UNIVERSITI PUTRA MALAYSIA

GENETIC DIVERSITY OF SELECTED COMMERCIAL FRESHWATER FISHES BASED ON PHOSPHOLIPASE C ZETA EXPRESSION AND MUSCLE PROTEIN PROFILING

NOOR AZIMAH BINTI NORBIDIN

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By

NOOR AZIMAH BINTI NORBIDIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Master of Science.

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science.

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Chairman : Sabrina bt. Sukardi, PhD

Faculty : Medicine and Health Sciences

Egg activation is important to help releases the egg from meiotic arrest and blocks polyspermy. It is linked with an increase in intracellular egg calcium ions (Ca^{2+}) in almost all species studied and current studies imply that the mammalian sperm factor involved is a sperm-specific phospholipase C zeta, PLCζ. Here, we first reported the identification of PLCζ in the testis and egg of Lampam Jawa. Our findings provide the evidence that PLCζ is present in the species of male and female Lampam Jawa *(Barbonymus gonionotus)*. For this study, six types of commercial freshwater fish were selected i.e. Red Tilapia (*Oreochromis sp. Red Tilapia*), Black Tilapia (*Oreochromis mossambicus*), Catfish or Keli (*Ictalurus punctatus*), Silver Catfish or Patin (*Pangasius pangasius*), Snakehead Fish or Haruan (*Channa striata*) and Silver Barb or Lampam Jawa (*Barbonymus gonionotus*). The objectives of this study were to isolate the mRNA from the gonads of freshwater fishes, to identify and amplify the phospholipase C zeta (PLCζ) gene fragments, to sequence the purified DNA fragments and to compare the PLCζ sequence to other PLCζ sequence available in NCBI database, to characterize muscle protein of selected commercial freshwater fish and lastly, to compare phylogenetic trees of 16S rDNA generated. In addition, protein profiles can be used as indicators of evolutionary relatedness. The differences and similarity aspects of fish muscle protein were measured and the relatedness based on protein profile was compared with the relatedness of fishes obtained from 16S rDNA sequences alignment by using dendrogram. The methods used for the expression of PLCζ were RNA extraction, spectrophotometric quantitation of RNA, Two-Step RT-PCR reaction, agarose gel electrophoresis, gel documentation, gel extraction, sequencing and dendrogram. The methods used for muscle protein profiling were sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), gel viewing, muscle's protein banding profile analysis, genetic diversity and lastly comparison of protein profile and 16S phylogenetic tree generated by using dendrogram. For the study of PLCζ expression, male and female Lampam Jawa showed bands at around 420bp in agarose gel electrophoresis that indicated the

presence of PLCζ gene and no significant bands were found in other types of fishes used in this study. For muscle protein profiling, the multiple bands of proteins obtained from SDS – PAGE showed similar protein contents among different fish species used in this study. The dendrogram showed the highest percentage of similarity is between Tilapia Hitam and Tilapia Merah which is 84% followed by Haruan and Patin which exhibited less than 84% similarity. Keli had 67% similarity with Haruan, Patin, Tilapia Merah and Tilapia Hitam while Lampam Jawa showed less than 60% similarity with Keli, Patin, Haruan, Tilapia Merah and Tilapia Hitam.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

KEPELBAGAIAN GENETIK IKAN – IKAN KOMERSIAL AIR TAWAR TERPILIH BERDASARKAN EKSPRESI FOSFOLIPAS C ZETA DAN PROFIL PROTEIN OTOT

