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Research Article

Phylogenetic analysis of selected cyprinids inferred from sequencing of a mitochondrial *cytochrome c oxidase* I (COI) gene

Yuzine B. Esa[1*], Siti Shapor Siraj[1], Khairul Adha A. Rahim[2], Siti Khalijah Daud[3] and Tan Soon Guan[4]

[1]*Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia 43400 Serdang Selangor DE, Malaysia. *email: yuzine@putra.upm.edu.my*

[2]*Department of Aquatic Science, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak.*

[3]*Biology Department, Faculty of Science, Universiti Putra Malaysia 43400 Serdang, Selangor DE, Malaysia.*

[4]*Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia 43400 Serdang Selangor DE, Malaysia.*

ABSTRACT. This study examines the phylogenetic relationships of 21 selected freshwater cyprinids using sequence analysis of mitochondrial DNA *cytochrome c oxidase* I (COI) gene (464 base pairs). The phylogenetic study supported the monophyletic status between genus *Tor* and *Neolissochillus*, although their positioning within the mahseer clade (together with mahseers from South Asia) remained unresolved. Thus, the current result supported their taxonomic distinction and further erected the reclassification of *Neolissochillus stracheyi* from the genus *Tor* (previously classified as *Tor soro* into the genus *Neolissochilus* based on morphological characters such as the absence of the median lobe. The phylogenetic results also showed that the genus *Barbus* (represented by *Barbus barbus*) was the closest taxa to the genus *Tor*, followed by *Cyprinus carpio* and *Barbonymus gonionotus*. Another interesting finding was that *B. gonionotus* was phylogenetically distinct from its morphologically similar species, *Barbonymus schwanenfeldii* (K2P distance value = 15.1%) and did not group together in a single *Barbonymus* clade. The high genetic divergence observed between *B. schwanenfeldii* and *B. gonionotus* recommends the taxonomic revision of the latter barb from its current position within the genus *Barbonymus*. However, shortcomings of our results are clearly recognized and data should be treated with great caution, since it was based on a limited number of samples and a single maternally inherited gene (COI). Overall, the study managed to provide an insight into the phylogenetic relationships among cyprinids under study.

Keywords: Cyprinidae, freshwater fish, mtDNA, COI, phylogeny.

INTRODUCTION

Freshwater fishes of the Family Cyprinidae form the largest family of freshwater fishes in terms of number of genera and species worldwide, including in Malaysia (Zakaria-

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Ismail, 1990). To date, more than 500 indigenous cyprinid species have been described and recorded from Peninsular Malaysia and Borneo Island (Mohsin & Ambak, 1983; Roberts, 1989, Kottelat *et al.*, 1993; Inger & Chin, 2002). Among the cyprinids, fishes of the genus *Tor* Gray (subfamily Cyprininae), commonly known as mahseer, are one of the most important freshwater fishes (Litis *et al.*, 1997; Ng, 2004).

Unfortunately, phylogenetic studies of cyprinids fishes of Southeast Asia including in Malaysia are scarce and the taxonomy is poorly resolved. Nevertheless, new technology such as molecular genetics technique has facilitated in resolving the taxonomic and phylogenetic problems commonly faced by non-genetic (morphological and meristic) characters since molecular data are heritably transmitted and are highly unlikely to be influenced by environmental factors (Avise, 1994).

Among molecular phylogenetic studies of Cyprinidae include those by Briolay *et al.* (1998) on the cyprinids of Central and South America using cytochrome b (*cyt* b) sequences; and Liu & Chen (2003) on the East Asian cyprinids using control region (D-loop). No molecular phylogenetic study has been reported on the cyprinids of Southeast Asia. On a genetic level, Ryan & Esa (2006) examined the phylogenetic relationship among freshwater fishes of the genus *Hampala* in Malaysia using partial *cyt* b sequences and Nguyen *et al.* (2008) recently examined the phylogenetic relationships of selected mahseer throughout Asia using three mitochondrial DNA gene regions (16S rRNA, *cyt b* and ATPase6-8).

The present study aimed to clarify the phylogenetic relationships among selected cyprinids of Malaysia by utilizing direct sequencing of the mitochondrial COI gene and examining their phylogenetic relationship with the mahseer. Among the indigenous cyprinids included were from the genus *Barbodes, Barbonymus, Cyclocheilichthys, Hampala* and *Puntius*. In addition, a few sequences of cyprinids originated outside the Southeast Asia region (e.g. goldfishes and Chinese carps) and two mahseer species from South Asia were also included in the analysis to provide a better insight into the cyprinids phylogeny under the present study.

MATERIALS AND METHODS

Sample sources and DNA extraction

The indigenous fish samples used in the study were obtained from various river systems in Peninsular Malaysia, Sarawak and Sabah (Table1). The specimens were collected with cast-nets, pole-nets or electro-fishing apparatus and fish was preserved in 95% ethanol. Full samples were morphologically recognized by using keys provided by Mohsin & Ambak (1983), Kottelat *et al.* (1993) and Inger & Chin (2002). Total DNA was extracted from muscle tissue using CTAB (Grewe *et al.*, 1993). The quality and approximate yield of DNA were determined by electrophoresis in a 1% agarose gel containing ethidium bromide run at 90V for 30 minutes and visualized under UV light.

