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GENETIC VARIABILITY AND DIFFERENTIATION OF FERAL AND CULTURED POPULATIONS OF ASIAN SEA BASS (*Lates calcarifer*) IN MALAYSIA INFERRED BY MICROSATELLITES

by

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Thesis submitted in fulfillment of the requirements for the degree of Master of Science

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LIST OF ABBREVIATION

bp	Base pair (s)
$CaCl_2$	Calsium chloride
°C	Celcius
dNTP	Deoxyribonucleotide triphosphates
DNA	Deoxyribose nucleic acid
EDTA	Ethylene diaminetetraacetic acid
Kbp	Kilobase pair (s)
$MgCl_2$	Magnesium Chloride
T_m	Melting temperature
TEMED	N, N, N',N' - tetramethylethylenediame
OD	Optical density
PCR	Polymerase chain reaction
P	Probability
rpm	Revolution per minutes
NaCl	Sodium chloride
SDS	Sodium Dodecyl Sulfate
S.E.	Standard error
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TNES	Tris-NaCl-EDTA-SDS
UV	Ultraviolet
V	Volt
v/v	Volume/volume
w/v	Weight/volume

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1.1 Choay-Hoong, Lai and Othman, A.S. Utilisation of molecular analysis towards the improvement of sea bass, *Lates calcarifer* breeding programme in Malaysia. (Proceedings of the KUSTEM 5th Annual Seminar, 2-3rd May 2006, Terengganu). BIODIVERSITY AND CONSERVATION: POSTER PRESENTATION

1.2 Choay-Hoong, Lai , Othman, A.S. and Siti Azizah M.N. Data mining for simple sequence repeats (SSRs) in genome survey sequences (GSSs) from Asian sea bass, *Lates calcarifer*. (Proceedings of 7th National Congress on Genetic, 5-7th May 2007, Kelantan). BIODIVERSITY AND CONSERVATION: POSTER PRESENTATION

1.3 Choay-Hoong, Lai , Othman, A.S. and Siti Azizah M.N. Neighbour-Joining and Factorials Correspondence Analysis (AFC) for discriminating feral and cultured Asian sea bass . (Proceedings of 2nd Regional Conference on ECOMOD 2007, 28-30 August 2007, Penang) ANALYSIS: ORAL PRESENTATION

KEVARIABELAN DAN PERBEZAAN GENETIK POPULASI-POPULASI FERAL DAN KULTUR IKAN SIAKAP (*Lates calcarifer*) DI MALAYSIA MENGGUNAKAN MIKROSATELIT

ABSTRAK

Sepuluh lokus mikrosatelit telah digunakan untuk menyiasat kevariabelan dan pembezaan genetik tiga populasi feral (Pulau Sayak, Semerak and Tanjung Piandang) dan enam populasi (Pulau Sayak, Sungai Linggi, Merchang, Sungai Pendas, Punang and Sematan) kultur ikan siakap, *Lates calcarifer* di Malaysia. Tahap Kekayaan alel (*Ar*) adalah dalam julat 2.0-11.3 manakala keheterozigotan yang dijangka (*He*) adalah dalam julat 0.234-0.875. Semua populasi feral menunjukkan kevariabelan genetik yang tinggi dan hampir sama. Kekayaan alel (*Ar*) sebar rendah dalam tiga daripada lima populasi ikan siakap pusat akuakultur berbanding dengan sampel daripada populasi feral. Populasi kultur Sematan mempamerkan anjakan mod dalam frekuensi taburan alel yang mencadangkan berlakunya "bottleneck". Kesignifikanan anggaran pembezaan genetik berpasangan di antara populasi feral adalah rendah (F_{ST} = 0.0310-0.0899) tetapi sederhana antara populasi kultur (F_{ST} = 0.0252-0.1637). Ini mencadangkan berlakunya hanyutan genetik dalam pusat pembiakan akuakultur dengan setiap daripadanya mengamalkan rejim pengurusan yang berbeza. Ini menunjukkan keperluan untuk penambahan bahan genetik yang sihat, pengawasan genetik dan program pembiakan yang efektif bagi memastikan kesihatan genetik ikan siakap di Malaysia. Analisis STRUCTURE menunjukkan bilangan nombor kluster (*K*) yang paling tepat untuk sembilan populasi dalam kajian ini adalah enam. Merujuk kepada keputusan nilai F_{ST} berpasanga dan Ujian Penagihan Individu, kedua-dua populasi Pulau Sayak-F and Semerak telah menerima kemasukan populasi-populasi sama ada daripada ke enam-enam kluster atau adalah mewakili leluhur species ini menyebabkan pemerhatian populasi yang bercampur.

GENETIC VARIABILITY AND DIFFERENTIATION OF FERAL AND CULTURED POPULATIONS OF ASIAN SEA BASS (*Lates calcarifer*) IN MALAYSIA INFERRED BY MICROSATELLITES

ABSTRACT

Ten microsatellite loci were used to investigate genetic variability and differentiation of three feral (Pulau Sayak, Semerak and Tanjung Piandang) and six cultured (Pulau Sayak, Sungai Linggi, Merchang, Sungai Pendas, Punang and Sematan) populations of Asian sea bass, *Lates calcarifer* in Malaysia. Level of allelic richness (*Ar*) ranged from 2.0-11.3 while expected heterozygosities (H_e) ranged from 0.234 to 0.875. All feral populations exhibited almost similar and high levels of genetic variation. Genetic variation in terms of expected heterozygosity (H_e) and allele richness was slightly lower in three (Sungai Pendas, Punang and Sematan) of five hatchery samples than in samples of the feral populations. The Sematan cultured population exhibited a mode shift in its allele frequency distribution which suggests a recent bottleneck has occurred. Pairwise estimates of genetic differentiation between feral populations were low (F_{ST} = 0.0310-0.0899) but moderately high among cultured populations (F_{ST} = 0.0252-0.1637), suggesting occurrence of genetic drift in the hatcheries with each hatchery practicing different management regime. This demonstrates the need for introduction of healthy genetic materials, genetic monitoring and effective breeding programs to ensure the genetic health of the Asian sea bass in Malaysia. STRUCTURE analysis suggests that the most likely number of cluster (*K*) for nine the populations in this study was equal to six. Based on results of pairwise F_{ST} values and Individual Assignment Tests, both Pulau Sayak-F and Semerak were suggested to have received either introductions of populations representing all the six clusters or represent the ancestral population of the species, thus contributing to the observed admixture in the populations.

CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION

The Asian sea bass (*Lates calcarifer*) is one of the most economically important species among our native Malaysian fishes. Most Asian sea bass are produced by commercial aquaculture in many Asian countries such as Malaysia, Taiwan, Thailand and Indonesia. Mature wild sea bass can only be captured during certain mating season and this also depends on the weather. Severe declines in landing Asian sea bass supplies throughout the year have led farms to grow this species intensively to meet consumer's demand.

Domestication of Asian sea bass using cage nets has been established by the Fisheries Department in Malaysia since 1970s (Awang, 1986a). However, the number of founding populations and detailed information on mating scheme were not known. A number of private hatcheries in Malaysia have also been utilising broodstock from neighbour countries such as Thailand to produce larvae besides local strains. In addition, several private hatcheries have even imported fingerlings directly from Thailand instead of producing their own fingerlings or purchasing from Fisheries Department in Malaysia. Despite a long aquaculture history, the genetic structure of these local stocks has not been determined. After more than 30 years of aquaculture activities, guidelines based on population genetics are essential for founding and maintaining cultivated stocks and more importantly to avoid genetic erosion.

Aquaculture practices may inadvertently reduce the levels of genetic variation present in farmed stocks by breeding only small numbers of founding broodstocks. Selective breeding programs can also lead to inbreeding when they utilize only a small number of "superior" individuals (Thai *et al*., 2007). There is often a high probability of selecting related individuals as parents for constructing the next generation and hence increasing inbreeding if pedigree records are not properly maintained (Norris *et al*., 1999).

Breeding program may also intentionally introduce divergent stocks and utilize crossbreeding programs to increase diversity and productivity (Hulata, 1995). Hence, to which extend different broodstocks disseminated are important for effective management of aquaculture species should also be addressed. Meanwhile, there is also a need to evaluate the status of wild stocks in aquaculture species since uncertainty in origin of these fish can lead to negative effects on the native strains (Cross, 2000).

It is highly probable that the present native populations of Asian sea bass may be contaminated with hatchery escapees given the lack of proper management control. Whether the release is accidental or intentional (restocking) the consequence is often that the indigenous gene pools are compromised by loss of diversity within populations, introgression and eventually extinction of local populations (Ryman *et al*., 1995 and references therein). Identification of wild stocks is also crucial to provide an available source of wild genetic diversity in domestication and selective breeding programs.

In the past few years, the use of genetic markers in fisheries management and aquaculture has escalated for addressing some of these questions in population genetics.

The approach in this study to uncovering cryptic population structure of the sea bass is the utilization of microsatellite markers. Microsatellites are sequences made up of a short single sequence motif that are tandemly repeated (Hancock, 1999). They have been the marker of choice for various types of genetics studies because of their high polymorphism level. Over the pass decade, microsatellite markers have been widely used in fisheries population genetic studies of various species including Atlantic salmon (Mcconnell *et al*., 1997), oysters (Li *et al*., 2006), bay scallop (Wang *et al*., 2007b), common carp (Thai *et al*., 2007) and more. These markers have also gained popularity in population genetic studies of several Malaysian species such as Asian Arowana (Tang *et al*., 2004), green-lipped mussel (Ong, 2007) and *Tor douronensis* (Nguyen, 2008).

Preliminary population genetic study on total 62 samples from three wild and four cultured populations *L. calcarifer* in Peninsular Malaysia has been done by Sim (2004) using 19 microsatellite loci. The previous study has provided the basis for future investigation on genetic variation and genetic structure of Asian sea bass in Malaysia. In addition, analysis of genetic variation of *L. calcarifer* using mitochondrial DNA markers based on 156 samples from two culture and five wild populations in Peninsular Malaysia (Norfatimah, 2007). However, both preliminary studies only referred to a small number of individuals.

The objectives of this study are:

(1) To quantify genetic variation within and among cultured and feral populations of Asian sea bass, *Lates calcarifer*; and

(2) To investigate the current population differentiation of Asian sea bass in Malaysia.

CHAPTER TWO

LITERATURE SURVEY

2.1 Asian sea bass, *Lates calcarifer*

2.1.1 Taxonomy, Species Identification and Genetic Records

Lates calcarifer, locally called 'Siakap' or Asian sea bass (as compared to the European sea bass - *Dicentrarchus labrax*) is an economically important finfish in Malaysia. This species was first described by Bloch in 1790 from a specimen received from Dutch merchants returning from the Indo-Pacific region (Grey, 1986). Due to its wide geographical coverage, *L. calcarifer* is known by various common names such as 'barramundi' in Australia, 'giant perch' and 'anama' in Papua New Guinea, 'sea bass' and 'bhekti' in India, 'sea bass' in Thailand and Philippines, 'akema' in Japan and 'sea bass' in Indonesia, to list a few (Dunstan, 1962; Rabanal and Soesanto, 1982).

Lates calcarifer belongs to the family Centropomidae which comprises of 9 species. Members from this family inhabit waters from coastal marine, estuaries to freshwater including mangrove estuaries and rocky to coral reefs. Some of its species are popular and sought-after with high economic importance (Larson, 1999). *Lates calcarifer* is commercially one of the most important coastal finfish and angling species within this family. It is marketed as fresh and frozen. The taxonomic classification and description of *L. calcarifer* is given below (FAO, 1974; Grey, 1986; BOLD systems, 2006).

