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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. schwanenfeldii*) 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 (Avise, 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; Avise, 1994; Avise & 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	Swk	Sbh		
Family: Cyprinidae	Cyprininae	*Barbodes collingwoodii*	Kepiat		√	√	2	AY243348, DQ366151
	Cyprininae	*Barbonymus gonionotus*	Lampam jawa			√	2	DQ366152, DQ366153
	Cyprininae	*Barbonymus schwanenfeldii*	Tengadak/Lampam sungai	√	√		2	AY355438, AY355426
	Cyprininae	*Cyclocheilichthys apogon*	Boeng/Cemperas		√		2	AY243347, DQ366154
	Cyprininae	*Hampala bimaculata* Type A	Juak/Barop		√		2	AY697362, AY697375
	Cyprininae	*Hampala bimaculata* Type B	Juak/Barop		√	√	1	AY697383
	Cyprininae	*Hampala* intermediate	Barop			√	2	AY697396, AY697397
	Cyprininae	*Hampala macrolepidota*	Adong/Sebarau	√	√		2	AY697310, AY697345
	Cyprininae	*Hampala sabana*	Barop			√	1	AY697406
	Cyprininae	*Hypsibarbus wetmorei*	Krai	√			1	DQ366155
	Cyprininae	*Lobocheilos bo*	Kulong		√		2	DQ366156, DQ366157
	Cyprininae	*Neolissochilus hexagonalepis*	Kejor/Tengas	√			1	DQ366150
	Cyprininae	*Neolissochilus stracheyi*	Kelah	√			2	DQ366168, DQ366169
	Cyprininae	*Osteochillus hasseltii*	Bantak/Pait/Terbul		√		2	AY243346, DQ366160
	Cyprininae	*Osteochillus spilurus*	Bantak/Pait/Terbul			√	2	DQ366161, DQ366162
	Cyprininae	*Osteochillus sp.*	Bantak		√		2	DQ366158, DQ366159
	Cyprininae	*Puntioplites bulu*	Mengalan/Tengalan		√		2	AY243349, DQ366163
	Cyprininae	*Puntius binotatus*	Sisik tebal/Bangah	√	√		2	AY697411, AY365025
	Cyprininae	*Puntius bramoides*	Kachong/Salap			√	1	DQ366164
	Cyprininae	*Puntius sealei*	Mata merah		√	√	2	DQ366165, DQ366166
	Cyprininae	*Tor douronensis*	Semah/Kelah/Pelian		√	√	2	AY243356, DQ366167
	Cyprininae	*Tor tambroides*	Kelah/Empurau	√			1	DQ366170
	Danioinae	*Leptobarbus hosii*	Sayan		√		1	AY243350
Outgroup (Helostomatidae)		*Helostoma temminckii*	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 YCGTT G- 3' known as GluDG-L (Palumbi *et al*., 1991) and 5'-CCCTC AGAAT GATAT TTGTC CTCA- 3' known as CB2-H. Approximately 50-100ng of DNA template was amplified in a 25µl reaction mixture containing 50mM 10X Buffer, 2mM $MgCl_2$, 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 goramy) 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* intermediate	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 hosii*	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	*Neolissochilus stracheyi*	0.5												
14	*Osteochillus* sp.	16.1	16.1											
15	*Osteochillus hasseltii*	15.3	15.3	11.1										
16	*Osteochillus spilurus*	16.5	16.5	12.3	12.0									
17	*Puntioplites bulu*	13.3	13.3	12.2	12.4	14.8								
18	*Puntius binotatus*	16.0	16.0	13.5	15.1	13.4	12.3							
19	*Puntius bramoides*	15.2	15.2	15.3	16.5	16.5	13.9	14.9						
20	*Puntius sealei*	15.0	15.7	18.0	16.1	16.4	13.4	12.1	16.0					
21	*Tor douronensis*	6.2	6.5	17.3	15.6	16.1	14.7	17.1	15.0	16.6				
22	*Tor tambroides*	5.4	5.9	16.8	15.9	17.1	13.6	16.7	14.2	16.3	6.2			
23	Leptobarbus hosii	17.8	18.5	15.7	15.8	16.1	14.0	18.1	16.7	17.2	16.8	15.4		
24	*Helostoma temminckii*	25.7	25.5	26.6	26.2	25.1	24.6	26.8	28.4	27.1	26.1	25.7	24.5	