Polymerase chain reaction (PCR) and DNA sequencing

A 500 bp segment of the *cytochrome c oxidase* I gene was amplified with the oligonucleotide primers COIf (5' CCTGCAGGAGGAGGAG AYCC 3', forward) and COIe (5' CCAGAGATTAGAGGGAATCAGTG 3', reverse) as described by Palumbi *et al.* (1991). Approximately, 50-100 ng of the template DNA was amplified in a 25 l reaction mixture containing 50 mM 10X buffer, 2 mM $MgCl_2$, 0.2 M of each dNTP (Promega), 0.1 M of each primer, and 0.5 units of *Taq* DNA polymerase (Promega). The cycle parameters consisted of 35 cycles of denaturation (95°C, 30 seconds), annealing (45°C, 30 seconds), and extension (72°C, 60 seconds). The amplified products were visualized on 2% agarose gel containing ethidium bromide, ran for approximately 30 min at 90 V and photographed under UV light. 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 (COIf). A sequencing reaction using the reverse primer (COIe) was subsequently carried out on some of the samples (haplotypes) to verify the polymorphism in the DNA sequence initially detected using the forward primer.

Statistical analysis

Sequences of 16 cyprinids retrieved from GenBank were added into the phylogenetic analysis (Table 1). This includes sequences of two mahseer from South Asia (two sequences of each *Tor khudree* and *Tor malabaricus*, respectively). Trees were rooted using sequences of *Rasbora sumatrana*

Table 1. Samples of indigenous cyprinids, mahseer from South Asia, other cyprinids and outgroup species used in this study. *n* = Number of sample

Category	Species	*n*	GenBank Accession Number
Indigenous species	*Tor douronensis*	6	EF192444, EF192445, EF192449, EF192451, EF192454, EF192456
	Tor tambroides	5	DQ532827, EF192458, DQ532856, EF192460, EF660859
	Neolissochillus stracheyi	3	EF192462, EF192463, DQ366196
	Barbonymus schwanenfeldii	4	DQ532805, FJ464383-FJ464386
	Barbonymus gonionotus	3	FJ464387-FJ464389
	Barbodes colingwoodii	1	FJ464396
	Puntius bramoides	2	FJ464392-FJ464393
	Cyclocheilichthys apogon	2	FJ464390-FJ464391
	Hampala bimaculata	1	FJ997244
Mahseer from South Asia	*Tor khudree*	2	DQ520926, DQ520927
	Tor malabaricus	2	DQ520928, DQ520929
Other cyprinids	*Barbus barbus*	1	AB238965
	Puntius tetrazona	1	EU287909
	Carassius carassius	1	AY714387
	Carassius auratus auratus	1	AB111951
	Carassius cuvieri	1	AB045144
	Carassius auratus langsdorfi	1	AB006953
	Ctenopharyngodon idella	1	EU391390
	Hypophthalmichthys molitrix	1	EU315941
	Hypophthalmichthys nobilis	1	EU343733
	Cyprinus carpio	1	AP009047
Outgroup species	*Rasbora sumatrana*	1	EF452882
	Salmo salar	1	EU524353

(Rasboranidae) and *Salmo salar* (Salmonidae), obtained from GenBank (Table 1).

Multiple alignments of the sequences were conducted using the ClustalX programme (version 2.0.10; Larkin *et al.*, 2007), and subsequently aligned by eye. The pairwise genetic distance between each cyprinid was calculated using the Kimura two-parameter evolution model (Kimura, 1980) implemented in MEGA (version 4.0; Tamura *et al.*, 2007). Saturation test for all codon was done using DAMBE version 5.0.66 (Xia & Xie, 2001).

Phylogenetic relationships were inferred using four different methods of analysis: neighbour-joining (NJ) (Saitou & Nei, 1987), maximum parsimony (MP), maximum likelihood (ML) and Bayesian analysis. Modeltest 3.06 PPC (Posada & Crandall, 1998) was used to identify the best model of evolution for the COI dataset. The model with the best maximum likelihood (ML) score using the Akaike Information Criterion (AIC) was chosen (Akaike, 1973). The best model suggested by the analysis was subsequently used in maximum-likelihood (ML) and Bayesian analyses.

A distance analysis using the neighbour-joining (NJ) method was done using a close-neighbour-interchange (CNI) option implemented in MEGA (version 4.0; Tamura *et al.*, 2007). The NJ clustering was performed using the Kimura two-parameter evolution model (Kimura, 1980). Phylogenetic confidence was estimated by bootstrapping (Felsenstein, 1985) with 1000 replicate data sets.

A maximum parsimony (MP) tree was estimated using heuristic searches, as implemented in PAUP* v4.0b10 (Swofford, 2001). Heuristic searches were implemented using random addition sequence (100 repetitions) and tree bisection-reconnection (TBR) branch swapping procedure. Bootstrap trees (Felsenstein, 1985) were computed using 1000 replicates.

