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Molecular Data from the Cytochrome *b* for the Phylogeny of Channidae (*Channa* sp.) in Malaysia

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Abstract: Snakeheads are freshwater fish which are distributed wildly in Asia and Africa. In Malaysia, Channa genus were identified in six species named *Channa lucius*, *C. striata*, *C. micropeltes*, *C. gachua*, *C. marulioides* and *C. melasoma*. In previous studies, *Channa* species were mainly classified based on morphology. Phylogenetic relationships from 36 individuals of 6 *Channa* species distributing in Malaysia were analyzed over the sequence of cytochrome *b* gene. Primers named forward L14841 and reverse H15149 amplified the gene. The results indicated that the species belonged to two major clades. The first clades was only *C. lucius*. The second clade comprised of species named *C. striata*, *C. micropeltes*, *C. gachua*, *C. marulioides* and *C. melasoma*.

Key words: Snakehead fish, *Channa*, cytochrome *b*, sequencing, mitochondrial, genome, mtDNA

INTRODUCTION

The Channidae is one of the most widespread families of freshwater fish. The natural range of this family encompasses all of Asia and Africa; however, members of this family are now present in America, Europe and Australia due to releases by fish hobbyists. In Malaysia, snakeheads, members of the Channidae, are common food fish. The species found in Malyasia include *Channa striata* (ikan haruan), *C. micropeltes* (toman), *C. marulioides* (toman bunga) and *C. lucius* (bujuk): two other species, *C. gachua* and *C. melasoma*, have no common Malaysian names. The systemics of the Channidae is a matter of continuing debate, especially because of the similarities in their external morphology and internal anatomy: morphological and physiological data have produced inconclusive taxonomies. Genetic characters may provide accurate and unambiguous indicators of taxonomic divergence (Wilson *et al*., 1995).

Molecular biologists have ignored mitochondria because they did not immediately recognize the far-reaching implications and applications of the discovery of the mitochodrial genome (mtDNA). It took time to accumulate a basic database of sufficient scope and content to address many challenging questions related to anthropology, biogenesis, disease, evolution and more. Mitochondria and their genomes have been studied with respect to DNA replication, DNA repair, transcription, RNA edition, translation and mitochondrial protein import (Scheffler, 2001). The mitochondrial genome is also ideal for evolutionary and phylogeneticstudies s. The mitochondrial genes for 16S ribosomal RNA and cytochrome *b* have proved a powerful tool in phylogenetic studies, providing information on the systematics of terrestrial and marine vertebrates (Allard *et al*., 1992; Milinkovitch *et al*., 1993) and on the systematics of marine invertebrates (Cunningham *et al*., 1992; Geller *et al*., 1993; Rumbak *et al*., 1994; Bucklin *et al*., 1995; Canapa *et al*., 1996).

In the present study, partial sequences of the mitochondrial gene for Cytochrome *b* were analyzed for six channid species *Channa marulioides*, *C. melasoma*, *C. lucius*, *C. gachua*, *C. micropeltes* and *C. striata* to determine the phylogenetic relationships amongst these species.

MATERIALS AND METHODS

Sample collection. Flesh from 6 individuals of six species, *Channa marulioides*, *C. melasoma*, *C. lucius*, *C. gachua*, *C. micropeltes* and *C. striata* were collected from Peninsular Malaysia. The flesh of each individual was preserved in 95% ethanol before DNA extraction.

DNA preparation: Total DNA from flesh of the snakedhead species was extracted using the phenol-chloroform method of Taggart *et al*. (1992) with the following modifications.

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PCR amplification: The DNA was amplified using the universal oligonucleotide primers: L14841 and H15149 primers for the cytochrome *b* gene (Kocher *et al*., 1989). The sequences of these primers are as follows:

L14841: 5'-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TCA AA-3'

H15149: 5'-AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3'

These primers amplify a 309 bp region of the cytochrome b gene from the mitochondrial DNA of most vertebrates (Kocher *et al*., 1989). The amplified fragments are usually 376 bp long including the two primer sequences (Kocher and White, 1989).

