UNIVERSITI PUTRA MALAYSIA

ISOLATION, CHARACTERIZATION AND GENETIC LINKAGE MAPPING OF MICROSATELLITE MARKERS IN MYSTUS NEMURUS

HOH BOON PENG.

ISOLATION, CHARACTERIZATION AND GENETIC LINKAGE MAPPING OF MICROSATELLITE MARKERS IN *Mystus nemurus*

By

HOH BOON PENG

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

December 2005

To my dearest wife……

YAN LI

Abstract of thesis presented to the Senate of University Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

ISOLATION, CHARACTERIZATION AND GENETIC LINKAGE MAPPING OF MICROSATELLITE MARKERS IN *Mystus nemurus*

By

Hoh Boon Peng

July 2005

Chairperson: Associate Professor Siti Shapor Siraj, PhD

Faculty: Science

The river catfish (*Mystus nemurus*) is of great potential importance as an alternative fish protein source, notably in Malaysia and Thailand. The seed supply is seasonal and inability to reproduce in captivity is a hindrance to mass produce this species. Molecular breeding and genetic mapping with the use of DNA based markers have paved ways in overcoming conventional breeding to improve important economic traits.

The main objectives of this study were to isolate and develop microsatellite markers for *Mystus nemurus*, and apply these markers on genetic linkage map for this species. Two methodologies were used to isolate the microsatellite loci in *M. nemurus*, namely Random Amplified Hybridizing Microsatellites (RAHMs) and 5' anchored Polymerase Chain Reaction (5' anchored PCR). A total of 236 repeat sequences were identified. Of these, 13 were developed from RAHMs and 223 were developed from 5' anchored PCR.

A total of 177 primer pairs were developed. Of these 64 have been screened for polymorphisms on 90 unrelated fishes collected from six locations (Kedah, Selangor, Perak, Johor, Terengganu and Sarawak), where each location consisted of 15 individuals. Forty four primer pairs were found to be polymorphic. The highest heterozygous marker was MnBp5-1-12a (0.8714) while the lowest heterozygosity was observed in MnBp8-1-25a (0.0000). A dendrogram was constructed from the 44 polymorphic loci. An additional 34 polymorphic markers, previously generated, were combined in order to construct another dendrogram, and it was found that the dendrogram stabilized at 50 polymorphic microsatellite loci.

Induced breeding was carried out to generate two known fish family groupings. The first family grouping was generated from the parents chosen randomly from the Terengganu broodstock. One hundred progeny were produced. The second family grouping was generated from the male chosen from Terengganu population and the female chosen from Pahang population. Fifty progenies were used for the analysis of Mendelian segregation and linkage mapping.

A total of 70 microsatellite markers isolated for this species were screened and evaluated for the Mendelian Inheritance ratio on the two fish families, mentioned of which 31 showed segregation in either one of the families. These markers were analyzed with contingency chi-square analysis. Of these, 13 pair markers were grouped into 8 groups. Strategy "Pseudo-testcross" was applied in the linkage mapping analysis. Analysis of linkage using LOD score was carried out and a total of 7 linkage groups

were generated from the 2 fish families under the LOD of 1.2. Additional analysis of LOD was performed on the family data of 44 dominant markers done previously. Three linkage groups were obtained at the LOD of 1.2 and fractionated into 6 groups when the LOD score was increased to 2.0.

This is the first map generated for *M. nemurus* which provides information on genetic linkage for this species. It is a stepping-stone for carrying out more intensive genetic mapping and QTL analysis on this species in the future. Eventually, marker assisted selection (MAS) or isolation of economically important genes could be made possible and these would benefit the Malaysian aquaculture industry

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Falsafah Kedoktoran

PEMENCILAN, PEMBANGUNAN, DAN ANALISIS PEMETAAN PENANDA MIKROSATELIT UNTUK *Mystus nemurus*

Oleh

HOH BOON PENG

Julai 2005

Pengerusi: **Professor Madya Siti Shapor Siraj, PhD**

Fakulti: **Sains**

Ikan baung (*Mystus nemurus*) adalah penting sebagai sumber protein ikan alternatif, terutamanya di Malaysia dan Thailand. Bekalan benih ikan adalah bermusim dan ketidakeupayaan spesies ini membiak dalam kurungan menyebabkan pengeluaran spesies ini tidak dapat dihasilkan secara besar-bsearan. Pembiak kacukan molekul dan pemetaan genetik dengan penggunaan penanda genetik berasaskan DNA membuka jalan bagi memperbaiki kelemahan perbiakbakaan konvensional untuk mendapatkan trait-trait ekonomi yang lebih baik.