Phylogenetic tree was also estimated using the maximum-likelihood (ML) approach also implemented in PAUP* v4.0b10 (Swofford, 2001). Bootstrap values were estimated using the same method as above but with 100 replicates and branch swapping. Bayesian analyses were performed using MrBayes version 3.0 (Ronquist & Huelsenbeck, 2003). The Markov Chain Monte Carlo (MCMC) process was set to 4×10^6 generations with trees being sampled every 100 generations.

RESULTS

The characteristics of COI dataset

The COI dataset analysed using Modeltest 3.06 PPC (Posada & Crandall, 1998) resulted in the General Time Reversible model plus gamma (GTR + Γ) as the assumed model of DNA evolution. The model was further used in the ML and Bayesian analyses. The estimated nucleotide frequencies were: A= 0.265, C= 0.228, G= 0.195 and T= 0.313. ML scores in Bayesian analysis was (LnL = -7314) and the shape parameter of the discrete gamma distribution was $\Gamma = 0.9772$. Thus the "burnin" (the time that is needed to reach a "steady state" of ML scores) was 7314, so the first 7314 generations were regarded as being uninformative for the analysis. The identical sites accounted for 130 (27.8%) of the 468 bp of the COI sequences in all taxa, excluding the two outgroups (*Rasbora sumatrana* and *Salmo salar*), while 338 (72.2%) were variable with 287 (61.3%) parsimony informative sites. Saturation tests done to sequences at each codon especially the third codon which usually had a faster rate of transition and transversion showed little saturation (Figure 1) but was still useful for phylogenetic analysis.

Phylogenetic relationships among samples

The NJ analysis produced tree topology (Figure 2) slightly different compared to the Bayesian, ML and MP analyses which produced tree topologies almost similar to each

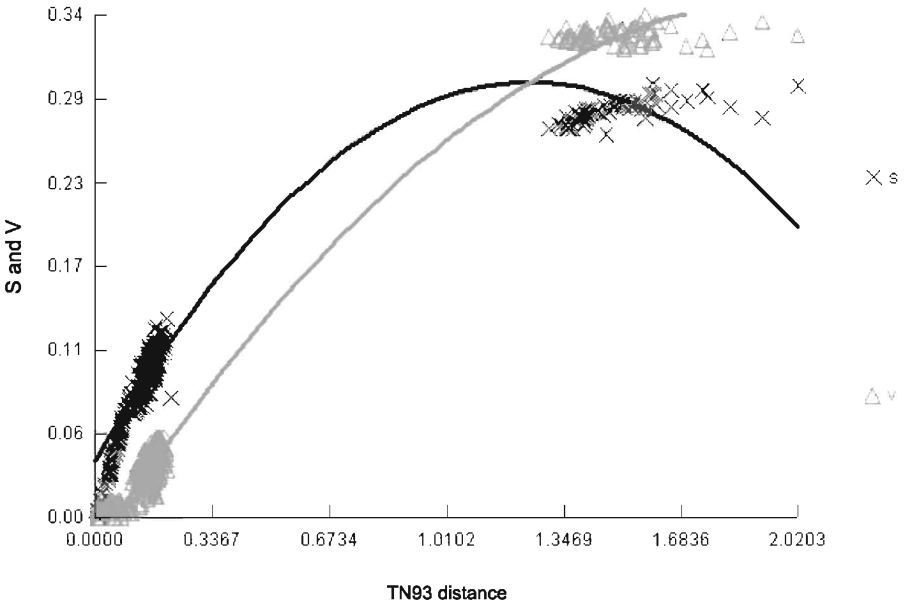

Figure 1. Plot of transition (s) and transversion (v) against divergence using Tamura and Nei (1993) distance method onto the third codon position shows little saturation of the codon in the COI gene.

other, particularly the clustering and positioning of the mahseers (genus *Tor* and *Neolissochillus*) (Figures 3, 4 & 5). The phylogeny appeared to match major groupings currently recognized in the taxonomy but there was no support for nearly all the higher level groupings. It is clear further work will be needed to clarify the relationships between many of these genera. Nevertheless, there are some interesting insights in relation to some of the species and genus under study.

All samples of *N. stracheyi* were grouped together with strong bootstrap support (99% posterior probabilities (pP), 100% bootstrap from ML and MP, 95% from NJ). The other mahseer samples formed a resolved *Tor* lineage with low to high support (pP= 93%, 65%, 58% and < 50% from ML, MP and NJ analysis, respectively). The ML and Bayesian analyses placed *T. malabaricus* as sister taxa to *T. douronensis* and *T. khudree* as sister taxa to *T. tambroides*. The MP analysis, however, positioned *T. khudree* as basal to the other *Tor* group. On the other hand, the NJ method grouped *T. malabaricus* closer to the *T. khudree* and *T. tambroides* clusters. Interestingly, all methods of analysis positioned *Barbus barbus* as basal to the mahseer group with high bootstrap support.

All the goldfish samples were clustered together in a *Carrasius* lineage and the three Chinese carp samples (*Hypophthalmichthys molitrix*, *Hypophthalmichthys nobilis* and *Ctenopharyngodon idella*) were clustered together in a same group. Interestingly, the carnivorous *Hampala bimaculata* was grouped together with *Puntius tetrazona*, a small cyprinid well-known as an ornamental fish. All methods of analysis did not position two cyprinids of the genus *Barbonymus*, *Barbonymus gonionotus* (Java barb) and *Barbonymus schwanenfeldii* (Tinfoil barb) under the same clade, and their level of genetic divergence was substantially high (15.1%, Table 2). However, *Cyprinus carpio* (Common carp) was always positioned as basal to the other cyprinid groups under study from all methods of analysis.