The PCR reactions were made up containing 1X PCR buffer (Bioron), 5 mM $MgCl_2$ (Bioron), 0.2 mM dNTP mix (Bioron), 0.5 μM of each primers (Research Biolabs) and 50 ng of DNA template. The reaction mix was denatured at 98°C for 5 min. Taq polymerase from Bioron (0.5 U) was then added to give a final reaction volume of 15 μL.

The PCR reactions were amplified using a Perkin Elmer Cetus Gene Amp PCR System 2400. Amplification conditions were as follows (35 cycles): 94°C, 1 min (denaturation), 55°C, 1 min (annealing) and 72°C, 2 min (elongation). A final extension for 5 min at 72°C was conducted at the end of the 35 cycles. This protocol was applied after optimizing $MgCl_2$ concentration (1-5 mM), primer concentration (0.1-1.0 μM) and annealing temperature (55-60°C).

Purification of double-stranded PCR products: PCR products were purified using a Qiagen purification kit immediately aftter amplification. Buffer PB (50 μL) was added into a 0.2 mL PCR flat tube containing 10 μL of PCR product. This mixture was placed into a QIAquick Spin column (QQC) and then centrifuged for 60 sec at 13,000 rpm. Buffer PE (0.75 mL) was added into QQC, centrifuged for 60 sec at 13,000 rpm and the eluent discarded. The QQC was placed into a new 1.5 mL tube and 30 μL of EB was added into the center of the QQC. The tube was left to equilibrate for 5-10 min and then centrifuged for 60 sec at 14,000 rpm. The purified product in the eluent was stored at -20°C.

DNA sequencing: The purified product was incubated for 15 min at 37°C and then for 15 min at 80°C. Automated sequencing was performed using the Big Dye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) and a ABI PRISM 377 DNA sequencer. The sequencing reactions were performed using 35 cycles of 96°C for 10 sec, 55°C for 5 sec and 60°C for 4 min. The products were precipitated using ethanol and sodium acetate precipitation.

Analysis of DNA sequences: Phylogenetic trees were produced using the neighbor-joining and parsimony. In the case of neighbor-joining, evolutionary distances were determined using maximum likelihood and were corrected using the equations of Jukes and Cantor (1969), Kimura (1980) and Tajima and Nei (1984) and using PHYLIP version 3.5c software (Higgins *et al*., 1992; Felsenstein, 1993; Thompson *et al*., 1994).

Maximum-parsimony trees were produced using branch-and-bound search by attributing equal weight to transition and transversions and by transversion parsimony, where the four nucleotides are reduced to two character states, purines and pyrimidines. Bootstrap values, indicating robustness of nodes in neighbor-joining and parsimony trees were determined using 1000 replications.

RESULTS

Genomic from 6 snakehead species is shown in Fig. 1. The samples having sharp and clear band were sequenced. The list of six species is presented in Table 1. The specimen used were fresh flesh.

DNA sequencing: Information on base compositional bias is important because phylogenetic tree-building methods may be unreliable when applied to taxa which differ greatly in base composition (Steel *et al*., 1993). Most analytical methods such as neighbour-joining, parsimony and maximum likelihood tend to group sequences of similar base composition together regardless of their evolutionary history (Lockhart *et al*., 1994).

Table 1: Species used for the phylogenetic analysis

Family	Species	Note
Channidae	*Channa marulioides* (Bleeker, 1851)	Fresh specimen
	Channa micropelites (Cuvier and Valenciennes, 1831)	Fresh specimen
	Channa lucius (Cuvier and Valenciennes, 1831)	Fresh specimen
	Channa melasoma (Bleeker, 1851)	Fresh specimen
	Channa gachua (Hamilton, 1822)	Fresh specimen
	Channa striata (Bloch, 1797)	Fresh specimen

Table 2: Nucleotide percentage composition and length of cytochrome *b* sequences

Species	A	T	C	G	A and T	C and G
Channa marulioides	25.1	27.2	30.0	17.7	52.2	47.8
Channa micropeltes	26.7	26.1	30.1	17.1	52.5	47.5
Channa lucius	24.3	26.7	31.4	17.6	51.1	48.9
Channa melasoma	27.2	27.2	29.2	16.4	54.4	45.6
Channa gachua	24.5	32.2	26.0	17.3	56.5	43.5
Channa striata	25.6	28.7	28.5	17.2	54.3	45.7
Average	25.6	28.1	29.2	17.2	53.5	46.5
SD	1.1	2.2	1.8	0.4	1.9	1.9

