MOLECULAR CHARACTERIZATION OF EST MICROSATELLITES AND ANALYSIS OF POPULATION GENETIC STRUCTURE IN STRIPED SNAKEHEAD FISH, *CHANNA STRIATA*

SARA TAKALLOO

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Name of Candidate: **SARA TAKALLO** I/C/Passport No: **L18556081**
Regisration/Matric No.: **SGF110020**
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ABSTRACT

The Striped snakehead fish, *Channa striata* is a freshwater air breathing fish species indigenous to Malaysia. It is locally known in Malaysia as Haruan. *Channa striata* is economically playing a major role in the aquaculture and fisheries industries. *Channa striata* is important especially as a food source in South East Asia. It has been broadly used for medical and pharmaceutical purposes due to its therapeutic and recuperative qualities. Among the many DNA marker systems available, EST microsatellites have a number of advantages due to its high level of transferability, high reproducibility, codominant inheritance and low cost for development compared to other molecular markers.

The present study employed 20 EST microsatellites designed from RNA transcriptome sequencing. Out of the 15 loci which were able to be amplified, four loci were found to be polymorphic. The polymorphic EST-SSR loci were applied to investigate the level of genetic variation and differentiation of four geographical populations of *C.striata*. The populations involved in this study included Johor, Kedah, Pahang and Sarawak.

The number of alleles per locus ranged from one to five. The highest value of heterozygosity was observed in Pahang population, while the lowest value corresponded to Kedah. No evidence of significant deviation from Hardy Weinberg Equilibrium was detected in all populations. There was no pattern on linkage among specific pairs of loci at all sites indicating no physical linkage is likely to be present among the loci analyzed. The value of population pair-wise F*st* in this study indicates significant differentiation among populations. Kedah and Pahang are the most similar populations whereas Sarawak and Johor are the most different populations among them.

Cross-species amplifications of *C.striata* primers were conducted on 10 species including *Amblyrhynchicthy truncates, Barbichthys laevis, Barbonymus chwanenfeldii, Cirrhinus caudimaculatus, Hypsibarbus wetmorei, Osteochilus hasselti, Thynnichthys thynnoides, Pangasius nasutus, Hampala macrolepidota* and *Channa micropeltes*. Out of the 10 species employed in cross-species amplification, *Channa micropeltes* was the only species in which the primers could work well. Among the 15 primers amplified in *Channa striata*, only 11 primers produced distinct and clear bands in *Channa micropeltes*. Seven out of these 11 primers could detect the same repeats indicating these primers were conserved in *C.striata* and *C.micropeltes*, which belong to the same family. This conservation of microsatellites can save time and cost in other Channidae family members since it is not necessary to develop microsatellite markers for each species.

ABSTRAK

Ikan 'Striped snakehead', *Channa striata* ialah sejenis ikan air tawar penghirup udara yang merupakan spesies asli di Malaysia. Ia juga dikenali oleh penduduk tempatan di Malaysia sebagai 'Haruan'. Dari segi ekonomi, *Channa striata* memainkan peranan utama dalam akuakultur dan industri perikanan. *Channa striata* adalah penting terutamanya sebagai sumber makanan di Asia Tenggara. Ia telah digunakan secara meluas untuk tujuan perubatan dan farmaseutikal disebabkan kualiti terapeutik dan penyembuhan yang dimilikinya. Di antara kebanyakan sistem penanda (marker) DNA yang wujud, mikrosatelit EST mempunyai beberapa kelebihan kerana tahap pemindahannya yang tinggi, kebolehulangan yang tinggi, kodominan serta kos pembangunan yang rendah berbanding dengan penanda molekular yang lain.

Kajian ini menggunakan 20 mikrosatelit EST yang direka dari jujukan transkriptom RNA. Daripada 15 lokus yang dapat diamplifikasikan, 4 lokus didapati polimorfik. Lokus EST-SSR polimorfik ini telah dimanfaatkan untuk mengkaji tahap variasi dan pembezaan gen dalam populasi *C.striata*. Antara populasi yang terlibat dalam kajian ini termasuklah dari Johor, Kedah, Pahang dan Sarawak.

Bilangan alel per lokus adalah dari 1 hingga 5. Nilai tertinggi heterozigositi dapat dilihat dalam populasi Pahang manakala nilai terendah dalam populasi Kedah. Tiada bukti yang menunjukkan keseimbangan Hardy Weinberg dikesan dalam semua populasi. Tidak ada corak pada hubungan antara pasangan lokus tertentu di semua tapak menunjukkan bahawa tiada hubungan fizikal yang mungkin hadir antara lokus yang dianalisis. Nilai Fst secara berpasangan di kalangan populasi dalam kajian ini menunjukkan perbezaan yang signifikan

di kalangan populasi. Populasi Kedah dan Pahang adalah yang paling serupa manakala populasi Sarawak dan Kedah adalah yang paling berbeza di antara semua.

Amplifikasi spesies-silang primer *C. striata* telah dijalankan pada 10 spesies termasuk *Amblyrhynchicthy truncates*, *Barbichthys laevis*, *Barbonymus chwanenfeldii*, *Cirrhinus caudimaculatus*, *Hypsibarbus wetmorei*, *Osteochilus hasselti*, *Thynnichthys thynnoides*, *Pangasius nasutus*, *Hampala macrolepidota* dan *Channa micropeltes*. Daripada 10 spesies tersebut yang digunakan dalam amplifikasi spesies-silang, *Channa micropeltes* merupakan satu-satunya spesies yang primernya boleh berfungsi dengan baik. Antara 15 primer yang diamplifikasikan dalam *Channa striata*, hanya 11 primer menghasilkan jalur yang berbeza dan jelas dalam *Channa micropeltes*. Tujuh daripada 11 ini primer dapat mengesan jujukan berulang yang sama, menunjukkan bahawa primer ini telah dipulihara dengan tingginya dalam *C.striata* dan *C.micropeltes* yang tergolong dalam keluarga yang sama. Pemuliharaan mikrosatelit ini boleh menjimatkan masa dan kos dalam keluarga channidae yang lain kerana tidak perlu lagi untuk membangunkan mikrosatelit untuk setiap spesies.

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LIST OF SYMBOLS AND ABBREVIATION

µg	microgram
µl	microliter
^{o}C	degree Celsius
10X	ten times
1X	one time
A	adenosine
bp	base pair
C	cytosine
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymidine triphosphate
ddH2O	Double distilled water
DNA	Deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
F*st*	Fixation index
g	gram
G	guanosine
h	hour
He	Expected heterozygosity
Ho	Observed heterozygosity
HWE	Hardy Weinberg equilibrium
kb	kilobase
kg	kilogram
LD	Linkage disequilibrium
M	Molar
mg	milligram
mg/ml	milligram per milliliter
MgCl 2	Magnesium chloride
min	minute

ml	milliliter
PCR	Polymerase Chain Reaction
PIC	Polymorphism Information Content
RNA	ribonucleotide acid
Rpm	Rotations per minute
s	second
SSR	Simple sequence repeat
T	thymine
Ta	Annealing temperature
TBE	tris-borate-EDTA buffer
U	unit
UV	ultraviolet
V	Volt
VNTR	Variable Number Tandem Repeats

CHAPTER 1

INTRODUCTION

The snakehead, *Channa striata* is a freshwater air-breather fish species indigenous to Malaysia (Mat Jais, 2007a). It can be found in a variety of habitats such as lakes, rivers, rice fields, reservoirs, ponds, swamps and canals. *C.striata* is an economically important food source in Malaysia and many countries of the Asia Pacific due to it contains high level of protein (Lee and Ng, 1994; Hossain et al., 2008).

It is rich in essential amino acids and fatty acids as well as dietary minerals such as magnesium, copper, calcium, manganese, zinc and iron. This fish has been extensively used as remedy due to its medical and pharmaceutical properties and having anti-fungal, anti-microbial and anti-inflammatory activities (Zuraini et al., 2006).

Aquaculture activity of *C.striata* is not significant in Malaysia due to its presumed affluence in the nature. However, due to several anthropogenic factors, which cause a drastic damage to its natural habitats, the wild populations of *C.striata* in Malaysia are on gradual decline (Nagarajan et al., 2006; Hossain et al., 2008). Hence, providing vital information about population genetics is essential, especially through evaluation of genetic diversity in order to management and conservation of both wild and cultivated populations (Beaumont and Hoare, 2003).

Genetic diversity within population is required to maintain due to its necessity for evolution and the correlation between heterozygosity and population compatibility (Li et al., 2007). In

this respect, genetic markers such as microsatellites are powerful tools to assess the genetic diversity and population structure (Gupta and Varshney, 2000).

Microsatellites or simple sequence repeats (SSR) are short tandem repetitive repeats of two to six nucleotide units, which are flanked by conserved sequences and widely dispersed throughout the genomes. Microsatellites are considered as the informative markers in various aspects of molecular genetic studies due to their co-dominance and high level of polymorphism (Byrne et al., 1996; Oliveira et al., 2006). However, the development of microsatellite from genomic DNA requires considerable efforts such as construction genomic DNA libraries, cloning and sequencing which are time consuming and expensive.

Developing SSR from expressed sequence tags database is an alternative strategy can overcome limitations. Highly reproducible, greater transferability across species, low cost of development and providing a high polymorphism information are important advantages making EST-SSR the most interesting source in population and conservation studies (Gupta et al., 2003; Gupta and Rustgi, 2004).

In addition, the flanking sequences of SSR derived from transcribed regions are located in more conserved regions across related species compared to genomic SSR, which allows EST-SSR to apply for heterospecific amplification. Therefore, in recent years, EST-SSR developed in one species can be applied in relate species (Decroocq et al., 2003; Pashley et al., 2006).

The objectives of this study were

To develop EST-SSR markers for *Channa striata* and apply them to compare the genetic diversity of populations of *Channa striata* using EST-SSR markers, and also using EST microsatellite markers developed in *C.striata* for cross amplification to investigate their conservation among other species.

CHAPTER 2

LITERATURE REVIEW

2.1. Snakehead

Snakeheads belong to the family Channidae are air breathing freshwater fishes distributed in Africa and Asia continent including two genera. The Asian genus, *Channa* comprises of 26 species and the African genus, *Parachanna* comprises of only three species (Musikasinthorn, 2003; Li et al., 2006).

According to Kottelat et al. (1993), Lee and Ng (1994), Mohsin and Ambak (1992), snakeheads are classified into

Kingdom :	Animalia
Phylum :	Chordata
Subphylum :	Vertebrata
Class :	Actinopterygii
Order :	Perciformes
Suborder :	Channoidei
Family :	Channidae

Species of the genus *Channa* has a wide distribution, extending from southeastern Iran and eastern Afghanistan eastward through Pakistan, India, southern Nepal, Bangladesh, Myanmar, Thailand, Laos, Malaysia, Sumatra, Indonesia, Vietnam, Korea, and China northward into Siberia (Li et al., 2006; Vishwanath and Geetakumari, 2009). The three species of the genus *Parachanna* are only confined to the Central West Africa (Li et al., 2006).

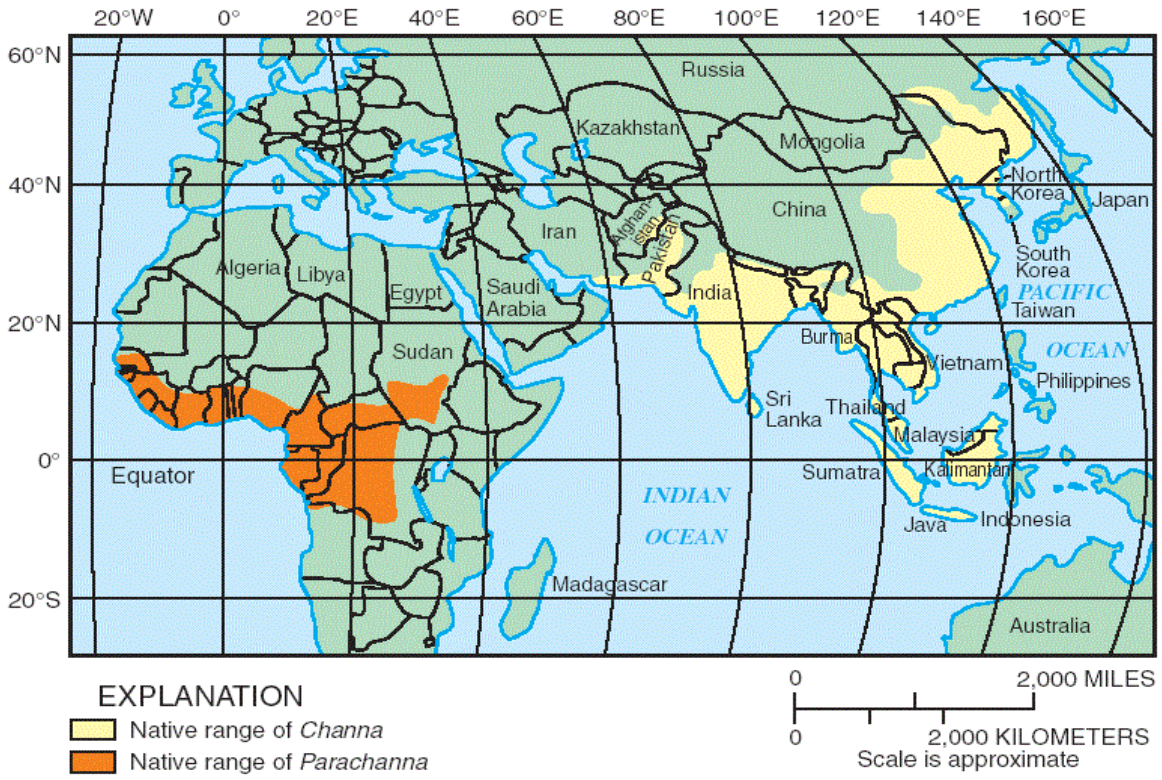


Figure 2.1. Native distribution of the family Channidae (USGS, 2004).