Oleh

NOOR AZIMAH BINTI NORBIDIN

Julai 2014

Pengerusi : Sabrina bt. Sukardi, PhD

Fakulti : Perubatan dan Sains Kesihatan

Pengaktifan telur penting untuk membantu membebaskan telur daripada tangkapan meiotik dan menghalang polispermi. Ia dikaitkan dengan pertambahan ion – ion kalsium (Ca^2) dalam intrasel telur dalam hampir semua spesies yang telah dipelajari dan kajian terkini menunjukkan bahawa faktor mamalia sperma yang terlibat ialah sperma – spesifik fosfolipas c zeta, PLCζ. Di sini, kami telah melaporkan pengenalan PLCζ di dalam testis dan telur Lampam Jawa. Kajian kami telah menyediakan bukti bahawa PLCζ hadir dalam spesies jantan dan betina Lampam Jawa (*(Barbonymus gonionotus)*). Untuk kajian ini, enam jenis ikan komersial air tawar telah dipilih iaitu Tilapia Merah *(Oreochromis sp. Red Tilapia)*, Tilapia Hitam *(Oreochromis mossambicus,* Keli *(Ictalurus punctatus,* Patin *(Pangasius pangasius,* Haruan *(Channa striata,* dan Lampam Jawa *(Barbonymus gonionotus)*. Objektif – objektif kajian ialah untuk mengasingkan mRNA daripada gonad ikan air tawar, untuk mengenal dan menguatkan serpihan gen fosfolipas c zeta (PLCζ), untuk mengurut serpihan DNA yang telah disucikan dan untuk membandingkan urutan PLCζ dengan urutan PLCζ lain yang terdapat di dalam pusat data NCBI, untuk mencirikan protein otot ikan komersial yang terpilih dan akhirnya untuk membandingkan pokok filogenetik 16S rDNA yang telah dihasilkan. Tambahan pula, profil protein boleh digunakan sebagai petunjuk persamaan evolusi. Aspek – aspek perbezaan dan persamaan protein otot ikan diukur dan kaitan berdasarkan profil protein dibandingkan dengan kaitan ikan yang diperoleh daripada urutan penjajaran 16S rDNA menggunakan dendrogram. Kaedah – kaedah yang digunakan untuk ekspresi PLCζ ialah ekstrak RNA, kuantiti spektrofotometrik RNA, reaksi Dua – Langkah RT – PCR, elektroforesis gel agaros, dokumentasi gel, ekstrak gel, urutan dan dendrogram. Kaedah – kaedah yang digunakan untuk profil protein otot ialah natrium dodesil sulfat poliakrilamid gel elektroforesis (SDS – PAGE), melihat gel, analisis profil band protein otot, kepelbagaian genetik dan akhirnya perbandingan profil protein dan pokok filogenetik 16S yang terhasil menggunakan dendrogram.

Untuk kajian ekspresi PLCζ, Lampam Jawa jantan dan betina menunjukkan band pada sekitar 420bp di dalam elektroforesis gel agaros yang menunjukkan kehadiran gen PLCζ dan tiada band signifikasi dijumpai di dalam spesies ikan jenis lain yang digunakan di dalam kajian ini. Untuk profil protein otot, pelbagai band protein diperoleh daripada SDS – PAGE yang menunjukkan kandungan protein sama di antara spesies ikan berbeza yang digunakan di dalam kajian ini. Dendrogram menunjukkan peratus persamaan tertinggi di antara Tilapia Hitam dan Tilapia Merah iaitu 84% diikuti dengan Haruan dan Patin yang menunjukkan kurang daripada 84% persamaan. Keli mempunyai 67% persamaan dengan Haruan, Patin, Tilapia Merah dan Tilapia Hitam sementara Lampam Jawa menunjukkan kurang daripada 60% persamaan dengan Keli, Patin, Haruan, Tilapia Merah dan Tilapia Hitam.

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I certify that a Thesis Examination Committee has met on 10 July 2014 to conduct the final examination of Noor Azimah Binti Norbidin on her thesis entitled "Genetic Diversity of Selected Commercial Freshwater Fishes based on Phospholipase C Zeta Expression and Muscle Protein Profiling" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The committee recommends that the student be awarded the Master of Science.

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Faculty of Medicine and Health Science
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Senior Lecturer
Faculty of Medicine and Health Science
Universiti Putra Malaysia
(Internal examiner)

Mahanem Mat Noor, PhD
Associate Professor
Universiti Kebangsaan Malaysia
(External examiner)

ZULKARNAIN ZAINAL, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Sabrina bt. Sukardi, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Cheah Yoke Kqueen, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	percent
°C	degree Celsius
µg	micro gram
µl	micro liter
µM	micro molar
BLAST	Basic Local Alignment Search Tool
bp	basepair
Ca^{2+}	calcium ion
Ca2þ	calcium ion
cm	centimeter
cDNA	Complementary deoxyribonucleic acid
cRNA	copy ribonucleic acid
ddH_2O	double - distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
EtBr	ethidium bromide
EST	expressed sequence tag
g	gram
GAP	Good Agriculture Practices
GMP	Good Manufacturing Practices
h	hour
IP_3	inositol 1,4,5-trisphosphate
IP_3R1	inositol 1,4,5-trisphosphate receptor 1
kb	kilo-basepair

kDA	kilo Dalton
kg	kilogram
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
mM	mili molar
mRNA	messenger Ribonucleic Acid
NCBI	National Center for Biotechnology Information
NLS	nuclear localization signal
ng	nanogram
nm	nanometer
PCR	polymerase chain reaction
P_F	forward primer
pg	pikogram
PLC	phospholipase C
PLCZ1	Phospholipase C Zeta 1
PLCζ	Phospholipase C Zeta
PN	pronuclei
P_R	reverse primer
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction

s	second
SDS–PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
sp.	species
TBE	Tris-Borate-EDTA
T_m	melting temperature
UV	ultraviolet light
V	volt

CHAPTER 1

INTRODUCTION

A phenomenon where unfertilized eggs are arrested at certain stage of meiotic cell division in a species-specific manner is called egg activation and they are free from the arrest by fertilization (Miyazaki, 2006). The fundamental signal for egg activation is the rise in calcium ions (Ca^{2+}) at fertilization, hence responsible for triggering early embryogenesis (Stricker, 1999). It is linked with an increase in intracellular egg Ca^{2+} in almost all species studied and in mammals this occurs as a series of Ca^{2+} oscillations that begin right after fertilization and can continue for several hours.