Taxonomic classification:

Phylum	: Chordata
Subphylum	: Vertebrata
Class	: Actinopterygii
Subclass	: Teleostomi
Order	: Perciformes
Family	: Centropomidae
Genus	: *Lates*
Species	: *Lates calcarifer* (Bloch)

Taxonomic description:

The species has a compressed and elongate body with a deep caudal peduncle (Figure 2.1). The head is pointed with a concave dorsal profile becoming convex in front of the dorsal fin. Its mouth is large, slightly oblique with its upper jaw reaching behind the eye; the teeth are villiform with no canines present. The lower edge of the pre-operculum has a strong spine; the operculum has a small spine with a serrated flap above the origin of the lateral line. Its lower first gill is arched with 16 to 17 gill rakers. Scales are large, ctenoid. The dorsal fin has 7 to 9 spines and 10 to 11 soft rays with a very deep notch almost dividing the spiny from soft part of the fin. The pectoral fin is short and rounded with several short, strong serrations above its base.

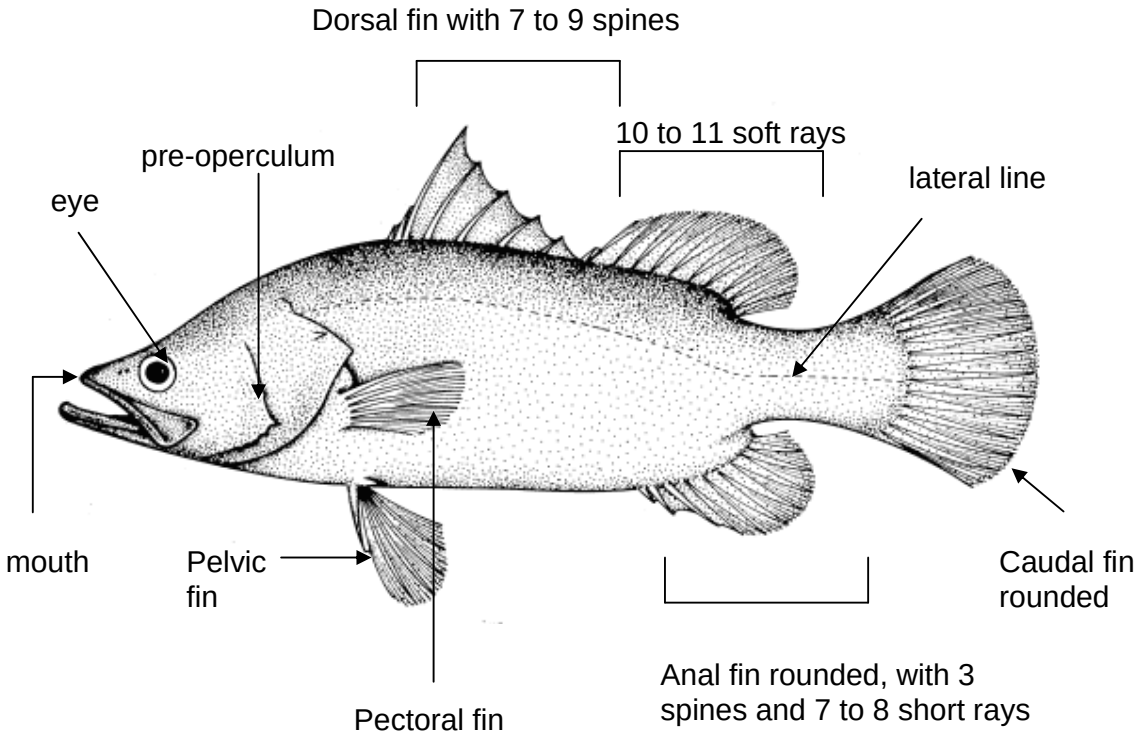

Figure 2.1 Morphological characteristics of *Lates calcarifer* (Modified from FAO, 2008).

The dorsal and anal fins both have scaly sheaths. The anal fin is rounded, with 3 spines and 7 to 8 short rays. The caudal fin is rounded. The colour of this fish is divided into two; phases, either olive brown above with silver sides and belly (usually juveniles) or green/blue above and silver below. No spots or bars are present on the fins or body (FAO, 1974; Grey, 1986).

Lates calcarifer has one of the smallest genomes among food fish species (Carrey and Mather, 1999). Its genome is compact (~700 Mb) (Wang *et al*., 2007a) with chromosome numbering 2n= 48 (Arkhipchuk, 1999; Carrey and Mather, 1999). The complete mitochondrial DNA (mtDNA) nucleotide sequence of *L. calcarifer* is 16,535 bp in length containing 13 protein coding genes, 22 transfer RNAs, 2 ribosomal RNAs, and one major noncoding control region (Lin *et al*., 2006). More recently, the first generation genetic linkage map of *L. calcarifer* was successfully mapped into 24 linkage maps using 240 microsatelite markers (Wang *et al*., 2007a). The map provides a pivotal resource for further study of this species.

2.1.2 Distribution, habitat and biology

Lates calcarifer can be found in coastal, estuaries and fresh water habitats. It has a very extensive range in tropical and semi-tropical areas of indo-West Pacific. Its distribution ranges from western India, around Sri Lanka to Bay of Bengal, and through the whole of Southeast Asia to eastern Papua New Guinea and northern Australia (Greenwood, 1976; Moore, 1980; Blaber, 2002). This highly opportunistic, fecund species has dominated many tropical rivers throughout its range due to a dynamic and flexible biology.

The Asian sea bass is a protandrous hermaphrodite species (Moore, 1979, 1980; Moore and Reynolds, 1982; Reynolds and Moore, 1982; Russell and Garrett, 1983, 1985; Davis, 1982, 1985). The gonads of *L. calcarifer* are dimorphic and complete reorganization of gonad structure and function takes place after 6 inversions, probably under the influence of hormones. Male *L. calcarifer* spawn several years before sex inversion. The sex reversal is initiated as the testes ripen for the last time, and the change to ovary takes place rapidly within a month of spawning. The change to female usually takes place at about 7 years of age and a body length of about 800 mm, but is apparently more related to age than to body length. The body length at which sex change occurs varies somewhat across its extensive geographic range, probably due to habitat, food and genetic differences (Blaber 2002). Moore (1980) postulated that protandrous sex reversal in *L. calcarifer* allows the larger and more successful females to ensure greatest contribution to the gene pool of a particular population.