Objektif utama kajian ini ialah untuk memencilkan dan membangunkan penanda mikrosatelit untuk *M. nemurus*, dan mengaplikasikan penanda-penanda ini dalam analisis pemetaan genetik untuk spesies ini. Dua cara telah digunakan untuk memencilkan lokus mikrosatelit bagi *M. nemurus*, iaitu "Random Amplified Hybridization Microsatellites (RAHMs)" dan "5' anchored PCR". Sejumlah 236

jujukan mikrosatelit telah dikenalpasti. Daripada jumlah itu, 13 dapat dipencilkan daripada RAHMs manakala 223 dipencilkan daripada 5' anchored PCR.

Sebanyak 177 pasang primer spesifik telah direka, 64 daripadanya telah digunakan dalam penentuan polimorfisme ke atas 90 ikan yang disampel dari 6 lokasi (Kedah, Selangor, Perak, Johor, Terengganu dan Sarawak), di mana setiap lokasi mengandungi 15 individu ikan. Didapati 44 pasangan primer adalah polimorfik. Penanda yang mempunyai heterozigositi tertinggi ialah MnBp5-1-12a (0.8714), manakala heterozigositi yang terendah ialah MnBP8-1-25a (0.0000). Dendrogram dibina berasaskan kepada 44 lokus polimorfik tersebut. Penambahan 34 penanda polimorfik yang dibangunkan terdahulu digabungkan untuk membina satu lagi dendrogam. Didapati bahawa dendrogram ini adalah stabil pada jumlah lokus mikrosatelit polimorfik 50.

Pembiakbakaan aruhan dilakukan untuk menghasilkan dua kumpulan famili ikan. Kumpulan pertama dihasilkan daripada induk yang dipilih secara rawak dari stok Terengganu. Sebanyak 100 progeni ikan dihasilkan. Untuk kumpulan kedua, induk jantan dipilih dari stok Terengganu dan induk betina dipilih dari stok Pahang. Sebanyak 50 progeni telah digunakan untuk analisis segregasi Mendel dan pemetaan genetik.

Sejumlah 70 penanda mikrosatelit yang dipencilkan khusus untuk spesies ini telah disaring dan dinilai untuk Nisbah Pewarisan Mendel terhadap kumpulan dua famili ikan, yang mana 31 penanda menunjukkan segregasi dalam salah satu daripada

kumpulan famili tersebut. Penanda ini dianalisis dengan Analisis Kontengensi Khi-kuasa dua. Daripada ini, 13 pasang penanda dikumpulkan kepada lapan kumpulan. Strategi "Pseudo-testcross" diaplikasikan untuk analisis pemetaan rangkaian. Analisis rangkaian pemetaan dengan menggunakan skor LOD telah dilakukan dan sejumlah tujuh kumpulan rangkaian telah dihasilkan daripada dua kumpulan famili ikan apabila skor LOD ditentukan pada 1.2. Analisis tambahan LOD dilakukan terhadap data yang diperolehi daripada 44 penanda dominan yang telah dikaji sebelum ini. Tiga kumpulan rangkaian telah diperolehi pada LOD 1.2. Ini adalah peta genetik pertama bagi M. nemurus yang memberi informasi tentang rangkaian genetik untuk spesis ini. Kajian ini merupakan batu loncatan untuk menghasilkan pemetaan genetik yang lebih intesif dan analisis QTL bagi spesies ini pada masa hadapan. Akhirnya, Pemilihan Berbantukan Penanda (MAS) atau pemencilan gen yang berkepentingan ekonomi mungkin boleh direalisasikan dan ini akan menguntungkan industri akuakultur Malaysia.

