LIST OF ABBREVIATION

Bp	Base pair (s)
cM	centiMorgan
$CaCl_2$	Calsium chloride
°C	Celcius
dNTP	Deoxyribonucleotide triphosphates
DNA	Deoxyribose nucleic acid
EDTA	Ethylene diaminetetraacetic acid
ESTs	Expressed Sequenced Tags
GSS	Genome Sequenced Survey
LOD value	Logarithm of Odd Value
HWE	Hardy Weinberg Equilibrium
$MgCl_2$	Magnesium Chloride
MAS	Marker assisted Selection
NaCl	Sodium chloride
NGS	Next Generation Sequencing
OD	Optical density
PCR	Polymerase chain reaction
QTL	Quantitative Trait Loci
Rpm	Revolution per minutes
SDS	Sodium Dodecyl Sulfate
T_a	Annealing temperature
T_m	Melting temperature
TBE	Tris-borate-EDTA
TEMED	N, N, N',N' - tetramethylethylenediame
TE	Tris-EDTA
V	Volt
v/v	Volume/volume
w/v	Weight/volume

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1.1 ZULAIHA, A.R., CHOAY-HOONG, L., ROZIANA, M.K., **SHAHRIL, A.R.** & OTHMAN, A.S. 2010. Development and Characterization of Expressed Sequence Tags (ESTs) Derived Microsatellite Marker of the Asian sea bass, *Lates calcarifer*. *Molecular Ecology Resources*, doi: 10.1111/j.1755-0998.2010.02970.x

1.2 ZULAIHA, A.R., CHOAY-HOONG, L., ROZIANA, M.K., **SHAHRIL, A.R.** & OTHMAN, A.S.. Isolation and Characterization of Novel Microsatellite Loci for Asian Sea Bass, *Lates calcarifer* from Genome Sequence Survey (GSS) database. *Journal of Genetic*. Accepted for publication.

PETA UNTAIAN GENETIK IKAN SIAKAP, *Lates calcarifer* DENGAN MENGGUNAKAN PENANDA DNA MIKROSATELIT UNTUK APLIKASI LOKUS TRAIT KUANTITATIF (QTL)

Abstrak

Peta untaian genetik ikan siakap telah dibina dengan menggunakan satu panel yang terdiri daripada sepasang induk (satu jantan dan satu betina) beserta 160 anak mereka. Panel pemetaan ini diperoleh daripada penentuan induk daripada program massa pembiakan dengan menggunakan penanda DNA mikrosatelit. Dalam tugasan penentuan keturunan, sejumlah 24 pasang penanda mikrosatelit telah digunakan dalam proses penyaringan semua induk pembiakan. Keputusan analisis menunjukkan bilangan alel untuk setiap lokus berjulat daripada 3 hingga 14 dengan purata sebanyak 6.65 alel untuk setiap lokus dan nilai keheterozigotan yang diperhatikan dan yang dijangka masing-masing berjulat dari 0.121 hingga 1.000 dan dari 0.198 hingga 0.857. Purata untuk PIC bernilai 0.515 dengan julat 0.186 hingga 0.826. Daripada analisis ini, hanya lima penanda mikrosatelit digunakan untuk penentuan keturunan kerana kelima-lima penanda ini menunjukkan polimorfisme yang tinggi. Dalam analisis keturunan, 94.9% individu anak telah berjaya ditentukan kepada satu atau dua pasang induk dan hanya 51 (5.1%) individu yang tidak berjaya ditentukan kerana individu ini hanya berjaya digenotip kurang daripada dua lokus. Satu pasang induk dan progeni mereka telah dipilih dan digunakan sebagai panel pemetaan dalam pembinaan peta untaian genetik. Dalam analisis untaian, sebanyak 273 penanda mikrosatelit telah digunakan. Daripada 273 penanda ini, hanya 210 (76.9%) penanda mikrosatelit telah berjaya dipetakan kedalam 24 kumpulan untaian dengan tetapan minimum 3.0 nilai LOD dan jarak maksimum 50.0 cM (sentiMorgan). Penanda mikrosatelit yang selebihnya tidak dapat dipetakan kerana menunjukkan kehadiran

alel nol, penanda tak beruntaian dan monomorfik. Bilangan penanda mikrosatelit dalam kumpulan untaian bejulat dari 2 hingga 16 dengan purata 8.75 untuk setiap kumpulan untaian. Peta ini merangkumi 961.9 cM dengan purata jarak antara penanda sebanyak 4.811 cM. Peta ini bukan hanya boleh digunakan untuk pemetaan lokus trait kuantitatif (QTL), bahkan boleh membekalkan sumber baru dalam pemahaman evolusi genom ikan.

A GENETIC LINKAGE MAP OF ASIAN SEABASS *Lates calcarifer* USING DNA MICROSATELLITE MARKERS IN APPLICATION TO QUANTITATIVE TRAIT LOCI (QTL)

Abstract

Genetic linkage maps of Asian sea bass have been established using a mapping panel containing a pair of parent (1 male and 1 female) and their 160 progenies. This mapping panel was obtained from parentage assignment from mass breeding programme using DNA microsatellite markers. In parentage assignment, a total 24 pairs of microsatellite markers have been used to screen all broodstock. The analyses result showed the number of alleles per locus ranged from 3 to 14 with an average of 6.65 alleles per locus and the values of the observed and expected heterozygosities ranged from 0.121 to 1.000 and from 0.198 to 0.857 respectively. The average of PIC was 0.515 with range from 0.186 to 0.826. From this analysis, only five microsatellite markers were used in parentage assignment since these 5 markers show high polymorphism level. In parentage analyses, 94.9% were successfully assigned to a single or double parent pair and only 51 (5.1%) individual progenies were left unassigned since these individual were genotyped for fewer than 2 loci. A single parent pair with their progeny have been selected and used as a mapping panel in linkage mapping construction. In linkage analyses, a total of 273 microsatellite markers have been used. Out of 273 markers, only 210 microsatellite markers (76.9%) were successfully mapped into 24 linkage groups (labelled LG1-LG24) at minimum 3.0 LOD value and maximum distance at 50.0 cM (centiMorgan). The rest of the markers were not mapped in our analysis because they showed the presence of null alleles, unlinked markers and monomorphic. Numbers of

marker in Linkage Group were range from 2 to 16 with an average 8.75 markers per linkage group. The map covers 961.9 cM, with an average inter-marker distance of 4.811 cM. The map will not only enable the mapping of quantitative trait loci (QTL), but also provide new resources for understanding the evolution of fish genomes.