The genus *Channa* is one of the most common food fish sources in Thailand, Vietnam and other East Asian countries, where they are extensively cultured. In addition, of their importance as a food fish, they are used as a therapeutic to help wound healing and have efficacy on immune system (Adamson, 2010). These fishes are economically important species with great potential for aquaculture and fisheries throughout southern and southeastern Asia (Vishwanath and Geetakumari, 2009).

Snakeheads cannot tolerate seawater. Their habitats vary by species. Most of the species of snakehead live in streams and rivers, they are also found in swamps, rice paddies, ponds and ditches (Ng and Lim, 1990; Lee and Ng, 1991).

Snakehead are distinguished by some distinct morphological characteristics such as elongated and cylindrical body, broad flattened head, which is similar to snake with large eyes situated in the frontal part of the head, large mouth with well developed teeth on both upper and lower strong jaws, fin rays in all fins, rounded caudal fin, long dorsal and anal fins and an accessory respiratory organ known as the suprabranchial chamber developing in a labyrinth form located behind the gills in the head region enabling them to breathe atmosphere air (Berra, 2001; Musikasinthorn, 2003).

Different development of the accessory respiratory organ in *parachanna* and *channa* result in differentiate these two genera by the morphology of air breathing apparatus, which is less developed in the genus *Parachanna* with the simple structure compared to the genus *Channa* with the chambers which are bordered by two plates, one from the epibranchial of the first gill arch and the other as an expansion of the hyomandibular unlike the simple cavity of the *Parachanna* not involving processes from the first epibranchial or hyomandibular. These chambers are not labyrinthic (Berg, 1947; Courtenay and Williams, 2004).

Identifying different species of snakehead fish is not feasible by only relying on morphological and meristic features. Genetic methods have great potential in order to distinguish distinct populations or stocks of fish (Cadrin et al., 2005).

The taxonomy and phylogeny of genus *channa* have not completed. According to the phylogenetic study, which has been performed in 2010 by Adamson and colleagues, there is a probability of entity of more unidentified species of *channa* in South East Asia. Among these species, *channa striata* (haruan), *channa micropeltes* (toman), *Channa marulioides*

(toman bunga)**,** *channa lucius* (bujak*), channa gachua*, *channa melasoma* and *channa bankanensis* are discovered in Malaysia (Ambok et al., 2007; Rahim et al., 2009).

2.2. *Channa striata (C.striata)*

The snakehead murrel, *Channa striata* (common snakehead, chevron snakehead, and striped snakehead) locally known as "Haruan" in Malaysia, is a native tropical freshwater fish species belongs to the Channidae family

Kingdom :	Animalia
Phylum :	Chordata
Class :	Actinopterygii
Order :	Perciformes
Family :	Channidae
Genus :	*Channa*
Species :	*C.stiata*

Figure 2.2. Images of *Channa striata* adapted from (DOF, 2011).

Channa striata has the ability to adapt rapidly to various environments and withstand the harsh environmental condition. Its habitats preference, are freshwater ponds and streams. Nevertheless, several reports indicate the existence of this species in rivers, swamps, canals, drains, rice fields and mining pools in Malaysia (Lee and Ng, 1991; Ambok et al., 2007). *C.striata* is a carnivorous fish that feeds on snakes, frogs, snails, insects, prawns, worms and fishes (Lee and Ng, 1994). It is categorized as a large fish, attaining up to 100 cm in length however, this size is rarely found in nature due to fishing threat.

Channa striata is important especially as food source in South East Asia (Froese and Pauly, 2008). It has been broadly used in medical and pharmaceutical due to its therapeutic and recuperative qualities especially for internal and external wound healing as a result of high content of arachidonic acid (Michelle et al., 2004; Yaakob and Ali, 1992; Zakaria et al., 2004). It also has effect in suppressing inflammation of arthritic joint result in applied as a complementary remedy in osteoarthritis and joint disorder (Ng et al., 2004). It can also facilitate the recuperation of many skin-related problems due to its great nutraceutical components. An essential fatty acid, Docosahexaenoic acid (DHA) found in *C.striata,* can act as an inhibitor factor on dermal complications (Mat Jais, 2007b).

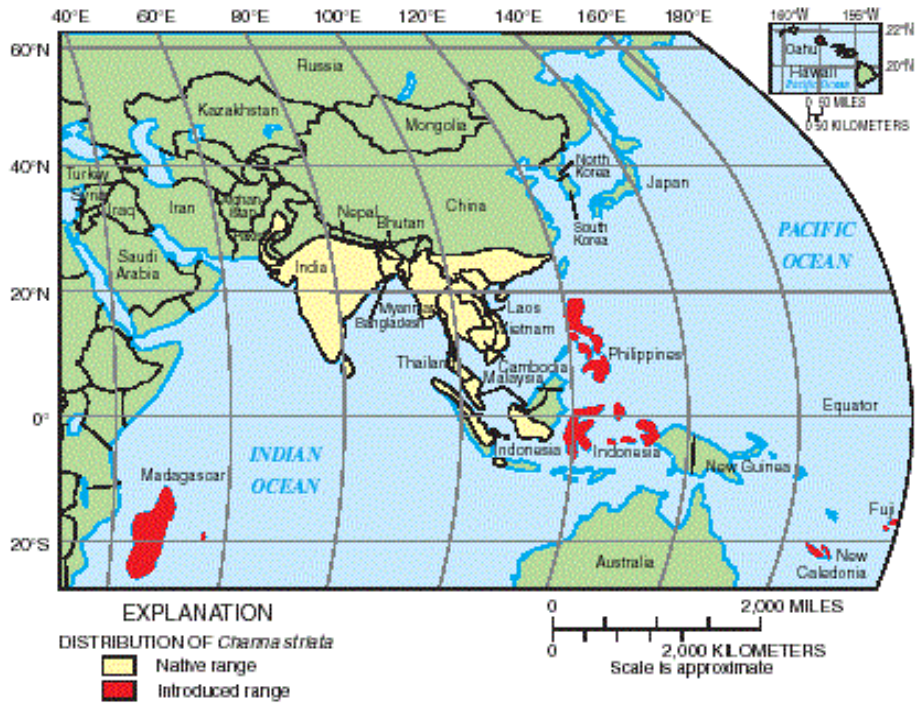


Figure 2.3. Distribution of *Channa striata* (USGS 2004).

Channa striata is the most widely naturally distributed snakehead species among the Channidae family, which expands from Pakistan, Myanmar, Laos, Vietnam, Thailand, Malaysia, and Indonesia to Southeast China. *C.striata* is also the most commonly introduced species of snakehead, which has been established in tropical island of Hawaii, Madagascar, the Philippines and Mauritius (Hossain et al., 2008; Courtenay and Williams, 2004).

2.3. *Channa micropeltes*

Giant snakehead, *Channa micropeltes*, which better known to locals as "toman", belongs to the family Channidae. The red snakehead is commonly used for juveniles of this species its color before getting mature.

Kingdom :	Animalia
Phylum :	Chordata
Class :	Actinopterygii
Order :	Perciformes
Family :	Channidae
Genus :	*Channa*
Species :	*C.micropeltes*

C.micropeltes has been considered as the largest and fastest growing species among other species of Channidae family due to its extended body, which can go over 1 meter in length and 20 kg in weight (Lee and Ng, 1991; Talwar and Jhingran, 1992).

C.microplete lives mainly in the lakes, rivers, canals, large streams and reservoirs (Lee and Ng, 1991). It can also found in muddy and swampy areas due to its ability to crawl in such area, and able to breathe atmosphere air which is prepared by accessory respiratory organ.

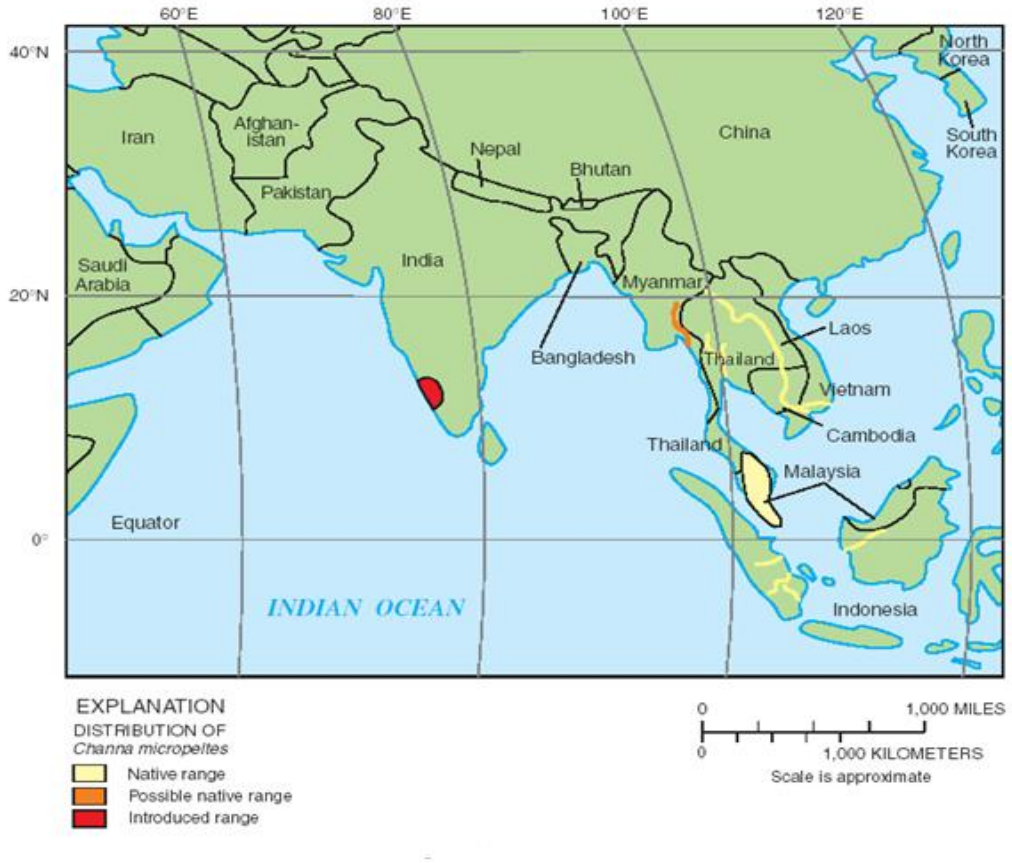


Figure 2.4. Distribution of *Channa micropeltes* (USGS, 2004).

C.micropeltes has a disjunctive distribution. It is distributed both, in Southeast Asia and Southwest India, which are far apart (Talwar and Jhingran, 1992). It is assume that the population, which inhabit in India is not included of a native range. The introduced range is not only limited to southwest of India, there are some population live in US states such as Wisconsin, Maryland, Virginia and Massachusetts.

This fish has elongated body with dark longitudinal stripes. Its head is pointed and flattened. It has a large mouth with two strong jaws. The lower jaw is covered by enlarged and cutting teeth. It has a long dorsal fin and a rounded caudal fin. The pectoral fin is about two times larger than pelvic fin.

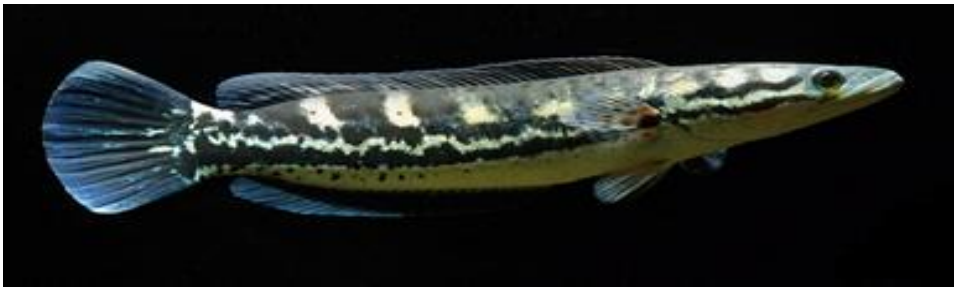
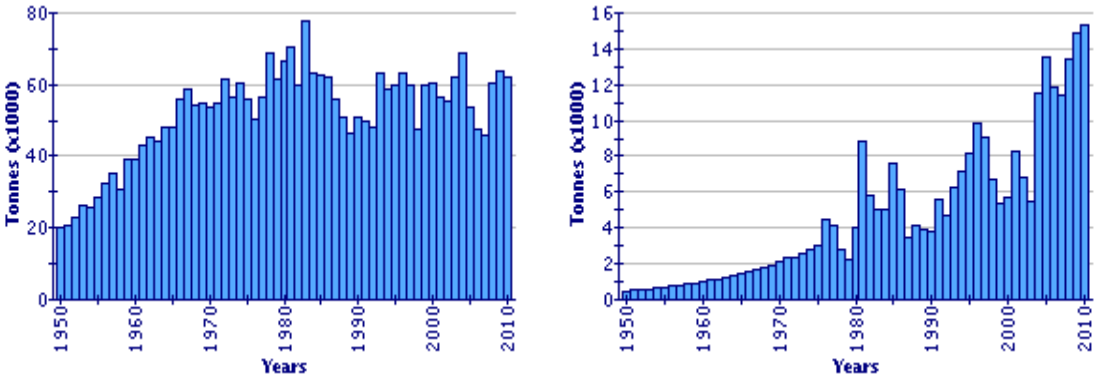


Figure 2.5. Images of *Channa micropeltes* adapted from (USGS, 2004).

