UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT AND ISOLATION OF DNA MICROSATELLITE MARKERS FOR THE CHARACTERISATION AND IDENTIFICATION OF MYSTUS NEMURUS (C & V)

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DEVELOPMENT AND ISOLATION OF DNA MICROSATELLITE MARKERS FOR THE CHARACTERISATION AND IDENTIFICATION OF *Mystus nemurus* (C & V)

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Mystus nemurus or better known as 'ikan Baung' is an indigenous species in Malaysia. The popularity of this freshwater fish among the locals had made it an economically important aquaculture candidate. Of the so many DNA marker systems available, microsatellites are particularly useful for population studies and have been proven to be the most efficient due to their codominant modes of inheritance.

Two methods were used to isolate microsatellites in *M. nemurus*, namely Random Amplified Hybridisation Microsatellites (RAHMs) and Random Amplified Microsatellites (RAMs). A total of 88 microsatellite sequences and 18 cryptic simple regions were produced. The majority (90%) of these microsatellites were detected by the RAMs method while the RAHMs method required further optimisation and modification. The isolation of microsatellites resulted in 33 primer pairs being designed.

Of these, a total of 15 microsatellite loci were employed in a genetic variation study of *M. nemurus* from seven different locations in Malaysia. The populations involved in this study included Perak, Kedah, Terengganu, UPM, Sarawak, Johor (Layang-layang) and Johor (Kahang River). The number of alleles per locus ranged from 4 to 13 with an average of 8.5. The highest value of observed heterozygosity was 0.3914 (UPM) and the lowest value was 0.2356 (Terengganu). The F_{is} values indicated heterozygote deficiencies in all the populations studied. Besides, the chi-square goodness of fit test and the G log-likelihood ratio test showed that the majority of loci deviated significantly from Hardy-Weinberg Equilibrium (HWE). The results obtained indicated that the small sample sizes caused the deviations from HWE and the deficiencies of heterozygotes. The presence of null alleles was another reason for such results since their occurrence was high (23.5%). The cluster analysis showed that the Perak and Terengganu populations were the closest and that the majority of the clusterings was in accordance with the geographical regions from which the populations were obtained.

Cross-species amplification studies of *M. nemurus* primers were conducted on *Pangasius micronemus* and *Clarias batrachus*. The successful cross-species amplifications indicated that microsatellite loci were conserved among catfish species in other family taxa. This conservation of microsatellites in other catfish species will save time and valuable resources since the development of microsatellite markers for each catfish species is not necessary. A cluster analysis was also performed to investigate the genetic relationships among the three catfish species. The results showed that *M. nemurus* was closer to *P. micronemus* based on the calculated genetic distance values.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai keperluan ijazah Master Sains

PEMBANGUNAN DAN PEMENCILAN BAGI PENANDA MIKROSATELIT DNA UNTUK PENCIRIAN DAN PENGENALPASTIAN BAGI *Mystus nemurus* (C & V)

Oleh

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Mystus nemurus atau lebih dikenali sebagai "ikan Baung" merupakan spesies asli di Malaysia. Popularitinya ikan air tawar ini di antara penduduk tempatan menjadikannya calon akuakultur yang penting dari segi ekonomi. Bagi kebanyakan sistem penanda DNA yang tersedia, mikrosatelit sangat berguna untuk kajian populasi dan ia terbukti adalah paling efisien disebabkan oleh sifat perwarisan kodominannya.

Dua kaedah telah digunakan untuk memencil mikrosatelit dalam *M. nemurus*, iaitu "Random Amplified Hybrisation Microsatellites (RAHMs)" dan "Random Amplified microsatellites (RAMs)". Sejumlah 88 jujukan mikrosatelit dan 18 "cryptic simple regions" telah dihasilkan. Majoriti (90%) mikrosatelit telah dikesan dengan kaedah RAMs manakala kaedah RAHMs perlu dioptimumkan dan diubahsuaikan selanjutnya. Pemencilan mikrosatelit ini mengakibatkan 33 pasangan primer direka.