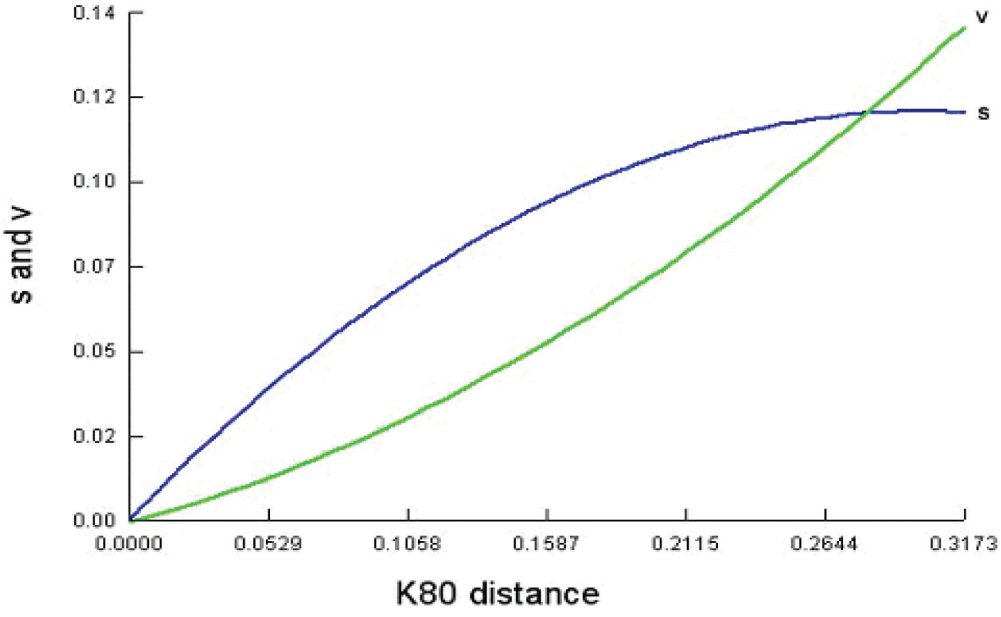

Fig.1: Plot of transition (s) and transversion (v) against divergence using Tamura and Nei (1993) distance method onto the third codon position showing saturation of the transition codon of the Cyt b *gene*

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

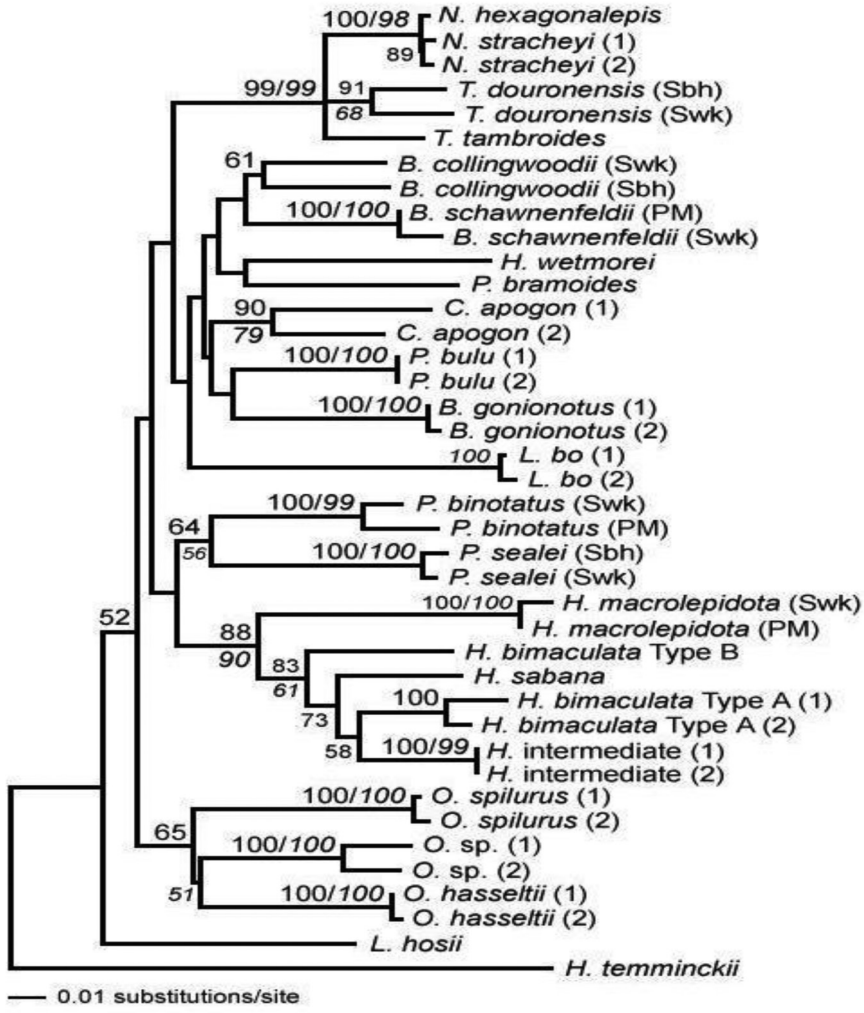

Fig. 2: Phylogenetic relationships of indigenous fishes under study based on the Cyt b *gene of the mtDNA. The values on the branches represent both NJ and MP bootstrap estimates (italic values represents MP analysis), based on 1000 replicates. Only the bootstrap values >50% are shown (PM=Peninsular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