2.4. Aquaculture

Aquaculture is the fastest growing food production system in the world. Most aquaculture production is carried out using wild stock recently captured from natural environments (Lymbery, 2000). Many populations of wild fish have been reduced over last century. There are several factors for this decline such as over-fishing, rapid increase in pesticide usage, destruction of habitat, migratory routs barriers and so on. Since genetic variation enhances adaptation to changing environment conditions, its assessment can provide valuable information in understanding and managing populations and also it applies as an efficient approach for conservation of species (Franklin, 1980).

Aquaculture of freshwater fish in Malaysia plays an important role in protein food supply. *C.striata* is one the most common freshwater species that is harvested as food source across its native range. The unique specifications of this species enable it to be cultured in wild rang. Moreover, the therapeutic properties of wild *C.striata* remain on the cultured fishes (Mat Jais, 2007b). In recent years, anthropogenic factors such as environmental pollution, loss of habitat, over fishing, which result in degradation in harvest of wild *C.striata* along with fast growth rate, high tolerance to adverse condition and therapeutic properties have led to progress in culture of the fish.



(a) (b)

Figure 2.6. (a) Global capture production for *Channa striata* 1950-2010. (b) Global aquaculture production for *Channa striata* 1950-2010 (FAO Fishery Statistic, 2011).

2.5. Molecular markers in aquaculture

Development of molecular markers has played a pivotal role in genetic detection of individuals, population or species. DNA molecular marker technologies have provided many

advances in various aspects of aquaculture genetics research (Liu and Cordes, 2004; Lakra, 2001).

Molecular markers have revolutionized the genetic diversity investigations. This resulted in obtaining valuable information, which can be applied in research on evolution, conservation, management of genetic resources. Various important scientific information in aquaculture practices such as species identification, population structure study, wild and hatchery populations comparison and genetic variation have been provided by utilizing molecular markers (Chaunhan and Rajiv, 2010).

Genetic methods play an important role to distinguish fish species when the stocks have not been identified by morphological and meristic characters (Cadrin et al., 2005). Molecular markers are practical genetic tools in an increasing necessity which can be used to distinguish species during breeding and improvement (Qiu et al., 2010).

Molecular markers have become useful and robust tools in almost all aspects of genetic analysis. Correct identification of species, accurate recognition of genetic relationship and diversity, efficient classification, mapping of desirable genes are the important issues in genetic studies require molecular markers (Wang et al., 2011). Molecular markers have been effectively used to access of significant amounts of data in genetic diversity and population structure, which are important in applying, assessing and conserving genetic resources of species. Polymorphic, reproducible, co-dominant, fast and inexpensive detection are the characteristic of molecular marker lead them to become robust and beneficial molecular tools (Bai, 2011).

2.6. Types of molecular marker in aquaculture

The first generation of genetic marker, represented by protein coding loci, could be applied to the management program of fish stocks. Development of allozyme analysis resulted in the first application of genetic markers in aquaculture studies. Although allozyme assay remained one of the most common technique for several fisheries and aquaculture applications due to its simplicity and general applicability, it cannot be assumed to be selectively neutral (Karl and Avise, 1992). On the other hand, some changes in DNA sequence are concealed at protein level and lead to reduction in detectable variations.

In addition to low level of polymorphism revealed in many allozyme studies of fish population (Crawford et al., 1990), sensitivity of the amount and quality of samples required, has limited application of allozyme in aquaculture genetics. Hence, further investigations need to be done in order to achieve a better genetic marker, which can overcome the deficiencies (Ferguson, 1994).

Direct examination of DNA molecule via development of DNA amplification using PCR (Polymerase Chain Reaction) technique, which has led to progressive rise to genetic marker technology have revolutionized aquaculture genetics research.

Initial molecular genetics analysis of fish mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) provide several advantages over protein markers. Analysis of mtDNA in being progressively applied in phylogeny, evolution and population structure of fishes (Meyer, 1993). Since mtDNA is inherited maternally, phylogenies and population structure derived from mtDNA data may not reflect those of nuclear genome. Furthermore, mtDNA molecule

should be considered a single locus in genetic analysis due to its non-Mendelian mode of inheritance (Liu and Cordes, 2004).

The advantages of nuclear DNA markers assemble in their abundance in the genome, high level of polymorphism and mendelian inheritance. Most of nDNA markers contain non coding regions of nuclear genome which evolve more rapidly than coding regions. This allows these sequences more appropriate to serve in genetic structure studies at species level. Nuclear genetic marker is a fragment of genomic DNA with the known location, which has been applied to identify species and individuals. A variety of molecular marker, including restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphism (AFLP), Random amplification of polymorphic DNA (RAPD), Variable number tandem repeat (VNTR), Single nucleotide polymorphism(SNP), Short tandem repeat (STR), Single feature polymorphism (SFP), Diversity Arrays Technology (DART) have been developed for different application (Varshney and Graner, 2005).

Due to number of advantages of microsatellites over other molecular markers, it has replaced allozymes and mtDNA (Luikart and England, 1999). Microsatellites are powerful marker with great differentiating power and utilized extensively to provide a great wealth of information on various aspects of aquaculture research such as species, strain identificatin, genetic diversity and resource analysis of aquaculture stocks, parentage assignments and reproductive contribution, mapping quantitative trait loci (QTL) and marker-assisted selection (MAS) (Chistiakov et al., 2006).

Molecular makers are classified into two types due to their association with different regions of genome including recognized genes (Type I) and unknown genomic regions (Type II) (Chauhan and Rajiv, 2010).

Table 2.1 summarizes the basic properties of some of the markers that are currently being used in fisheries. The choice marker to be used depends on what issues need to be assessed.

Table 2.1. Comparison of different molecular markers (Liu, 2004).

Marker type	**Mode of inheritance**	**Type**	**Likely allele number**	**Polymorphism**	**Major application**
Allozyme	Mendelian codominant	Type I	2-6	Low	Linkage mapping population studies
mtDNA	Maternal inheritance		Multiple haplotypes		Maternal lineage
RFLP	Mendelian codominant	Type I or Type II	2	Low	Linkage mapping
RAPD	Mendelian dominant	Type II	2	Intermediate	Fingerprinting for population studies, hybrid dentification
AFLP	Mendelian dominant	Type II	2	High	Linkage mapping, population studies
SSR	Mendelian codominant	Mostly type II	Multiple	High	Linkage mapping Population studies paternity analysis
EST	Mendelian codominant	Type I	2	Low	Linkage mapping Physical mapping comparative mapping
SNP	Mendelian codominant	Type I or Type II	2	High	Linkage mapping Population studies

2.7. Microsatellites

Microsatellites are short DNA segments consist of nucleotides that are repeated numerous times in the genome. The number of repeats can vary between individuals of same species. Simple sequence repeats (SSRs), short tandem repeats (STRs), variable number tandem repeats (VNTRs), simple sequence length polymorphisms (SSLPs) are alternative terms that represent microsatellites (Liu and Cordes, 2004).

Microsatellite can be differentiated according to their different combination of their repeats. The perfect or uninterrupted microsatellite are single type repeat unit classified as mono-, di-, tri-, penta- and hexa- nucleotide repeats, while imperfect microsatellite are disrupted by base substitutions; insertion is another mechanism is caused the interrupted microsatellite, and composed microsatellite consist of different type of repeated sequence (Dieringer and scholotterer, 2003).

Table 2.2. Different combination of microsatellite repeats.

perfect microsatellites:	
-Mononucleotide: (T)13	TTTTTTTTTTTTT
-Dinonucleotide: (AC)9	ACACACACACACACACAC
-Trinucleotide: (CCT)4	CCTCCTCCTCCT
-Tetranucleotide: (CTGA)5	CTGACTGACTGACTGACTGA
-Pentanucleotide: (CAGGTA)4	CAGGTACAGGTACAGGTACAGGTA
-Hexanucleotide: (CCTGGA)4	CCTGGACCTGGACCTGGACCTGGA
Imperfect microsatellites	ACACACACACACGACACACAC
Interrupted microsatellites	ATATATATGGGATATATAT
Compound microsatellites	AGAGAGAGCTCTCTCTCT

Microsatellites are DNA regions with variable number of short repeats flanked by a unique sequence. They are typically co-dominant, high reproducible, highly polymorphic and transferable across species (Qu et al., 2012).

Microsatellite do not usually code for protein, which means they do not influence the characteristic of organisms. If microsatellite does not code for protein, a mutated allele is more likely to be retain in population because it is not under selection pressure. Mutation on genes that code for protein, are usually harmful and because of that individual selected against the population and they typically do not survive to reproduce and pass their genetic material on the next generation but if you have microsatellite mutation which does not influence the fitness of individuals that mutation can stay in population because that individual not being selected against. Microsatellite mutation can survive in population. Alleles of microsatellite can be much more variable than alleles of genes. Polymorphism in microsatellite has been attributed to different alleles contain variable number of repeats (Liu and Cordes, 2004).

High degree of polymorphism in microsatellite is due to high mutation rates in repeated sequence (Peakall et al 1998). High rates of mutation in microsatellite occur through two possible mechanisms. The first mechanism, which is the most common one is slippage replication affecting microsatellites by gain or contraction of one or more repeat units**.**

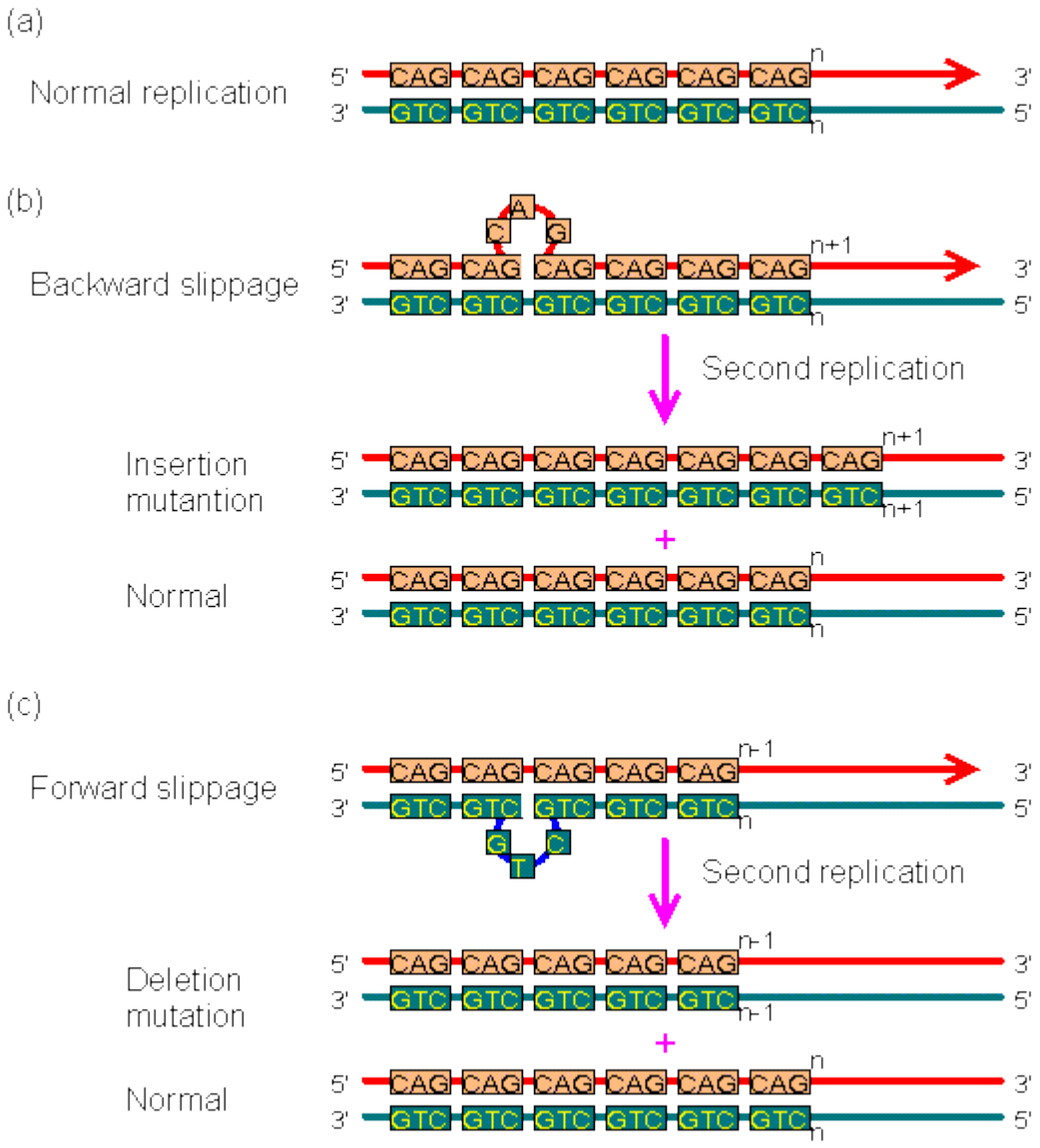


Figure 2.7. Mutation by replication errors. (a) Normal replication. (b) Backward slippage, resulting in the insertion mutation. (c) Forward slippage, resulting in the deletion mutation (Molecular Biology Web Book). In this figure, mispairing involves only one repeat. In fact, the slippage could cause several repeats to become unpaired.