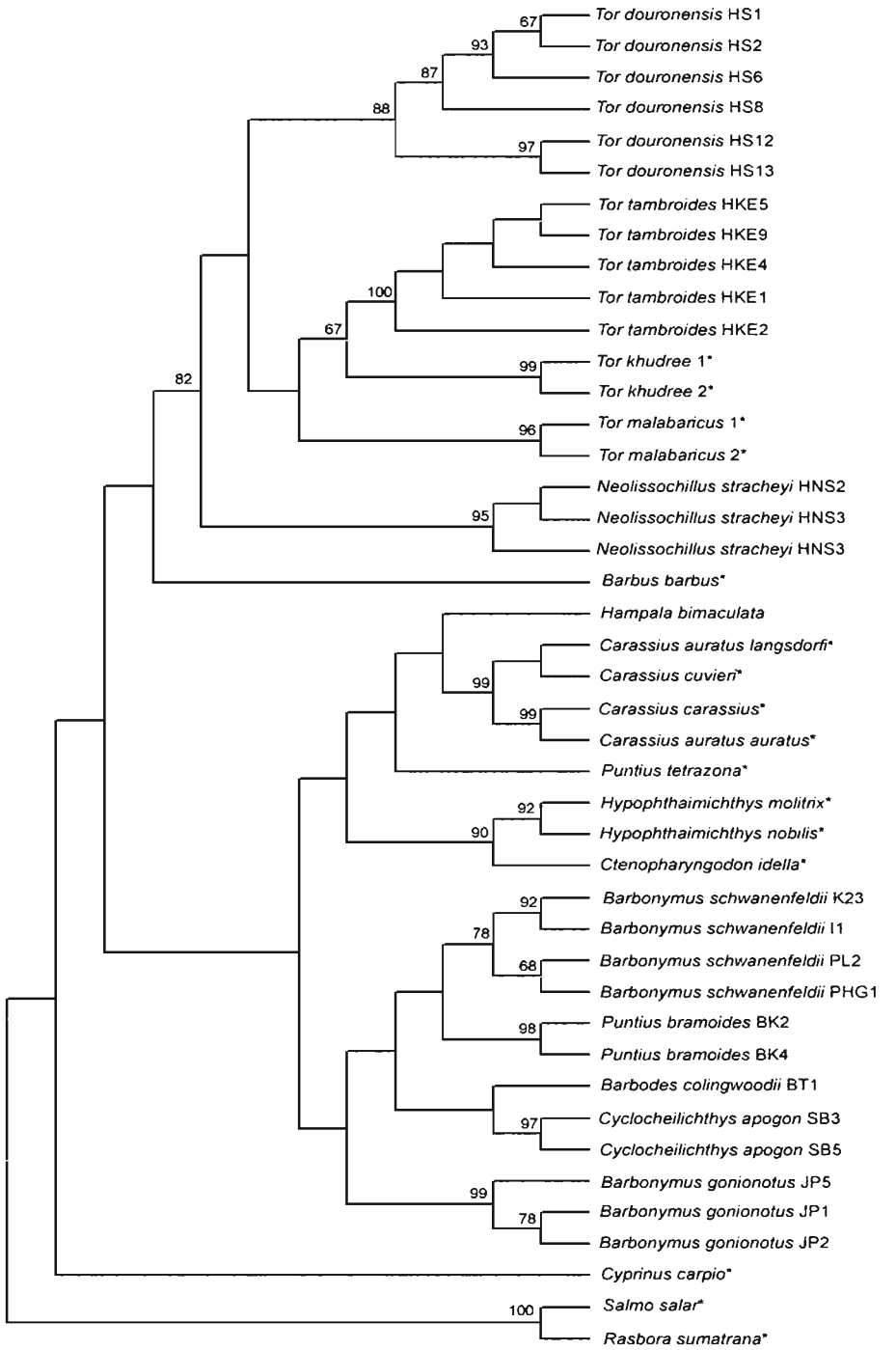

Figure 2. Neighbour-Joining (NJ) tree (consensus tree) showing relationships among the cyprinids. The number at each node represents the bootstrap value (%) based on 1000 pseudoreplications for NJ analysis.

