UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF PCR-BAS ED DNA MARKERS TO IDENTIFY AND CHARACTERISE MALAYSIAN RIVER CATFISH, MYSTUS NEMURUS (C & V) : RAPD AND AFLP

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By

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Thesis submitted in fulfillment of the requirements for the degree of Master of Science in the Faculty of Science and Environmental Studies, Universiti Putra Malaysia

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LIST OF ABBREVIATIONS

Chemicals

EDTA	Ethylenediamine tetraacetate
KCl	Potassium chloride
$MgCl_2$	Magnesium chloride
SDS	Sodium dodecyl sulphate
TBE	Tris-borate-EDTA buffer

Units

bp	base pair
°C	Celsius
hr	hour
kb	kilobase
min	minute
mM	millimolar
ng	nanogram
nmole	nanomole
OD	optical density
pmole	picomole
sec	second
ug	microgram
ul	microliter
uM	micromolar
V	volt
w/v	weight / volume

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science.

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Chairman: Professor Tan Soon Guan, Ph.D.

Faculty: Science and Environmental Studies

The main objectives of this study are to evaluate the usefulness of a variety of recently developed DNA markers to identify and characterise different populations of Malaysian river catfish, *Mystus numerus*.

In the initial methodology development, 111 primers comprising of 40 single RAPD primers, 64 pairs of AFLP primers and 7 pairs of African catfish microsatellite primers were screened against *M. nemurus* samples. RAPD and AFLP primers gave positive results. Both techniques were also successfully used in examining the genetic diversity present in five populations of *M. nemurus* originating from Kedah, Perak, Johor, Sarawak and one UPM culture population.

However, none of the microsatellite primers developed for the African catfish was suitable for typing our local *M. nemurus*. Therefore, this technique was not used for the population studies and emphasis was only given to RAPD and AFLP markers in this thesis.

Nine RAPD primers and four AFLP primers detected a total of 42 and 158 polymorphic markers respectively. The modes of inheritance of the bands produced by four out of the nine RAPD primers and two out of the four AFLP primers were studied using family samples. The results showed that 9 of the RAPD markers and 24 of the AFLP markers used in population studies segregated as stable Mendelian loci while 2 RAPD markers and 13 AFLP markers showed unusual segregation. The rest of the markers could not be examined because no segregation was found in the families.

A correlation coefficient value of 0.69 between the RAPD and AFLP distance matrices indicated that the data generated by both methods are not similar but showed agreement to a certain extent. Both types of markers revealed high genetic diversities within the UPM and Sarawak populations. Both types of markers also showed a low level of genetic variability in the Kedah population. In addition, RAPD and AFLP showed small genetic distances among the UPM, Kedah and Perak populations. Based on the position of each population in the dendrogram, the

Sarawak population, which is located in East Malaysia, clustered by itself, thus isolated from the rest of the populations located in Peninsular Malaysia.

In comparison, the AFLP technique showed higher resolving power than RAPD. Individuals that could be discriminated from one another by AFLP could not be discriminated by RAPD. Three subgroups each from the Kedah, Perak and Sarawak populations were revealed by AFLP but not by RAPD.

Overall, AFLP would be advantageous over RAPD in investigating a large number of loci within a few genotypes in a minimal time while RAPD would be advantageous in rapid screening of a large number of genotypes since it is cheaper and less time is required for template DNA preparation.

The results of this study suggest that RAPD and AFLP analysis, if carried out carefully, give a good indication of the separation between individuals of different populations and are suitable for identification of closely related genotypes.

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PERKEMBANGAN PENANDA DNA YANG MENGGUNAKAN PCR UNTUK MENGECAM AND MENGENALPASTI IKAN BAUNG, *MYSTUS NUMERUS* (C & V) DI MALAYSIA: RAPD DAN AFLP

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Tujuan penyelidikan ini ialah untuk menilai kegunaan berbagai jenis penanda DNA terbaru yang menggunakan tindak balas rantaian polimerase (PCR) untuk mengecam dan mengenalpasti populasi-populasi ikan baung tempatan, *M. nemurus*.