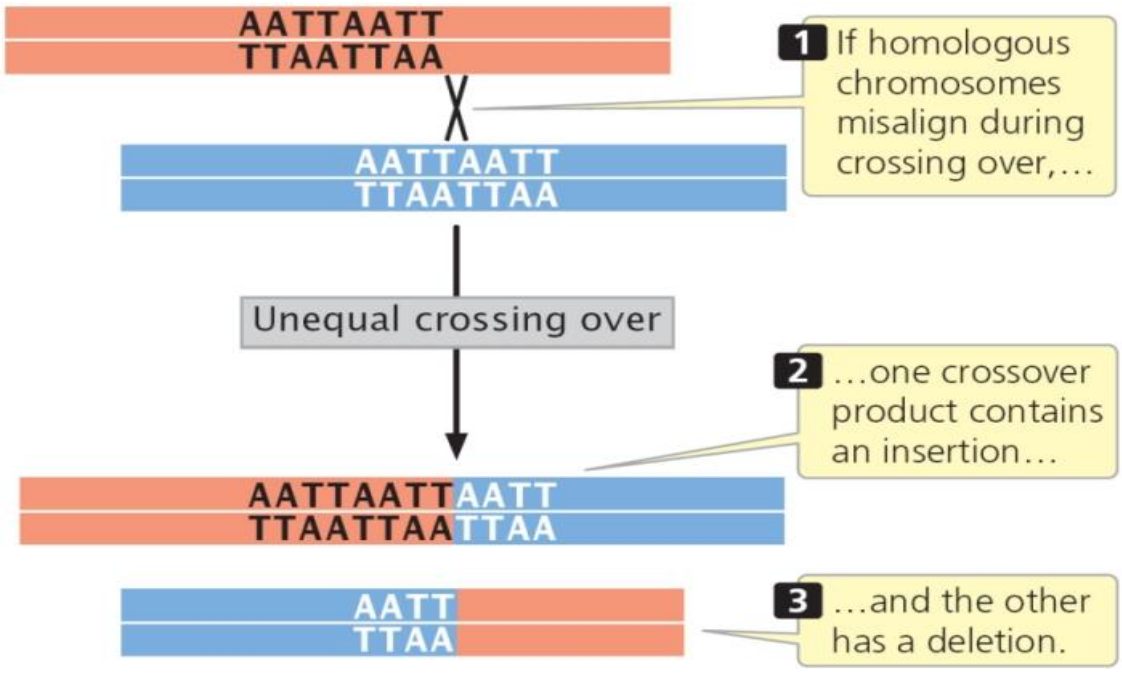


Figure 2.8. Unusual crossing over produces insertions and deletions. Mutation may also happen by crossing over during meiosis. Crossing over is exchange of DNA fragment between homologous chromosome. This process increases genetic variability and lead to different number of repeats in microsatellite (Benjamin, 2010).

Table 2.3. Microsatellite applications.

Application	Field	Genetic Scale
Forensic	Criminology	Individual
Disease Detection	Biomedical	Individual
Parentage analysis	Evolution and Ecology	Within Population
Population structure	Evolution and Ecology	Among Population
Phylogenetics	Evolution and Ecology	Among Species

Due to the high variability of microsatellite loci, these markers had been widely applied in the genetic studies. Microsatellites have been used as an informative genetic marker in investigation of genetic linkage due to have a high heterozygosity (Dieringer and scholotterer, 2003).

2.8. EST-SSR

Since the development of microsatellite involves in construction of sequence genomic libraries has been limited by the time consuming, labor intensive and costly requirement (Edwards and Barker, 1996). The next generation technologies, transcriptome sequencing has provided the efficient and rapid method in genetic marker development. SSR derived from expressed sequence tag can prepare a utile source of important molecular marker which requires less time and money to develop (Gupta and Rustgi, 2004).

EST-SSRs have a number of advantages over SSR, which make it the better choice in genetic map-based analyses and population genetic studies (Borrone et al., 2007). Locating in more conserved coding region of genome lead to the higher level of transferability of EST-SSR between species result in make it more informative and robust than SSR marker to detect polymorphisms and genetic diversity studies (Scott et al., 2000, Decroocq et al., 2003).

EST-SSR has been employed in comparative genetic mapping and construction of genetic linkage map and breeding applications due to its universality among species (Cordeiro et al., 2001). EST-SSR are being increasingly widely applied as powerful genetic marker in many aspects of genetic studies on numerous species due to their association to genes with known function, high level of transferability, show high reproducibility, co dominant inheritance and low cost for development compared to other molecular marker (Varshney et al., 2005).

2.9. Cross species amplification

Since the isolation and characterization of DNA markers for each species are time-consuming and expensive due to necessity of several steps such as designing of primer, optimizing, cloning and sequencing which are required for separate development for each species (Barbara et al., 2007; Lin et al, 2008). There is an indirect way can facilitate development of markers across species and overcome drawbacks to save time and efforts. This alternative strategy, which is called cross-species amplification, is based on utilizing PCR primers developed in one species to amplify same loci in other related species (Chang et al., 2008; Gemmell et al., 1997; Holmen et al., 2009).

High level of transferability of microsatellite markers to closely related species makes them possible to amplify same loci in different species (Pinheiro et al., 2009). Slower rate of mutation in flanking regions of microsatellite than the microsatellite sequences, together with the evolution conservation of flanking regions, allows them to serve as primer templates for closely related species and apply for heterospecific amplification (Tong et al., 2002; Holmen et al., 2005; Jarne and Lagoda., 1996).

The success of applying the marker developed for on species in different ones is highest in species with short phylogenetic distance, hybrid or out crossing breeding system (Barbará et al., 2007; Steinkellner et al.,1997).

Application of cross species amplification of microsatellites has been restricted due to several limitations. (i) The success of SSR primer to amplify the same loci is obtained in species belonging to same genus. (Scribner and Pearce 2000). (ii) Amplification of given

microsatellite may fail or may not be polymorphic (Morin et al., 1998). (iii) Microsatellite loci developed from a given primer with same PCR product length may have different sequences which is called size homoplasy which is arise from the mutation in the flanking regions or discontinuity of repeats. (Anmarkrud et al., 2008; Hempel and Peakall, 2003).

Although the transferability of SSR markers is high across species belonging to same genus, but the rate of transferability of SSR markers reduce across a broader taxonomic range. EST-SSR are likely to be more conserved and have higher rates of transferability compared to genomic SSR markers due. This is due to the fact that the EST-SSR are derived from transcribed regions of the genome (Pashley et al., 2006; Scott et al., 2000).

CHAPTER 3

METHODOLOGY

3.1. Sample collection

For cross species amplification study, ten different species were applied. All species were collected from the rivers in Malaysia. For analysis of population genetic structure of *Channa striata*, samples were collected from four location: Johor, Kedah, Pahang and Sarawak.

Figure 3.1. Map of Malaysia showing the sample locations used in this study.

Table 3.1. List of species are applied in cross species study (Tao et al., 2013).

Order	Family	Genus	Species	Local name
Cypriniformes	Cyprinidae	Amblyrhynchicthys	*Amblyrhynchicthy truncatus*	Tempuling
Cypriniformes	Cyprinidae	Barbichthys	*Barbichthys Laevis*	Bentulu
Cypriniformes	Cyprinidae	Barbonymus	*Barbonymus chwanenfeldii*	Lampam Sungai
Cypriniformes	Cyprinidae	Chirrinus	*Cirrhinus Caudimaculatus*	Selimang batu
Cypriniformes	Cyprinidae	Hypsibarbus	*Hypsibarbus wetmorei*	Kerai Kunyit
Cypriniformes	Cyprinidae	Osteocheilus	*Osteochilus hasselti*	Terbul
Cypriniformes	Cyprinidae	Thynnichthys	*Thynnichthys thynnoides*	Lomah
Siluriformes	Pangasidae	Pangasius	*Pangasius nasutus*	Patin buah
Cypriniformes	Cyprinidae	Hampala	*Hampala macrolepidota*	Sebarau
Perciformes	Channidae	Channa	*Channa micropeltes*	Toman

3.2. Primer Design

Primers were designed using IQDD (Meglectz et al., 2010). The reference sequences used for primer design were derived from RNA transcriptome sequencing. Primers were designed for 20 different microsatellite region of *C. striata.*

Table 3.2. EST Microsatellite primer pairs developed for *C.striata.*

Primer Name	Primer Sequence
EST 01	**L: GCG CAA AAC ATA AAA TGT GAA** **R: ACC AAC TGG ACA GAT GAG GC**
EST 02	**L: ATA GGC CGA CTG CTG TGA CT** **R: GCT CTC AAC ACC AGC CTT TC**
EST 03	**L: GGA GAG GCA GCT TTA GCC TT** **R: ACC CAA CTC AGG ACA GCT TG**
EST 04	**L: CTG GTC CCT CAA TGA TTC GT** **R: TCG AAG TGC AGG TTC TGA TG**
EST 05	**L: AGA CGG CAG CTC TCG CTT** **R: CCA AGG CCA GCC AAG ATG**
EST 06	**L: TTG TTG TGT ATT GGG GCT GA** **R: GTC CAG GCC GTG TTA TTT GT**
EST 07	**L: GCA GCA ATT GAG TGT CAG GA** **R: TGT GTT TCT GCT GAT GAA GC**
EST 08	**L: AAG ATC GAC TGC AAC CTG CT** **R: AAG CTA GGG CTT GGG AAC AT**
EST 09	**L: CAC CTT GCT TCT GTT TCG GT** **R: TTT CCC AAA ACA ATC CGC T**
EST 10	**L: AGC AAA GTG GAT GAC CTG CT** **R: GTG CTC TCC CTC CTC TTC CT**
EST 11	**L: AAC GAC AGG TAT CGT CCT GG** **R: GTC CAG AGC GAC GTA ACA CA**
EST 12	**L: GCC AGA GTT TGG CAT TTC AT** **R: TGG CAT CTT GGA CTT CAC AG**
EST 13	**L: CAC AGA GAT CGG GGA ACT GT** **R: TGC GGC TGT ACT GAA AAG TG**
EST 14	**L: TGT TTG AAA ACG GGT GTG AA** **R: CCC TGT TGG TTT ATG GGA AA**
EST 15	**L: AGA ATC GCC TAG CCA GGA AC** **R: CTC AAT TTC TCG GCG AAC TC**
EST 16	**L: GAC GTC AGT CCC TTC AAC AT** **R: CCC GAA GTA TCG GTC AGT GT**
EST 17	**L: GGC TGT CAG TGG AAG GAG AG** **R: TCT CGA TGC AGC AGC ATA TC**
EST 18	**L: CAG TGT CCT CAG CAG CTT TG** **R: GAG CTT GAT CTT CCT CTC GC**
EST 19	**L: TAA CCT TCT GCC TCG GCT TA** **R: GGA GCT CCT AAT GTT TCC AA**
EST 20	**L: GCT GGT CTG TCT GAT GCA GT** **R: CAT TGG GCT TCA AAA CCT GT**

3.3. Cross species amplifications

20 EST microsatellites DNA marker developed for the *Channa striata* were tested for cross amplification of microsatellite loci in ten different species and four populations of *channa striata.*

3.4. Preparation of genomic DNA

3.4.1. Genomic DNA isolation

After dissection, piece of muscle tissue was removed and placed in to the tube and then they have been kept in -80 ºC. Genomic DNA from muscle tissue was extracted using CTAB method. 100 mg of tissue was cut and placed in to pre-chilled mortal. Tissue was ground to a fine powder under liquid nitrogen by using pestle. The powder was carefully transferred to a 2 ml tube, then 1000 µl of CTAB extraction buffer was added to the tube to continue homogenizing the sample tissue. The mixture was treated by 20 µl RNAase and incubated at 60ºC for 15 minutes. This is followed by the addition of 40 µl of proteinase K to the tube and vortexing before being incubated at 65 ºC for 2 hours until the tissue was completely lysed. The tube were vortexed during incubation. Following this step, the tube centrifuged at 13,000 rpm for 10 minutes, then 1000 µl of supernatant was carefully transferred in new tube. After that the equal volume of isoamyle chloroform was added and mixture was mixed by vortexing. The next step was transferring the top aqueous layer into a new tube without disturbing the interphase after centrifugation at the speed of 8.000 rpm for 15 min. Two volumes of 100% ethanol were added to precipitate DNA and sample were kept in -20 ºC for 20 min. After this sample was spun at the max speed (13,000 rpm) for 15 min to pellet the

DNA. The supernatant was again carefully discarded from the tube and the pellet was washed with 1000 µl of 80% ethanol by inverting few times followed by centrifugation at 13,000 rpm for 5min. The supernatant was then removed intently by pipetting it out of the tube and the DNA pellet was allowed to air dry at room temperature for 30 min. The last step of DNA extraction was resuspending the DNA in 50 µl of TE buffer to dissolve the DNA pellet. The extracted DNA was then stored at -20 ºC for the further investigation.

3.4.2. DNA qualification

The presence of DNA extracted was detected by using both gel electrophoresis and Nano drop. Agarose gel electrophoresis confirmed the presence of DNA extracted from the scale samples. For electrophoresis , 3µl of DNA was properly mixed with 2µl of loading dye and the loaded into 1 % agarose gel. After gel electrophoresis and staining , the gel was viewed by UV illumination for bands. The concentration of the DNA was determined by measurement of UV absorbance using a ratio of the OD 260nm/280nm is an indicator of DNA purity.

To check the quality and purity of extracted DNA, Nanodrop was applied. The absorbance of DNA were measured at 260 nm and 280 nm wavelengths. The ratio of the reading at 260 nm and 280 nm reflected an estimate of purity of the nucleic acid. DNA with ratio between 1.8 and 2.0 is considered as pure.

Concentration was also measured by Nanodrop. Low concentration cause to fail the PCR due to lack of template. Too much DNA itself would most likely inhibit the PCR amplification. If the concentration of DNA is too high, the DNA should be diluted with deionized water.