CHAPTER ONE

1.0 Introduction

Asian sea bass, *Lates calcarifer* belongs to Centropomidae family, is one of the most commercially important finfish cultured and marketed in Southern Asia. Asian sea bass has been placed under several families by various authors in the past. However, Centropomidae is the normally accepted family name of this species, and the recognized generic name is *Lates*. In Malaysia, Asian sea bass is known as Siakap. Asian sea bass or also known as Barramundi in Australia is native to the tropical waters of northern Australia, Southeast Asia, and southern China (FAO, 2011a, 2011b).

In the early 1970s, breeding programme of Asian sea bass was successfully done in Thailand. This successful programme provides a platform for Malaysian farmers to involve in aquaculture sector. Locally produced fry in Malaysia were only being made available by The Malaysian Fisheries Department in mid 1980s (Norfatimah et al., 2009). The popularity of Asian sea bass has increase rapidly due to their reasonable market price. Their fecundity and fast growing characteristics have increased the aquaculture production of this species. In the 1980s and 1990s, *L. calcarifer* aquaculture industry began to take footings intensively in China, India, Indonesia, Malaysia, the Philippines, Singapore Taiwan, Vietnam and Australia (Petersen et al., 2011).

Over the years, statistics released by FAO have recorded steady increase in Asian sea bass production since the 1970s. Statistics released by the Malaysia

Department of Fisheries, have recorded the total estimation of aquaculture production from brackish water culture system for 2010 was approximately 425,649.78 tonnes. From the total production, *L. calcarifer* aquaculture production for the year 2010 has recorded approximately 20,021.52 tonnes or 4.7% from total aquaculture production of brackish water culture system.

In Malaysia, since 1980s, two approaches in fish breeding have been successfully practiced that is natural spawning (Mass Breeding Programme) and induce spawning (Awang, 1986). Globally, instead of a long history of aquaculture production system, systematic and efficient breeding programs to improve economically important traits in the farmed species have rarely been utilized until recently. Efficient breeding is not only aimed at reaching the yield production goal but also to reduce production cost, improve disease resistance, improve utilisation of feed resources and improving product quality. The potential benefits of using molecular markers linked to genes of interest in breeding programmes have been explored for many decades (Ruane and Sonnino, 2007). However, in Malaysia, there was no recorded breeding programme associated with molecular markers linked to genes of interest.

The development of molecular markers has had dramatic changes on animal genetics studies. Molecular marker is a fragment of DNA sequence that is associated to a part of the genome. The common genetic markers in the aquaculture research include allozymes, mitochondrial DNA, RFLP, RAPD, AFLP, microsatellite and SNP markers. These entire markers have their own specific potential power, advantage, disadvantage, limitation, requirement and purpose of studies (Liu and

Cordes, 2004). The application of DNA markers have enable wide scales of aquaculture research include determination of genetic variability and inbreeding level, parentage assignments, species and strain identification, Marker-assisted selection (MAS) in breeding programme and the construction of genetic linkage and QTL (Quantitative Trait Loci) maps for aquaculture species (Liu and Cordes, 2004, Chistiakov et al., 2006). In this study, a set of microsatellite loci were developed from GSS and EST database. Then, these developed microsatellite markers will be use in downstream studies, parentage assignment and linkage mapping construction of Asian sea bass.

Although applications of genomics research in aquaculture such as parentage assignment and genetic variability assessment for this species have been found great utility to date, the key component for future is QTL mapping. Aquaculture genomics is especially interested in performance and production traits that are unique to individual species. Beside, mapping of specific QTL associated with production and performance trait in targeted species will greatly accelerated when coupled with comparative genomics of model species in order to established the genomic information data. This shows the significance of comparative genomic studies. However, well-constructed QTL mapping required high density of linkage mapping before we can integrate it into QTL mapping (Liu and Cordes, 2004, Chistiakov et al., 2006).

While there was already linkage mapping available for this species, the further linkage map will aid in enrichment of QTL mapping, selective breeding programme and comparative genomics studies (Wang et al., 2007). Beside, we are

expected that the result will be different from the previous study since we have used different set of microsatellites and breeding panel. Thus, the objectives of this study are:

1. To mining Expressed Sequenced Tags (ESTs) and Genome Sequenced Survey (GSS) databases for development of microsatellite markers.
2. To assign progeny from breeding mass program to their identified parent pairs using DNA microsatellite markers.
3. To construct a linkage map of Asia sea bass, *Lates calcarifer* using DNA microsatellite markers.

CHAPTER TWO

LITERATURE REVIEW

2.1 Asian sea bass, *Lates calcarifer*

2.1.1 Introduction

Asian sea bass (*Lates calcarifer*) is an economically important food fish in Malaysia. Asian sea bass was described by Bloch in 1970s. Occupying both fresh and saltwater areas, *L. calcarifer* is commonly found in the coastal, estuarine and freshwater areas in India, Myanmar, Sri Lanka, Bangladesh, the Malaysian Peninsula, the Island of Java, Borneo, Celebes, Philipines, Papua New Guinea, northern Australia, southern China and Taiwan (Greenwood, 1976, Moore and Reynold, 1982). Due to its wide and extensive geographic distribution, this species is known by various local names such as (Rabanal and Soesanto, 1982):

English : Giant perch, white seabass, silver seaperch, giant perch, palmer

India : Begti, bekti, dangara, voliji, fitadar, todah

East Bengal : Kora, baor

Sri Lanka : Modha koliya, keduwa

Thailand : Pla kapong kao, pla kapong

Malaysia : Siakap, kakap

North Borneo : Ikan salung-sung

Vietnam : Ca-chem, cavuot

Kampuchea : Tvey spong

Philippines : Kakap, apahap, bulgan, salongsong, katuyot, matang pusa

Indonesia : Kakap, pelak, petcham, telap

Australia and Papua New Guinea: Barramundi

Despite the presence of morphological variations in variables such as localities, life stages and others, Greenwood (1976) proposed that *Lates* found throughout the Indo-Pacific region actually belongs to a single species.

