UNIVERSITI PUTRA MALAYSIA

ISOLATION, CHARACTERIZATION AND APPLICATION OF MICRO SATELLITE MARKERS IN THE SOUTHEAST ASIAN RIVER CATFISH (BAUNG) MYSTUS NEMURUS (C & V)

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By

SAHAR USMANI

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Chairman: **Prof. Dr. Tan Soon Guan**

Faculty: **Science and Environmental Studies**

In Malaysia 70% of the dietary protein comes from fish. The popularity of the river catfish *Mystus nemurus* throughout Malaysia and also Southeast Asia has made it an important aquaculture candidate. Among the vast choice of molecular markers available to carry out a population study including stock identification and improvement, microsatellites have proven to be the most efficient.

The main objectives of this study were: (1) to develop efficient microsatellite isolation protocols for *Mystus nemurus* followed by a population study using the

newly developed microsatellite markers and (2) to test two new techniques, direct amplification of length polymorphism (DALP) and random hybridising microsatellites (RAHM), as potential DNA fingerprinting markers for population studies.

Microsatellites were isolated using new techniques, which proved superior to the conventional method of microsatellite isolation. Three methodologies were developed for the isolation of microsatellites in *M. nemurus* namely direct amplification of length polymorphism (DALP), random hybridising microsatellites (RAHM) and randomly amplified microsatellites (RAM). A total of 176 microsatellites were isolated with the maximum number being isolated using RAM. For RAM, once the PCR and cloning conditions have been optimised, several microsatellite sequences can be isolated in a short time frame of only 2 weeks compared to the more conventional approaches which take an average time of 5 to 6 weeks. Another major advantage offered by RAM is that with specific primers being used a genomic library enriched for only a certain class of microsatellite (di, tri or tetranucleotide) can be easily constructed.

Primers were developed for 78 microsatellite loci. These are the first set of microsatellite markers developed for *M. nemurus*. Samples from three family were used in a model system to test the inheritance patterns of 20 microsatellite markers. All the microsatellite loci tested in the family study segregated with the expected Mendelian ratios. This demonstrated the suitability of microsatellite markers for use in population genetic studies.

Twenty polymorphic microsatellite loci were used as markers for population characterization of *M. nemurus* from six different geographical locations in Malaysia (Perak, Kedah, Johor, UPM, Sarawak and Terengganu). The number of alleles per locus ranged from 2 to 11 with 6.3 as the average number of alleles per locus. Characterization of the populations showed relatively high levels of genetic variation compared to previous studies using allozymes markers. The highest genetic similarity was found between Perak and Kedah, while the highest genetic distance was found between Terengganu and Kedah. The majority of the clustering was in accordance with the geographical locations and the histories of the populations. Microsatellite analysis demonstrated that the Sarawak population might be genetically closer to the Peninsular Malaysian populations than has been previously indicated by other molecular marker studies.

DALP and RAHM were also used to conduct population studies of three populations of *M. nemurus* and both techniques proved to be effective marker systems. DALP generated 13 codominant loci and RAHM revealed 28 polymorphic zones. The clustering analysis based on RAHM was in agreement with the geographical locations of the populations.

The microsatellite markers developed under this study will be very useful for carrying out an informed breeding programme. The production of *M. nemurus* can be planned to be sustainable and this could help ensure that the natural gene pool is conserved.

Key words: M. nemurus, DALP, RAHM, RAM, microsatellites and population study.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai keperluan ijazah Doktor Falsafah

PEMENCILAN, PENCIRIAN DAN PENGGUNAAN PENANDA MIKROSATELIT DALAM IKAN DEDURI (BAUNG) SUNGAI ASIA TENGGARA, *MYSTUS NEMURUS* (C & V)

Oleh

SAHAR USMANI

June 2002

Pengarusi: **Prof. Dr. Tan Soon Guan**

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Di Malaysia 70% sumber protein diet datangnya daripada ikan. Kemasyhuran ikan baung *Mystus nemurus* di Malaysia telah menjadikannya sebagai calon penting untuk akukultur. Diantara pelbagai pilihan penanda molekul yang sedia ada untuk kajian populasi termasuk pengenalpastian stok dan pembaikan, mikrosatelit terbukti yang paling berkesan.

Objektif utama kajian adalah: (1) membangun kaedah pemencilan mikrosatelit yang paling berkesan untuk *Mystus nemurus* disusuli dengan kajian populasi menggunakan penanda mikrosatelit yang dihasilkan. (2) menguji dua kaedah baru

iaitu *direct amplification of length polymorphisms (DALP)* dan *random hybridising microsatellites (RAHM)* sebagai penanda capjari DNA berpotensi dalam sesuatu kajian populasi.