3.5. PCR optimization

The PCR optimization was performed for the ten individuals which belong to different species. Each primer set was optimized for annealing temperature and $MgCl_2$ concentration. The PCR was carried out in 10µl reaction volume containing 1x green PCR buffer (Promega,USA), 2.5 to 3.75 mM of $MgCl_2$ (Promega,USA), 1.0 unit of Taq polymerase (Promega,USA), 1mM each of dATP, dCTP, dGTP and dTTP, 100mM of each primer (1st Base), 100 ng of genomic DNA and deionised water was added.

Table 3.3. PCR Reaction Mixture.

Reagents	Amount
DH2O (µl)	2.5
5X Green Buffer (µl)	3.0
MgCl2 (µl)	1.2
dNTP mix (µl)	0.25*4
Forward Primer (µl)	0.5
Reverse Primer (µl)	0.5
Taq (µl)	0.3
DNA (µl)	1.0
Total (µl)	10.0

The PCR amplification was performed by using a Gradient Thermocycler starting with a 3 minutes pre-denaturation at 95°C. This step was followed by 35 cycles of denaturation at 94°C for 40 seconds, annealing temperature ranging from 44 to 54 for 40 seconds and an

elongation step at 72 °C for 40 sec. The final extension step was carried out at 72 °C for 7 min. finally it was held at 4 °C.

Table 3.4. PCR Conditions.

Cycle	Temperature	Time	PCR Cycle
1	95^0C	3 minutes	Initial Denaturation
35	94^0C	40 seconds	Denaturation
35	45-65^0C	40 seconds	Annealing
35	72^0C	40 seconds	Elongation
1	72^0C	7 minutes	Final Elongation
1	4^0C	Forever	Hold

3.6. Gel electrophoresis

The PCR product were separated on 1% Agarose gel. Agarose Powder was mixed with 1X TBE buffer. Running condition is depended on the gel concentration and the number of running gel at the same time

The mixture was heated in a microwave until the mixture was completely dissolved. Then it was poured in to the gel tray having a comb and it was left for about 1 hour to solidify. After that the tray included solid the comb was removed and the gel was transferred to the electrophoresis tank and immersed with the buffer used for its preparation.(1X TBE buffer).Then 5µl of the PCR products were loaded into each well of the gel. 1µl of DNA

ladder (Promega,USA) which was mixed with 3µl loading dye was used to estimate the size of PCR products.

The electrophoresis was carried out at 75 volts. 180 A, for approximately half an hour. This step was followed by staining using ethidium bromide (EtBr) solution (0.1 mg/ml) for 30 min. Once electrophoresis was completed, gel were removed from the glass plate and transferred in to the a container filled with ethidium bromide and were immersed in solution. After then, the gel was visualized and detected under a 365 nm UV light and documented using gel documentation machine, AlphaImager (Alpha Innotech; CA, USA).

3.7. Gel excision and purification

DNA gel extraction was performed by using Gel extraction Kit (MACHEREY-NAGEL) .The gel slice containing the expected DNA band was excised using a sharp and clean scalpel under ultraviolet light. After cutting the expected DNA band, the gel was weighted and transfer into a 1.5 ml microcentrifuge tube. Then for each 100 mg of agarose gel, 200 µl of buffer NT was added in order to resuspend the gel in buffer by vortexing the mixture every 2-3 minutes while It was heated in water bath at 50 °C.

The mixture then was loaded into column placed into the 2 ml collection tube. Then, the tube was centrifuged at 11.000 x g for 1 min. The flow-through is discarded and the column is placed back into the collection tube. 700 µl buffer NT3 is added to the column and centrifuged at 11,000 x g for 1 min followed by discarding flow-through and placing back the column into collection tube. For complete removal of buffer NT3 and drying the silica membrane, another centrifuge was done at 11,000 x g for 2 minutes. This step was performed to remove residual ethanol from Buffer NT3 which might inhibit subsequent reaction. In

addition to centrifugation, total removal was achieved by incubation at room temperature over night.

Finally, the NucleoSpin Extract II Column in placed into clean 1.5 ml microcentrifuge tube and 15-50 µl Buffer NE was added to elute DNA. The tube was incubated at room temperature for 1 min to increase the yield of eluted DNA. After incubation, the tube was centrifuged for 1 min at 11,000 x g.(pre heated)

3.8. Testing DNA purification

In order to confirm the occurrence of DNA in purified samples, the purified samples must be test. Therefore, the gel was made by dissolving 0.4 mg of agarose powder into 40 ml of 1 X TBE solution (Tris-borate-EDTA: 90mM Tris, 90mM borate and 1mM EDTA). After making the gel, the gel was placed into the tank containing buffer medium (1 X TBE). Then, 3µl of purified sample was mixed with 3 µl of loading dye (bromophenol blue) and loaded into the gel. As well as, 2.5 µl of 100bp was loaded into one well. Then , the gel was run by voltage 80v and current 200 AMP for 40 min. Then, the gel was stained by ethidium bromide (working concentration 0.5 µg/ml; 10 mg/ml stock) for 15 min and lastly , the picture of the gel showed the occurrence of DNA in purified sample.

3.9. DNA Sequencing

After testing DNA purification, the purified DNA were sequenced in both forward and reverse direction on an ABI 3730XL automated sequencer at first base laboratory.

3.10. Check for Polymorphism

The PCR products are checked for polymorphism on 4% metaphor Agarose gel at 75 volts in 1x Tris borate-EDTA buffer for approximately two hours. Gels were stained and visualized following the method described above. If the size polymorphisms were present, two distinct bands were observed in heterozygous individuals.

3.11. Analysis of Microsatellite DNA using Genetic Analyser (Fragment Analysis)

If the locus was polymorphic, fragment analysis was performed using a Genetic Analyser (Applied Biosystems, USA). The microsatellite forward primers were fluorescently labelled using 5' FAM dye and were used to amplify the same locus in the individuals of other populations. As these primers are light sensitive, extra care was taken to protect them from light.

After PCR using fluorescent primers, the amplified fragments were diluted 10 times in ddH_2O which was achieved by adding 9 µl ddH_2O to 1µl of the PCR product. 1µl of the diluted PCR product was transferred in a separate tube and 10µl of HiDi Formamide (Applied Biosystems) and 0.2 µl of GeneScan™ 500 LIZ™ Size Standard (Applied Biosystems, USA) were subsequently added. The tubes were vortexed for 2 mins and then centrifuged. They were then heated at 95 °C for 5mins in an Eppendorf Thermal Cycler to denature double stranded DNA and immediately kept in an icebox for 5 minutes and then transferred to a 96 well plate and covered. Care was taken that air bubbles were not be trapped inside the wells of the plate. The plate was loaded into the ABI 3031*xl* Genetic Analyser. The analysis of full 96 wells took around six hours. The electropherograms were applied to analyze and score the results using Peak Scanner.

Fragment analysis was performed to determine the variation of EST microsatellite allele in size at each locus.

3.12. Data Analysis

The results from Genetic Analyser were retrieved and scored using the software peak scanner v1.0 (ABI Applied Biosystems). This software allows visualisation of the chromatograph result generated by the ABI sequencer, and shows the fragment analysis data in terms of peaks. These peaks were observed and compared with the GeneScan™ 500 LIZ™ size standard. The peaks correspond to the exact length in base pairs of the amplified fragment. The scored microsatellite loci were collated in a Microsoft Excel before statistical analysis was undertaken using various software packages. The software called MICROCHECKER was used to check for the presence of stuttering, null alleles and long allele dropouts (van Oosterhout, et. al., 2004). After that, the scored Excel file was converted to an ".ARLEQUIN" file using the software called CONVERT (Glaubitz 2004).

Arlequin software was used to test for Hardy-Weinberg Equilibrium (Guo & Thompson, 1992), Linkage disequilibrium (Slatkin, 1994), and to calculate the number of alleles and the allelic range. The HWE test compares allele and genotype frequencies with the expected frequencies retrieve from the ideal population (random mating, no mutations, no selection, no genetic drift, no gene flow and meiotic drive). The population in equilibrium, the frequency of genotypes depends on the frequency of the genes and both are remain constant over generations. Violation of one or more HWE assumptions result in deviation of Hardy Weinberg Equilibrium in a given population. Arlequin was used to compute the genetic distance, Hardy-Weinberg equilibrium, linkage disequilibrium and pairwise

differences which were achieved by calculation of the observed heterozygosity (Ho) and expected heterozygosity (He) values per locus in each population.

Population structure analysis (*FST*) was also performed in Arelquin software. This analysis generates a table containing pair-wise *FST* values among all populations, and associated significance values. *FST* analysis uses the formula $FST = (HT - HS) / HT$, where HT is the average expected heterozygosity of all the populations and HS is the average expected heterozygosity of each population (Slatkin 1991, 1995).

CHAPTER 4

RESULTS

4.1. DNA extraction

DNA had been extracted from the samples of individuals using CTAB method. The DNA concentration was determined using Nanodrop 2000 (Thermo Scientific) and also confirmed by gel running (1% agarose gel). The range of extracted DNA concentration was 300-1000 ng/µl.

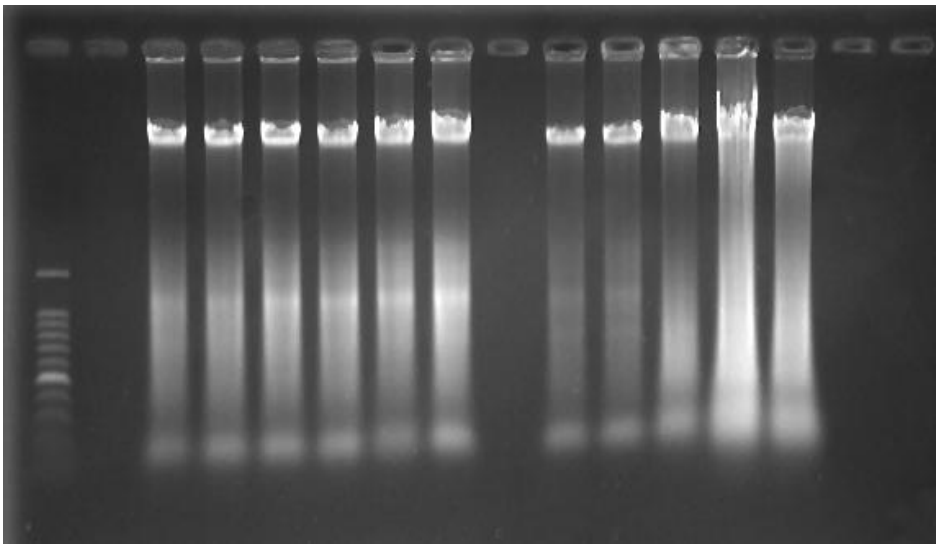


Figure 4.1. DNA was extracted successfully by CTAB method.

4.2. Population study of *Channa striata*

Microsatellite primer pairs designed from *Channa striata* RNA sequencing data were used in the population study of *C.striata*. The primers were checked for amplification and optimization before applying in population study.

4.3. Screening of EST microsatellite primer pairs

Microsatellite primer pairs were optimized at different PCR conditions. Annealing temperature and $MgCl_2$, which are the most important factors for high-quality PCR amplification were changed by gradient format to find the optimum temperature and $MgCl_2$ concentration to create clear and distinct bands.

After PCR amplification, the amplicons were visualized on 1% agarose gel. The results of the EST microsatellites screening showed that five primer pairs out of 20 primer pairs were beyond optimization at various PCR conditions. These primer pairs gave no PCR amplification after many trials at different annealing temperatures. The rest of the primers successfully amplified DNA product at their optimized annealing temperature.

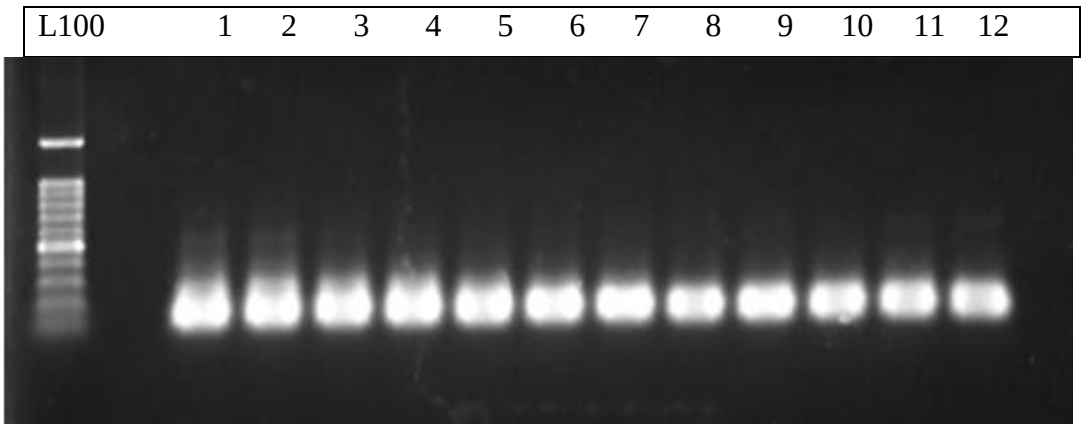


Figure 4.2. Optimization of primer EST-01F and 01R. The gradient temperature was 45 °C (lane 1) to 55 °C (lane 12).

4.4. Check for the polymorphism

Polymorphic microsatellite markers were determined by variation of banding pattern. PCR products were run on Metaphore Agarose gels to check for polymorphism. Out of 15 microsatellite primer pairs which obtain successful PCR amplification, 6 primers showed multiple bands detected on Metaphore Agarose gel. The screening process of markers resulted in only 6 primer pairs are polymorphic and being used for population study.