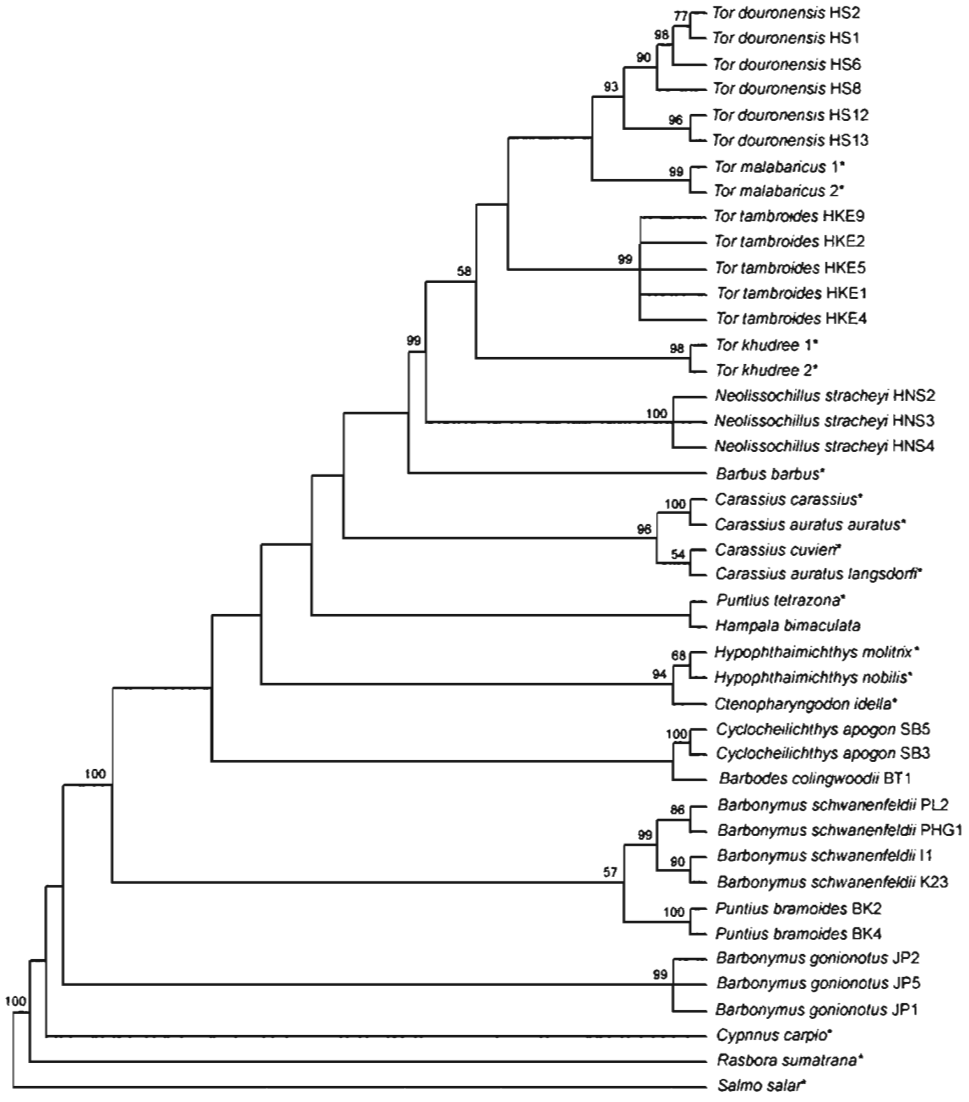

Figure 3. Maximum-Parsimony (MP) tree showing relationships among the cyprinids. The number at each node represents the bootstrap value (%) based on 1000 pseudoreplications of the dataset.