2.1.2 Taxonomy and morphometrics diagnostic

Lates calcarifer has been placed under several families by various authors/organization. However, there was a consensus previously placing the genus *Lates* under the family Centropomidae together with two other genera, *Centropomus* and *Psammoperca* (Otero, 2004). Similarly, FAO (1985) also placed *L. calcarifer* under the family Centropomidae which is the same family classification of *L. calcarifer* under the Integrated Taxonomic Information System (ITIS). The ITIS taxonomic classification of *L. calcarifer* is given below (ITIS, 2011):

Kingdom : Animalia

Phylum : Chordata

Subphylum : Vertebrata

Superclass : Osteichthyes

Class : Actinopterygii

Subclass : Neopterygii

Infraclass : Teleostei

Superorder : Acanthopterygii

Order : Perciformes

Suborder : Percoidei

Family : Centropomidae

Subfamily : Latinae

Genus : Lates

Species : *Lates calcarifer* (Bloch, 1790)

Otero (2004) in agreement with Mooi and Gill (1995), proposed moving *L. calcarifer* from family Centropomidae to Latidae. Otero (2004) contended that the family Centropomidae is monogeneric (*Centropomus*) and the family Latidae consists of three genera namely *Lates* (which include *L. calcarifer*), *Psammoperca* and the fossil genus *Eolates*. However, Li et al. (2011) conducted a study which combines dense taxon sampling within Centropomidae and related taxa with extensive molecular character sampling with the goal of testing the monophyly of Greenwood's (1976) Centropomidae and assessing the placement of this family among percomorphs. In total, sequence data from 13 genes, including mitochondrial 16S and twelve nuclear markers were analyzed to investigate the limits and relationships of the centropomids. The study of Li et al. (2011) concluded that the phylogeny of the family Centropomidae is *Lates, Psammoperca* and *Centropomus*, a conclusion which confirmed Greenwood taxonomial classification of Centropomidae in 1976 and reject Otero (2004) attempt at reclassification mentioned above.

The morphometrics diagnostic of *L. calcarifer* (Figure 2.1) as provided by the Food and Agriculture Organization of the United Nations (FAO, 2011a) identify the fish as:

i) Possessing elongated body, compressed, with a deep caudal peduncle.

ii) Head pointed, with concave dorsal profile becoming convex in front of dorsal fin.

iii) Mouth large, slightly oblique, upper jaw reaching to behind eye; teeth villiform with no canines present.

iv) Lower edge of pre-operculum with a strong spine; operculum with a small spine and with a serrated flap above origin of lateral line.

v) Lower first gill arch with 16 to 17 gillrakers.

vi) Scales large, ctenoid.

vii) Dorsal fin with 7 to 9 spines and 10 to 11 soft rays; a very deep notch almost dividing spiny from soft part of fin; pectoral fin short and rounded, several short, strong serrations above its base; dorsal and anal fins both have scaly sheaths. Anal fin rounded, with 3 spines and 7 to 8 short rays. Caudal fin rounded.

viii) Colour in two phases, either olive brown above with silver sides and belly (usually juveniles) or green/blue above and silver below. No spots or bars present on fins or body.

