Faculty of Resource Science and Technology

PHYLOGENETIC RELATIONSHIPS BETWEEN FOUR CATFISH SPECIES (FAMILY CLARIIDAE) INFERRED FROM ANALYSIS OF CYTOCHROME B MITOCHONDRIAL DNA GENE

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Declaration

I hereby declare that the thesis is based on my original work except for citation which has been acknowledged. I also declared that it has not been previously or concurrently submitted for any other degrees at UNIMAS or any other institutions of higher learning.

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List of Abbreviations

^{0}C	Degree Celsius
%	Percent
mg	Milligram
µl	Micro Liter
DNA	Deoxynucleoside-5'-triphosphate
rpm	Rotation Per Minute
PCR	Polymerase Chain Reaction
Cyt b	Cytochrome b Gene
mtDNA	Mitochondrial DNA
bpp	Bayesian posterior probability
MEGA	Molecular Evolutionary Genetic Analysis
PAUP	Phylogenetic Analysis Using Parsimony
MP	Maximum Parsimony
NJ	Neighbour Joining
ML	Maximum Likelihood
NaCl	Sodium Chloride

dNTPs	Dioxyribonucleotidetriphosphate
EtBr	Ethidium Bromide
CTAB	Hexadecyltrimethylammonium bromide

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Phylogenetic Relationships between Four Catfish Species (Family Clariidae) Inferred from Analysis of Cytochrome b Mitochondrial DNA Gene

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ABSTRACT

This study examined 405 base pairs of the mitochondrial cytochrome b gene from 13 individuals consist of four species (*Clarias batrachus, Clarias intermedius, Clarias leiancanthus* and *Clarias nieuhofii)* from the family Clariidae. The samples were taken from Balai Ringin, Sarawak. Phylogenetic reconstruction was accomplished by Maximum Likelihood, Maximum Parsimony, Neighbour Joining analyses, and Bayesian Analysis methods. Phylogenetic analysis supported the monophyletic status between all the four species of catfish. Overall, the partial sequence of the mitochondrial DNA cytochrome b gene was useful for resolving the phylogenetic relationships among Clariidae family.

Keywords: Cytochrome b (Cyt b), phylogenetic relationship, mtDNA

ABSTRAK

Kajian ini mengkaji 405 pasangan bes gen mitokondria cytochrome b daripada 13 individu terdiri daripada empat spesies (Clarias batrachus, Clarias intermedius, Clarias leiancanthus dan Clarias nieuhofii) dari keluarga Clariidae. Sampel yang digunakan diambildari Balai Ringin, Sarawak. Hubungan filogenetik telah dibina semula menggunakan kaedah "Maximum Likelihood", "Maximum Parsimony","Neighbour-Joining" dan "Bayesian". Analisa filogenetik membuktikan hubungan kesemua empat spesis ikan keli adalah berstatus monophyletic. Secara keseluruhan, penggunaan separuh gen mitokondria DNA cytochrome b boleh digunakan untuk menyelesaikan hubungan filogenetik di kalangan keluarga Clariidae.

Kata kunci: Sitokrom b (Cyt b), hubungan filogenetik, mitokondrial DNA.

1.0) INTRODUCTION

Fish are any aquatic vertebrate animal that are cold-blooded (ectothermic) with fin, scale and backbone. The fossil record shows that fish were among the first kind of animal that appear on the planet. The earliest known was a jawless fish that presumably roamed the seabed sucking up invertebrates. Fish meat contains abundant of protein that human and animal body required. This is why the demand for fish protein in Malaysia has increased to approximately 55 kg per person per year (Anon, 2002). This is due to the fish and fish byproducts that increasingly becoming important commodities to the world community (Noordin, 2002). Catfish farming is currently the largest food fish aquaculture industry in the USA based on total production (285 million kg), with a market value of over 480 million dollars (USDA, 2005).

Catfish (Order Siluriformes) are a diverse group of fish representing more than 3,000 species, 478 genera and 36 families (Ferraris and Pinna, 1999). Most catfish are tropical, but only two families are marine. All catfishes lack normal scales, although some may have armour of bony plates covering the body instead of being naked (Steward and Watkinson, 2004). Catfishes also have spines at the leading edge of the dorsal and pectoral fins which potential venom produced by the cells in the skin covered by the spines. Most of catfishes have barbels or "whiskers" around the mouth which help them to to locate food (Steward and Watkinson, 2004).

Clariidae are one of the about 31 families belonging to the teleostean order Siluriformes (Teugels, 2003). The family consists of fifteen genera, 12 endemic to Africa and two endemic to Asia, comprising a total of 93 species (Sabaj *et al.*, 2004). This family is recognize by an

elongated body, long dorsal and anal fins, especially the presence of suprabranchial organ, formed by arborescent structures originating from the second and the fourth gill arches and enabling direct air-breathing from the atmosphere. Its representatives can be found all over Africa, the Middle East and parts of Asia (Teugels, 2003). This adaptation and their thick skin with mucous pores could explain their distribution in swamps, flood plains and periodically dry pools (Burgess, 1989). *Clarias* is the only genus occurring on both African and Asian continents and can be divided into seven subgenera (Teugels, 2003).