Lates calcarifer are carnivorous. They feed on fishes as well as some small crustaceans mostly prawns. It has a complex life history which occupies various habitats at different stages of their life cycle (Figure 2.2). It is a euryhaline and catadromous species. It grows to maturity in the upper reaches of freshwater rivers and streams. Adults will then move downstream especially during tidal or flooding, to estuaries and coastal waters for spawning (Keenan, 1994). According to Moore and Reynolds (1982), migrations of Asian sea bass can be up to 300 km along the coast away from the influence of fresh water to suitable spawning habitats.

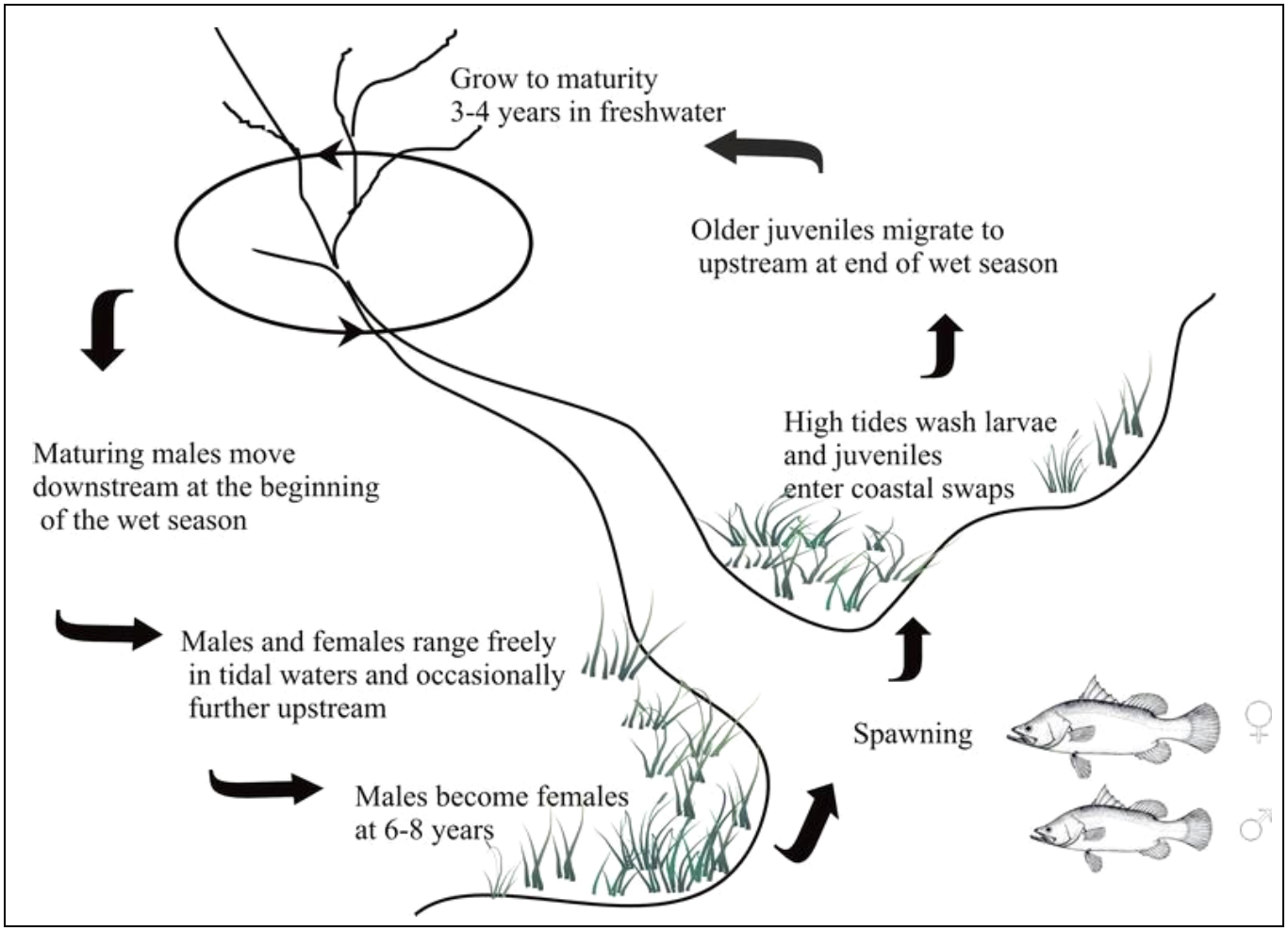

Figure 2.2 Life cycle of Asian sea bass, *L. calcarifer* (Modified from Blaber, 2002).

The Asian sea bass spawn after the full and the new moons during the spawning season. Figure 2.2 shows its life cycle. Movement to spawning sites takes place at the end of the dry season or early in the wet season (Blaber, 2002). Highly fecund adults spawn at river mouths, lakes, lagoons or open coastal areas (Moore, 1982) where salinity ranges between 28 to 32 PSS (practical salinity scale) (Keenan, 1994). The eggs and larval stages can tolerate a narrower range of salinity and temperature than adults (Russell and Garrett, 1983). The juvenile fry has been reported to meet optimized growth at temperatures from 27 to 36°C (Katersky and Cater, 2005). Juveniles enter and remain for shelter in flooded black swamps and floodplains before they dry up during the early part of the dry season. The monsoon pattern in Australia and Asia thus has a strong link towards the reproductive cycles of *L. calcarifer* (Russell and Garrett, 1983).

At the end of the wet season, older juveniles disperse into permanent tidal creeks and estuaries and migrate upstream where they remain until they reach maturity (William *et al*., 2004). Sea bass becomes sexually mature between 3 to 4 years of age. Maturing males typically move downstream at the onset of the wet season to tidal waters to spawn. Males and females range freely in tidal waters and occasionally further upstream (Russell and Garrett, 1983).

2.1.3 Fisheries Production in Malaysia

Fishery is a major sector for many developing countries promoting economic growth including Malaysia. The fisheries sector has played an important role as a major supplier of animal protein to the Malaysian population (FAO, 2008). The sector consists of two components, namely capture fisheries and aquaculture. The greater bulk of fish

landings have always come from the capture fisheries and the rest coming from aquaculture.