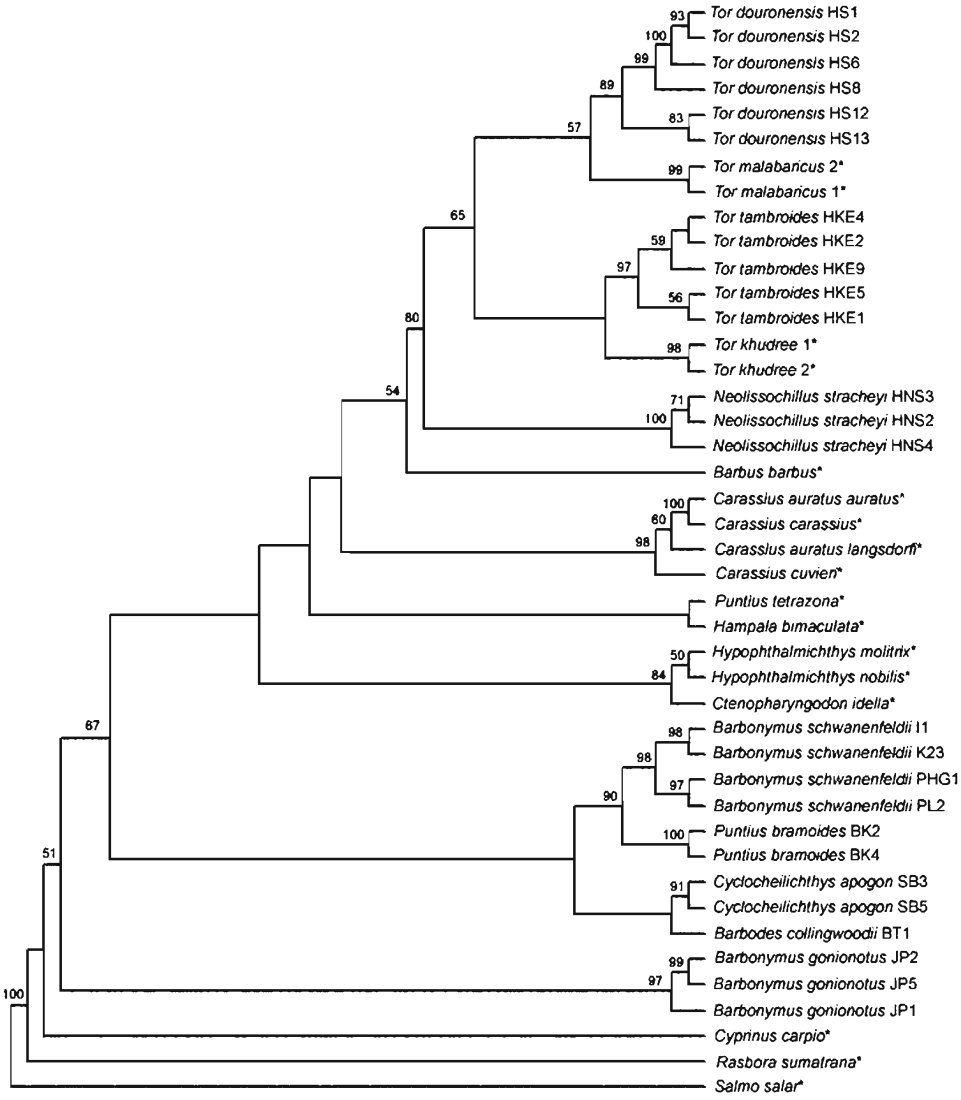

Figure 4. Maximum-Likelihood (ML) tree showing relationships among the cyprinids. The number at each node represents the bootstrap value (%) based on 1000 pseudoreplications of the dataset.

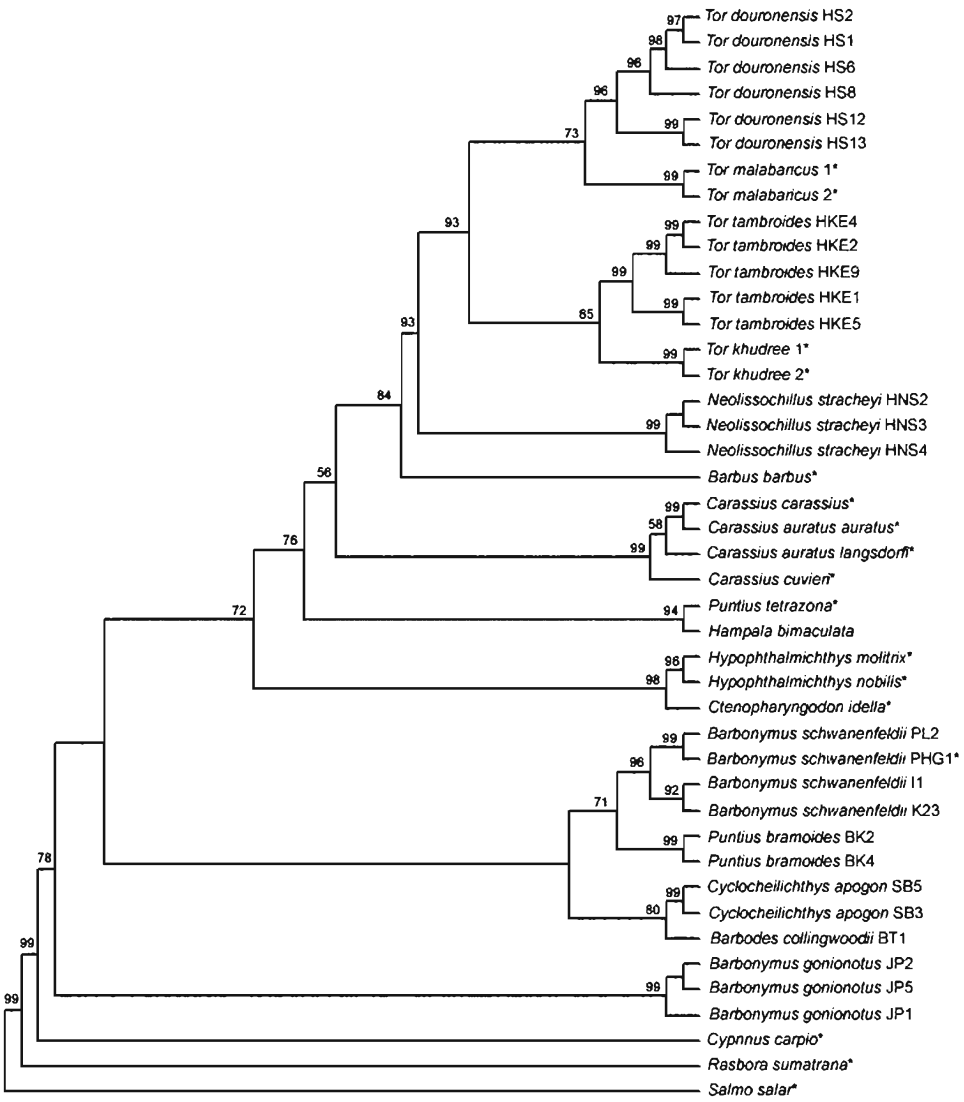

Figure 5. Bayesian tree showing relationships among the cyprinids. The number at each node represents the posterior probabilities (in percentage) of the dataset.