DNA sequence data play an essential role in the reconstruction of evolutionary relationships among organisms, resulting in insights in genetic affinities that may confirm or conflict with traditional taxonomy. Because of its attractive properties, ribosomal DNA (rDNA) is popular for examining phylogenetic relationships and for studying genetic variability and divergence within and between species. Such properties are secondary structure features, differential rates of evolution between different regions, and tandemly repeated genes (Arnheim, 1983; Gebri, 1985).

The importance of this study is there are currently very little works utilizing in catfish family especially from Borneo. Moreover, molecular data can help to strengthen the taxonomy of three distinct families which are Clariidae, Bagridae and Pangasiidae and can assist in resolving taxonomic status among some of the problematic species. This study was utilized sequences analysis of the mitochondrial Cyt b gene to examine the phylogenetic relationships of several species among the family of catfish.

OBJECTIVES

- To amplify Cyt b mtDNA gene in four species of Catfishes from family Clariidae.
- To determine the phylogenetic relationships among the four species of catfishes in Borneo, using Cyt b mtDNA gene.

2.0) LITERATURE REVIEW

2.1) Distribution, Morphology and Taxonomy of Catfish Families

Clariidae are one of the about 31 families belonging to the teleostean order Siluriformes (Teugels, 2003). The family consists of fifteen genera, 12 endemic to Africa and two endemic to Asia, comprising a total of 93 species (Sabaj *et al.*, 2004). Some species are distributed in Syria, southern Turkey and throughout Southeast Asia, but their diversity is the greatest in Africa (Teugels and Adriaens, 2003). *Clarias* is the only genus occurring on both African and Asian continents and can be divided into seven subgenera (Teugels, 2003).

Clariid catfishes are characterized by an elongate body, four pairs of barbels, and by the unique presence of a suprabranchial respiratory organ, formed by arborescent structures derived from the second and fourth gill arches (Teugels and Adriaens, 2003). The dorsal fin of most catfishes technically has two spines which the first being very short and forming a locking mechanism for the second spines. The catfish body is naked or covered with bony plates which normally up to four pairs of the barbells on the head, on the nasal and chin barbells may be variously absent.

The Clariidae belong to a group of catfishes that exploit a wide range of habitats from streams, rivers and freshwater lakes (Eccles, 1992; Agnése and Teugels, 2005). Several species of the catfish are known to be poisonous or venomous (Perriere and Gaudey, 2003). They can cause severe wounds by using their spines which they can inject a poison produced by glandular cells in the epidermal tissue covering the spines (Nelson, 2006). But most of the species are passive stinger.

Barbels or “whiskers” around the mouth which help them to to locate food in a long distance (Steward and Watkinson, 2004). This is because they don’t really smell good, but they have an extraordinary sense of smell. They have smeller right up front in two little pits located in front of the eyes and behind the upper jaw (Sutton, 2000). Each olfactory pit has two nostrils which one for incoming water and the other one for outgoing water. These pits are lined with sensitive tissue wrinkled into a series of folds to provide the maximum surface area for smelling (Sutton, 2000).

Most fishes’ taste buds are found in the mouth which are on tongue, palate and so on. But in cafish, the mouth is packed with taste buds. Besides, when the water flow across the gills, the gills rakers which facing water flow also are loaded with a lot of taste buds (Sutton, 2000). The taste buds also can be found outside of their body for example fins, back, belly sides and also their tail.

2.2 Mitochondrial DNA and Cyt b Gene

Mitochondrial DNA (mtDNA) has been one of the most widely used molecular markers for phylogenetic studies in animals because of its simple genomic structure (Avise, 2004). Mitochondria have extremely important cellular functions (Brown *et al*., 1979). This is because the life of animals is crucially dependent on mitochondrial function one would expect mitochondrial evolution to be highly constrained. Moreover, mtDNA is of higher stability and occurs in a much higher number of copies than nuclear DNA (Prusak *et al*., 2005).

Cytochrome *b* (cyt-*b*) has been considered one of the most useful genes for phylogenetic work, and is probably the best-known mitochondrial gene with respect to structure and function of its protein product (Esposti *et al.,* 1993). Cyt-*b* gene contains both slowly and rapidly evolving codon positions, as well as more conservative and more variable regions or domains overall. The cytochrome b gene of mtDNA has been found to be a powerful indicator for identifying the species with DNA analysis techniques (Zehner *et al.,* 1998, Parson *et al.,* 2000) also used in studies of molecular evolution([Kocher *et al.,* 1989, Montgelard *et al.,* 1997, Prusak *et al*., 2004 and legal medicine [Barlett and Davidson, 1992]. Besides, this gene also have been used for a diversity of systematic questions which from deep phylogeny to the population and recent divergence levels.