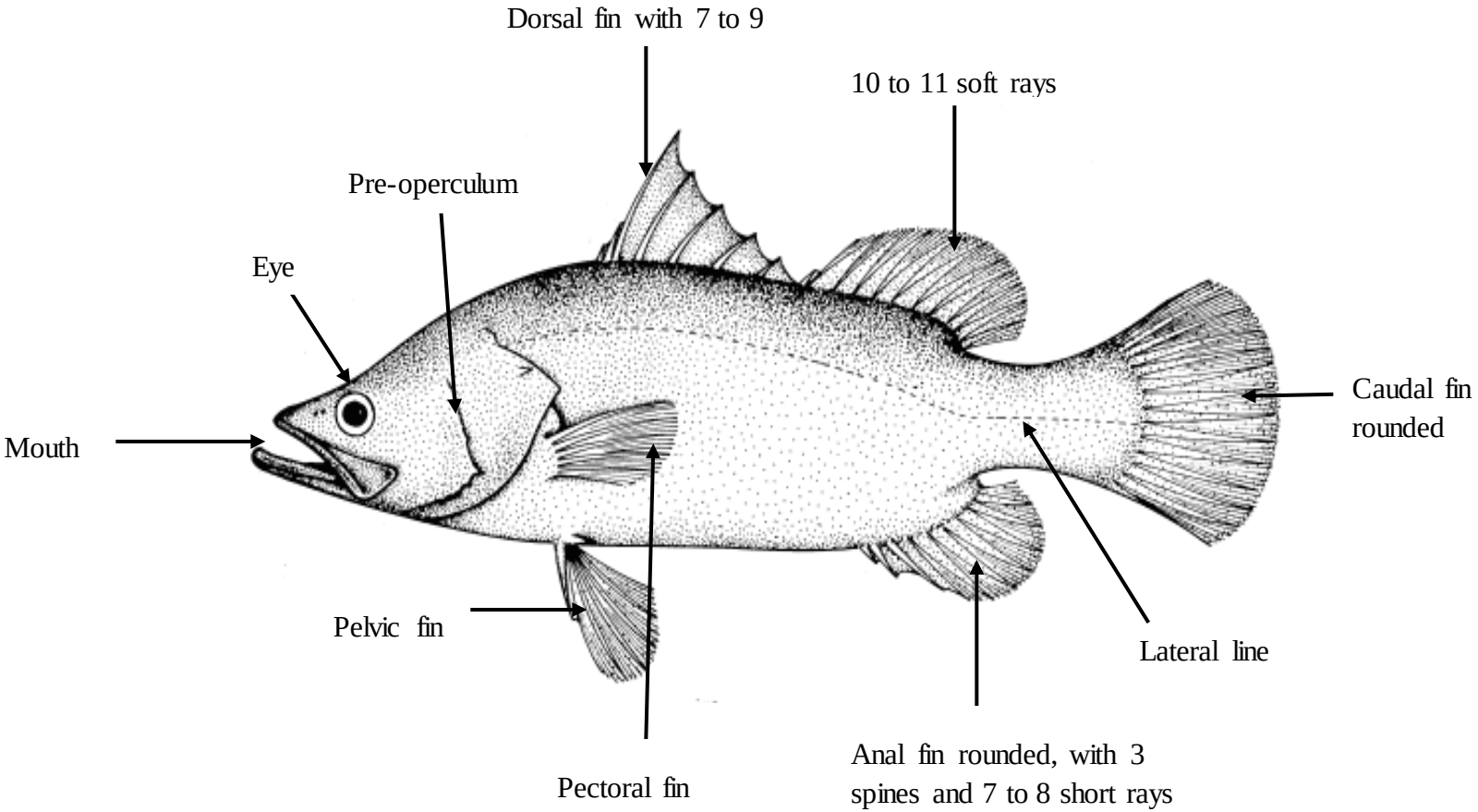

Figure 2.1 Morphological characteristics of *Lates calcarifer* (Modified from FAO, 2011a).

2.1.3 **Distribution and biology**

The natural range of *L. calcarifer* encompasses the tropical and subtropical areas of the western Pacific and Indian Ocean (Figure 2.2). Its range of distribution includes the areas from Australia, Southeast Asia, the Philippines, the Japanese Sea, countries bordering the Arabian Sea which also include part of eastern Africa (FAO, 2011a, 2011b).

In South and Southeast Asia and Australia, feral populations are commonly found in tropical and subtropical estuaries and coastal waters (Blaber, 2000) at a temperature range of 26-29º C and between the depths of 10-40 m. *L. calcarifer* is a euryhaline fish that inhabits fresh water, brackish water and marine coastal areas at different stages in its life cycle. A hermaphrodite species, individuals *L. calcarifer* first mature into males in year two to four of its life in fresh water before moving to coastal water and mature into females in year 3-7 of its life (Le Francois et al., 2010).

The species can grow up to 200 cm in length but commonly the fish sizes are between 25 and 100 cm (FAO, 2011a). Fish with size less than 80 cm are generally males, while fish with size over than 100 cm are usually females. Normally, most fish are females after 5 years (Tucker, 1998). *L. calcarifer* is a catadromous species and reproduction and early life occurs in seawater (Le Francois et al., 2010).

Spawning seasons vary according to latitudes, presumably in response to varying water temperature. *L. calcarifer* in northern Australia spawn between September and March. In the Philipines, *L. calcarifer* spawn from late June to late

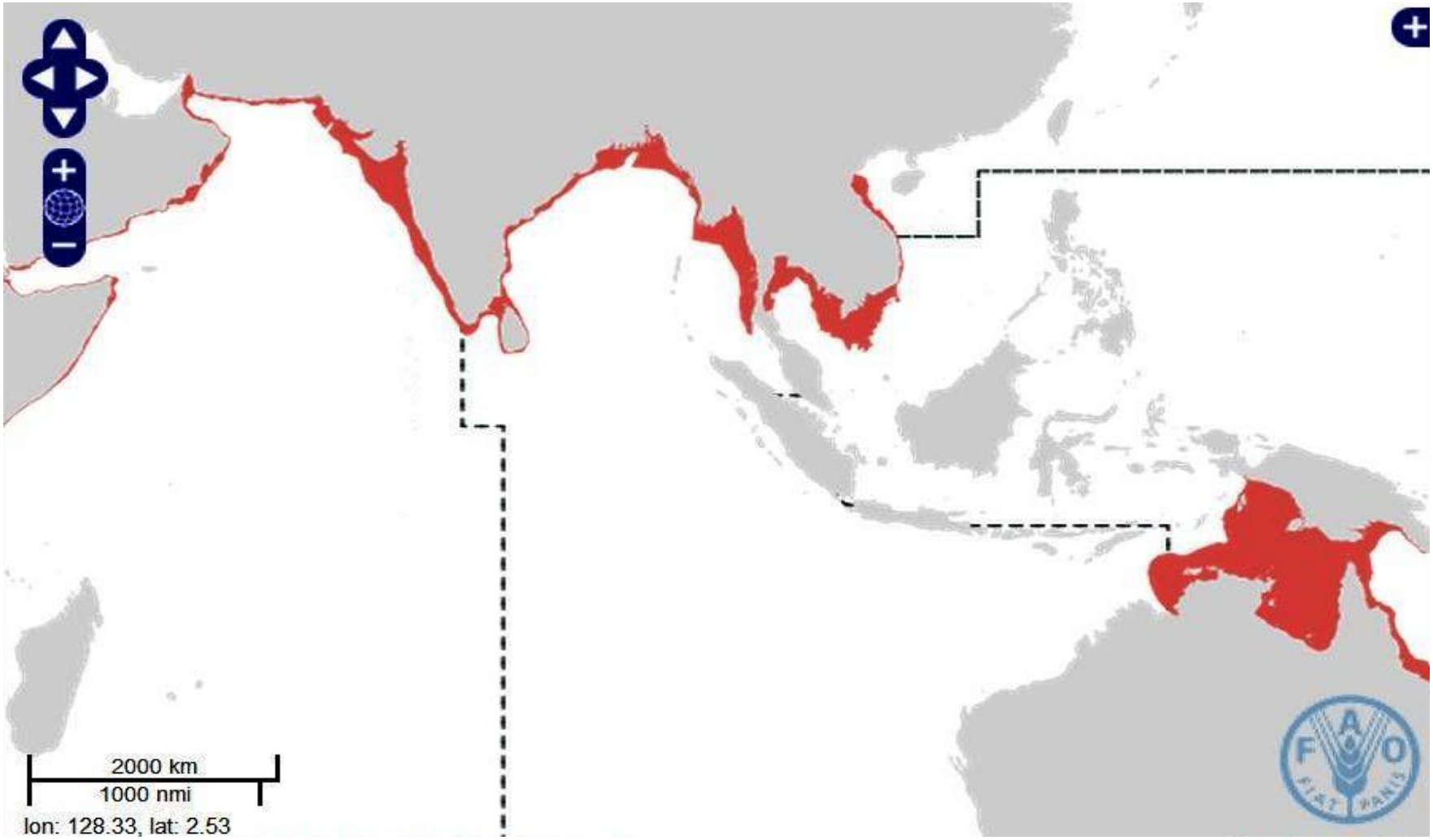

Figure 2.2: Map of global *L. calcarifer* distribution (Source: FAO 2011a)
Red shaded represent distribution of *L. calcarifer*

