

Phylogenetic Relationships among Several Freshwater Fishes (Family: Cyprinidae) in Malaysia Inferred from Partial Sequencing of the Cytochrome *b* Mitochondrial DNA (mtDNA) Gene

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ABSTRACT

The phylogenetic relationships among 23 species of Malaysian freshwater fishes in the family Cyprinidae was inferred by partial sequencing of the Cytochrome *b* (Cyt *b*) mitochondrial gene. Samples were collected from various localities in Sarawak, Sabah and Peninsular Malaysia. The inferred phylogeny appeared to match major groupings currently recognized in the taxonomy but no support was evident for nearly all the higher level groupings. Nevertheless, some interesting insights were gained in relation to the phylogenetic relationships among some genera under study. Meanwhile, the phylogenetic relationship among Mahseer fishes (genus *Tor* and *Neolissochilus*) were poorly resolved using the current data alone, but the taxonomic revision of other genera particularly for the genus *Puntius* could improve this. The current study suggest that *P. binotatus* and *P. sealei* could be representative of the genus *Puntius*, while any other species identified as belonging to the genus *Puntius* should cluster with this group. The study also revealed that two morphologically similar *Barbonymus* species (namely, *B. gonionotus* and *B. schwanefeldii*) were phylogenetically distinct (13.0% K2P genetic distance). This indicated that a taxonomic revision of *B. gonionotus* would be required from its current position within the genus *Barbonymus*. The results of the current study also revealed two interesting findings for *Hampala*; (1) the Borneo endemic *Hampala* forms are distinct from the widespread *H. macrolepidota*, and (2) two distinct lineage were evident in *H. bimaculata* from Sarawak. In general, the sequence analysis of the cytochrome *b* mtDNA region has been proven to be useful for assessing phylogenetic relationships among indigenous freshwater fishes in Malaysia.

Keywords: Cytochrome *b*, sequence, mitochondrial DNA, molecular phylogeny

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INTRODUCTION

The Southeast Asian region, which includes Peninsular Malaysia and the island of Borneo, has one of the highest diversity of freshwater fishes in the world (Zakaria Ismail, 1990). According to FishBase, more than 600 species of freshwater fish are recorded in Malaysia (Froese & Pauly, 2004). In Peninsular Malaysia, about 400 freshwater fish species have been described (Mohsin & Ambak, 1983), while more than 350 species of freshwater fishes have been recorded in Borneo (Inger & Chin, 1962; Roberts, 1989; Kottelat *et al.*, 1993). The Family Cyprinidae forms the largest family in terms of number of genera and species, and it dominates almost every water body in the region (Mohsin & Ambak, 1983; Zakaria Ismail, 1990). Fishes in the sub-family Cyprininae (i.e. genera *Barbodes*, *Barbonymus*, *Cyclocheilichthys*, *Hampala*, *Osteochillus*, *Puntius* and *Tor*) are the most speciose species in Malaysia (Inger & Chin, 1962; Mohsin & Ambak, 1983).

Indigenous freshwater fishes in Malaysia play an important role not only as the main source of protein for rural populations but also as an important source of livelihood in terms of fish trading (Khan *et al.*, 1996, Litis *et al.*, 1997). Apart from their importance as a food source, many freshwater fishes (e.g. carps) are also favoured as ornamental fishes (Mohsin & Ambak, 1983; Ng, 2004).

Since indigenous freshwater fish are an important source for the region, a comprehensive study on their systematic relationships, particularly of the cyprinids, will assist appropriate management and conservation. Unfortunately, systematic studies of Malaysian cyprinid fishes are scarce and their taxonomy is poorly understood. Current systematic classifications are based solely on their morphological, physiological and other assayable external phenotypic characteristics (Inger & Chin, 1962; Mohsin & Ambak, 1983; Roberts, 1989; Kottelat *et al.*, 1993). Meanwhile, conventional systematic characters can often be unreliable because they can be influenced by environmental and non-genetic

factors (Vrijenhoek, 1998). On the other hand, DNA-based characters are unlikely to be influenced by environmental pressures (Briolay *et al.*, 1998). Furthermore, they are heritable traits, and confidence can therefore be placed on the amount and nature of the genetic information obtained (Awise, 1994).