However, many problems have been encountered when using cyt-*b*, including base compositional biases, rate variation between lineages, saturation at third codon positions, and limited variation in first and second codon position, resulting in little phylogenetic information for “deep” evolutionary questions, or few informative sites for the third codon position at the population levels (Meyer, 1994). The phylogenetic utility of the cyt-*b* gene has been studied at

several taxonomic levels among vertebrate taxa and particularly in fish taxa (Zardoya and Doadrio, 1999).

According to Meyer (1994), there are several good reasons for the continued use of cytochrome b as a phylogenetic marker. Although it is slow in amino acid substitutions, the rate of evolution for the silent substitutions in third codon positions is similar to that of other mitochondrial genes (Meyer, 1994). Cytochrome b is probably the best-known mitochondrial gene with respect to its structure and functional constrains on the gene product.

2.3 Phylogenetic Studies of Catfish

Amnuay *et al*. (2007) studies on the complete mitochondrial DNA sequence of the Mekong giant catfish (*Pangasianodon gigas*) and the phylogenetic relationships among Siluriformes. The Mekong giant catfish is the largest scale-less freshwater fish in the world, and a critically endangered species. The phylogenetic studies was conducted based on the mitochondrial protein which was the combination of nucleotide sequences of mitochondrial 12S and 16S rRNA genes data sets in order to further clarify the relative phylogenetic relationships among 15 out of the 33 families of Siluriformes.

A phylogenetic study of the African catfish from the family Mochokidae was done by Vigliotta (2008). The hypothesis of the phylogenetic relationships was based on the maximum parsimony analysis of 92 morphological characters. The results of the analyses were The Mochokidae are a monophyletic group, sister to a group composed of the South American Doradidae and Auchenipteridae. groups within the Mochokidae are largely defined by a combination of characters pertaining to the teeth, oral jaws, and suspensorium. Changes in the

jaw and suspensorium are a key theme in mochokid evolution. The results of this analysis indicate the need for the revision of several mochokid groups. Higher-level systematics of the Mochokidae can be greatly improved with continued emphasis on broader, lower-level taxonomic work.

The recent studies conducted by Wong *et al.* (2011) on the title of DNA Barcoding of catfish species authentication and phylogenetic assessment. They developed and evaluated DNA barcodes for the use in differentiating United States domestic and imported catfish species. They sequenced 651 base-pair (bp) barcodes from cytochrome oxidase 1 (CO1) gene from individuals of 9 species of imported and domestic catfishes. As a result the CO1 region of the entire sample was successfully amplified by using PCR. According to the Maximum Parsimony (Mp) tree, the species in this study were clustered independently within their corresponding genera.

3.0) MATERIALS AND METHODS

3.1) Samples Collection and Preservation

The samples of catfish with 14 individuals from four species of Clariidae family were collected from Balai Ringin, Serian. The collected specimens were immediately frozen or preserved in ethanol, and subsequently stored at -20^{0}C until required for genetic analyses. The samples were identified through their morphological characteristics measurements using the keys provided by Inger and Chin (1962) and Mohsin and Ambak (1983).

Table 1: Description of sample location and size of species.

Species	Source	Sample size
Clarias intermedius	Balai Ringin	3
Clarias leiancanthus	Balai Ringin	3
Clarias nieuhofii	Balai Ringin	3
Clarias batrachus	Balai Ringin	5

Figure 1: *Clarias intermedius*

Figure 2: *Clarias batrachus*

Figure 3: *Clarias nieuhofii*

Figure 4: *Clarias leianchanthus*

3.2) DNA Extraction.

Total DNA was extracted from muscle tissue using a CTAB method (Standard hexadecyl trimethyl ammonium bromide) following Grewe *et al.,* (1993). Firstly, a total of 1-2 cubid millimeter of the tissue sample was added into each of 1.5 ml microcentrifuge tube that containing 600 µl CTAB buffer followed by 15 µl Proteinase K (20mg/ml). The tubes was briefly mixed and incubated at 65^0C for 2-3 hours until tissue completely dissolved.

Six hundred micro liter of chloroform: isoamyl alcohol (24:1) was added into each tube and mixed briefly for 2 minutes before centrifuged at 13,000rpm for 20 minutes. The only upper layer of the supernatant was taken and transferred to a newly labeled tube. Then, an equal volume of cold absolute ethanol was added, mixed well and the tubes sit on the bench for a few minutes. The sample was centrifuged at 13,000rpm for 15 minutes and the ethanol was discarded.

Next, approximately 70% of cold absolute ethanol with 25 µl 3M NaCl was added. Then, centrifuged at 13,000rpm for 15 minutes and the solution were discarded carefully after centrifugation. The tube was air dried for about 15 minutes at room temperature. Finally, the DNA pellet was suspended in 100 µl of water (ddH_2O) after the pellet was air dried. Finally, the DNA extraction was kept in the freezer (-20^oC).

3.3) Running Gel Electrophoresis

The samples need to be visualised to see whether the extraction, PCR, or the purification is success or not. A good result will depend on the band of the DNA that can be seen on the agarose gel. The most suitable concentration to visualize the DNA is 1 %. 0.5g of agarose