In Malaysia, the fish is normally captured during its spawning season from February to October (Ali, 1986a) depending on weather conditions. With the development of aquaculture capacity the global and Malaysia's production of *L. calcarifer* have increased (Figures 2.3 and 2.4; Appendix 1.1 and 1.2) to meet consumer's demand.

According to the FAO Fishery Statistics (2008), Thailand is the main aquaculture producer of Asian sea bass besides Saudi Arabia, Hong Kong, Indonesia, Malaysia, Singapore, Brunei, Taiwan, Australia and French Polynesia. Asian sea bass is sold fresh and also in chilled form. In Malaysia, most Asian sea bass are marketed at 500-900 g/piece. Small numbers of larger fish (1-3kg) are also sold.

Normally, wild Asian sea bass fetch relatively better price than cultured type. For live cultured fish, the current prices range from RM13.00 to RM15.00/kg whereas wild Asian sea bass captured from the open sea can fetch around RM16.00/kg or higher especially during festive and Monsoon seasons. The retail prices in supermarket of the whole chilled Asian sea bass form usually cost lower ranging from RM10.00 to RM 13.00/kg.

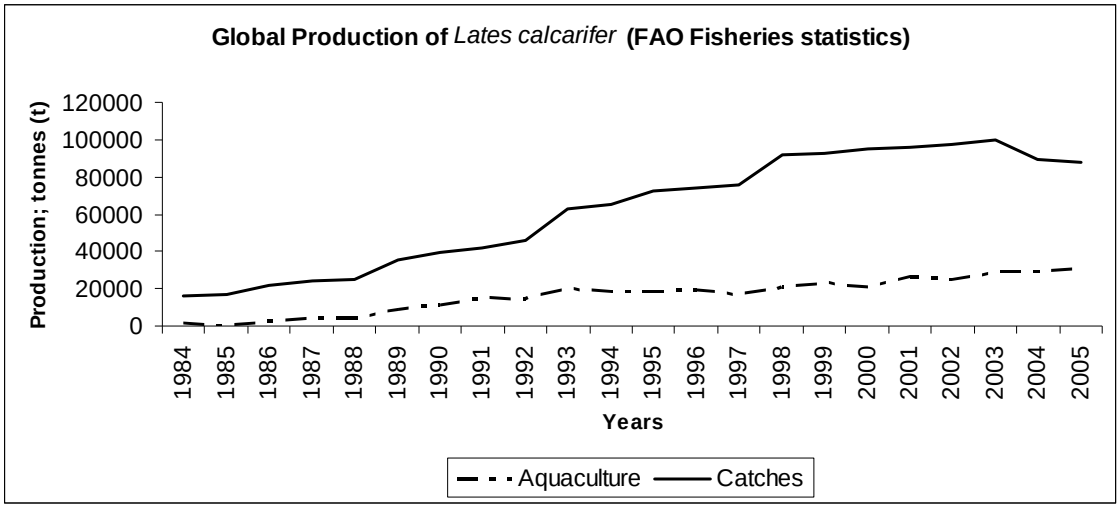

Figure 2.3 FAO global production of *Lates calcarifer*. (Source: http://www.fao.org/figis/servlet/SQServlet?file=/usr/local/tomcat/FI/5.5.23/figis/webapps/figis/temp/)

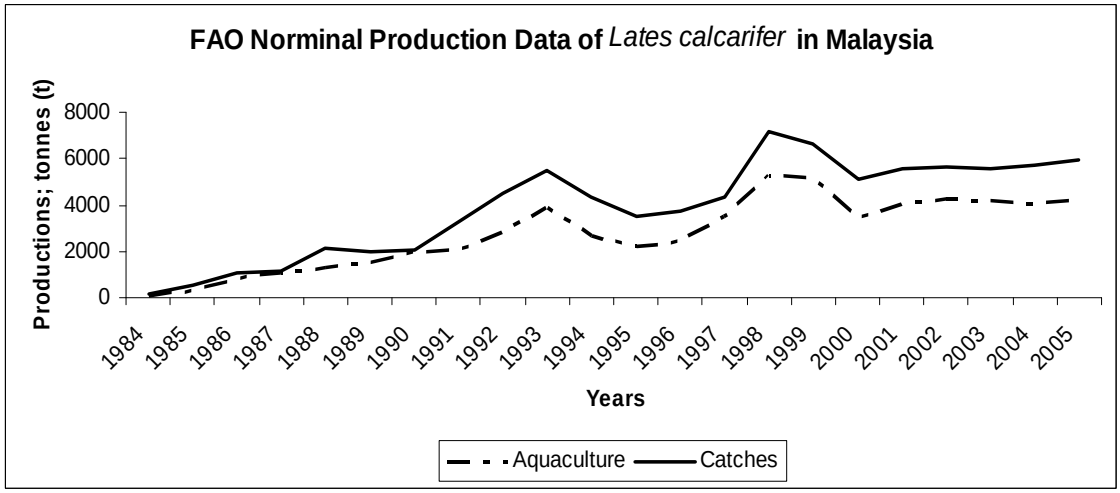

Figure 2.4 FAO norminal production of *Lates calcarifer* in Malaysia. (Source: http://www.fao.org/figis/servlet/SQServlet?file=/usr/local/tomcat/FI/5.5.23/figis/webapps/figis/temp/)

2.1.4 Status of the Asian sea bass aquaculture in South East Asia

The Asian sea bass cage culture was first studied at the Songkla Fisheries station, Thailand in 1971. In 1973, wild Asian sea bass were successfully induced to spawn (Maneewong, 1986). The successful breeding of Asian sea bass in Thailand allowed Malaysian's farmers to start their culture of this species using imported fries from Thailand. Since then, this has encouraged the popularity of culturing Asian sea bass in Malaysia for grow-out. As the Asian sea bass culture expanded in the late 1970s the supply of seeds from this source was found to be inadequate and inconsistent (Awang, 1986a).