The development of appropriate molecular marker has strengthened the genetic, taxonomic and systematic studies of fish (Stepien & Kocher, 1997). Meanwhile, the advent of polymerase chain reaction (PCR) technology has greatly facilitated the examination of genetic variation in natural populations (Amos & Hoelzel, 1992). The combination of PCR, with the availability of "universal primers" (e.g. Kocher *et al.*, 1989) has enabled a rapid amplification of specific sequences without the need for cloning procedures. The analysis of DNA sequence polymorphisms can provide the highest resolution of genetic variation in cytoplasmic markers with mitochondrial DNA (mtDNA), now a popular tool for constructing phylogenetic relationships. This marker has been applied to resolve questions in biodiversity, conservation genetics and molecular systematic studies (Amos & Hoelzel, 1992; Awise, 1994; Awise & Hamrick, 1995; Stepien & Kocher, 1997).

The present study therefore attempted to construct a molecular-based phylogeny for a number of freshwater fish taxa in Malaysia, particularly from the Cyprinidae family, using partial DNA sequencing of the Cytochrome *b* (Cyt *b*) mtDNA gene.

MATERIALS AND METHODS

Sample Sources and DNA Extraction

The samples were obtained from various river systems in Sarawak, Sabah and Peninsular Malaysia (Table 1). The full samples were recognized morphologically using keys provided by Inger and Chin (1962), Mohsin and Ambak (1983), and Kottelat *et al.* (1993). A total of twenty-three species of cyprinid fishes, representing twelve genera, were examined in this study. The specimens were collected using

TABLE 1
Scientific and local names of cyprinid fishes and outgroup, sampling location, sample size and GenBank accession numbers used in this study

Subfamily	Scientific name	Local name	Location		Sample size	GenBank Accession No.
			PM	Sbk		
Cyprininae	<i>Barbodes collingwoodii</i>	Kepiat	✓	✓	2	AY243348, DQ366151
Cyprininae	<i>Barbonyms gonionotus</i>	Lampam jawa		✓	2	DQ366152, DQ366153
Cyprininae	<i>Barbonyms schwanenfeldii</i>	Tengadak/Lampam sungai	✓		2	AY355438, AY355426
Cyprininae	<i>Cylocheilichthys apogon</i>	Boeng/Cemperas	✓	✓	2	AY243347, DQ366154
Cyprininae	<i>Hampala bimaculata</i> Type A	Juak/Barop	✓	✓	2	AY697362, AY697375
Cyprininae	<i>Hampala bimaculata</i> Type B	Juak/Barop	✓	✓	1	AY697383
Cyprininae	<i>Hampala intermediae</i>	Barop		✓	2	AY697396, AY697397
Cyprininae	<i>Hampala macrolepidota</i>	Adong/Sebarau	✓	✓	2	AY697310, AY697345
Cyprininae	<i>Hampala sabana</i>	Barop		✓	1	AY697406
Cyprininae	<i>Hypsibarbus wetmorei</i>	Krai	✓		1	DQ366155
Cyprininae	<i>Lobocheilos bo</i>	Kulong		✓	2	DQ366156, DQ366157
Cyprininae	<i>Neolissochilus hexagonalepis</i>	Kejor/Tengas	✓		1	DQ366150
Cyprininae	<i>Neolissochilus stracheyi</i>	Kelah	✓		2	DQ366168, DQ366169
Cyprininae	<i>Osteochillus hasseltii</i>	Bantak/Pait/Terbul		✓	2	AY243346, DQ366160
Cyprininae	<i>Osteochillus spilurus</i>	Bantak/Pait/Terbul		✓	2	DQ366161, DQ366162
Cyprininae	<i>Osteochillus sp.</i>	Bantak		✓	2	DQ366158, DQ366159
Cyprininae	<i>Puntioptiles bulu</i>	Mengalan/Tengalan		✓	2	AY243349, DQ366163
Cyprininae	<i>Puntius binotatus</i>	Sisik tebal/Bangah	✓	✓	2	AY697411, AY365025
Cyprininae	<i>Puntius bramoides</i>	Kachong/Salap		✓	1	DQ366164
Cyprininae	<i>Puntius sealei</i>	Mata merah		✓	2	DQ366165, DQ366166
Cyprininae	<i>Tor douronensis</i>	Semah/Kelah/Pelian		✓	2	AY243356, DQ366167
Cyprininae	<i>Tor tambroides</i>	Kelah/Empurau	✓		1	DQ366170
Danioinae	<i>Leptobarbus hosii</i>	Sayan		✓	1	AY243350
Outgroup (Helostomatidae)	<i>Helostoma temminckii</i>	Biawan/Tebakang		✓	1	AY697412
Total					40	

PM= Peninsular Malaysia; Swk= Sarawak; Sbh= Sabah

cast-nets, pole-nets or were electro-fished with whole fish preserved in 95% ethanol. The total DNA was extracted from the muscle tissue using a CTAB method (Grewe *et al.*, 1993). The quality and approximate yield of DNA were determined through electrophoresis in a 1% agarose gel containing ethidium bromide run at 90V for 30 minutes and visualized under UV light.