Pada permulaan perkembangan methodologi, 111 primer yang terdiri daripada 40 primer tunggal RAPD, 64 pasang primer AFLP and 7 pasang primer mikrosatelit 'African catfish', diuji terhadap beberapa sampel *M. nemurus*. Primer RAPD and AFLP memberi keputusan yang positif. Kedua-dua teknik ini juga telah berjaya digunakan untuk memeriksa kepelbagaian gen dalam lima populasi baung tempatan yang berasal daripada Kedah, Perak, Johor, Sarawak dan juga satu

populasi kultur populasi UPM Walau bagaimanapun, tidak ada satu pun primer mikrosatelit yang diperiksa sesuai digunakan untuk baung tempatan kita. Oleh itu, teknik ini tidak digunakan dalam kajian populasi dan tumpuan hanya diberikan kepada penanda RAPD and AFLP dalam tesis ini

Sembilan primer RAPD dan empat primer AFLP masing-masing mengesan sejumlah 42 and 158 penanda polimorfik Keturunan empat daripada sembilan primer RAPD dan dua daripada empat primer AFLP dikaji dengan sampel-sampel sekeluarga. Keputusan menunjukkan 9 daripada penanda RAPD dan 24 daripada penanda AFLP yang digunakan dalam kajian populasi diasing secara Mendelian manakala 2 daripada penanda RAPD and 13 daripada penanda AFLP menunjukkan pengasingan luar biasa Penanda yang lain tidak dapat diperiksa keturunannya kerana tidak ada pengasingan yang ditunjukkan oleh sampel-sampel sekeluarga.

Perhubungan antara jarak genetik RAPD dan AFLP yang bernilai 0 69 menunjukkan data yang dihasilkan oleh kedua-dua teknik ini tidak seratus-peratus sama tetapi mematuhi antara satu sama lain dalam sesuatu tahap Kedua-dua jenis penanda mempamerkan kepelbagaian gen yang tinggi yang terkandung dalam populasi dari UPM and Sarawak Kedua-dua jenis penanda juga memberi variasi gen yang rendah di kalangan populasi dari Kedah. Tambahan pula, RAPD and AFLP menunjukkan nilai jarak genetik yang kecil antara populasi dari UPM, Kedah dan

Perak Berdasarkan kedudukan populasi-populasi dalam dendrogram, populasi daripada Sarawak, yang berasal dari Malaysia Timur, disingkan daripada populasi-populasi lain di Semenanjung Malaysia

Daripada perbandingan kedua-dua teknik tersebut, teknik AFLP menunjukkan daya beza jelas yang lebih tinggi daripada RAPD Individual ikan baung yang dapat disingkan antara satu sama lain oleh petanda AFLP tidak dapat disingkan oleh penanda RAPD Tiga sub-kumpulan yang setiap satunya daripada populasi Kedah, Perak dan Sarawak juga dapat dipamerkan oleh AFLP tetapi tidak dapat dipamerkan oleh RAPD

Pada keseluruhannya, AFLP adalah lebih berfaedah daripada RAPD untuk menyisat banyak lokus yang terkandung dalam beberapa genotip dalam masa yang singkat Sebaliknya, RAPD adalah lebih berfaedah untuk menyisat sekumpulan besar genotip kerana teknik ini lebih murah dan kurang masa yang diperlukan untuk menyediakan templat DNA

Keputusan kajian ini menunjukkan bahawa analisis RAPD dan AFLP, jika dijalankan dengan berhati-hati, dapat memberikan keputusan yang baik dalam mengecam dan mengenalpasti sampel daripada populasi-populasi yang berbeza Ia juga sesuai untuk mengecam genotip yang mempunyai perhubungan yang rapat

CHAPTER I

INTRODUCTION

This project is part of the research program entitled '*Population Genetics in Management and Conservation of Aquatic Resources and DNA fingerprinting in fishes*' headed by Prof Dr Tan Soon Guan of UPM and funded by the Malaysian Ministry of Science, Technology and Environment under the IRPA (Intensification of Research in Priority Areas) scheme

Identification and characterisation of population units are imperative for fisheries management because efficient resource utilisation can best be achieved when managing at the population level In some countries, genetic markers have been widely used in fisheries management (Martin *et al* , 1992, Ferguson *et al* , 1995) but this approach is still new in Malaysia Not much work has been done on the genetic characterisation of local fish species through the use of molecular markers Also, the number of loci used in such studies were small and mainly on protein electrophoresis (Daud *et al* , 1989, Patimah *et al* , 1989, Siraj *et al* , 1998) The possible neglect of genetic diversity in fisheries management decisions might have been due to the lack of tools for the determination of genetic variation and lack of expertise in this field of study Therefore emphasis had been placed on environmentally influenced characters such as morphological characters instead of molecular genetic markers for evaluating variation among populations As such, it

is hoped that the findings of this research will provide a better understanding of our local aquatic resources with respect to genetic diversity so as to improve fisheries management.

The fish species that was studied here is the local river catfish, ikan baung with a scientific name of *Mystus numerus*. It is a freshwater fish that can be found in the wild. Commercialisation as well as farm-raising of this fish are still not widely established yet compared to the other fishes such as tilapia mainly because of the limited research data on this species. Little is known about the genetic background of the natural populations and the cultured stocks.