Table 2. Pairwise Kimura-two-Parameter (K2P) genetic distances among the 21 cyprinids used in this study.

Species	1	2	3	4	5	6	7	8	9	10	11
1 *Tor douronensis*	-										
2 *Tor tambroides*	0.0662	-									
3 *Tor khudree*	0.0561	0.0452	-								
4 *Tor malabaricus*	0.0633	0.0412	0.0373	-							
5 *Neolissochillus stracheyi*	0.0774	0.0693	0.0550	0.0639	-						
6 *Barbonymus schwanenfeldii*	0.1515	0.1598	0.1531	0.1571	0.1666	-					
7 *Barbonymus gonionotus*	0.1603	0.1713	0.1514	0.1616	0.1868	0.1511	-				
8 *Cyclocheilichthys apogon*	0.1654	0.1658	0.1475	0.1584	0.1639	0.1230	0.1497	-			
9 *Puntius bramoides*	0.1655	0.1648	0.1697	0.1770	0.1740	0.0948	0.1577	0.1311	-		
10 *Barbodes collingwoodii*	0.1637	0.1699	0.1717	0.1600	0.1727	0.1028	0.1436	0.1064	0.1272	-	
11 *Barbus barbus*	0.1450	0.1375	0.1453	0.1401	0.1372	0.1268	0.1686	0.1569	0.1663	0.1463	-
12 *Puntius tetrazona*	0.1765	0.1650	0.1729	0.1677	0.1584	0.1685	0.1880	0.1641	0.1694	0.1490	0.1554
13 *Carassius carassius*	0.1598	0.1454	0.1501	0.1511	0.1604	0.1406	0.1611	0.1317	0.1648	0.1299	0.1185
14 *Carassius auratus auratus*	0.1598	0.1454	0.1501	0.1511	0.1604	0.1406	0.1611	0.1317	0.1648	0.1299	0.1885
15 *Carassius cuvieri*	0.1543	0.1460	0.1446	0.1426	0.1426	0.1512	0.1580	0.1201	0.1523	0.1329	0.1098
16*Carassius auratus langsdorfi*	0.1633	0.1427	0.1535	0.1514	0.1454	0.1516	0.1522	0.1290	0.1526	0.1422	0.1246
17 *Ctenopharyngodon idella*	0.1655	0.1362	0.1529	0.1493	0.1695	0.1674	0.1836	0.1381	0.1398	0.1563	0.1566
18 *Hypophthalmichthys molitrix*	0.1612	0.1599	0.1523	0.1518	0.1473	0.1439	0.1604	0.1233	0.1242	0.1371	0.1622
19 *Hypophthalmichthys nobilis*	0.1892	0.1644	0.1713	0.1644	0.1853	0.1495	0.1905	0.1383	0.1331	0.1678	0.1785
20 *Hampala bimaculata*	0.1945	0.1743	0.1824	0.1834	0.1677	0.1574	0.1711	0.1469	0.1790	0.1346	0.1443
21 *Cyprinus carpio*	0.1583	0.1670	0.1410	0.1603	0.1677	0.1457	0.1586	0.1548	0.1437	0.1365	0.1483

Table 2. continued

Species	12	13	14	15	16	17	18	19	20	21
1 *Tor douronensis*										
2 *Tor tambroides*										
3 *Tor khudree*										
4 *Tor malabaricus*										
5 *Neolissochillus stracheyi*										
6 *Barbonymus schwanenfeldii*										
7 *Barbonymus gonionotus*										
8 *Cyclocheilichthys apogon*										
9 *Puntius bramoides*										
10 *Barbodes collingwoodii*										
11 *Barbus barbus*										
12 *Puntius tetrazona*	-									
13 *Carassius carassius*	0.1285	-								
14 *Carassius auratus auratus*	0.1285	0.0000	-							
15 *Carassius cuvieri*	0.1314	0.0411	0.0411	-						
16 *Carassius auratus langsdorfi*	0.1287	0.0463	0.0463	0.0387	-					
17 *Ctenopharyngodon idella*	0.1572	0.1314	0.1314	0.1373	0.1376	-				
18 *Hypophthalmichthys molitrix*	0.1363	0.1518	0.1518	0.1428	0.1371	0.0910	-			
19 *Hypophthalmichthys nobilis*	0.1454	0.1490	0.1490	0.1460	0.1834	0.1677	0.1574	-		
20 *Hampala bimaculata*	0.1393	0.1199	0.1199	0.1170	0.1229	0.1726	0.1537	0.1850	-	
21 *Cyprinus carpio*	0.1493	0.1459	0.1459	0.1337	0.1463	0.1348	0.1488	0.1401	0.1629	-