DNA Sequencing

A set of primers were used to partially amplify the Cyt *b* gene; 5'-TGACT TGAAR AACCA YCGTTG-3' known as GluDG-L (Palumbi *et al.*, 1991) and 5'-CCCTC AGAAT GATAT TTGTC CTC A-3' known as CB2-H. Approximately 50-100ng of DNA template was amplified in a 25µl reaction mixture containing 50mM 10X Buffer, 2mM MgCl₂, 0.2 µM each dNTP (Fermentas), 0.1 µM of each primer, and 0.5 units of *Taq* DNA Polymerase (Fermentas). The cycle parameters consisted of 25 cycles of denaturation (95°C, 30 sec), annealing (47°C, 30 sec), and extension (72°C, 60 sec). The PCR products were further purified using DNA purification kits (Fermentas and Promega) according to the manufacturers' instructions. The purified PCR products were directly sequenced using the BigDye® Terminator v3.0 Cycle Sequencing kit (ACGT) on an ABI 377 automated sequencer (PE Applied Biosystem) using only the forward primer (GluDG-L).

Statistical Analysis

Multiple alignments of the sequences were conducted using ClustalX software (version 1.81; Thompson *et al.*, 1997), and aligned subsequently by eye. The pairwise genetic distance between each cyprinids was calculated using the Kimura two-parameter evolution model (Kimura, 1980) implemented in MEGA version 4.0 (Tamura *et al.*, 2007). Meanwhile, the saturation test for all the codons was done using DAMBE version 5.0.66 (Xia & Xie, 2007), and the phylogenetic relationships were inferred using two methods, namely distance analysis

using the neighbour-joining method (NJ) and the unweighted maximum parsimony (MP) analysis using close-neighbour-interchange, CNI option) implemented in MEGA. Phylogenetic trees inferred from the Cyt *b* sequences were rooted with *H. temminckii* (family: Helostomatidae, kissing gouramy) as the outgroup. Phylogenetic confidence was estimated by bootstrapping (Felsenstein, 1985) with 1000 replicate data sets.

RESULTS AND DISCUSSION

Forty sequences were obtained from twenty-four species (total length of 408 base pairs) and were used for phylogenetic analyses (two individuals of *Leptobarbus hosii* with slightly shorter sequences of 393 and 396 bp respectively were also typed). From the aligned sequences, 186 sites were variable and 144 were phylogenetically informative. The base compositions of sequences were similar to that of the previously reported fish Cyt *b* sequences (Cantatore *et al.*, 1994). Across the cyt *b* sequences, the nucleotide composition among the cyprinid fishes screened showed an anti-G bias, which is the characteristic of this mitochondrial gene (Cantatore *et al.*, 1994; Briolay *et al.*, 1998). The saturation test done onto the sequences at each codon, specifically the third codon which is known to have a faster rate of transition and the transversion showed that the transition at the third codon position was saturated (Fig. 1). The estimated transition : transversion ratio is approximately 1.7:1.

The genetic distances among the species were estimated with the Kimura two-parameter model (Kimura, 1980). Table 2 shows the genetic distances among the twenty-three fish species analyzed. *Neolissochilus hexagonalepis* was closely related to its sister taxa *N. stracheyi* - distance value of 0.5% (Table 2). Table 3 further summarizes the average genetic distances among the fish genera. The lowest genetic distance between the genera was observed between Genus *Neolissochilus* and Genus *Tor* (6.2%), while the highest genetic distance was between the Genus *Hampala* and Genus *Hypsibarbus* (18.8%). All the cyprinid sequences were

TABLE 2
Pairwise distance (%) among twenty-three species of cyprinid fishes analyzed based on the Cyt *b* gene. The distances were calculated using Kimura's two-parameter model of nucleotide substitution