ACKNOWLEDGMENT

I would like to thank my supervisors Assoc. Prof. Dr. Siti Shapor Siraj, Prof. Dr. Tan Soon Guan and Prof. Dr. Datin Khatijah Yusoff for their continuous support, direction, cooperation and interest in my research and their comments and suggestions on many aspects of this work.

I gratefully acknowledge the financial support from the National BioDirectorate grant 01-02-04-0074, the Ministry of Science, Technology and Innovative Malaysia, headed by Assoc. Prof. Dr. Siti Shapor Siraj. I am especially indebted to her for suggesting the problem and guiding me throughout the entire period of this project and for giving me the perfect environment to work in.

To Dr. Chong Lee Kim, thank you for providing me and sharing the data and made my research successful.

My thanks also goes to my fellow laboratory members, Dr. Sahar Usmani who is now in Yale University, USA and Mr. Chan Soon Choy, Miss Siti Nor Hajjar, Mr. The Chee Hong and Miss Ong Ching Ching, for their advice, friendship, helps and sharing. They had made my stay in the laboratory a wonderful experience. To Dr. Vijaya Kumar who is now in Universiti Sabah Malaysia, I specially appreciate for his comments and his help in using the PHYLIP analysis software. Additional thanks to Mr. Azmi Yaakub and Mr. Lee Kok Kuan for helping me with the fish breeding part of my research. To

Dr. Subha Bhassu, I appreciate for her advice and comments given to me throughout my entire research life.

Thanks also goes to my students, Chew Wee Chee, Norwahidayanty Kenasipan, Tham Poh Theam, and Khoo Chin Fui, who made teaching an exciting experience for me.

To Dad, Mum and my younger sister, Wei Nee, without their emontional support, love, and confidence, and trust in me I would never have been gone through all the hard times.

Last but not least, to my beloved wife, Yan Li, who had never complained for all the lonely minutes and seconds I had made her gone thru during my entire research life. Thank you for being so understanding and trust to me.

HOH BOON PENG

I certify that an Examination Committee met on 30th December 2005 to conduct the final examination of Hoh Boon Peng on his Doctor of Philosophy thesis entitled "Isolation, Characterization and Genetic Linkage Mapping of Microsatellite Markers in *Mystus nemurus*" 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:

Aziz Arshad, PhD
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Chairman)

Jothi M. Panandam, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Siti Khalijah Daud, PhD
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Internal Examiner)

Mahani Mansor Cktde, PhD
Professor
Department of Science and Technology
Universiti Kebangsaan Malaysia
(External Examiner)

HASANAH MOHD. GHAZALI, PhD
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

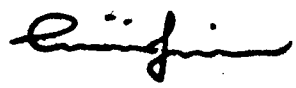
Date: 27 MAR 2006

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. Members of the supervisory committee are:

Siti Shapor Siraj, PhD
Associate Professor
Faculty of Science
Universi Putra Malaysia
(Chairperson)

Tan Soon Guan, PhD
Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

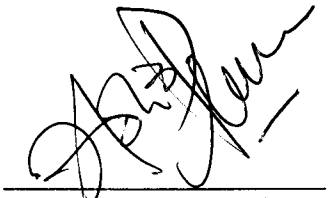
Khatijah Yusoff, PhD
Professor
Faculty of Biotechology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)



AINI IDRIS
Professor/ Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 13 APR 2006

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.



HOH BOON PENG

Date: **30 December 2005**

Table of Contents

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

α	alpha
AFLP	Amplified Fragment Length Polymorhism
ATP	adenosie triphosphate
β	beta
bp	base pairs
Ci	Curie
dH2O	distilled water
ddH2O	deionzed distilled water
dNTPs	deoxyribonucleotide
g	gravity
kb	kilobase
λ	Lambda
LOD	Logarithm of odds
LB	Luria Bertani
μ	micro
μg	microgram
μl	microliter
μM	micromolar
MAS	Marker Assisted Selection
mM	milimolar
mtDNA	mitochondira DNA
ng	nanogram

nmol	nanomole
OD	optical density
PCR	Polymerase Chain Reaction
ρmol	picomole
QTL	QuantitativeTrait Loci
RAHMs	Random Amplified Hybridizing Microsatellites
RAPD	Random Amplified Polymorphic DNA
rpm	revolution per minute
UV	ultra violet
V	volts

CHAPTER 1

INTRODUCTION

It has been estimated that the world's population will approach seven billion by the year 2010. Thus, the worldwide demands for food will also increase and will therefore, cause a shortfall of 19 million tonnes of fish and aquaculture productions (FAO, 1997). Consequently, the deficit in seafood production of the world is expected to increase.