October, while in Thailand, spawning correlated with monsoon season, with highest spawning activity detected during the northeast monsoon (August – October) and the southwest monsoon (February – June)(FAO, 2011c). In Malaysia, the spawning season starts from the beginning of March to the end of June (FAO, 2011d).

Spawning takes place around the mouth of a river or lagoon (FAO, 2011b) and occurs on the incoming tide in the evening and for several days following the new and full moon (Le Francois et al., 2010). This correlation between spawning activity and incoming tides is attributable to the fact that the incoming tides apparently assist the movement of eggs and larvae into the estuary (FAO, 2011c).

Fecundity of *L. calcarifer* and its size are correlated (FAO, 2011b) with large females of over 120cm producing over 40 million eggs on each spawning (Le Francois et al., 2010). When the tide rises, the spawned eggs and larvae are pushed inshore with the rising tide into flooded coastal feeding areas; the fries migrate upstream as the wet season ends and the wetlands dry (Le Francois et al., 2010).

Larvae will stay in the estuarine nursery swamps where they remain for several months before they move out into the freshwater reaches of coastal rivers and creeks. Juveniles of *L.calcarifer* remain in freshwater habitats until they are three–four years of age (60–70 cm total length) when they reach sexual maturity as males, and then move downstream during the breeding season to participate in spawning. Because *L.calcarifer* are euryhaline, they can live in a range of salinities (10–31 ppt), from fresh to seawater. When they are six–eight years old (85–100 cm total length), Australian *L. calcarifer* change sex to female and will be female for the rest of their

lives. In Asian populations of this species, mid life sex change is less prominent and primary females are common (FAO, 2011c).

The adult fish is regarded as carnivorous, but juveniles are omnivorous. Analysis of stomach content of wild seabass (1 to 10 cm) found 20 % phytoplankton and the rest are small fish and shrimp. The larger fish consists of 100 % animal prey, 70 % crustacean and 30 % small fish (FAO, 2011b). Most fish of this species remain in their original river system for their entire lives and move only short distances, although some *L. calcarifer* has been recorded as undertaking extensive movements between river systems. The almost non-existence migration between river system had resulted in limited exchange of individuals *L. calcarifer* from different river system which in turn contributed to the development of genetically distinct group of *L. calcarifer* in northern Australia, where there are six distinct strains in Queensland, and a further ten in the Northern Territory and Western Australia (FAO, 2011c).

2.1.4 Significance of *L. calcarifer* to fishery industry

2.1.4.1 Capture production of *L. calcarifer*

According to statistic released by FAO (2011a), the global capture production of *L. calcarifer* topped 80,000 tonnes in year 2009, a decrease from previous year (Figure 2.3). In Malaysia, statistic released by the Department of Fisheries puts the figure of Malaysian capture production of *L.calcarifer* in 2009 at 1,430 metric tonnes (Jabatan Perikanan Malaysia, 2011a).

2.1.4.2 Aquaculture production of *L. calcarifer*

Lates calcarifer is farmed throughout most of its range, with most production in Southeast Asia, generally from small coastal cage farms. Often these farms will culture a mixture of species, including barramundi, groupers (Family Serranidae, Subfamily Epinephelinae) and snappers (Family Lutjanidae) (FAO, 2011c). According to statistics released by FAO (2011a), the global aquaculture production of *L. calcarifer* is more than 45,000 tonnes in year 2009, recording a steady increase in production since the 1970s (Figure 2.4).

Thailand is credited as the original developer of the sea bass farm rearing techniques which was developed in the early 1970s (Wongsomnuk and Manevonk, 1973 cited in Cheong, 1989, Petersen et al., 2011*). L. calcarifer* is a fast growing species making it a popular species for aquaculture. In the 1980s and 1990s, *L. calcarifer* aquaculture industry began to take footings intensively in China, India, Indonesia, Malaysia, the Philippines, Singapore Taiwan, Vietnam and Australia (Petersen et al., 2011).

L. calcarifer is a highly adaptable species that can be aqua farmed using intensive and extensive methods whether indoors or outdoors and can be reared over the wide range of water salinity (10–31 ppt) from fresh water to seawater including inland brackish bore water i.e. salty ground water which accumulates in aquifers from seepage of rain and rivers through layers of soil and rock (Le Francois et al., 2010). Cultured fish spawns during the warmer months though caged *L. calcarifer* has been conditioned to produce eggs during any month.