	1	2	3	4	5	6	7	8	9	10	11
1 Barbodes collingwoodii											
2 Barbodes gonionotus	12.0										
3 Barbonymus schwanenfeldii	8.7	12.9									
4 Cyclocheilichthys apogon	10.4	11.4	11.9								
5 Hampala macrolepidota	17.3	17.2	19.9	19.0							
6 Hampala bimaculata Type A	17.2	14.8	16.5	17.1	11.5						
7 Hampala bimaculata Type B	14.7	17.2	15.4	15.9	13.3	9.6					
8 Hampala sabana	15.7	15.8	16.0	16.4	14.5	8.1	8.2				
9 Hampala intermedia	17.1	16.1	16.0	17.5	13.2	6.9	8.2	6.8			
10 Hypsibarbus wetmorei	12.9	13.7	13.4	12.9	20.3	18.6	16.1	18.1	19.1		
11 Lobocheilos bo	15.2	15.0	15.6	14.7	20.6	17.6	18.3	16.6	18.0	17.1	
12 Neolissochilus hexagonalepis	12.3	14.5	13.4	13.3	18.9	15.9	12.9	13.4	14.1	17.3	17.2
13 Neolissochilus stracheyi	12.3	14.5	13.4	13.3	19.5	16.2	12.8	13.4	14.1	17.3	17.0
14 Osteochillus sp.	14.8	17.5	14.3	15.3	19.1	17.4	15.9	15.7	15.7	16.7	16.6
15 Osteochillus hasseltii	13.9	15.4	15.8	13.5	19.5	19.5	17.4	15.4	17.7	16.5	16.9
16 Osteochillus spilurus	16.3	18.0	16.9	16.8	19.9	17.2	17.0	16.4	17.2	16.4	15.4
17 Puntioplites bulu	9.5	10.1	10.0	10.8	19.1	18.1	15.6	17.3	17.3	13.0	15.1
18 Puntius binotatus	13.6	17.4	14.3	14.8	15.6	17.0	16.2	17.4	16.4	15.1	17.8
19 Puntius bramoides	10.7	14.4	11.7	12.7	17.1	15.1	16.3	16.9	17.2	12.7	15.8
20 Puntius sealei	14.1	15.8	15.1	16.9	17.0	14.7	13.9	14.7	15.2	18.1	17.7
21 Tor douronensis	12.6	16.0	13.7	14.5	18.7	15.7	15.3	15.2	16.3	17.0	16.8
22 Tor tambroides	12.6	12.9	13.7	12.9	16.1	15.2	14.1	14.7	14.8	16.3	17.2
23 Leptobarbus hostii	15.1	15.5	15.1	16.8	16.2	18.6	15.6	16.6	15.9	16.8	17.4
24 Helostoma temminckii	23.9	25.6	26.1	27.1	28.6	28.3	27.9	26.7	27.2	31.3	29.8

Table 2 (continued)

	12	13	14	15	16	17	18	19	20	21	22	23	24
13 <i>Neolissochilus stracheyi</i>	0.5												
14 <i>Osteochillus</i> sp.	16.1	16.1											
15 <i>Osteochillus hasseltii</i>	15.3	15.3	11.1										
16 <i>Osteochillus spilurus</i>	16.5	16.5	12.3	12.0									
17 <i>Puntioplites bulu</i>	13.3	13.3	12.2	12.4	14.8								
18 <i>Puntius binotatus</i>	16.0	16.0	13.5	15.1	13.4	12.3							
19 <i>Puntius bramoides</i>	15.2	15.2	15.3	16.5	16.5	13.9	14.9						
20 <i>Puntius sealei</i>	15.0	15.7	18.0	16.1	16.4	13.4	12.1	16.0					
21 <i>Tor douronensis</i>	6.2	6.5	17.3	15.6	16.1	14.7	17.1	15.0	16.6				
22 <i>Tor tambroides</i>	5.4	5.9	16.8	15.9	17.1	13.6	16.7	14.2	16.3	6.2			
23 <i>Leptobarbus hosii</i>	17.8	18.5	15.7	15.8	16.1	14.0	18.1	16.7	17.2	16.8	15.4		
24 <i>Helostoma temminckii</i>	25.7	25.5	26.6	26.2	25.1	24.6	26.8	28.4	27.1	26.1	25.7	24.5	