Table 3: Paiwise distance matrix for the cytochrome *b* gene fragment

Species	*Channa marulioides*	*Channa micropeltes*	*Channa lucius*	*Channa melasoma*	*Channa gachua*	*Channa striata*
Channa marulioides	-	21.3	19.7	15.2	14.6	32.8
Channa micropeltes	0.85	-	21.8	15.6	23.1	30.9
Channa lucius	0.67	0.93	-	14.5	21.7	29.5
Channa melasoma	1.41	0.91	0.79	-	14.8	33.0
Channa gachua	1.38	0.96	0.81	056	-	31.4
Channa striata	0.72	0.95	0.82	0.58	0.49	-

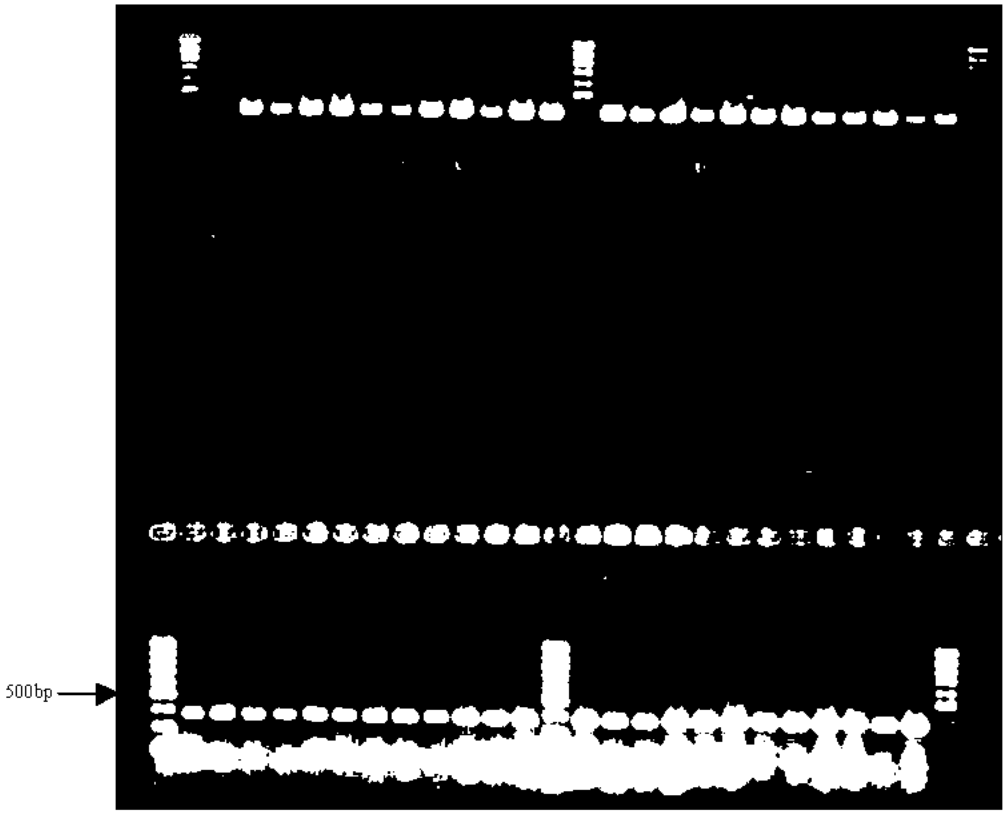

Fig. 1: PCR products amplified by cytochrome *b* gene. Lane M: 100 bp DNA ladder. Lane 1-12 Terengganu, lane 13-24: Perlis, lane 25-36: Perak and lane 37-48 Johore

The base compositions are reported in Table 2. The rooted and unrooted trees are shown in Fig. 2 and 3.

The examination of transition: Transversion ratio in the data indicated the declines in this ratio with increase genetic distance (Fig. 4).