Current study has proposed the start of egg activation is caused by sperm-specific phospholipase C named phospholipase C zeta or PLCζ in mammals which initially identified in the mouse, and subsequently been recognized in the monkey, human, and pig and in chickens, the latter finding suggesting a role for this protein during egg activation in other vertebrate groups. However, the mechanism by which PLCζ activated the egg activation and later fertilization is unknown in non-mammalian species.

A number of evidence supports the view that phospholipase C zeta (PLCζ) is the physiologic egg activation factor as injection of mouse recombinant RNA or protein into mouse oocytes induces identical Ca^{2+} oscillations as observed in fertilization. Meanwhile, sperm protein extracts that are immunodepletes of endogenous PLCζ eliminate the ability of fractionated sperm extracts to trigger Ca^{2+} oscillations in mouse oocytes correlates with the presence of PLCζ and also the Ca^{2+} releasing ability in mouse oocytes and sea urchin egg homogenates.

However, many remains unclear about functional role of PLCζ during fertilization and their mechanism of action as the unsolved puzzles are how PLCζ is packaged within sperm and whether it's pattern of localization changes during sperm maturation and the events preceding gamete fusion. The evidence that the protein was present throughout the perinuclear theca of the sperm head, where the endogenous egg activation factor is thought to reside, as well as in the sperm tail was found through a study using immunofluorescence to study the localization of PLCζ in mouse sperm, but the specificity of these localizations was not substantiated by the use of blocking peptides or appropriate quantitation.

Most study has recognized a pattern of localization for PLCζ in the equatorial segment as well as the post-acrosomal region in non-capacitated sperm of bull and mouse. Nevertheless, nonspecific immunostaining has complicated the latter finding of this study and besides, neither of these studies investigated whether any dynamic

changes took place in the localization of the PLCζ protein during the important processes of capacitation and the acrosome reaction.

The finding that may illustrate the presence of phospholipase C zeta (PLCζ) in some species' oocytes has so far questioned the ability of a sperm stimulus that may trigger the oocyte, despite the fact that the mass of scientific opinion agrees that PLCζ is the endogenous sperm factor. In this study we are going to first reveal PLCζ gene fragments that is present in the gonads of freshwater fishes and this finding later will be of great help to have PLCζ genome fully sequenced and annotated, and been added to the list of PLCζ in the non-mammalian species especially the aquatic.

In addition, freshwater fishes are commercially available fish that lives in freshwater such as rivers and lakes with salinity less than 0.05% with the most obvious difference from marine conditions is the salinity's level. The fishes require varied adaptation of physiology so as to maintain balanced ion concentration in their bodies to live in freshwater. Of all known fish species, mostly are found in freshwater and mainly caused by rapid speciation of the scattered habitats. Still, native fishes in the world faced a common threat in the genetic integrity and survival through the introduction of exotic freshwater fishes.

There are six species of commercial freshwater fishes selected for this study which are Tilapia Merah (*Oreochromis sp. Red Tilapia*), Tilapia Hitam (*Oreochromis mossambicus*), Catfish or Keli (*Ictalarus punctatus*), Silver Catfish or Patin (*Pangasius pangasius*), Snakehead Fish or Haruan (*Channa striata*) and Silver Barb or Lampam Jawa (*Barbonymus gonionotus*). Selection of these types of fishes is based on numerous factors such as commonly found in Malaysia and easily accessible in several places such as at wholesale markets, night markets, supermarkets, fish farms and aquarium pet shops. The freshwater fishes are the economically important food due to the high nutritional value especially protein and also good taste. Furthermore, they also contain the highest crude protein and the lowest crude fat compared to beef, pork and chicken and they also contain higher average percentage of the lean portion than beef and pork.

The increasing demand nowadays has made the freshwater fishes a well-known fish consumed in Malaysia and also worldwide. It is highly sought after for the high healthy protein content, lower fat content compared to other type of meat and delicious taste. These have made freshwater fishes a very suitable candidate for the study. They are on threat of near extinction though they are highly on demand as not much study has been done to save them. Haruan and Tilapia are well-known worldwide and this has help in saving these fishes from extinction, but they are not the only one in the group as there are more than hundred types of freshwater fishes in the world. Moreover, fishes caught in the wild are very much different from fishes bred in captivity, so does their meat quality, sexual maturity and beneficial contents.