In 1985, larval propagation of *L. calcarifer* was first achieved at the Fisheries Research Institute, Penang (Awang *et al*., 1985). The success in Asian sea bass propagation has subsequently overcome the insufficient supply of fries in private hatcheries. These fries are obtained at nominal cost (Ong, 1986). At the moment, researches on spawning of this species are still being carried out at the Pusat Pengeluaran and Penyelidikan Ikan Laut in Tanjung Demong, Terengganu.

The juveniles are usually cultured in floating or fixed nursery cages in rivers, coastal areas or directly in freshwater or brackishwater nursery tanks (Ali, 1986b). Production of cultivated Asian sea bass has increased rapidly over the years in Malaysia. Although most farmers are interested on culturing fish species with higher price, such as groupers, Asian sea bass is still an ideal candidate for aquaculture as it grows rapidly, reaching a harvestable size (350 g – 3 kg) in six months to two years (Boonyaratpalin, 1991; FAO, 2008). Universally, Asian sea bass is regarded as a fine table fish and has

the uncommon ability to synthesis long chain omega-3 fatty acids, whose importance to human health has been increasingly recognised (Peet, 2006). It is a relatively hardy species that tolerates crowding and has wide physiological tolerances. The high fecundity of female fish provides plenty of eggs for hatchery production of seed. Besides, Asian sea bass feed well on pellet diets, and juveniles are easy to wean to pellets (FAO, 2008).

The main culture problem of this species at the early larval stage is the cannibalistic behaviour of the fries. Most of the mortality during the later larviculture stages is due to cannibalism among frys when they are about 12-15 days old (about 5 mm TL) (Awang, 1986b; Maneewong, 1986; Suteemechaikul and Petchrid, 1986). However, cannibalism can be reduced by grading the fish at regular intervals (usually at least every 3-5 days) to ensure that the fish in each cage are similar in size (Awang, 1986b; Maneewong, 1986).

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Numerous diseases that infect Asian sea bass have been reported such as vibriosis, edwardsiellosis and haemorrhagic septicaemias. The causative agents include parasitic organisms, bacteria, fungal, viruses, malnutrition and environmental stresses such as extremes of temperature, low dissolved oxygen, or poor handling of the fish (Chonchuenchob *et al*., 1986; Humphrey and Langdon, 1986; Barlow, 1997; Moullac *et al*., 2003). Diseases control was achieved on a combination of three factors: diagnosis, prevention and treatment. Preventive approach involves vaccination; maintenance of water quality; reduction in environmental stress such as low dissolved oxygen, temperature extremes, regulations to prevent transfer of pathogens from one host

population to another; chemical prophylaxis; control of hatchery sanitation and disinfection (Ruangpan, 1988).

2.2 DNA Microsatellites

2.2.1 General Characters of Microsatellites

Microsatellite loci were discovered in the late 80s. Microsatellites, also called simple sequence (Tautz, 1989) and short tendem repeats (STRs; Edwards *et al*., 1991) have probably become the most popular and powerful method for identifying highly polymorphic Mandel markers (Hancock, 1999; Li *et al*., 2002; Scribner and Pearce, 2002). Their wide applicability span over areas namely populations genetics, parentage and kinship analysis, genome mapping, forensic DNA studies, identifying individuals, reconstruction of human origin, hybridization studies, molecular epidemiology and pathology (O'Connell and Wright, 1997; Goldstein and Schlötterer, 1999 and references therein; Liu *et a*l., 1999; Chistiakov *et al*., 2006).

Microsatellites are made up of 1-6 base pairs sequences which are tandem repeated and typically span between twenty to a few hundred bases (Beckmann and Weber, 1992; Hancock, 1999; Schlötterer, 2000). It has been detected within every organism so far investigated, in eukaryotic as well as prokaryotic genomes. These loci appear to be highly abundant and dispersed throughout the genome (Weber and Wong, 1993).

Microsatellite generally has higher mutation rates than non-repetitive DNA. Mutation rates of microsatellite are estimated at 10^{-3} to 10^{-4} per locus per gamete per

generation 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; Primmer *et al*., 1996; Goldstein and Pollock, 1997; Schug *et al*., 1997).

Microsatellites demonstrate high levels of polymorphism (Litt and Luty, 1989; Tautz, 1989; Weber and May, 1989). The variability is mostly due to changes in the number of copies of the microsatellite repeat. In general, mutation in a microsatellite allele generates changes in size of one repeat, but sometimes several repeat units could be changed (Weber and Wong, 1993; Di Rienzo *et al*, 1994; Primmer *et al*., 1996).

Molecular markers can be divided into type I (coding) markers which associated with genes of known functions and type II (non-coding) markers which associated with anonymous genomic sequences (O'Brien, 1991). In general, most microsatellites represent type II markers as they are commonly located in non-coding intergenic regions. However, there are also markers developed from coding regions and they are more difficult to develop (Liu *et al*., 1999). Non-gene sequences are free to mutate, causing higher levels of polymorphism. Sequences within protein-coding regions generally show lower levels of polymorphism due to functional selection pressure (Chistiakov *et al*., 2006).

Although all microsatellites are composed of repeated arrays of a specific DNA sequence, they may be further differentiated by the specific composition of their core sequence. They can thus be divided into four categories (Schlötterer and Zangerl, 1999) according to the composition of their core sequence:

a) Uninterrupted microsatellite (consists of single repeat type only), e.g.

GTGTGTGTGTGTGTGTGT

b) Interrupted microsatellite (where the core repetitive unit is interrupted by base substitutions), e.g.

GTGTGTGTG**A**GTGTGTGT

c) Composite microsatellite (consist of different types or lengths of tandem repeated sequences), e.g.

GTGTGTGTGT**CTCTCTCT**

d) Cryptic simple sequence (consists of many interruptions including the addition of a few different motifs), e.g.