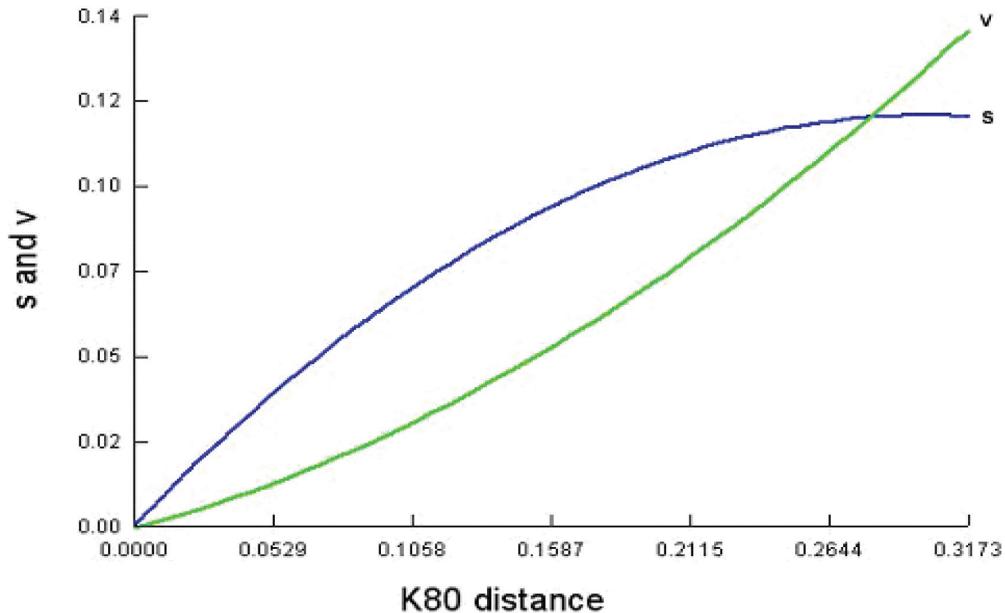


Fig.1: Plot of transition (s) and transversion (v) against divergence using Tamura and Nei (1993) distance method onto the third codon position showing saturation of the transition codon of the Cyt b gene

distantly related from the outgroup species, *H. temmincki* (Family Helostomatidae), with the distance values ranging from 23.9% to 31.3%, respectively (Table 3).

Fig. 2 presents the phylogenetic tree recovered from the partial Cyt b sequences of thirty-nine cyprinid individuals and one outgroup species, constructed using both NJ and MP methods (only the NJ tree is presented since tree topologies are very similar). Although the phylogeny appeared to match the major groupings currently recognized in the taxonomy, no support was evident for nearly all higher level groupings. Thus, it is clear that further work is needed to clarify the relationships between the many genera.

The phylogenetic analysis grouped the two genera of Mahseer fishes that exist in Malaysia, namely, Genus *Neolissochilus* and Genus *Tor*. The relationship between the two species in the genus *Tor* (*T. douronensis* and *T. tambroides*) was poorly resolved using the NJ and MP methods. In contrast, a close relationship between *N.*

hexagonalepis with *N. stracheyi* is supported by a strong bootstrap value (>99%). Nonetheless, a more variable mtDNA marker (e.g. control region or COI genes) or longer sequence of mtDNA genes may be required to further resolve systematic relationship among Mahseer species and populations.

A recent revision on the taxonomic classification of fishes within the genus *Puntius* has shown that some previously recognized taxa have been assigned to new genera: *Puntius collingwoodii* (Kottelat *et al.*, 1993) to *Barbodes collingwoodii* (Martin-Smith, 1996), *Puntius javanicus* (Davidson, 1975) to *Barbonymus gonionotus* (Kottelat, 2001), *P. schwanenfeldii* (Vidthayanon *et al.*, 1997) to *Barbonymus schwanenfeldii* (Kottelat, 2001), *P. daruphani* (Vidthayanon *et al.*, 1997) to *Hypsibarbus wetmorei* (Kottelat, 2001) and *P. bulu* (Kottelat *et al.*, 1993) to *Puntioplites bulu* (Kottelat & Whitten, 1996). The phylogenetic analysis using both NJ and MP has shown that all previously described species in the genus *Puntius* are

TABLE 3
 A summarized of average pairwise genetic distance (%) among the cyprinids fishes and the outgroup (*Helostoma*) of the Cyt *b* gene. The distances were calculated using Kimura's two-parameter model of nucleotide substitution