In this study, six different species of fish muscle proteins are isolated for extraction and made ready for electrophoresis separation, run on polyacrylamide gel and lastly analyzed. The proteins size is verified and compared between the different fish species. Thus, a most important method is the total protein extraction especially preserving middle abundance proteins while eliminating the high abundance proteins which consist mostly of muscle fiber proteins.

The study of variation in the nucleotides (adenine, thymine, cytosine, and guanine), genes, chromosomes or whole genome of organisms is called genetic diversity that will provide a mechanism for analysis of population relatedness to adjust in environment that change constantly and several molecular markers has been used in studies of genetic diversity and conservation biology recently. This study is performed to analyze the fitness value of natural or captive populations and also to define priorities to the management of threatened species or populations to develop demographic models of small or fragmented populations.

16S rDNA or 16S ribosomal DNA has a structural role like acting as a scaffold defining the position of the ribosomal protein and it is a component of the 30S subunit of prokaryotic ribosome. Between different species of organism, the 16S rDNA gene is highly conserved thus it is used for phylogenetic studies. In this study, 16S rDNA is the most suitable gene to be used as well as 18S and 23S. Even though it is more commonly used in prokaryotic, it is also can be used for phylogenetic study in fishes as it provides an effective means by which the phylogenetic relationship of a large group of organisms can be investigated.

In addition, electrophoresis is used mainly to separate charged biomolecules for example DNA, RNA and proteins. It is commonly used to separate molecules based on their size, shape and charge. With the matrix acting as molecular sieve, where smaller molecules move faster than larger molecules, the samples are loaded into a gel matrix of agarose or polyacrylamide. Since molecules transfer at different rates through the gel, this has allowed researchers to verify how big the molecules are, the differences or similarities between the samples as well as how many different molecules there are in a sample.

Moreover, to study protein structure and function, electrophoresis of protein is utilized as the technique used commonly by the Protein Profiler that moves beyond DNA and to generate protein profiles from the muscle both distantly and closely related species of fish, the SDS-PAGE or sodium dodecyl sulphate polyacrylamide gel electrophoresis is used. The comparison of the different species profiles can be done from the result obtained to test the hypothesis that protein profiles can be indicators of evolutionary relatedness.

1.2 Objective

The general objective of this study is to detect and isolate the sequence of phospholipase C zeta (PLCζ) as well as to characterize muscle protein and genetic diversity of commercially available freshwater fishes.

The specific objectives are:

1. To isolate the mRNA from the gonads of freshwater fishes.
2. To identify and amplify the phospholipase C zeta (PLCζ) gene fragments.
3. To sequence the purified DNA fragments and
4. To compare the PLCζ sequence to other PLCζ sequence available in NCBI database.
5. To characterize muscle protein of selected freshwater fish.
6. To compare phylogenetic trees of 16S rDNA generated.

1.3 Hypothesis

Phospholipase C zeta is present in the gonads of freshwater fishes and fish relatedness, differences and similarity aspects of fish muscle protein can be determined.

1.4 Justification

The aim of this study is to contribute to the knowledge of the existence of phospholipase C zeta (PLCζ) in aquatic species as well as to compare the relatedness based on protein profile with the relatedness of fishes obtained from 16S rDNA sequences alignment.

1.5 Problem Statement

Phospholipase c zeta (PLCζ) is phospholipase C (PLC) of sperm-specific expressed as mRNA in testis as shown from former studies of bird, medaka fish and mammalian (Cox et al., 2002; Saunders et al., 2002; Coward et al., 2005; Ito et al., 2008; Mizushima et al., 2009; Young et al., 2009; Coward et al., 2011). In sperm, PLCζ is expressed as protein (Fujimoto et al., 2004; Grasa et al., 2008; Young et al., 2009; Coward et al., 2011) as well as in their spermatogenic precursors (Heytens et al., 2009, 2010; Coward et al., 2011) matching the hypothesis of soluble sperm factor (Swann, 1990; Coward et al., 2011). Here, we want to demonstrate the presence of PLCζ in the commercial freshwater fishes such as Lampam Jawa, Haruan, Keli, Patin, Tilapia Hitam and Tilapia Merah. There is currently no PLCζ study done in commercial freshwater fishes especially in Malaysia, therefore, it provides a platform for future study in fish PLCζ.

Muscle protein profiling will utilize protein relatedness where we look for similarities between different fish species through their protein contents. Genetic diversity is important to maintain diversity among species and without it the cycle can break and the cycle will be rule by a single species community. We also want to demonstrate the freshwater fish's relatedness through their proteins content to see whether the fishes are related or not.

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