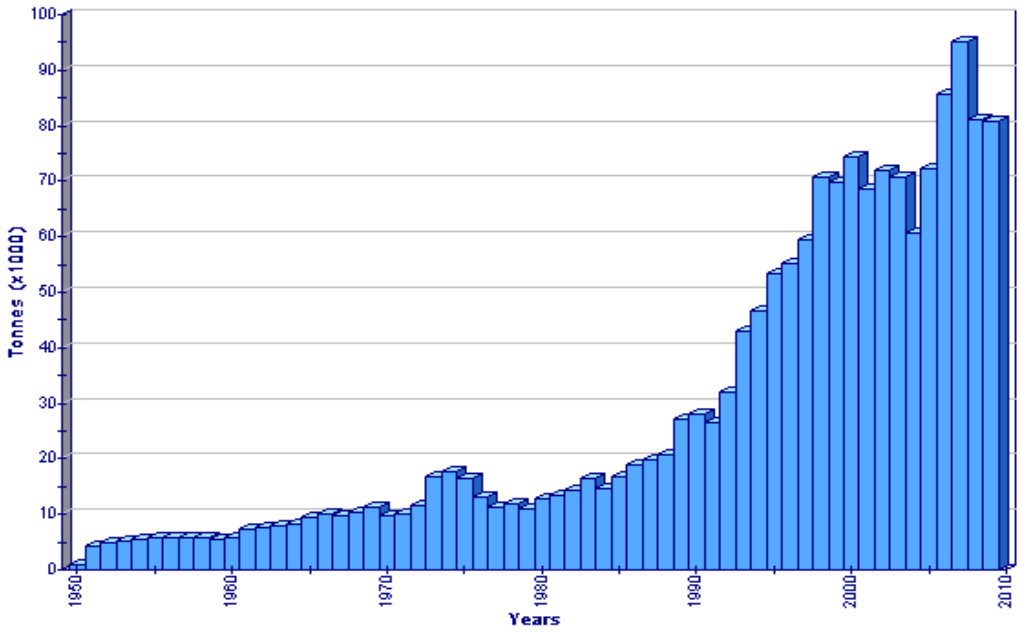

Figure 2.3: Graph of global capture production of *L. calcarifer* (Source FAO, 2011a)

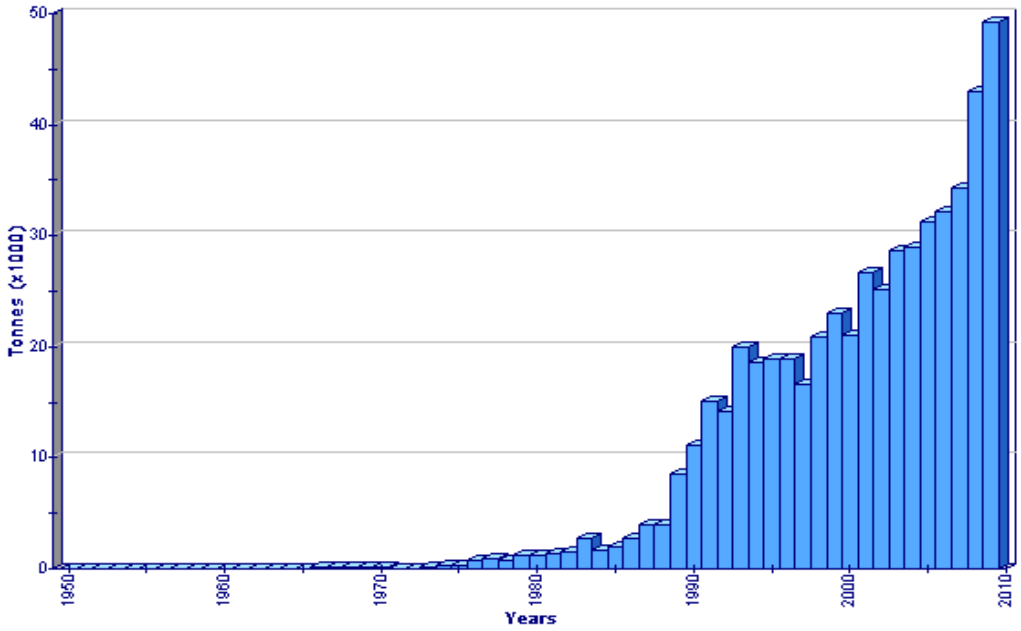

Figure 2.4: Global Aquaculture production for *L. calcarifer* (Source: FAO, 2011a)

Approximately a quarter million eggs per kg body weight (e.g. 6.8 million/16.5 kg) can be harvested via induced ovulation (Tucker, 1998).

The hatchlings are harvested when they grow over 25mm in length, which is normally reached in 21 days. The nursery phase starts when the fish are 10-25mm in length. In Asia, the common methods are nursery cages and ponds and such nursery cages and ponds may be coastal, brackish or fresh water cages and ponds (Le Francois et al., 2010). At this stage, *L. calcarifer* are fed with compound feeds (special weaning diets) which are recommended over crumbles and small portions of grower diets (Le Francois et al., 2010). *L.calcarifer* are normally weaned from live feeds to commercial diets around the time of metamorphosis. *L.calcarifer* are ready for grow out after 30-45 days when they reach 5-10cm in size. This nursery phase lasts for 30 to 45 days; once the fingerlings have reached 5–10 cm TL they can be transferred to grow-out ponds (FAO, 2011a).

Major cause of mortalities during the nursery phase and during early grow-out phase is cannibalism because *L. calcarifer* will cannibalise fish of up to 61–67 percent of their own length. Cannibalism may start during the later stages of larval rearing and occurred mostly to fish with total length less than 150 mm. Cannibalism however is not a significant problem in larger fish. Cannibalism can be reduced by ensuring that fishes are of uniform size and this is achieved by grading the fish at regular intervals (usually at least every seven–ten days) to ensure that the fish in each cage are similar in size (FAO, 2011a).

The last phase of *L. calcarifer* aqua-farming is the grow-out phase. In this phase, both floating and fixed cages are used to rear the fish. In Australia and the United States of America, a number of *L. calcarifer* farms have been established using recirculation freshwater or brackish water systems with a combination of physical and biological filtration. These farms may be located in regions where *L. calcarifer* could not otherwise be farmed because of consistently low temperatures (southern Australia, north-eastern United States of America). The major advantage of such systems is that they can be sited near to markets in these areas, thus reducing transport costs for the finished product (FAO, 2011a).