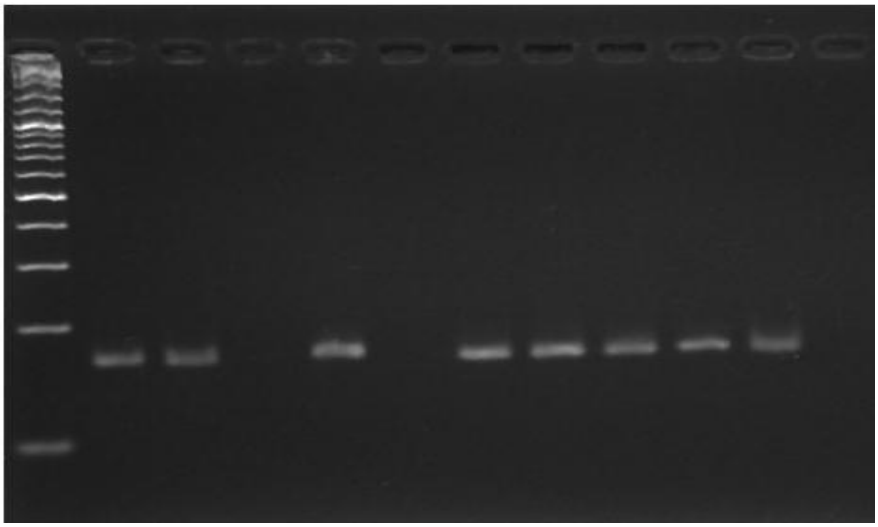


Figure 4.3. The PCR product of primer 6L and 6R on Metaphore Agarose gel.

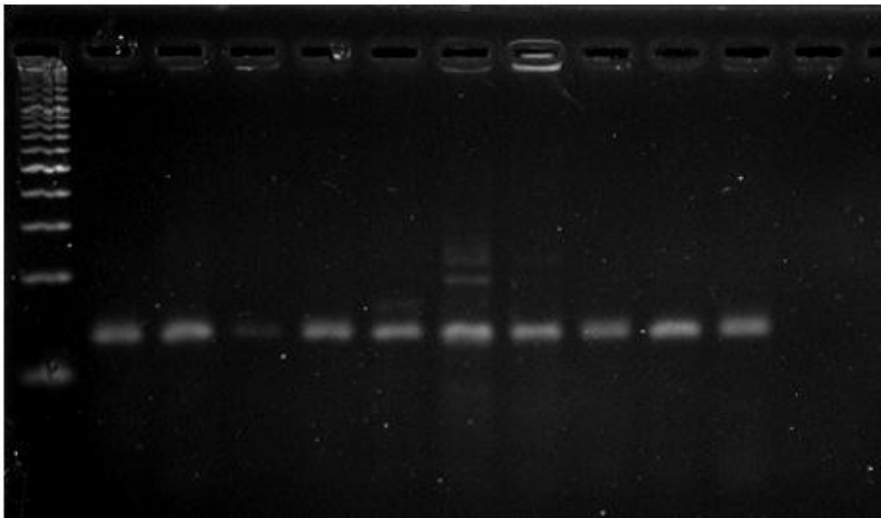


Figure 4.4. The PCR product of Primer 1L and 1R on Metaphore Agarose gel.

Further elucidation of polymorphism for identified 6 loci, were analyzed via fragment analyzer. The number of genotypes that can screen by the polymorphic markers is necessity. The alleles are scored according to PCR product size, which are considered as the molecular size marker. Multi-allelic genotypes was successfully determined using Peak Scanner. The peaks showed the exact size in nucleotide bases of the amplified fragments and revealed the presence or absence of heterozygosity. Figues 4.1-4.4 illustrate results of the fragment analysis for one individual at four loci.

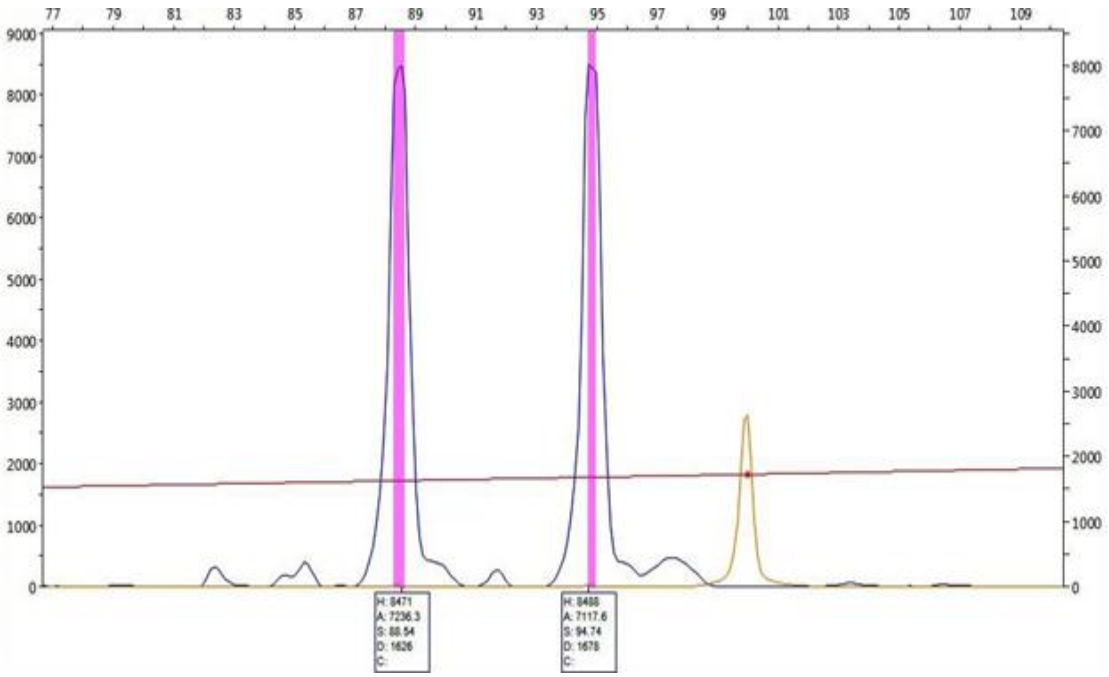


Figure 4.5. Fragment analysis peaks of primers 18L and 18R. Two distinct peaks shows amplification at two sites i.e. heterozygosity. The x-axis and y-axis corresponds to the size of fragments (bp) and the fluorescent intensity of each peak respectively.

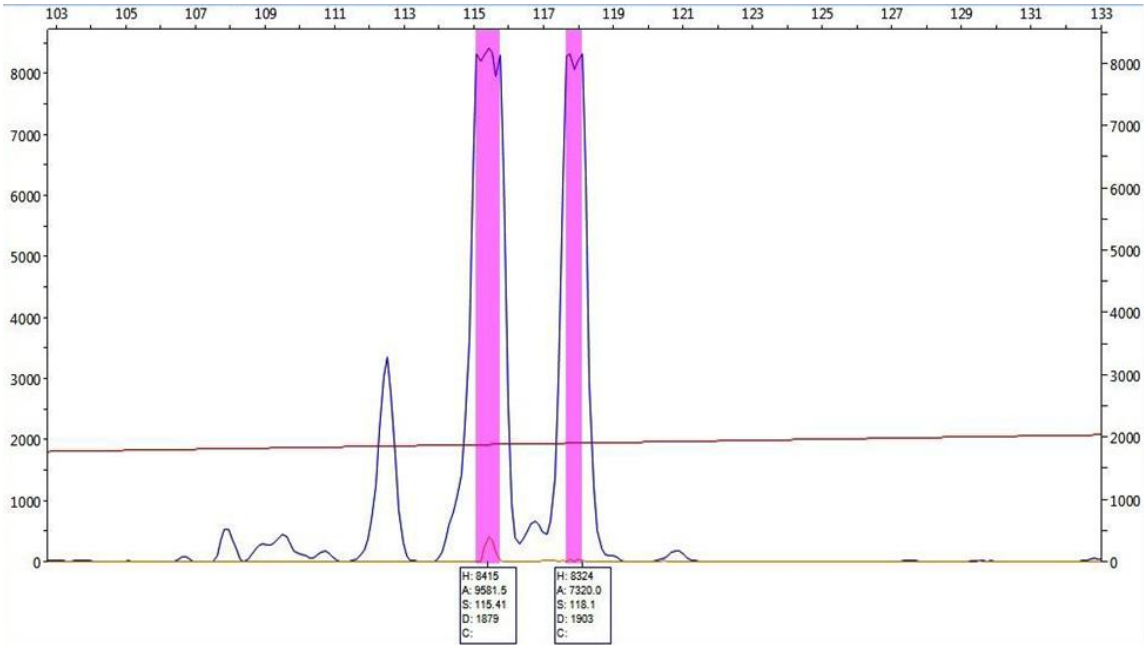


Figure 4.6. The fragment analysis peaks of primer 8L and 8R. Two distinct peaks shows amplification of two sites i.e. heterozygosity.

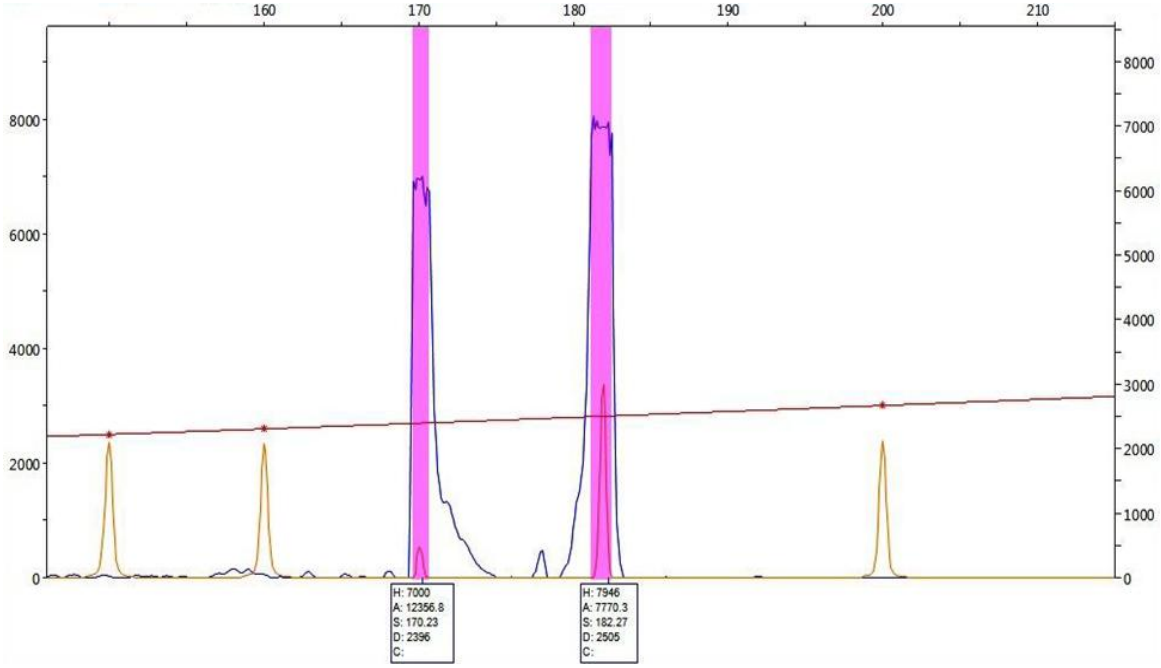


Figure 4.7. The fragment analysis peaks of primer 12L and 12R.

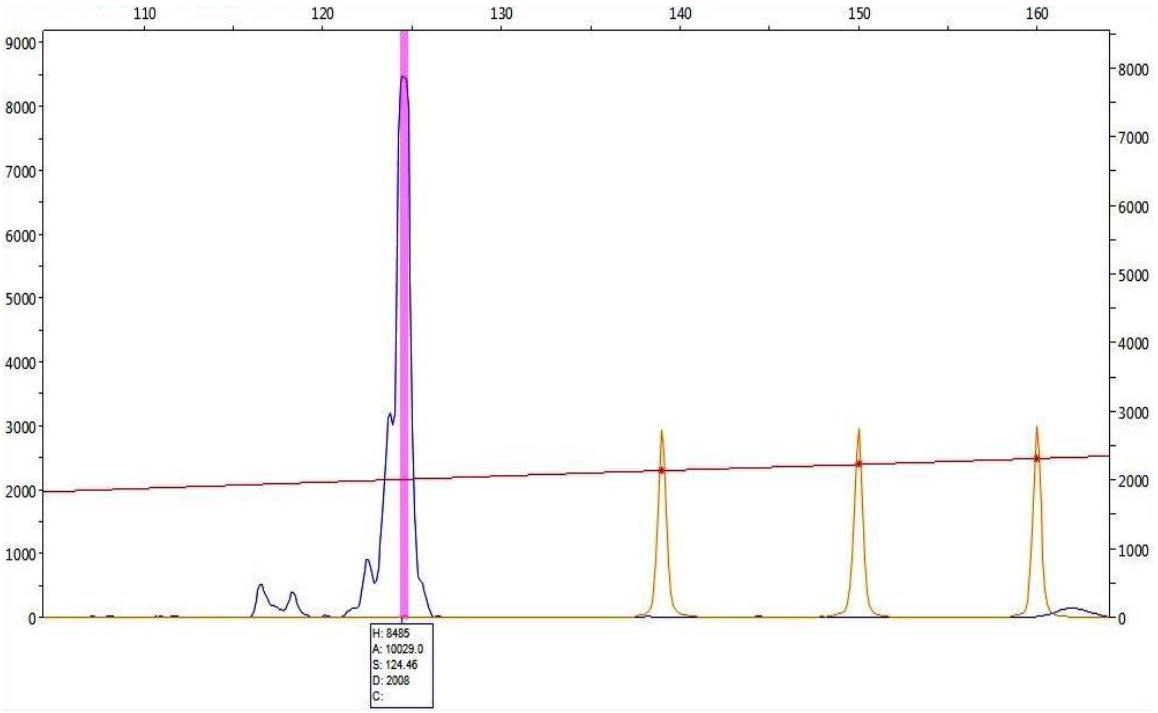


Figure 4.8. The fragment analysis peaks of primer 1L and 1R. Only one clear shows amplification at one site i.e. homozygosity.