One way of increasing the seafood supply is to increase marine fishing activities. However, the world's fish stocks are now struggling from overfishing. Almost two-thirds of marine fishes and other edible aquatic organisms from the Pacific and Atlantic Oceans had been fully exploited (reviewed by Liu and Dunham, 1998). The gap between the world's demand for fish and the ability of the ocean to provide sufficient food has widened. The solution to this problem lies in the development of sustainable aquaculture and hence, the introduction of more aquaculture species is an immediate necessity.

The river catfish, or Asian Red-tailed Catfish (*Mystus nemurus*; locally known as Ikan Baung) is of great potential importance as an alternative fish source to the world, especially to Southeast Asian countries. Its good flesh quality and taste, with high protein content but low in fat (Kamarudin *et al.*, 1987) makes it popular among consumers from this region. Therefore, this fish has been recognized as one of the favorite aquaculture species in Malaysia.

In some other countries, such as the United States, aquaculture products have become the third largest contributor to the trade balance of the country, and the channel catfish (*Ictalurus punctatus*) accounts for more than 50% of all the aquaculture production (Liu and Dunham, 1998). Similarly, *Mystus nemurus* has the potential to become the most important aquaculture product in this region, like the channel catfish is in the United States. However, most fish of this species available in the market currently comes from the various rivers of Malaysia. Their inability to reproduce on a large scale in captivity and the seasonal seed availability has led them to achieve high price in the market. Hence, commercialization and finding ways to increase production efficiency through genetic studies should be the immediate goal of the local aquaculture industry.

As traditional selective programs succeed, more effective selection programs need to be developed for traits that cannot be easily measured, such as disease resistance, growth, harvesting, oxygen tolerance, reproduction, and body mass. Therefore, emphasis should be on the genetic improvement of the species rather than on environmentally influenced characters. Marker Assisted Selection (MAS) and biotechnology offer great potential as alternatives to meet this challenge, but genetic linkage information of these traits must be obtained first. In other words, for the long term development and sustainability of aquaculture industry, it is important to construct a DNA marker-based genetic linkage map for application in selection programs through either the use of MAS, or isolation of economically important genes, in order to improve catfish broodstocks through biotechnology.

Genetic linkage mapping plays an important role for the purpose of understanding the organism studied. It reveals mechanisms of inheritance of phenotypes relevant to the markers. Correlation of recombination genome size with physical size of the genome makes it possible to estimate physical distances between markers. Comparative mapping among closely related species, particularly of catfish, will allow comparison of their genomic conservation and divergence, therefore, providing information on genomic evolution. The most important outcome of linkage mapping is that it reveals the physical distances among markers and the distances of markers to important traits which are useful in the isolation of the genes for the targeted traits.

The application of genetic markers in aquaculture research has increased dramatically in recent years. Several decades ago, protein level genetic markers such as isozymes were commonly used in the population characterization of fish, and identification of species or hybrids (Park and Moran, 1994). Later, with the discovery of various types of DNA level genetic markers, they have been widely accepted for use in various applications such as conservation, phylogenetic studies, construction of genetic linkage maps, studies of Quantitative Trait Loci (QTL) even in various breeding programs (Liu and Dunham, 1998). Markers such as Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Expressed Sequence Tagged sites (EST) and microsatellites have always been the favourite types of DNA markers in aquaculture research.

Many aquaculture species such as tilapia (Kocher *et al.*, 1998), rainbow trout (Young *et al.*, 1998), kuruma prawns (Li *et al.*, 2002), tiger shrimp (Wilson *et al.*, 2002),