Genetic divergence: The genetic divergence in intraspecies is low varied from 0.4-0.6%. Meanwhile, they show very high levels of interspecific variation at 16.9-19.2% (*C. lucius-C. melasoma*), 22.5-24.8% (*C. lucius-C. striata)*, 25.0-27.5% (*C. lucius-C. gachua*), 22.6-30.1% (*C. lucius-C. marulioides*) and 19.7-22.4% (*C. lucius-C. micropeltes*).

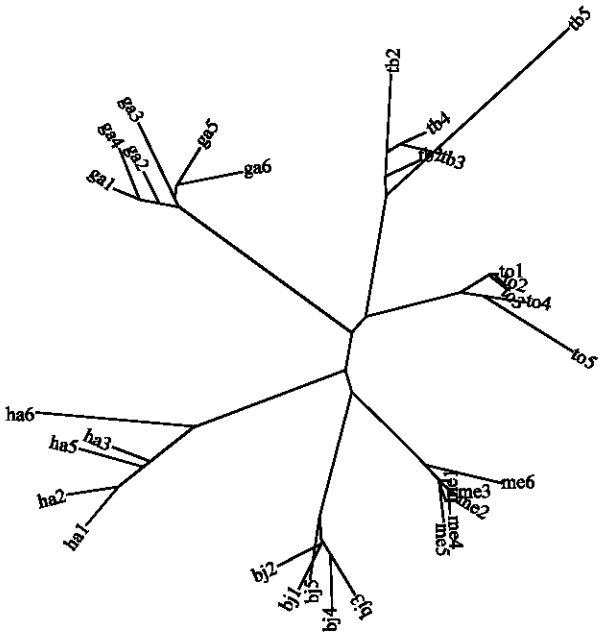

Fig. 2: Phylip unrooted tree of snakehead species. Ga: *Channa gachua*, Ha: *C. gachua*, Me: *C. melasoma*, Bj: *C. lucius*, Tb: *C. marulioides*, To: *C. micropeltes*

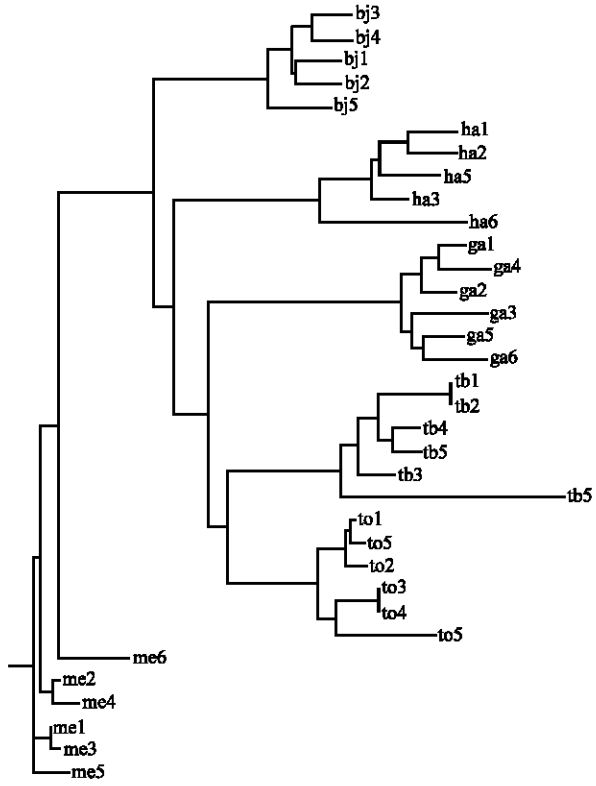

Fig. 3: Phylip rooted tree of snakehead species. Ga: *Channa gachua*, Ha: *C. gachua*, Me: *C. melasoma*, Bj: *C. lucius*, Tb: *C. marulioides*, To: *C. micropeltes*

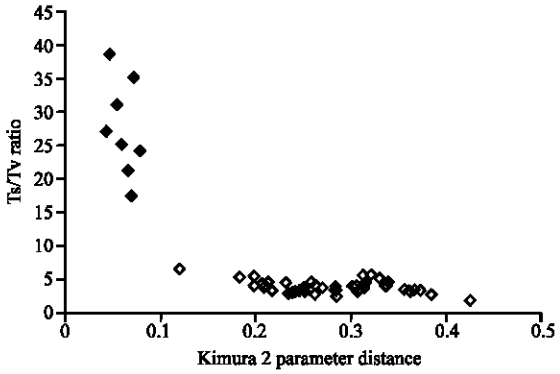

Fig. 4: Transition/transversion ratio versus corrected (Kimura two parameter) genetic distances

The distance matrix obtained from the analysis of the alignment of all sequences is shown in Table 3.