4.5. Microchecker Analysis

The amplified fragment sizes were checked for stuttering, presence of null alleles and large allelic dropouts using the software called Microchecker. But in all the populations, no evidence of stuttering, presence of null alleles and large allelic dropouts were observed.

4.6. Level of heterozygosity

To describe the polymorphism at each locus the number of alleles and allelic range were observed. The number of alleles per locus ranged from 2 to 4. The population from Kedah had the most number of alleles at each locus (average of 3 per polymorphic locus), while Johor had the least number of alleles (average of 2 per polymorphic locus).

The level of diversity as assessed by heterozygosity was observed in all populations. The estimated values of the observed heterozygosity and the expected heterozygosity for all the populations are shown in tables 4.1- 4.2.

The highest observed heterozygosity was found in the Pahang population with the average value of 1.0. Whereas Kedah population had the lowest observed heterozygosity. The Pahang population had the most difference between the observed and expected heterozygosity values while the other polpulations had the low difference.

Table 4.1. Statistic values of Sarawak population.

Locus	No of alleles	Allelic Range	Observed Heterozygosity	Expected Heterozygosity
EST 1	-	-	-	-
EST 8	2	4	0.44444	0.36601
EST 12	3	2	0.40000	0.67895
EST 14	2	1	0.66667	0.47059
EST 18	-	-	-	-

Table 4.2. Statistic values of Johor population.

Locus	No of alleles	Allelic Range	Observed Heterozygosity	Expected Heterozygosity
EST 1	2	1	0.05263	0.05263
EST 8	2	1	0.9000	0.50769
EST 12	2	1	0.25000	0.22436
EST 14	2	1	0.45000	0.45000
EST 18	2	1	0.00	0.09744

Table 4.3. Statistic values of Kedah population.

Locus	No of alleles	Allelic Range	Observed Heterozygosity	Expected Heterozygosity
EST 1	3	5	0.55000	0.44744
EST 8	3	4	0.15789	0.32290
EST 12	3	4	0.3000	0.34359
EST 14	2	1	0.05263	0.05263
EST 18	4	5	0.65000	0.52949

Table 4.4. Statistic values of Pahang population.

Locus	No of alleles	Allelic Range	Observed Heterozygosity	Expected Heterozygosity
EST 1	2	1	1.0	0.51282
EST 8	2	1	1.0	0.5282
EST 12	3	3	1.0	0.53718
EST 14	2	1	0.7500	0.51154
EST 18	3	4	1.0	0.55897

4.7. Hardy-Weinberg Equilibrium

No significant deviations from Hardy Weinberg were observed.

Table 4.5. Hardy Weinberg Equilibrium.

Locus	Sarawak	Johor	Kedah	Pahang
EST 1	-	1.0	0.50	1.0
EST 8	0.4000	1.0	0.60	1.0
EST 12	1.0	1.0	0.60	0.750
EST 14	1.0	1.0	0.1	1.0
EST 18	-	1.0	0.66667	0.60

4.8. Linkage disequilibrium

When two alleles of different loci do not associate independently in the population, it is said to be in linkage disequilibrium. The amount of linkage disequilibrium depends on the difference between observed allelic frequencies and those expected from a homogenous. There was no pattern on linkage observed among specific pairs of loci at all sites, indicating that no physical linkage is likely to be present among the loci analysed here. However, 8 of the 15 tests for linkage at Pahang were significant ($p<0.05$).

Table 4.6. Significance values of pair-wise tests for linkage for each population.

Linkage Pairs	Sarawak	Johor	Kedah	Pahang
(EST 1, EST 8)	1.0	0.98733	0.5376	0.00
(EST 1, EST 12)	1.0	1.0	0.68990	0.00
(EST 8, EST 12)	0.38218	0.36663	0.01109	0.00
(EST 1, EST 14)	1.0	0.57238	0.40901	0.03535
(EST 8, EST 14)	0.99901	0.57109	0.01069	0.3802
(EST 12, EST 14)	0.49554	0.14109	0.00	0.00406
(EST 1, EST 18)	1.0	1.0	0.37614	0.00
(EST 8, EST 18)	1.0	1.0	0.05644	0.00
(EST 12, EST 18)	1.0	1.0	0.31208	0.00
(EST 14, EST 18)	1.0	1.0	0.69733	0.05861

4.9. Population structure

For the study of the population structure, the *Fst* analysis was done. *Fst* analysis gives information about the level of differentiation among the populations. If the *Fst* value between two populations is significant and approaching to 1 then the populations are totally differentiated. If the magnitude of *Fst* value is near 0 or non-significant then populations are same. The population comparisons between Johor, Kedah, Pahang and Sarawak are given below:

Table 4.7. Results of pair-wise *Fst* analysis. *Fst* values presented below the diagonal, significance (p-values) above the diagonal.

Populations	Sarawak	Johor	Kedah	Pahang
Sarawak		<0.0001	<0.0001	<0.0001
Johor	0.56285		<0.0001	<0.0001
Kedah	0.57518	0.52321		<0.0001
Pahang	0.42937	0.33259	0.28906	

Above values show that all populations are significantly different from one another. Kedah and Pahang are the most similar populations (*Fst* = 0.28906, p<0.001) whereas Sarawak and Johor are the most different populations among all.

4.10. Cross-species amplification study

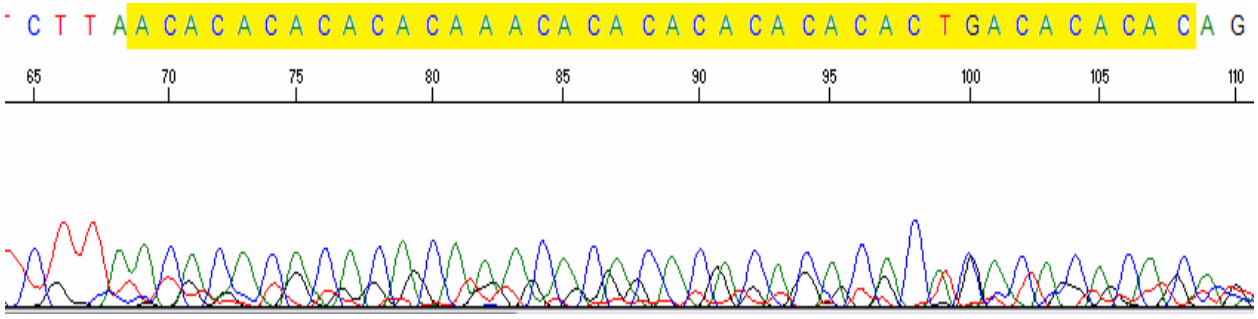
Among 20 loci of EST microsatellite markers developed in *C.striata*, those primers which were able to optimized chosen for use in cross-species amplification. The loci of EST microsatellites were cross-species amplified in DNA samples from 11 different species. Out of 10 species were screened for cross amplification, only one species produced distinct and clear bands. The clear bands were purified from the gel and were sent for sequencing to confirm the presence of microsatellite repeats. DNA sequencing involves determining the exact order of bases that form a DNA segment. Each base in the nucleotide sequence was detected and used laser by automatic sequencing machine. The results were analysed in Chromas or Sequence Scanner software.

Table 4.8. shows the comparison of microsatellite repeat motifs of *C.striata* and *C.micropeltes.* The results indicated successful-cross amplification in two species of channidae family which can prove highly conservation of microsatellite motifs in a different species.

Table 4.8. Comparison of microsatellite motifs in *C.striata* and *C.micropeltes*.

Primer Name	Repeat sequence (*C.striata*)	Repeat sequence (*C.micropeltes*)	Expected size (bp) *(C.striata)*	Fragment size (*C.micropeltes)*	Ta (°C)
EST 1	(AT)6	-	125	104	46-53
EST 3	(AGC)5	(AGC)3	126	105	53-56
EST 6	(AC)5	(AC)4 (TACA)4	168	147	55-60
EST 7	(AC)5	(A)10, (A)7	149	168	48-53
EST 8	(CAT)6	(CAT)2 TAT(CAT)9	109	102	55-60
EST10	(AG)5	(AG)3 GA(AG)2	282	226	60-65
EST 14	(AGC)5	(AGC)4	180	157	46-50
EST 16	(AC)6	-	119	120	52-57
EST 17	(AC)13	(AC)6AA(AC)8TG(AC)4	146	195	50-55
EST 19	(AC)19	-	129	96	52-57
EST 20	(AC)20	(AC)5	91	65	50-55

(A)



(B)

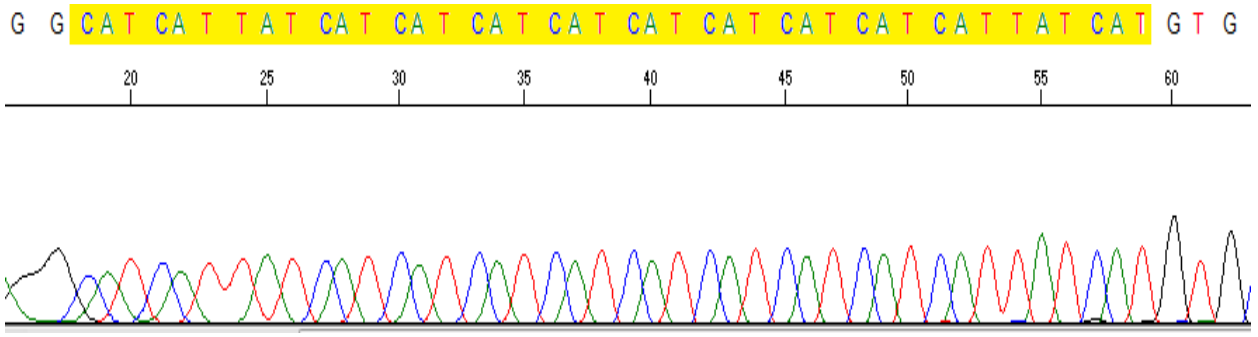


Figure 4.9. Microsatellite sequences. (A) Shows CA microsatellite repeats obtained from cross-amplification using primer EST-17, (B) Shows CAT microsatellite repeats obtained from cross-amplification using primer EST-8

CHAPTER 5

DISCUSSION

In this study, EST-SSR markers developed for *C.striata* were applied to analysis the population structure and screen for transferability across 10 species (Cross-species amplification).

Despite the lower level of polymorphism found in EST derived SSR compared to genomic SSRs, these markers have been investigated in recent years in many plant and animal species due to several factors which enhance the value of EST-SSR including, detection of variation in transcribed regions of the genome, higher transferability across closely related species and fast and cost effective development.

Primer pairs were optimized first for polymerase chain reaction (PCR) especially by changing annealing temperature. Of the 20 microsatellite primer pairs, 15 pairs were amplified successfully and gave PCR products of expected size. Among these microsatellite primers only 6 pairs were found to be polymorphic.

5.1. Level of heterozygosity

Since heterozygosity is associated with the amount of genetic variation, it is commonly applied to measure genetic diversity (Slate and Pemberton, 2002). According to the

assumption collected from several studies, observed heterozygosity tend to be higher than expected heterozygosity in wild population.

Deficiencies of heterozygosity have been reported in many studies (Appleyard et al., 2001). Some misclassification of heterozygous as homozygous may be caused from some difficulties associated with screening process such as presence of null alleles and appearance of stutter bands (Bagley, 1999). Null alleles can be the result of mutation in the flanking regions and cause them undetectable for primers. It is presumed that the heterozygote deficiencies were not attributed to null alleles or stutter bands since the results were checked using microchecker software.

Heterozygote deficiencies, which have been reported in several studies of fish species, may be resulted from other possibilities including isolation, genetic drift, the Wahlund effect and inbreeding (Bagley et al., 1999; Callen et al., 1993). Inbreeding was unlikely to happen in the wild populations where the outcrossing usually occurred.

In this study, the observed heterozygosity (Ho) was higher than the expected heterozygosity (He) in most of the loci in all four populations, and in the Pahang population was higher than in the other three populations. The absence of heterozygote deficiencies in the populations affirmed that the samples were good representative of wild stocks.

Slight excess of homozygosity was observed in some loci of the Sarawak and Kedah populations. Sample size is another factor which may affect the value of the observed heterozygosity (Norris et al., 2001). The highest averaged heterozygosity among all populations was observed in Pahang while Kedah showed the least heterozygosity.

Number of alleles, heterozygosity and analysis of molecular variance can evaluate levels of genetic diversity and population differentiation, which are critical for developing management and conservation strategies. For this reason, many studies rely on microsatellites to investigate the population structure due to their attractive features (Avise, 1996)

5.2. Hardy-Weinberg Equilibrium (HWE)

According to the results of microsatellite analysis, there was no evidence of significant deviation from Hardy Weinberg Equilibrium (HWE) for any population (Sarawak, Johor, Kedah and Pahang) at any locus.