	1	2	3	4	5	6	7	8	9	10	11	12	13
1 <i>Barbodes</i>													
2 <i>Barbonymus</i>	10.4												
3 <i>Cyclocheilichthys</i>	10.4	11.6											
4 <i>Hampala</i>	16.7	16.6	17.4										
5 <i>Hypsibarbus</i>	12.9	13.5	12.9	18.8									
6 <i>Lobocheilos</i>	15.2	15.3	14.7	18.4	17.1								
7 <i>Neolissochilus</i>	12.3	13.9	13.3	15.7	17.3	17.0							
8 <i>Osteochillus</i>	15.0	16.3	15.2	17.7	16.5	16.3	15.9						
9 <i>Puntioplites</i>	9.5	10.0	10.8	17.7	13.0	15.1	13.3	13.1					
10 <i>Puntius</i>	13.2	15.1	15.2	16.0	15.8	17.4	15.6	15.6	13.1				
11 <i>Tor</i>	12.6	14.3	14.0	16.0	16.8	16.9	6.2	16.4	14.4	16.4			
12 <i>Leptobarbus</i>	15.1	15.3	16.8	16.7	16.8	17.4	18.3	15.8	14.0	17.5	16.3		
13 <i>Helostoma</i>	23.9	25.9	27.1	27.8	31.3	29.8	25.6	26.0	24.6	27.2	26.0	24.5	

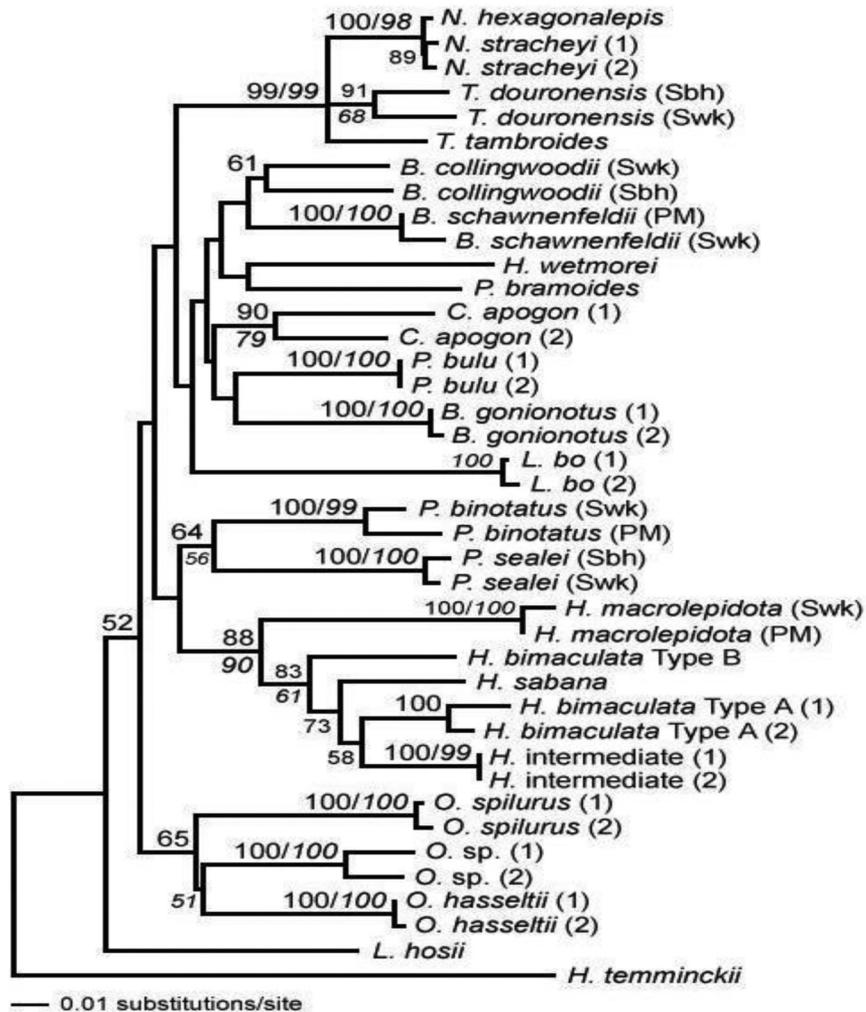


Fig. 2: Phylogenetic relationships of indigenous fishes under study based on the Cyt b gene of the mtDNA. The values on the branches represent both NJ and MP bootstrap estimates (italic values represents MP analysis), based on 1000 replicates. Only the bootstrap values >50% are shown (PM=Peninsular Malaysia; Swk=Sarawak; Sbh=Sabah)

divided into two sub-groups; the newly elevated *Puntius* species clustered randomly across the trees, while the remaining two *Puntius* fishes (*P. binotatus* and *P. sealei*) formed a distinct *Puntius* cluster with strong bootstrap support (>99%). Thus, the current molecular data suggest that *P. binotatus* and *P. sealei* could be the representative of the genus *Puntius*, and any

other species identified as belonging to the genus *Puntius* should cluster with this group.