Daripada jumlah tersebut, sejumlah 15 lokus mikrosatelit digunakan dalam kajian variasi genetik bagi *M. nemurus* dari tujuh lokasi yang berlainan di Malaysia. Populasi-populasi yang terlibat dalam kajian ini merangkumi Perak, Kedah, Terengganu, UPM, Sarawak, Johor (Layang-layang) dan Johor (Sungai Kahang). Bilangan alel per lokus berjulat daripada 4 hingga 13 dengan purata 8.5. Nilai tertinggi bagi keheterozigotan yang dicerap ialah 0.3914 (UPM) dan nilai terendah ialah 0.2356 (Terengganu). Nilai F_{is} menunjukkan kekurangan heterozigot dalam semua populasi yang dikaji. Selain itu, ujian "chi-square goodness of fit" dan "G log-likelihood ratio" menunjukkan majoriti lokus menyimpang dengan signifikan daripada keseimbangan Hardy-Weinberg (HWE). Keputusan yang diperolehi ini menunjukkan bahawa saiz sampel menyebabkan penyimpangan dari HWE dan kekurangan heterozigot. Kehadiran "null alelles" adalah sebab lain bagi keputusan seumpama ini kerana kewujudannya adalah tinggi (23.5%). Analisis kelompok menunjukkan bahawa populasi Perak dan Terengganu adalah terdekat dan majoriti kekelompokan adalah selaras dengan kawasan geografi.

Kajian amplifikasi merentasi-spesies dengan primer *M. nemurus* dilakukan ke atas *Pangasius micronemus* dan *Clarias batrachus*. Kejayaan amplifikasi merentasi-spesies menunjukkan bahawa lokus mikrosatelit dipelihara di antara spesies ikan deduri dalam takson famili yang lain. Pemeliharaan mikrosatelit ini akan menjimatkan masa dan sumber bernilai kerana perkembangan penanda mikrosatelit bagi setiap spesies ikan deduri adalah tidak perlu. Analisis kelompok juga dilakukan untuk menyelidik hubungan genetik di antara ketiga-tiga spesies ikan deduri. Keputusan ini menunjukkan bahawa *M. nemurus* adalah terdekat dengan *P. micronemus* berdasarkan kepada nilai jarak genetik yang dikira.

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LIST OF ABBREVIATIONS

α	alpha
γ	gamma
µg	microgram
µl	microlitre
ρmol	picomole
10X	ten times
1X	one time
A	adenosine
bp	base pair
C	cytosine
cM	centi Morgan
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
ddH_2O	double distilled water
dGTP	deoxyguanine triphosphate
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphate
dTTP	deoxythymine triphosphate
EDTA	ethylenediamine tetraacetic acid
g	gram
G	guanosine
h	hour
kb	kilobase
kg	kilogram
LB	Luria-Bertani
M	molar
mCi	milli curie
mg	milligram
mg/ml	milligram per millilitre
$MgCl_2$	magnesium chloride
min	minute
mL	millilitre
mM	millimolar
mm	millimetre
ng	nanogram
nm	nanometre
°C	degree celsius
OD	optical density
PCR	polymerase chain reaction
RNA	ribonucleic acid
x g	relative centrifugal force
s	second
SDS	sodium dodecyl sulphate
T	thymine
TBE	tris-borate-EDTA
U	unit

UV	ultraviolet
V	volt
v/v	volume per volume
W	watt

CHAPTER 1

INTRODUCTION

The total population of the world at present exceeds six billion and the figure is expected to increase year by year. Because of this, food is one of the main agenda discussed globally. Due to the high demand for food security and the shortage of meat protein supply, attention is now being focussed more on aquaculture as a source of food.

Mystus nemurus is an indigenous freshwater fish that is found in the rivers and lakes of the Southeast Asian region. In Malaysia, *M. nemurus* is a favourite food among the locals for its good quality meat and taste. According to Kamarudin *et al.* (1987), the fish has high crude protein content and it is low in crude fat compared to chicken, beef and pork. Salmon and tuna fish are imported to Malaysia as alternative sources of food which are healthier due to their polyunsaturated fatty acid contents. Problems arise when the prices in the wet market are unaffordable for low income consumers. Thus, *M. nemurus* is an economical solution to the problem at hand. Since *M. nemurus* has gained popularity among the locals, it has the potential to be commercially produced to sustain the high demand of food from aquaculture. Therefore, introducing this fish to the aquaculture industry is an essential step towards commercial production of this fish.

At present most of the fish in the markets are caught from the wild. Extensive fishing at the commercial level will result in a decline of the natural fish population. Thus, it adds up to the importance of *M. nemurus* as an aquaculture species. As it is with

most terrestrial animal and plant breeding programmes, the ultimate goal of aquaculture particularly fish breeding is to improve commercially important quantitative traits such as colour, protein content, body mass and disease resistance through a predetermined direction. However, modern breeding programmes have led to the erosion of the genetic base for future breeding which poses a threat to the survival of aquacultured species (Kincaid, 1983). Besides, inbreeding depression arising from poor breeding practices often result in loss of heterozygosity of the breeding population, eventually causing constrictions in the gene pool.