GAGTGT**CTTCTT**GTCTGTG**TTTT**G

2.2.2 Microsatellite Evolution

Although the physical mutational mechanism of microsatellite loci is not yet fully understood, such high rates of mutation in microsatellite can be explained by two potential mutational mechanisms: unequal crossing-over (UCO) or gene conversion (Smith, 1976; Jeffrey *et al*., 1994) and slipped-strand mispairing, SSM (also referred to as DNA polymerase slippage) (Levinson and Gutman 1987; Eisen, 1999; Hancock, 1999; Zane *et al.*, 2002). In unequal crossing-over, the two chromosome strands are misaligned during crossing-over, which results in a deletion in one DNA molecule and an insertion in the other. This happens most easily for tandem repeated sequences where

the recombination machinery cannot easily determine the correct annealing between two strands (Hancock, 1999).

In slipped-strand mispairing, the nascent DNA strand and the template strand temporarily dissociate from each other during DNA replication. This does not pose a problem when non-repetitive sequences are replicated. If this occur while a repeat region is being replicated, the nascent strand may re-anneal out of phase with the template strand. When replication is continued, the eventual nascent strand will be longer or shorter than the template, depending on whether the looped-out bases have occurred in the nascent strand or the template strand. If the looped-out bases occurred in the nascent strand, this will create a longer product whereas if the looped-out bases occurred in template strand, this will create a shorter product. In other words, a loop on the nascent strand will result in an insertion mutation while an excised loop on the template strand will create a deletion mutation (Hancock, 1999; Brohede, 2003) (Figure 2.5). Generally, length difference is recognized by the DNA mismatch repair system which will remove the mismatch. Yet, deficiencies in the mismatch repair system lead to increase of microsatellite instability, indicating that the mismatch repair system restores the original microsatellite length (Schlötterer and Pemberton, 1998).

2.2.3 Threoretical Models of Microsatellite Mutation

A complete understanding of the mutational process that shape microsatellite evolution is essential to utilize the information revealed by these markers. Although microsatellite loci have been used for numerous applications in evolutionary genetics, the mutational events in these markers is still not fully understood (Garza *et al.*, 1995).

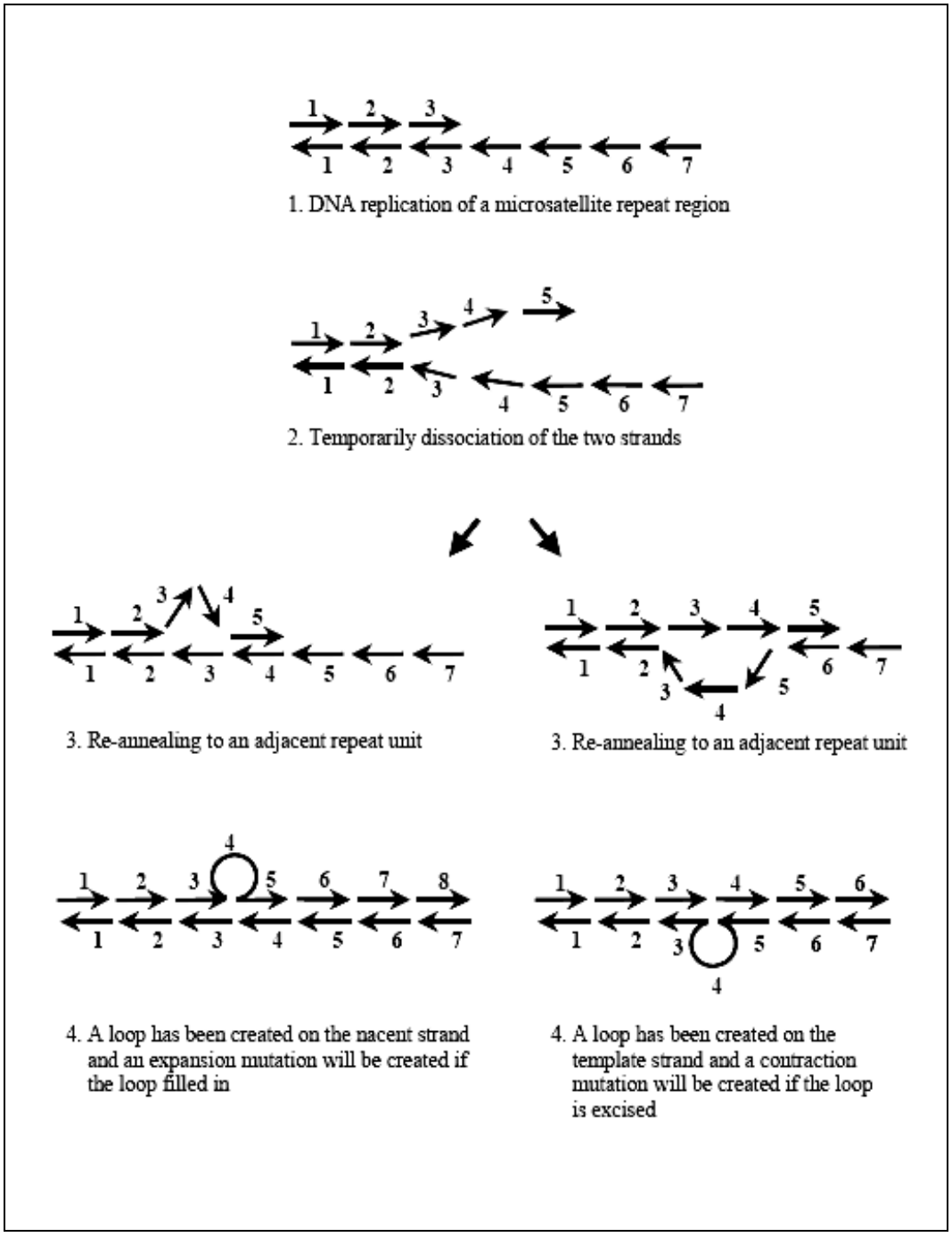

Figure 2.5 Microsatellite mutations by slipped strand mispairing (Adapted from Brohede, 2003).

Two classical models that have been suggested to explain microsatellite mutational evolutionary events are the Infinite Allele Model (IAM, Kimura and Crow, 1964) and Stepwise Mutational Model (SMM, Kimura and Ohta, 1978).