In addition, the phylogenetic analysis revealed that the two morphologically similar *Barbonymus* species, *B. gonionotus* and *B. schwanenfeldii* did not cluster in a single *Barbonymus* clade. Instead, *B. gonionotus* clustered with *P. bulu* and *Cyclocheilichthys*

apogon while *B. schwanenfeldii* formed a second clade with *B. collingwoodii*, *H. wetmorei* and *P. bramoides*. This result suggests that the morphological similarity between *B. gonionotus* and *B. schwanenfeldii* may result from the convergent evolution rather than co-ancestry. *B. gonionotus* is not native to Peninsular Malaysia, as it was introduced from Java at the beginning of the 19th century (Welcomme, 1981). In Malaysia, this particular exotic species has since bred well in ponds and in natural river systems where it was introduced. Nowadays, *B. gonionotus* is found living in sympatry with *B. schwanenfeldii* in many river systems. Nonetheless, some recent molecular studies using *Cyt b* mtDNA RFLP fragment analysis (Esa & Khairul, 2003) of the two species from the sites where they are sympatric in the Seriting River (Negeri Sembilan) did not find any evidence for hybrid introgression, supporting their genetic distinctiveness (distance value of 13.0%).

The genus *Hampala* was one of the main focuses of the current study. As indicated earlier on, the phylogenetic analysis produced slightly different NJ and MP topologies, particularly in relation to the relationships among *H. bimaculata*, *H. sabana* and an undescribed *Hampala* taxa (known in this study as the intermediate form). Two important findings were investigated further within the genus *Hampala*. First, the widespread *H. macrolepidota* was phylogenetically distinct from other Borneo endemic *Hampala* taxa and was an older lineage than the other forms. Secondly, this study identified two monophyletic *H. bimaculata* haplotypes, with different geographical distributions (Type A from Southern and Central Sarawak, Type B from Northern Sarawak and the West Coast of Sabah). In other words, they were found to represent distinct mtDNA lineages. Therefore, a thorough and more detailed molecular study on the phylogeography and phylogenetic relationships among *Hampala* fishes should provide better insights into the systematic (and

taxonomic) status and evolutionary history of this interesting genus.

Overall, the taxonomy and systematic of freshwater fishes in Malaysia are fragmented and poorly resolved. The current study has provided a robust attempt to reconstruct the phylogeny of a number of cyprinid taxa in Malaysia using the molecular approach. Indeed, the molecular data generated here have shown that a molecular approach could be very useful in clarifying the systematic status of the Malaysian freshwater fish species. In addition, the richness and high biodiversity of the fauna particularly freshwater fishes should be properly documented for appropriate management and conservation. Meanwhile, more molecular studies should be undertaken to examine larger datasets of cyprinids and related families in order to obtain a more comprehensive understanding of their systematic relationships.

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REFERENCES

- Amos B., & Hoelzel, A. R. (1992). Application of molecular genetic techniques to the conservation of small populations. *Biological Conservation*, 61, 133-144.
- Avise, J. C., & Hamrick, J. L. (1995). *Conservation Genetics: Case Histories from Nature*. New York: Chapman & Hall.
- Avise, J. C. (1994). *Molecular Markers: Natural History and Evolution*. New York: Chapman & Hall.