In Malaysia, *L. calcarifer* is a significant species reared for commercial harvesting. The *L. calcarifer* aquaculture industry traces its origin to 1976 under a Malaysian government programme whereby wild and imported fries of *L. calcarifer* were reared in floating net cages at the Setiu Lagoon in the eastern coast of Peninsular Malaysia. The programme was proven to be feasible and nowadays many private commercial hatcheries are now operating throughout Malaysia, with the main supplier of *L. calcarifer* hatchlings being the Fisheries Department of Malaysia through its Pusat Pengeluaran dan Penyelidikan Ikan Laut (PPIL) situated at Tanjong Demong, Terengganu (Norfatimah et al., 2009). Statistics released by the Fishery Department, Ministry of Agriculture and Agro-based Industry, Malaysia for the year 2009 estimated that aquaculture production from brackishwater culture system of *L.calcarifer* in Malaysia totalled 14,229.82 metric tonnes (Jabatan Perikanan Malaysia, 2011b).

2.2 Molecular Markers

2.2.1 Introduction

Molecular marker is a specific fragment of DNA that can be identified within the whole genome. Nowadays, molecular markers serve as valuable tools for researchers to solve a problem in genetic based research. Molecular markers allow us to quantify genetic diversity, track the movement of individuals, characterize new species, measure inbreeding, identify the remains of individuals and retrace historical pattern (Freeland, 2005).Types of molecular marker include allozymes, mitochondrial DNA, RFLP, RAPD, AFLP, microsatellite, and SNP. The abundance of marker is mainly determined by frequency at which the sites of interest occur within the genome. RFLPs and AFLPs generate abundant markers due to the large number of restriction enzymes available and the frequent occurrence of their recognition sites within genomes. Microsatellites were highly abundant within eukaryotic genomes. RAPD markers are even more abundant because numerous random sequences can be used for primer construction. In contrast, the number of allozyme markers is restricted due to the limited number of enzyme detection systems available for analysis.

Generally molecular markers can be divided into type I marker and type II marker. Type I are markers associated with genes of known function. Since represent coding region, this type of markers is conserved between species, possessing similar DNA/ protein sequences and similar physiological functions. In contrast, Type II molecular markers are not necessarily associated with genes. In general, most Type

II markers are commonly located in non-coding intergenic regions. Non-gene sequences are free to mutate, causing producing higher levels of polymorphism compared to coding region (Chistiakov et al., 2006). Besides, molecular markers also divided into dominant and codominant marker based on whether markers can discriminate between homozygotes and heterozygotes. Codominant markers allow us to identify all the present alleles at particular locus, which mean we can differentiate between homozygotes and heterozygotes, whereas dominant markers will reveal only single dominant alleles.

2.2.2 Molecular Markers in Fisheries and Aquaculture genetics

Over the years, the popularity of molecular markers has risen in aquaculture community. Molecular markers were extensively used in aquaculture genetic research include species identification, genetic diversity, gene flow and inbreeding level, parentage assignment, linkage and Quantitative Trait Loci (QTL) studies and Molecular Assisted Selection (MAS). The popular molecular markers in aquaculture genetic research include RFLP, AFLP, allozymes, mitochondrial DNA, microsatellite, and the most recently popular marker, SNP marker (Single Nucleotide Polymorphism) (Liu and Cordes, 2004). All these markers have their own principle, application, requirement, potential power, strength and weakness. Molecular markers were chosen based on their ability to resolve specific problem or selected task. The review of molecular markers application with their recommend markers were summarized in Table 2.1.

Table 2.1: Application of DNA markers in a aquaculture genetics (adapted from Liu and Cordes, 2004).

Task	Recommendation marker	Other useful marker types
Species identification	Microsatellites	RAPD, AFLP, isozymes
Strain identification	AFLP, microsatellites	RAPD
Hybrid identification	RAPD	AFLP, microsatellites, mitochondria[a]
Paternity determination	Microsatellites	
Genetic resources/ diversity analysis	AFLP, Microsatellites	RAPD
Genetic mapping	Type I markers, Microsatellites, SNP	AFLP, RFLP
Comparative mapping	Type I markers	ESTs, conserved microsatellites

[a]: Use of mitochondrial markers should also allow determination of maternity.

2.2.3 DNA Microsatellites

2.2.3.1 General Characteristic

Microsatellites, also known as simple sequence repeats (SSRs) are part of DNA sequence that consist of multiple copies of tandemly arranged simple sequence repeats that range from 1-6 base pair (bp) (Tautz, 1989). Microsatellites can be classed into mononucleotide repeat units, dinucleotide repeat units, trinucleotide repeat units, tetranucleotide repeat units and more others based on the repetition composition (e.g. CA, CCA or TATA). An example of microsatellite sequence for dinucleotide repeat $(AC)_{9,}$ which consist of nine repeats of AC sequence (ACACACACACACACACAC).

Generally, microsatellites are categorized or classified into simple microsatellites and composite microsatellites (Liu, 2007). Simple microsatellites (e.g. $(AC)_9$) are microsatellites that contains only one type of repeat whereas composite microsatellite (e.g. $(CA)_9(CCA)_{10}$) contains more than one type of motif repeats. Sometimes, there are cases where the core repetitive unit is interrupted by base substitutions. This type of microsatellite is known as interrupted microsatellite (e.g. GTGTGTGTG**A**GTGTGTGT). In contrast, microsatellite consists of many interruptions including the addition of a few different motifs known as cryptic simple sequence (e.g. GTGT**CTTCTT**GTCTGTG**TTTT**G).