The main difference between the two models is whether mutation results in unique alleles or not. The SMM holds that mutation of microsatellite alleles occur by loss or gain of a single tandem repeat and hence alleles may possibly mutate towards allele states already present in the population (Estoup and Cornuet, 1999). This infers that two alleles that differ by one repeat are more closely related (have a more recent common ancestor) than alleles that differ by many repeats. In other words, size matters when carrying out statistical tests on population substructuring. The genetic distance statistic that uses this model is called R_{ST}. The SMM is generally the preferred model when calculating relatedness between individuals and population substructuring, although there is the problem of homoplasy.

In contrast, The IAM describes that mutation of microsatellites involves any number of tandem repeats and always give rise to a new allele that is not previously encountered in the population (Estoup and Cornuet, 1999). An 18-repeat allele could be just as closely related to a 15-repeat allele as a 16-repeat allele. All that matters is that they are different alleles. In other words, size is not important. The statistic that uses this model is called F_{ST}. IAM is considered to be a more appropriate model when the mutational process is unknown (O'Connell and Wright, 1997).

Besides the two extremes models IAM and SMM, other mutation models that have been introduced are often found to be variants of SMM or IAM, for example the

two-phase model (TPM) (Di Rienzo *et al.*, 1994) and the K-allele model (KAM) (Crow and Kimura, 1970).

2.2.4 Application of Microsatellite in Fisheries and Aquacultures

Microsatellite markers have been successfully applied in a variety of research fields and practical disciplines because of their multi-allelic nature, codominant inheritance, small length, extensive genome coverage and relative abundance (Powel *et al*., 1996). The applications of microsatellite markers are relevant to the general audience in a wide range of fundamental and applied fields of biology and medicine. Table 2.1 shows the use of various techniques including microsatellites to study a vast range of issues. In general, DNA based methods such as mtDNA sequences analysis, RAPDs, DNA fingerprints and especially microsatellites are suitable for most of the purposes listed.

In the field of fisheries and aquaculture, microsatellites are useful for the characterization of genetic stocks, broodstock selection, constructing dense linkage maps, mapping economically important quantitative traits and identifying genes responsible for these traits and application in marker-assisted (MAS) breeding programmes. The genetic variability of microsatellites is expansively exploited in evolutionary studies of a wide variety of fish species (Chistiakov *et al*., 2006). Microsatellite markers can reveal much higher levels of genetic diversity than phenotypic or allozyme markers (Miller and Kapuscinski, 1996; Shaw *et al*., 1999; Triantafyllidis *et al*., 2002; Corujo *et al*., 2004).

Table 2.1 Methods available for genetically characterizing individuals and populations and their applicability to each issue. Techniques with + can be used for the purpose specified, with several + indicating the technique has high utility, ? are cases where the technique is useful in only some cases, while – indicates that the technique is not useful in this context (Adapted from Frankham *et al*., 2002).

Issue	Chromosomes	Allozymes	mtDNA	RAPD	Microsatellites	DNA fingerprint
Non-instrusive sampling	-	-	+++	++	+++	-
Forensics	-	+	+++	++	++	++
Population size	-	-	+++	+	+	?
Estimating N_e	-	++	++[a]	-	+++	?
Demographic history	-	-	++	-	+	?
Detecting and dating bottlenecks	-	++	++[a]	++	+++	?
Detecting selection	+	+	+++	+	+++	++
Migration and gene flow	-	++	+[a]	++	+++	++
Individual identification and tracking	-	-	++	+	+++	-
Population structure	-	++	+?	++	+++	++
Phylogeography	-	-	+++	-	+++	-
Source populations to recover endanger species	-	++	+	++	+++	+++
Introgression	+	++	+[a]	++	+++	++
Secondary contact	-	-	+++	-	+++	+
Taxonomic status	+++	++	++	+++	+++	+++
Sites for reintroduction	-	-	+	+	+++	-
Populations for reintroduction	-	++	+	++	+++	+++

Table 2.1 (continued)

Issue	Chromosomes	Allozymes	mtDNA	RAPD	Microsatellites	DNA fingerprint
Reproductive systems	-	++	-	+	+++	?
Paternity	-	+	-	+	+++	+++
Founder relationships	-	?	-	+++	++	+++
Sources for new founders for endangered populations	-	++	+	++	+++	++
Sexing birds		-	-	-	?	?
Detecting disease	-	-	++?	++	+	++
Diet	-	-	+++	++	++	++

Note: [a] Can detect only female contributions.

Rainbow trout was one of the first species to be investigated for within and among population variability using microsatellites (Nielsen *et al*., 1994). Their survey revealed similar patterns of differentiation for mtDNA and the microsatellite locus employed. Similar study has been performed on potadromous rainbow trout populations from Lake Ontario investigated using both microsatellite loci and mtDNA (Dueck, 1994; O'Connell *et al*., 1996). A comparison of marker sets revealed that the number of mtDNA haplotypes was similar to the number of alleles observed at microsatellite loci, although single and the combined microsatellite loci data revealed significantly higher levels of differentiation.

The almost random distribution of microsatellites and their high level of polymorphism greatly facilitate the construction of genetic maps (Dietrich *et al*., 1994). The mapping applications in several economically important fish using microsatellites include studies on the Nile tilapia (Cnaani *et al*., 2002), zebrafish (Knapik *et al*., 1998), Atlantic salmon (Koop and Davidson, 2005), rainbow trout (Sakamoto *et al*., 2000), channel catfish (Liu *et al*., 2001; Waldbieser *et al*., 2001; Karsi *et al*., 2002), European sea bass (Chistiakov *et al*., 2005). Of special interest to the present study Wang *et al*. (2007a) successfully constructed a first generation linkage map of Asian sea bass based on 240 microsatellites.

Microsatellites is also suitable for the investigation of kinship relationship and paternity analysis in understanding mating pattern in the wild and management of captive management (O'Connell and Wright, 1997; Schlötterer, 2000). By using just two microsatellite loci, Colbourne *et al*. (1996) examined parentage in bluegill sunfish (*Lepomis macrochirus*), from eggs deposited within a natural nest. The study