- Briolay, J., Gartier, N., Brito, R. M., & Bouvet, Y. (1998). Molecular phylogeny of Cyprinidae inferred from cytochrome *b* DNA sequences. *Molecular Phylogenetics and Evolution*, *9*, 100-108.
- Cantatore, P., Roberti, M., Pesole, G., Ludovico, A., Milella, F., Gadaleta, M. N., & Saccone, C. (1994). Evolutionary analysis of cytochrome *b* sequences in some perciformes: Evidence for a slower rate of evolution than in mammals. *Journal of Molecular Evolution*, *39*, 589-597.
- Davidson, A. (1975). *Fish and fish dishes of Laos*. Vientiane: Imprimerie Nationale.
- Esa, Y. B., & Khairul Adha, A. R. (2003). The Impact of Introduced Species (Non-Native and Exotic) on the Genetic Diversity of Native Freshwater Fishes in Malaysia. Los Banos: ASEAN Regional Centre for Biodiversity Conservation (ARCBC) publication.
- Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, *39*, 783-791.
- Froese, R., & Pauly, D. (2004). *FishBase. World Wide Web electronic publication*. Retrieved from www.fishbase.org, version (09/2004).
- Grewe, P. M., Krueger, C. C., Aquadro, C. F., Bermingham, E., Kincaid, H. L., & May, B. (1993). Mitochondrial variation among lake trout (*Salvelinus namaycush*) strains stocked into Lake Ontario. *Canadian Journal of Fisheries and Aquatic Science*, *50*, 2397-2403.
- Inger, R. F., & Chin, P. K. (1962). *The Freshwater Fishes of North Borneo*. Chicago: Chicago Natural History Museum.
- Khan, M. S., Lee, P. K. Y., Cramphorn, J., & Zakaria-Ismail, M. (1996). *Freshwater Fishes of the Pahang River Basin, Malaysia*. Malaysia: Wetlands International-Asia Pacific.
- Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, *16*, 111-120.
- Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pääbo, S., Villablanca, F. X., & Wilson, A. C. (1989). Dynamics of mitochondrial DNA evolution in mammals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Science, USA*, *86*, 6196-6200.
- Kottelat, M., & Whitten, A.J. (1996). *Freshwater fishes of Western Indonesia and Sulawesi: Additions and Corrections*. Hong Kong: Periplus Eds.
- Kottelat, M. (2001). *Fishes of Laos*. Colombo: WHT Pubs. Ltd.
- Kottelat, M., Whitten, A. J., Kartokasari, S. N., & Wirjorajmodjo, S. (1993). *Freshwater Fishes of Western Indonesia and Sulawesi*. Singapore: Berkeley Book Pte. Ltd.
- Kumar, S., Tamura, K., Jakobsen, I. B., & Nei, M. (2001). *MEGA2: Molecular Evolutionary Genetics Analysis software*. Arizona: Arizona State University.
- Litis, B. A., Sungan, S., Jugang, K., Ibrahim, M., & Bini, H. A. (1997). *Features of Indigenous Fish Species Having Potential for Aquaculture*. Sarawak, Malaysia: Inland Fish. Div. Dept. Agric.
- Martin-Smith, K. M. (1996). Length/weight relationships of fishes in a diverse tropical freshwater community, Sabah, Malaysia. *Journal of Fish Biology*, *49*, 731-734.
- Meyer, A., Kocher, T. D., Basasibwak, P., & Wilson, A. C. (1990). Monophyletic origin of Lake Victoria cichlid fishes suggested by mitochondrial DNA sequences. *Nature*, *347*, 550-553.
- Mohsin, A. K. M. and Ambak, M. A. (1983). *Freshwater Fishes of Peninsular Malaysia*. Selangor, Malaysia: Universiti Pertanian Malaysia Publication.
- Ng, C. K. (2004). *Kings of the rivers: Mahseer in Malaysia and the region*. Kuala Lumpur: Inter Sea Fishery (M) Sdn. Bhd. Pub.
- Palumbi, S., Martin, A., Romano, S., McMillan, W. O., Stice, L., & Grabowski, G. (1991). *The Simple Fool's Guide To PCR*. University of Hawaii: Dept. of Zool. & Kewalo Marine Laboratory Publication.
- Roberts, T. R. (1989). *The Freshwater Fishes of Western Borneo (Kalimantan Barat, Indonesia)*. San Francisco: California Academy of Science.

- Stepien, C. A., & Kocher, T. D. (1997). Molecular and Morphology in Studies of Fish Evolution. In Kocher, T. D, Stepien, C. A. (Eds). *Molecular Systematics of Fishes* (p.1-11). San Diego: California Academic Press.
- Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, 24, 1596-1599.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by the quality analysis tools. *Nucleic Acids Research*, 24, 4876-4882.
- Vidhayanon, C., Karnasuta, J., & Nabhitabhata, J. (1997). *Diversity of freshwater fishes in Thailand*. Thailand: Office of Enviromental Policy & Planning.
- Vrijenhoek, R. C. (1998). Conservation genetics of freshwater fish. *Journal of Fish Biology*, 53, 394-412.
- Welcomme, R. L. (1981). Register of international transfers of inland fish species. *FAO Fisheries Technical Paper*, 213, 120.
- Xia, X., & Xie, Z. (2001). DAMBE: Software package for data analysis in molecular biology and evolution. *Heredity*, 92(4), 371-373.
- Zakaria Ismail, M. (1990). Cyprinid fishes of the Genus *Cyclocheilichthys* in Peninsular Malaysia. *Malayan Nature Journal*, 44, 109-121.