Genetic variation of the natural population that had been introduced in the aquaculture industry needs to be assessed and managed. Variation is an important component of biodiversity and should be conserved for its intrinsic value (Ferguson *et al.*, 1995). The concept of the centre of diversity was introduced by the Russian geneticist, Nicolai I. Vavilov (Fairbanks and Andersen, 1999). This had led to the realisation of the importance of conserving genetic resources. The concept describes that a region of maximum variation, usually having a number of endemic forms and characteristics, can usually be considered as the centre of type-formation in the world. Indigenous fish species like *M. nemurus* has the most genetic diversity in Southeast Asia and needs to be conserved.

Conservation of natural populations of *M. nemurus* is essential to prevent the loss of genetic variation of the fish. Natural populations act as gene banks in nature. The genetic base of broodstock populations for the aquaculture industry must not be over exploited. By preserving the genetic diversity of natural populations, breeders will

always have the choice to select new broodstock populations that has superior quality for future selection and breeding programmes.

In fisheries, the use of DNA level markers for conservation and effective breeding programmes has gained considerable importance over the past decades. Polymorphisms reflect genetic variations and are detected at higher levels in the DNA when compared to other levels of markers such as isozymes and morphological markers. Of all the different DNA markers, microsatellites exhibit attributes that make them suitable for applications in aquaculture and fisheries research (O'Reilly and Wright, 1995). In some countries, genetic markers such as microsatellites have been widely used in fisheries management (Martin *et al.*, 1992; Ferguson *et al.*, 1995) to achieve efficient resource utilisation. This leads to the importance of identification and characterisation of population units by using genetic markers.

Broodstock management with the use of DNA level markers had not been widely realised and practised particularly in the Malaysian aquaculture industry. Most local farms still use the traditional approaches in breeding. In Malaysia, studies of local fish species through molecular markers are limited. For example, only limited studies on *M. nemurus* had been done at the DNA level (Chong *et al.*, 2000; Usmani, 2002). The studies that had been done were mainly at the protein level (Daud *et al.*, 1989; Patimah *et al.*, 1989; Siraj *et al.*, 1998). More studies on the fish population structure are therefore needed to give a better understanding of the species in order to encourage and establish more farms. Thus, it is essential to develop microsatellite markers for *M. nemurus* for more successful and effective breeding programmes.

Besides, microsatellite markers are applicable for careful monitoring of natural populations so as to ensure that the natural gene pool is conserved.

In this study, the objectives were:

1. to develop microsatellite markers for *M. nemurus*,
2. to characterise and evaluate the inheritance of these microsatellite markers from parents to offspring,
3. to employ these microsatellite markers for population study, and
4. to investigate the conservation of these microsatellites in closely related catfish species.

CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomy in suborder Siluroidei

2.1.1 *Mystus nemurus* (Cuvier and Valenciennes)

2.1.1.1 Taxonomy

The classification of "ikan Baung" is shown as below (Mohsin and Ambak, 1983):

Kingdom	: Animalia
Phylum	: Chordata
Superclass	: Pisces
Class	: Osteichthyes
Subclass	: Teleostomi
Superorder	: Ostariophysi
Order	: Cypriniformes
Suborder	: Siluroidei
Superfamily	: Bagroidae
Family	: Bagridae
Genus	: *Mystus*
Species	: *Mystus nemurus*

The catfishes possessed many generic names. It was first given the name *Mystus* by Gronow in 1763 and this was later validated by Scopoli in 1977. In the year 1856, Dumerill replaced *Mystus* with *Macrones*, which is still used in most books, and it is preoccupied in entomology. Several other names used before as synonyms for this fish are *Aoria*, *Hemibagrus*, *Hypselobagrus* and *Aspidobagrus* (Mohsin and Ambak, 1983).

Mystus nemurus, better known to the locals as "ikan baung", is one of the popular freshwater fish in Malaysia which belongs to the catfish group. This species gained its popularity due to the high nutritional value and good taste. "Ikan baung" or river catfish resembles the cat with four pairs of whiskers like barbels around the mouth (Figure 2.1). The barbels are divided into a pair of nasal barbels, which reached the eyes; a pair of maxillary barbels reaching to the far end of the anal fin; a pair of mandibulary barbels reaching the base of the pectorals and a pair of mental barbels which are shorter.

Its head morphologically looks broader than high with upper jaw slightly longer than lower jaw. It has a long or moderate adipose fin and a dorsal fin with a pungent spine that is serrated in its hind border. The pectoral fins also have pungent spine serrated behind. The caudal fin is deeply forked with the upper lobe more or less produced and pointed.

Mystus species is scaleless with size and colour variation occurs depending on the habitat of origin. The catfish found in Thailand are generally yellowish in colour and the ones that are found in Malaysia are grey or black. The body size is much larger in Thailand compared to Malaysia. These wide variations could be attributed to several factors such as environmental as well as genetic variation (Chong *et al.*, 2000).