DISCUSSION

After optimization, the amplification conditions were as follows: 35 cycles of denaturation at 94°C in 1 min., annealing at 55°C in 1 min and extension at 72°C in 2 min. The PCR reactions were 2 μL temple DNA, 1X PCR buffer, 5 mM $MgCl_2$, 0.2 mM dNTP mix and 0.5 μM of each primer, namely L14841 (5'-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TCA AA-3') and H15149 (5'-AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3') and 0.5 U *Taq* polymerase in 15 μL final reaction.

All *Channa* species were distinguished using cytochrome *b* sequencing. The complete sequences used in this study were deposited in GenBank (IntelliGenetics, Inc.). The average frequencies of nucleotides for all taxa were as follows: A: 25.5, C: 29.2, G: 17.2 and T: 28.1%. It can be seen from Table 3 that the order of the most represented base was C>T>A>G. This is similar to the order in the cytochrome b of Percid fishes (Song *et al*., 1998) and Rockfish *Sebastes* (Rocha-Olivares *et al*., 1999). There is a low percentage of G in the snakehead sequences. This is similar to results found in *Gasterosteus aculeatus* (Three-spined stickleback), *Acipenser transmontanus* (Sturgeon), *Anguilla rostrata* (American Eel), *Fundulus heteroclitus* (Killifish), *Gadus morhua* (Atlantic Cod) as reported in Meyer (1993). The percentage of G is lowest in *C. melasoma* and highest in *C. marulioides* with values of 16.4 and 17.7, respectively.

The amount of interspecific variation in base composition is reflected in the standard deviation values presented in Table 2. The highest standard deviation value is in T and the lowest is in G. The SD values were similar to that for other fish species listed in Lydeard and Roe (1997).

Transition: Transversion ratio: Transition is the substitution between two purines (adenine and guanine) or between two pyrimidines (thymine and cytosine). Transversion is the substitution between a purine and a pyrimidines. Transition usually predominates over transversion (Brown *et al*., 1982). The ration between transition and transversion of *Channa* is about 2:1. This is similar to findings reported by Meyer (1993). In contrast, the transition bias in Blue Marlin (Finnerty and Block, 1992) and other vertebrates (Kocher *et al*., 1989) is about 15:1.

Purine/pyrimidine transition: When two species diverge, the initial rate of transition is the same for the two classes of bases. The purines (A and G) show saturation at a lower level of divergence than the pyrimidine (T and C). It caused the frequencies of A and G to be much more unequal than the frequencies of C and T (Kocher and Carleton, 1997). The transitions between pyrimidines outnumber transitions between purines in *Channa* 2 to 1. This is similar to the 2 to 1 ratio in Blue Marlin *Makaira nigricans* (Finnerty and Block, 1992). This is higher than the 1 to 1 in Atlantic Cod *Gadus morhua* (Carr and Marshall, 1991) and the 1 to 2 in Rainbow trout *Oncorhynchus mykiss* (Beckenbach *et al*., 1990).

The matrix of sequence divergences estimated using the gamma-corrected (a = 056) Kimura two parameter model is shown in Fig. 4. Considering both transition and transversion, the corrected sequence divergence ranged from 16.9-30.1%. Sequence divergences were lowest between the species pairs *Channa micropeltes* and *C.marulioides*.

A phylogeny of the channid is useful for taxonomic purposes as well the investigation of evolutionary patterns and processes. Channids are a taxonomically difficult group, known as having a single common design but considerable morphological diversity. This diversity is linked to ecological factors in such a way that much homoplasy can be expected in morphological characters.

In general, the analysis of mitochondrial ribosomal genes of Channa evidences a high inter and intraspecific sequence variability.

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