The most abundant forms of microsatellite are dinucleotide repeats, followed by tri or tetra repeats but in some cases, as reported in the Japanese puffer fish *Takifugu rubripes*, tetranucleotide repeats appear more often compare to

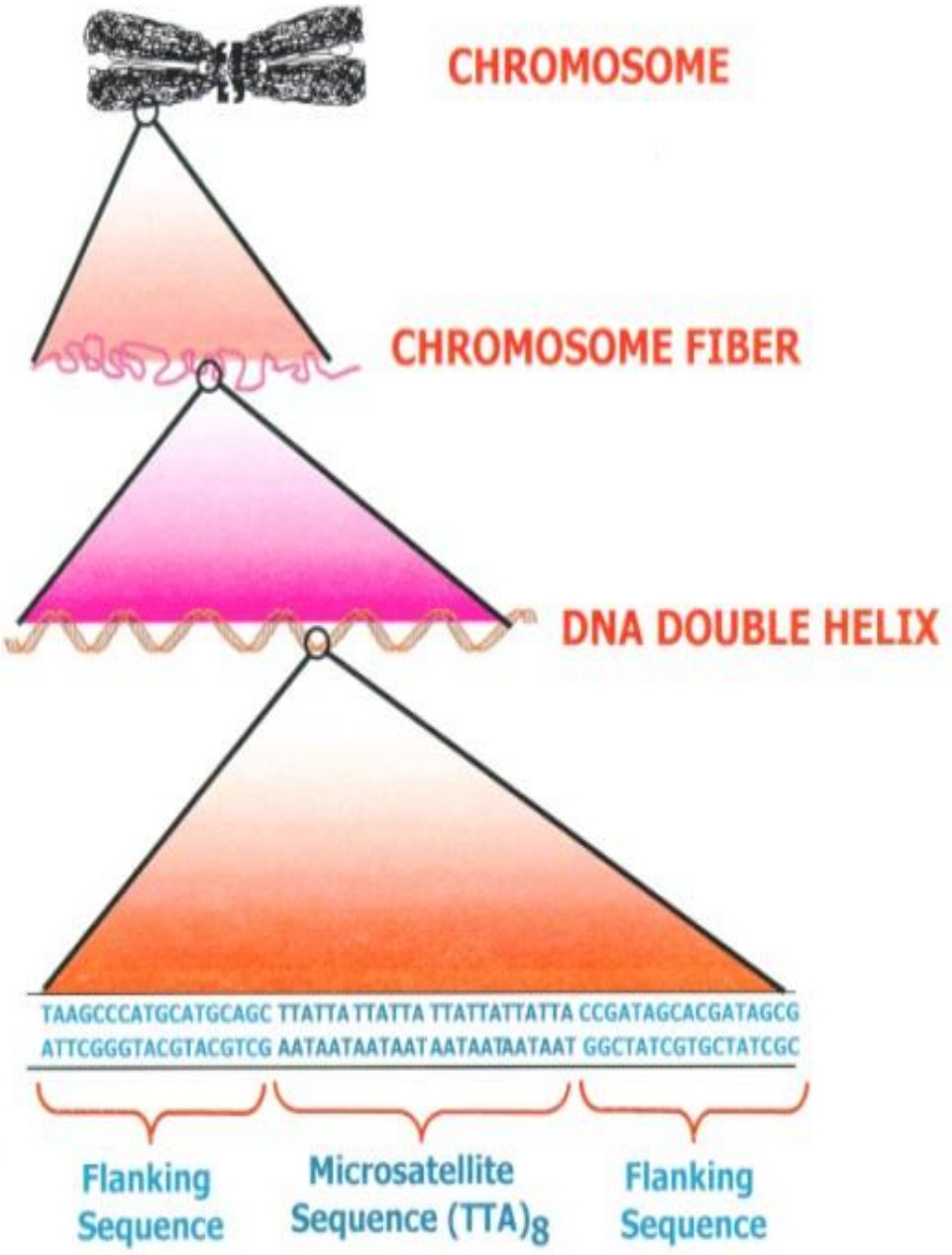

Figure 2.5: The anatomy of microsatellite.

(Source: http://www.asicoaquaticmarkers.com/AnatomyofaMicrosatellite.htm)

trinucleotide repeats (Edwards et al., 1998). Toth et al. (2000) also reported, (CA)n was the most common dinucleotide repeat, follow with (AT)n and then (CT)n.

Compared to non-repetitive DNA, microsatellite generally has higher mutation rates. The differences in repeat unit numbers were believe to be caused by polymerase slippage during DNA replication (Levinson and Gutman, 1987; Tautz, 1989). Microsatellite mutation rates have been reported as high as 10^{-2} per generation (Weber and Wong, 1993), whereas point mutation in non-repetitive DNA gives rates of the order of 10^{-9} to 10^{-10} (Weber and Wong, 1993; Jarne and Lagoda, 1996). This high rate of mutation will lead to the size homoplasy which means that ancestor-descendant relationship maybe difficult to solve using microsatellite data. The high mutation rates will produce high level of polymorphism and make them relatively good choice for inferring recent population genetic events (Chistiakov et al., 2006).

2.2.3.2 Microsatellite and its application in Fisheries and Aquacultures

Microsatellites represent locus specific markers, which have been widely used in a variety of fundamental and applied fields of life and medical sciences. Their special characteristic makes them suitable for a wide scale of research discipline. Microsatellites also have proven their benefit and give a great enthusiasm in aquaculture genetics.

Genetic identification of species or strains could be determined by microsatellites. Since microsatellites markers were based on species or closely related species, their identification is relatively straightforward. Microsatellites also serve as tools to evaluate inbreeding levels (F_{IS}). From inbreeding levels viewpoint, genetic structure of populations can be easily determine using *F*-statistics and genetic distances analysis. They are useful in demographic history analysis, assessment of population size effectiveness (N_e) and to assess the magnitude and directionality of gene flow between populations. As noted, Rainbow trout was one of the first species was used to study the population variability using microsatellite markers (Nielsen et al., 1994). That study has revealed similar patterns of differentiation for mtDNA and the microsatellite locus. Another study, population studies of potadromous rainbow trout from Lake Ontario has been performed using both microsatellites and mtDNA (Dueck, 1994, O'Connell et al., 1997). A comparison of marker sets revealed that the number of alleles observed at microsatellite loci was similar to the number of haplotypes revealed by mtDNA.

The inheritance pattern in microsatellites shows one allele was inherited from the male parent and another allele came from the female parent. This basic inheritance makes microsatellites very popular in parentage assignment. A unique profile can be generated for each individual using selected microsatellite loci. The genotype profile is highly discriminating, since microsatellite produce high variation even though in same species or population. Applications of microsatellites have been reported to determine paternity and reproductive contribution in wild and farmed populations of various economically significant species such as rainbow trout,(Herbinger et al. 1995, Bentzen et al.,2001) *Lepomis macrochirus* (Neff, 2001),