In previous isozyme studies of local *Mystus numerus*, only 4 polymorphic loci out of 17 loci and 16 polymorphic loci out of 24 loci were found by Tay (1997) and Siraj *et al.* (1998) respectively. Therefore, more effective techniques with respect to the ease of detecting genetic polymorphisms are required to support the existing isozyme results as well as to provide new information which is not found in the isozyme work. Hence, in our current studies, attempts were made to apply different types of PCR-based DNA markers to identify and characterise five populations of local *Mystus numerus*. These populations originated from Kedah, Perak, Johor and Sarawak state representing the wild groups, and one UPM cultured population whose parents were purchased from a fish farm in Selangor state.

The scopes of this study are divided into three parts:

(i) methodology development,

(ii) Mendelian inheritance study, and

(iii) population study.

In the first part of the study, 111 primers representing three types of PCR-based methods were tested. They are random amplified polymorphic DNA (RAPD, 40 single primers), amplified fragment length polymorphism (AFLP, 64 pairs of primer combinations), and microsatellites (7 pairs of African catfish, *Clarias gariepinus* primers,). Unfortunately, only RAPD and AFLP primers gave positive results while none of the microsatellite primers produced any true microsatellite fragments. Because of this, the microsatellite technique could not be applied in the subsequent segregation and population studies. Therefore, emphasis is only given for RAPD and AFLP analyses in this thesis.

The primers that gave positive results in the first part of the study will then be used to screen against the families of *M. nemurus* in order to evaluate the inheritance of the DNA markers from the parents to the offspring. This study will determine the usefulness of the primers in the breeding program.

In the third part of the study, five populations of *M. nemurus* provided by Unit Hatchery of UPM were used to evaluate the utility of the methods developed above in identifying and characterising different populations of *M. nemurus*.

Based on the scope of the study, the objectives of this project could be summarised as follows

(i) to develop DNA marker typing methods for *M. nemurus*,

(ii) to evaluate the inheritance of the DNA markers from the parents to the offspring,

(iii) to investigate the population structures of *M. nemurus* inhabiting five different geographical regions in Malaysia using the markers developed above

(iv) to identify specific gene markers (diagnostic markers) that would characterise the different *M. nemurus* populations, and

(v) to compare the utility as well as the data generated by different DNA markers

It is hoped that this thesis will provide some valuable information for future studies In addition, the population genetic data presented here together with the existing isozyme data could provide some sort of guidelines in the choice of stocks for breeding purposes

CHAPTER II

LITERATURE REVIEW

Mystus Species

Habitat and Distribution

Mystus is widely distributed in the East Indies (now Indonesia), and also in the Asian mainland such as Peninsular Malaysia, Indo-China, and Thailand (Smith, 1945). This species is also found in North Borneo (now Sabah) (Inger and Chin, 1962). It is normally found in rivers, swamps, lakes and other forms of water bodies. Inger (1955) reported that some specimens were found in the tidal zones at river mouths where it may actually live from the brackish water to the head waters of most water basins.

In Malaysia, it can be found in most of the states in Peninsular Malaysia such as Pahang, Selangor, Perak, Terengganu (Mohsin and Ambak, 1983) as well as in Sabah and Sarawak. It is locally known as 'ikan baung'.

Taxonomic Classification

The catfish is classified under the following (Smith, 1945; Inger and Chin, 1962; Mohsin and Ambak, 1983):

Phylum *Chordata*

Superclass *Pisces*

Class *Osteichthyes*

Subclass *Teleostomi*

Order *Cypriniformes*

Family *Bagridae*

Genus *Mystus*

Species *Mystus baramensis*
Mystus nigriceps
Mystus nemurus
Mystus wyckii
Mystus vittatus

The catfishes have borne with many generic names The earliest available name is *Mystus*, which was first used by Gronow in 1763 and was validated by Scopoli in 1977 Dumerill in 1856 replaced *Mystus* with *Macrones* which is still in use in most of the books The name *Macrones* is preoccupied in entomology Several other names i e *Aoria, Hemibagrus, Hypselobagrus and Aspidobagrus* are also used as synonyms for this fish (Mohsin and Ambak, 1983)

Morphology

River catfish or baung as it is locally known has cat-like small eyes with whisker-like barbels around the mouth somewhat resembling the cat, hence it has been given the name river catfish (Kamarudin *et al.*, 1987) Its head is broader than the high, and the upper jaw is slightly longer It has four pairs of barbels, nasal

barbels reaching the eyes, maxillary barbels reaching to the far end of the anal fin, mandibulary barbels reaching the the base of pectorals and mental barbels are shorter. It has a long or moderate adipose fin. The dorsal fin has a pungent spine which is serrated in its hind border and the pectoral fins have pungent spines which is serrated behind. The caudal fin is deeply forked with the upper lobe more or less produced and pointed. The body is scaleless and brown or black in colour (Mohsin and Ambak, 1983).