Mikrosatelit telah dipencilkan menggunakan teknik baru yang jauh lebih berkesan berbanding dengan kaedah pemencilan mikrosatelit konvensional. Tiga kaedah telah dibangunkan untuk memencil mikrosatelit untuk *M. nemurus* iaitu DALP, RAHM dan RAM. Sejumlah 176 mikrosatellit telah dipencilkan. Bilangan maksimum mikrosatelit telah dipencilkan melalui RAM. Dengan kaedah RAM, apabila keadaan PCR dan pengklonan telah dioptimumkan, beberapa jujukan mikrosatelit dapat dipencilkan dalam jangka masa yang singkat iaitu 2 minggu berbanding dengan kaedah konvensional yang mengambil masa 5 ke 6 minggu. Satu lagi kebaikan RAM ialah dengan menggunakan primer khusus, satu perpustakaan genomik yang diperkaya untuk sesuatu kelas mikrosatelit (di, tri, atau tetranukleotida) dapat dihasilkan. Primer telah direka untuk 78 lokus mikrosatelit. Ini merupakan set penanda mikrosatelilt yang pertama sekali di hasilkan untuk spesies *M. nemurus*. Tiga sampel keluarga telah digunakan dalam sistem model untuk mengkaji corak warisan 20 penanda mikrosatelit. Kesemua lokus mikrosatelit dalam kajian keluarga mengasing mengikut nisbah Mendel yang dijangka. Ini menunjukkan kesesuaian penggunaan penanda mikrosatelit ini dalam kajian populasi genetik.

Dua puluh lokus mikrosatelit ini telah digunakan sebagai penanda untuk kajian populasi *M. nemurus* dari enam lokasi geografi yang berbeza di Malaysia. (Perak, Kedah, Johor, UPM, Sarawak and Terengganu). Bilangan alel setiap lokus berjulat

daripada 2 hingga 11 dengan purata bilangan alel setiap lokus sebanyak 6.3. Pencirian populasi menunjukkan kadar variasi genetik yang tertinggi berbanding kajian sebelumnya yang menggunakan penanda alozim. Persamaan genetik yang paling tinggi didapati antara Perak dan Kedah manakala perbezaan genetik yang paling tinggi adalah antara di Terengganu dan Kedah. Kebanyakkan kelompokkan adalah bersesuaian dengan lokasi geografi dan sejarah populasi tersebut. Analisis mikrosatelit menunjukkan populasi Sarawak mungkin lebih dekat dengan populasi dari Semenanjung Malaysia daripada yang dijangka sebelum ini melalui kajian penanda molekul yang lain.

DALP dan RAHM juga telah digunakan dalam kajian populasi untuk tiga populasi *M. nemurus* dan kedua-kedua teknik ini didapati berkesan sebagai sistem penanda. DALP menghasilkan 13 lokus kodominan dan RAHM 26 zon polimorfik. Analisis kelompok berdasarkan RAHM menyetujui kedudukan geografi populasi tersebut.

Penanda mikrosatelit yang dihasilkan dalam kajian ini amat penting untuk menghasilkan program pembiakan yang teratur. Pengeluaran *M. nemurus* akan terjamin dan seterusnya dapat membantu pemuliharaan takungan gen alami.

Kata kunci: M. nemurus, DALP, RAHM, RAM, microsatelit dan kajian populasi.

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I certify that an Examination Committee met on 17th June, 2002 to conduct the final examination of Sahar Usmani on her Doctor of Philosophy thesis entitled "Isolation, Characterization and Application of Microsatellite Markers in the Southeast Asian River Catfish (Baung) *Mystus nemurus* (C & V)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

Sahar Usmani.

SAHAR USMANI

Date: June 28, '02

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ABBREVIATIONS

α	alpha
ATP	adenosine triphosphate
β	beta
bp	basepairs
Ci	Curie
dH_2O	distilled water
ddH_2O	double distilled water
deH_2O	deionized water
dNTP	deoxyribonucleotide
kb	kilobase
λ	lambda
mCi	milli curie
mM	millimolar
ng	nanogram
nmole	nanomole
OD	optical density
pm	picomole
μg	microgram
μl	microliter
μM	micromolar
V	volts
w/v	weight/volume
xg	centrifugal force

CHAPTER 1

INTRODUCTION

The worlds population crossed the 6 billion mark in the year 2000, an increase of 25% within a decade, representing a greater strain on food resources than ever before. As for other resources, the increase in the consumption of fish and seafood is leading to greater exploitation of the natural resources taking the yields from traditional marine and inland capture fisheries to dwindling levels. The need for new solutions for sustainable food production has assumed significant urgency.