The HWE test may be violated due to some perturbing factors such as small population, non-random mating, mutation, inbreeding and selection (Wigginton et al., 2005). Wild populations may be subjected to mutation, events involving genetic bottleneck, random genetic drift or natural selection which cause the populations to exhibit departure from HWE. Non-conformance to HWE is more likely to happen in cultured populations.

Most of the detected deviations from HWE were a result of heterozogout deficiencies. Excess of homozygotes, which can lead to an imbalance in the proportion of heterozygotes and homozygotes in a population may be caused by the presence of null alleles. Microchecker analysis did not identify any large allelic dropouts and null alleles. Occurrence of null alleles and small sample size contribute to heterozygote deficiencies which is the most common reason for deviation from HWE. Probably for highly polymorphic loci, bigger sample sizes are required to achieve HWE.

5.3. Linkage Disequilibrium (LD)

Linkage disequilibrium (LD) is a powerful tool for analysis population structure. Linkage disequilibrium can be influenced by some factors including: genetic linkage, selection, mutation, recombination, genetic drift in small population, non-random mating and population structure.

Many studies on different populations indicated that LD decreased with the marker distance only for closely linked loci. However, LD affected by only genetic linkage factor, would be lost after some generations depending on the rate of recombination between loci (Bozkaya and Kurar, 2005). A relatively small and constant population size and inbreeding are suggested result in higher LD, presumably because of genetic drift.

The LD pattern for microsatellite is generally high due to its high rate of mutation and high number of alleles per locus and able to reflect recent genetically important events, which occurred in population (Chapman and Wijsman, 1998)

According to LD test conducted in Sarawak, Johor and Kedah, which result in no significant linkage disequilibrium, it can be considered that the mentioned populations are in linkage equilibrium. Therefore, these polymorphic loci can be useful markers for analysis of population and genetic diversity. However, eight of 15 tests for linkage at Pahang population were significant ($p<0.05$), suggesting possibly that this sample does not constitute a single randomly breeding population, or that these loci may be under selection in the Pahang population. Although this population did not deviate significantly from H-W at any locus, in each case there was heterozygote excesses observed.

5.4. Genetic differentiation between populations (F-*statistic*)

Population structure study can be estimated from microsatellite allele frequencies. F statistics is the most common and informative tools for investigation of population structure and divergence between populations. To assess population differentiations, test of pair wise F*st* was conducted. It was calculated according to the variation of allele frequencies. F*st* has been applied in many studies to investigate the gene flow and linkage pattern among populations (Wright, 1978). Genetic structure of populations is affected by environmental barriers, climatic conditions, historical processes and life histories, which are contributed to shape the divergence between populations (Ball et al., 2000).

It is useful to examine variation at microsatellite loci for determining the genetic structure of populations (Scribner et al., 1996). Microsatellites are considered the most informative genetic marker to reveal genetic heterogeneity among geographic samples (O'Connell et al., 1998; Shaw et al., 1999). This is due to high allelic diversity of microsatellite, which results in possess discriminating power in detecting subtle population differentiation (Ross et al., 1999).

The interpretation of Fst values has suggested that the values near to zero indicate the lowest level of differentiation, moderate and considerable differentiation ranged from 0.05 to 0.15 and 0.15 to 0.25 respectively and the Fst values above 0.25 is noticed as very great genetic differentiation (Balloux and Lugon-Moulin, 2002).

In this study, pair-wise comparisons of Fst between populations revealed significant differentiation. The values of Fst in this study lying within the range of 0.29-0.57 indicative

of completely differentiation among populations. According to the results, Kedah and Sarawak were considered as the most different populations among all. This is mainly due to the long geographical distance between them as Kedah and Sarawak are located in the northwest of Peninsular Malaysia and the northwest of the island Borneo respectively. The smallest differentiation belongs to the comparison of Kedah and Pahang which indicated these two populations are the most similar populations between the others. However, the pair wise estimators of Fst suggested noticeable genetic differentiation in these two populations were considered as dissimilar genetic populations. This may be due to the geographical barrier of Titiwangsa Mountain Range that separates the west coast of Peninsular Malaysia from the east. Pahang is located on the east coast of Peninsular central while Kedah is situated on the northwestern area of the west coast.

Isolation by distance can differentiate populations genetically. Populations over long distances become more divergent than populations in close proximity. Genetic divergence is directly related to geographical distance between population localities (Gold and Turner, 2002) Isolation by distance is a crucial factor which can differentiate populations genetically.

Gene flow was limited in these populations due to some physical and biological parameters. Physical processes, natural selection, isolation by distance and stable migration routes are factors that potentially could prevent gene flow (Gold and Turner, 2002).

5.5. Cross-species amplification of *Channa striata* EST microsatellite loci

Several studies have shown the sequence conservation of the microsatellites flanking regions through evolution (Tong et al., 2002). According to this fact, the primers developed for one

species can be amplified in closely related species. The efficiency of cross-species amplification is inversely related to the phylogenetic distance between two species (Steinkellner et al., 1997).

The purpose of this study is to investigate if the primer pairs developed for *Channa striata* could be applied to amplify microsatellite loci in ten other species including *Amblyrhynchicthy truncates, Barbichthys laevis, Barbonymus chwanenfeldii, Cirrhinus caudimaculatus, Hypsibarbus wetmorei, Osteochilus hasselti, Thynnichthys thynnoides, Pangasius nasutus, Hampala macrolepidota*, which belong to different genus and also the *Channa micropeltes* species, which belongs to the same genus for analysis of the effect of evolutionary distance on cross-species amplification.

It has been assumed that microsatellite loci were more conserved for aquatic species due to some investigations, which applied heterologous microsatellite markers in aquatic organisms resulting in some evidence indicating the slower rate of mutation in aquatic species compared to terrestrial species (Moore et al., 1991; FitzSimmons et al., 1995).

According to the results, low level of transferability of microsatellite loci developed for *Channa striata* was found among the nine species. The failed amplification of microsatellite loci developed for *C.striata* in other species indicated lack of conserved flanking region in these species resulting in low transferability which can be attributed to phylogenetic distance and great diversity of source species. *Channa micropeltes* was the only species for which the primers gave very strong banding profile. The sequencing analysis results showed that out of 11 primers amplified in this species, 7 primers could detect the same microsatellite repeats. This similarity indicated the high conservation of microsatellites across species.

The use of heterologous markers on related species is a useful strategy for studies of species which lack sequence information available and facilities to isolate new microsatellite loci for particular species. Cross-species amplification can also save time and costs, which are needed for initial identification of markers. heterologous microsatellite markers have been applied in phylogenetic and population genetic studies, population divergence, paternity and kinship analysis. It can also be applied in species or hybrid identification studies. Application of cross-species amplification can be efficient in construction of genetic linkage map through hybridization of defined species with other closely related species due to high level of conservation of microsatellite loci, which is required for comparative genetic mapping.

CHAPTER 6

CONCLUSION

This study was conducted to assess the genetic variation among four populations of *C.striata* in Malaysia using EST-SSR markers. Among the 20 microsatellites, which were developed for *C.striata,* five microsatellite DNA markers were found to be polymorphic. These polymorphic DNA markers were employed for investigating the population genetic structure.

Data analysis of microsatellite markers revealed that the Pahang population has the highest heterozygosity among all populations, while the lowest heterozygosity was observed in the Kedah population. All the populations represented conformance to HWE. Further investigation detected significant differentiation between populations. According to pair-wise comparison of F*st*, Kedah and Sarawak were the most differentiated due to the geographical distance whereas the Kedah and Pahang are the most similar populations among all.

EST-SSR markers, which were designed for *C.striata* were applied for cross-species amplification in other species. Failed amplification in most species indicated low transferability between selected species except from one species, which belongs to the same family of *C.striata.*

Presence of the same Among all, 11 primer pairs in *Channa micropletes* produced distinct and clear bands. microsatellite repeats were confirmed through sequencing results in seven loci.

Successful cross-amplification indicated the highly conservation of microsatellite across these two species and it showed the slower evolution of microsatellite regions in species of the same family. Application of heterelogous microsatellite markers is an effective approach which can save considerable amounts of time and cost for developing new markers.

For future studies, comparison of variation levels between wild and cultured stocks can be conducted by applying these microsatellite markers on cultured population of *Channa striata* in Malaysia to investigate whether it is possible to maintain the genetic variation of the wild source within the cultured stocks. It will be useful to monitor the alteration of genetic diversity which is happening of every generation in order to get better efficiency in management and conservation approaches and also in developing microsatellite markers in the aquaculture industry.

APPENDIX

Microsatellite allele size of *C.striata* loci across ten individuals of Sarawak population

Population	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B
Sarawak	**EST1**		**EST8**		**EST11**		**EST12**		**EST14**		**EST18**	
1	125	125	121	133	308	308	186	186	182	182	94	94
2	125	125	121	133	308	308	182	182	*	*	94	94
3	125	125	133	133	308	308	182	182	179	182	94	94
4	125	125	133	133	308	308	178	182	182	182	94	94
5	125	125	121	133	308	308	182	182	179	182	94	94
6	125	125	133	133	308	308	178	178	182	182	94	94
7	125	125	121	133	308	308	182	186	179	182	94	94
8	125	125	133	133	308	308	178	186	179	182	94	94
9	125	125	*	*	308	308	182	186	179	182	94	94
10	125	125	133	133	308	308	178	178	179	182	94	94

Microsatellite allele size of *C.striata* loci across twenty individuals of Johor population

Population	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B
Johor	**EST1**		**EST8**		**EST11**		**EST12**		**EST14**		**EST18**	
1	123	125	112	115	308	308	178	178	179	182	94	94
2	123	123	112	115	308	308	178	178	179	182	94	94
3	123	123	112	115	308	308	178	178	179	182	91	91
4	123	123	112	115	308	308	178	178	179	179	94	94
5	123	123	112	115	308	308	178	178	179	182	94	94
6	123	123	112	115	308	308	178	178	179	179	94	94
7	123	123	112	115	308	308	178	182	179	182	94	94
8	123	123	112	115	308	308	178	182	179	179	94	94
9	123	123	112	115	308	308	178	178	179	182	94	94
10	123	123	112	115	308	308	178	178	179	182	94	94
11	123	123	115	115	308	308	178	178	179	182	94	94
12	123	123	112	115	308	308	178	178	179	179	94	94
13	123	123	112	115	308	308	178	178	179	179	94	94
14	123	123	112	115	308	308	178	178	182	182	94	94
15	123	123	112	115	308	308	178	178	182	182	94	94
16	123	123	112	115	308	308	178	178	179	179	94	94
17	123	123	112	115	308	308	178	182	179	179	94	94
18	123	123	112	115	308	308	178	182	179	179	94	94
19	*	*	112	115	308	308	178	178	179	182	94	94
20	123	123	115	115	308	308	178	182	179	179	94	94

Microsatellite allele size of *C.striata* loci across twenty individuals of Kedah population

Population	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B
Kedah	**EST1**		**EST8**		**EST11**		**EST12**		**EST14**		**EST18**	
1	123	123	118	118	308	308	182	182	179	179	88	88
2	123	123	118	118	308	308	182	182	179	179	88	94
3	123	125	115	118	308	308	182	182	179	179	88	94
4	123	123	118	118	308	308	182	182	179	179	82	88
5	115	123	118	118	308	308	170	182	179	179	88	94
6	123	125	118	118	308	308	182	182	179	179	82	88
7	123	123	118	118	308	308	182	182	179	179	88	88
8	115	123	118	118	308	308	182	182	179	179	88	88
9	123	125	118	118	308	308	182	182	179	179	88	88
10	115	123	118	118	308	308	182	182	179	179	88	94
11	115	123	118	118	308	308	182	182	179	179	88	94
12	115	123	118	127	308	308	170	182	179	182	88	94
13	123	123	118	118	*	*	182	182	179	179	88	94
14	123	123	118	118	308	308	170	182	179	179	88	88
15	123	123	127	127	308	308	170	182	179	179	79	88
16	123	123	118	118	308	308	182	182	179	179	88	94
17	123	125	115	118	308	308	182	182	179	179	88	94
18	123	125	*	*	308	308	170	182	179	179	94	94
19	115	123	118	118	308	308	170	182	179	179	88	94
20	123	123	127	127	308	308	186	186	*	*	88	88

Microsatellite allele size of *C.striata* loci across twenty individuals of Pahang population

Population	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B
Pahang	**EST1**		**EST8**		**EST11**		**EST12**		**EST14**		**EST18**	
1	115	123	112	115	308	308	170	182	179	179	88	94
2	115	123	112	115	308	308	170	182	179	182	88	94
3	115	123	112	115	308	308	170	182	179	182	82	88
4	115	123	112	115	308	308	170	182	179	182	88	94
5	115	123	112	115	308	308	170	182	179	182	88	94
6	115	123	112	115	308	308	170	182	179	182	88	94
7	115	123	112	115	308	308	170	182	179	182	88	94
8	115	123	112	115	308	308	170	182	179	182	88	94
9	115	123	112	115	308	308	170	182	179	179	88	94
10	115	123	112	115	308	308	178	182	182	182	88	94
11	115	123	112	115	308	308	170	182	179	182	88	94
12	115	123	112	115	308	308	170	182	179	182	88	94
13	115	123	112	115	308	308	170	182	182	182	88	94
14	115	123	112	115	308	308	170	182	179	182	88	94
15	115	123	112	115	308	308	170	182	179	182	88	94
16	115	123	112	115	*	*	170	182	179	182	88	94
17	115	123	112	115	308	308	170	182	179	182	82	88
18	115	123	112	115	308	308	170	182	179	182	88	94
19	115	123	112	115	308	308	170	182	179	179	88	94
20	115	123	112	115	308	308	170	182	179	182	88	94

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