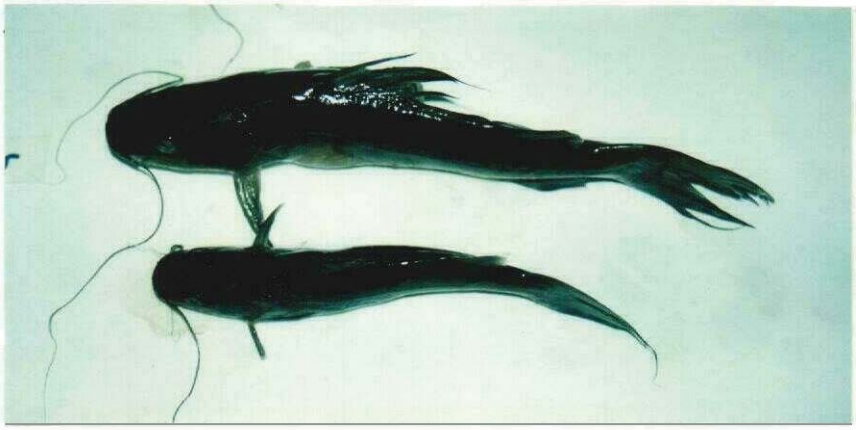

Figure 1: The morphology of *M. nemurus*

Importance

The river catfish is an economically important food that has gained popularity among consumers due to its high nutritional value and good taste. Three common Malaysian freshwater catfish namely *M. nemurus*, *C. batrachus* and *C. Macrocephalus* have higher average percentage of the lean portion than beef and pork (Kamarudin *et al*., 1987). Besides that, they contain the highest crude protein

and the lowest crude fat compared to beef, pork and chicken (Kamarudin *et al.*, 1987).

The river catfish are unable to reproduce in captivity and their seasonal seed availability cause them to achieve high prices in the market (Siraj *et al.*, 1995). The price of one kilogram of catfish is between RM10 to RM15 three years ago (Saberi Mawi from Pusat Penyelidikan Perikanan air Tawar, 1995). Recently, the price had been increased to RM18/kg. Due to its high potential in the market, the river catfish had gained popularity among fish aquaculturist and research had been carried out pertaining to reproductive biology (Khan *et al.*, 1990), diet (Khan *et al.*, 1993, 1994), nutrition (Kamarudin *et al.*, 1987) and recently genetics (Daud *et al.*, 1989; Dodson, 1995; Siraj *et al.*, 1995; Tay, 1997).

Molecular Techniques

Molecular techniques allowing differentiation between strains, genotypes, or individuals have become indispensable tools in population genetic survey. Several molecular techniques are available, but these differ in their ease of use and their power to resolve genetic differences and may sometimes produce contradictory results (Martivez *et al.*, 1997). For example, enzyme electrophoresis indicated two different breeding populations of minke whales from West Greenland and Iceland (Danielsdottir *et al.*, 1992), while restriction fragment analysis of mitochondrial and ribosomal DNAs did not reveal any significant differences (Palsboll, 1989).

Before the introduction of the polymerase chain reaction (PCR) technology, protein electrophoresis and classical hybridization-based technique using RFLP had been widely used in detecting polymorphic genetic markers (Ladizinsky *et al.*, 1984; Apuya *et al.*, 1988; Theilmann *et al.*, 1989). However, since the introduction of PCR (Saiki, 1988), a series of increasingly effective PCR-based techniques with respect to ease of detecting genetic polymorphisms have been developed. In general, these techniques used one or two small primers to amplify a region of DNA flanked by them. The amplification takes place in a thermocycler and is mediated by a thermostable DNA polymerase. In principle, if primers which flank a polymorphic region of the genome are available, then this region can be amplified and polymorphism can be scored by the presence versus absence of the amplified products as observed from gel electrophoresis. These polymorphisms are the result of point mutations or rearrangements in the DNA such as insertions or deletions. As the variations in the banding patterns would be a direct reflection of the genetic relationship between the organisms examined, these banding patterns can be considered as `genomic fingerprints` allowing numerical analysis for characterization (typing) and identification purposes (Janssen *et al.*, 1996).

In general, there are two types of PCR-based molecular techniques:

(i) ***PCR with arbitrary primers*** such as RAPD (Random Amplificd Polymorphic DNA; Williams *et al* , 1990), AP-PCR (Arbitary Primed PCR; Welsh and McClelland, 1990), DAF (DNA Amplification Fingerprinting; Caetano-Anolls *et*