In several countries, fish account for more than 50% of the dietary protein intake (FAO, 1997). Reports from the United Nations indicate that to meet the world's requirement for fish by 2020, the current supply of seafood products will have to increase by seven folds. These shortfalls in aquatic supplies need to be met from aquaculture (FAO, 1997). Even though many species are farmed through aquaculture, more species need to be introduced to meet the ever increasing demand for fish. If the exploitation of the natural populations is not checked, it will result in a complete loss of several species. It is thus of paramount importance that good and sustainable aquaculture practices be expanded and encouraged.

Malaysia has drawn up an aquaculture development plan for the period of 1996 – 2010 to meet the aspirations set out in its national aquaculture policy (NACA, 1996). The plan outlines strategies to develop commercial aquaculture in a sustainable

manner and to increase the current annual production of 133,000 mt to 350,000 mt by the year 2010.

Mystus nemurus is an indigenous, fresh water fish found in the rivers and lakes of Southeast Asia. The good meat quality makes it a favourite food among locals in Malaysia and other countries of Southeast Asia. It has high crude protein content and is low in crude fat (Kamarudin *et al.*, 1987). Most of the fish for the market comes from the rivers of various states in Malaysia. Extensive fishing at the commercial level will result in a decline of the natural fish population. *M. nemurus* has thus been recognized as an important species for aquaculture.

An increased and efficient aquaculture production is based on several factors. One of the most important factors is to endow the farmer with knowledge on the genetics of the brood stock.

As is with most terrestrial breeding programmes the ultimate goal of aquaculture is to improve or alter the economically important quantitative traits such as colour, protein content, body mass and disease resistance and to do it in a predetermined direction. For any selective breeding programme to be successful, it is imperative to maintain the genetic variation within and between the stock populations. Variation is an important component of biodiversity and should be conserved for its intrinsic value (Ferguson, *et al.,* 1995). Poor breeding practices where only a small number of superior individuals are selected for breeding or the continued use of a small broodstock population can result in the loss of heterozygosity of the breeding

population, eventually causing constriction in the gene pool (Kincaid, 1983) and giving rise to inbreeding depression. Therefore, it is important to assess the level of genetic distance between populations or know the level of heterozygosity of a population for each generation.

Long evolutionary processes lead to genetic variation between populations, breeds, strains and even species, while genetic variation within a population arises due to genetic mutation. Genetic variation in a natural population is maintained through random mating. Once the natural genetic variation is lost, it is impossible to recover it and this loss can prove to be deleterious. It can result in the overall extinction of the population or species.

Genetic variation can be assessed at different levels. The most basic is looking at morphological characters followed by detecting variation at the protein and DNA level. In fisheries, the use of molecular genetic techniques has increased dramatically over the past several years (Park and Moran, 1994). For the past two decades or more, fish geneticists have been using isozymes (protein level markers) as their primary tool to characterize population level genetic variation in various fish species (Park and Moran, 1994). DNA level markers have also gained considerable importance since the discovery of multi-locus DNA fingerprinting by Jeffreys *et al.* (1985). Polymorphisms detected at the DNA level are higher and give more information. Several types of DNA markers have gained wide acceptance in fisheries for several applications from species and hybrid identification to assessing the success of genomic application (Ferguson *et al.*, 1995). Of all the different DNA

markers, allozyme, mtDNA and minisatellites, microsatellites exhibit attributes that make them particularly suitable for numerous applications in aquaculture and fisheries research (O'Reilly and Wright, 1995). They have the power to solve a number of biological problems from basic population studies to QTL mapping. Because of their abundance in most genomes, they are now being used to construct dense linkage maps.

In Malaysia and most of Southeast Asia, 70% of the proteins come from fish. Fish constitutes a major portion of the Malaysian diet. With the increasing demand of *M. nemurus*, it is advisable to increase the production through the aquaculture industry rather than providing for the market through wild catches.

The concept of broodstock management with the use of molecular markers has not been widely realized and has not been put to much practical use. Most farms use approaches that are more traditional. Till today, very little genetic information is available for this commercially viable species. With the ever growing awareness of the success of molecular markers in breeding programmes, it becomes essential to develop powerful genetic markers for *M. nemurus*. As it is an indigenous species, conservation issues also need to be addressed. Careful monitoring of the natural populations is required to ensure that the natural gene pool is not compromised by addition of inbred or selected broodstocks from catfish farms.

It has been proven over time that microsatellites are best used to help resolve such problems. Microsatellite markers can help determine if the cultured population is