DISCUSSION

The phylogenetic, systematic and taxonomic studies of cyprinids in Malaysia, particularly among the indigenous taxa are still highly fragmented and poorly resolved. Among the mahseer, the taxonomic differentiation of *T. douronensis* and its related species, *T. tambroides* is highly controversial, with many conflicting descriptions among different authors (Roberts, 1989; Kottelat *et al.*, 1993; Rainboth, 1996; Zhou & Chu, 1996; Ng, 2004). Roberts (1999) classified them to be a single species, and a junior synonym to *T. tambra*. In general, *T. tambroides* can be easily identified morphologically based on the presence of a long median lobe character that is shorter in the other two mahseers (*T. douronensis* and *T. tambra*) (Kottelat *et al.*, 1993; Kottelat & Whitten, 1996; Rainboth, 1996). Nevertheless, mahseer samples generally resembling *T. tambroides* in other characters but exhibiting shorter or medium types of median lobes similar to its two congeners, has been reported on several occasions (Esa, 2009; Sungan, pers.comm). Likewise, *T. douronensis* exhibiting a long median lobe was also found, for example, in many samples from North Borneo (Sabah). In addition, the mahseer recognized as *T. tambroides* collected from Peninsular Malaysia tends to exhibit two types of colour, silver-bronze and reddish (Ng, 2004), but is indistinguishable using molecular markers (RAPD) (Siraj *et al.*, 2007). A similar situation also occurred in Sarawak where mahseer identified as *T. tambroides* tends to exhibit many varieties of colour (reddish, silver-bronze, silver-white and many others; Sungan, pers.comm).

Thus, species identification strictly on the basis of morphological characters alone is quite unreliable, because of considerable geographical and ecological variability (Tsigenopoulos & Berrebi, 2000) and the same situation also applies to the taxonomic identification of mahseer (Siraj *et al.*, 2007; Esa *et al.*, 2008). Molecular genetics characters (such as sequence analysis of COI mtDNA region) on the other hand are less likely to be influenced by environmental adaptations (Carvalho & Pitcher, 1995). Furthermore, they are heritably transmitted and therefore confidence can be placed on the amount and nature of the genetic information obtained (Avise, 2000).

Findings from the present study strongly supported the monophyletic status between the two mahseers of the genus *Tor* (*T. douronensis* and *T. tambroides*) and *N. stracheyi* representing the genus *Neolissochillus*. Thus, our phylogenetic results strongly supported the warranty of species status for all the three indigenous mahseer. However, the reciprocally monophyletic status between genus *Tor* and genus *Neolissochillus* could not be fully elucidated from the present study since not all described species from both genera were analysed under the present study. In addition, the current findings also supported the recent reclassification of *N. stracheyi* from the genus *Tor* (previously classified as *Tor soro* (Mohsin & Ambak, 1983) into the genus *Neolissochilus* (Rainboth, 1996) based on the absence of the median lobe.

The phylogenetic relationship between *T. douronensis* and *T. tambroides* was one of the important highlights of the present study. The reciprocally monophyletic status and substantial genetic distance (K2P value= 6.6%) clearly supported their genetic differences as showed by previous studies (Esa *et al.*, 2008; Nguyen *et al.*, 2008). However, the limited number of samples analysed hindered us from confirming their taxonomic status, but they should undoubtedly be recognised as different species under the phylogenetic species concept (PSC: Cracraft, 1989).

The close phylogenetic relationship between *T. tambroides* and *T. khudree* as suggested by the Bayesian, ML and NJ analyses supported the recent findings by Nguyen *et al.* (2008), which clustered them into one group with moderate support. However, the relationships between *T. malabaricus* with the other mahseer were still uncertain since the NJ analysis suggested it as

being closer to its South Asia counterpart (*T. khudree*), the MP analysis suggested it as basal to the other mahseer, while both the Bayesian and the ML analyses grouped *T. malabaricus* as a sister taxa to *T. douronensis*. Thus, further study using more mahseer samples, or the inclusion of additional mtDNA genes might resolve the problem.

For the other indigenous cyprinids, an interesting finding was that *B. gonionotus* was phylogenetically distinct from its morphologically similar species, *B. schwanenfeldii* (K2P distance value = 15.1%) and did not group together in a single *Barbonymus* clade. The high genetic divergence observed between *B. schwanenfeldii* and *B. gonionotus* suggests the taxonomic revision of the latter barb from its current position within the genus *Barbonymus*. *Barbonymus gonionotus* is originally a non-native species to Peninsular Malaysia and is believed to be introduced from Java during the early 19th century (Welcomme, 1981; Ryan *et al.*, 2007). Being native to Indonesian rivers, this species breeds well in ponds as well as in natural river systems where it was introduced. Today, *B. gonionotus* is found living in sympatry with *B. schwanenfeldii* in many river systems. However, recent molecular studies using RFLPs of cyt *b* mtDNA fragment (Esa & Khairul, 2003) on the two species from their sympatric sites in Serting River (Negeri Sembilan) did not find any evidence of hybridisation or introgression, suggesting an indication of reproductive isolation between them.

Overall, the current study managed to provide insights into the phylogenetic relationships among cyprinids under study, especially the important mahseer of Malaysia. However, shortcomings of our results were clearly recognised and data should be treated with great caution, since it was based on a limited number of samples and a single maternally inherited gene (COI). Indeed, further studies on their taxonomy, population structures and phylogeography are required based on larger sample sizes per population, samples from other areas of their geographical distributions, a more variable mtDNA region such as the control region (D-Loop) to reveal more variations at the inter and intra population levels, and data from nuclear markers such as single locus microsatellite markers to complement the mtDNA findings.

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