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Mitochondrial DNA Diversity of *Tor tambroides* Valenciennes (Cyprinidae) from Five Natural Populations in Malaysia

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Yuzine B. Esa, Siti Shapor Siraj, Siti Khalijah Daud, Khairul Adha A. Rahim, Jeffrine Rovie Ryan Japning, and Soon Guan Tan (2008) Mitochondrial DNA diversity of *Tor tambroides* Valenciennes (Cyprinidae) from five natural populations in Malaysia. *Zoological Studies* **47**(3): 360-367. In this study, we examined the genetic structure of *Tor tambroides* Valenciennes, an important indigenous freshwater fish species in Malaysia, using sequence analysis of 464 base pairs of the mitochondrial cytochrome *c* oxidase I (COI) gene. In total, 92 *T. tambroides* samples were analyzed from 4 locations on Peninsular Malaysia (*n* = 87) and a single population from Sarawak (Batang Ai, *n* = 5) on Borneo I., and 4 sequences of *Tor douronensis* from Sarawak were used for comparisons. In total, 9 haplotypes were found, with 7 haplotypes being unique and 2 haplotypes being shared among the 5 populations. The phylogenetic analysis using Neighbor-joining (NJ) and maximum-parsimony (MP) methods supported the monophyletic status between *T. tambroides* and *T. douronensis*, thus suggesting their status as different species. The clustering of all *T. tambroides* samples into a single clade suggested that their genetic identity belongs to a single species. The sharing of haplotype HKE4 between Batang Ai of Sarawak (*n* = 4) and Perak of Peninsular Malaysia (*n* = 3) reflects the historical connection of drainages between the regions possibly during Pleistocene glaciation periods. Limited variations were found among all peninsular *T. tambroides* populations. The low level of mitochondrial (mt)DNA differences currently found among *T. tambroides* populations is probably due to the high proportion of the HKE1 haplotype being found in all the populations (0.736 - 1.000), or the small number of samples used in the present study. Overall, the present study was able to shed light on the phylogenetic relationships and genetic structure of *T. tambroides* in Malaysia. http://zoolstud.sinica.edu.tw/Journals/47.3/360.pdf

Key words: *Tor tambroides*, Freshwater fish, mtDNA COI, Population structure.

Cyprinids of the genus *Tor* Gray, commonly known as Mahseer, are one of the important freshwater fishes in Malaysia (Mohsin and Ambak 1983, Roberts 1989, Litis et al. 1997, Ng 2004). *Tor tambroides* Valenciennes, locally known as "kelah" in Peninsular Malaysia (Mohsin and Ambak 1983) and "empurau" in Sarawak (Litis et al. 1997) is one of the important Mahseer used for food as well as the aquarium industry and game fishing (Ng 2004). It is the most common Mahseer found on Peninsular Malaysia and is also found in Sarawak state on Borneo I. *Tor tambroides* inhabits the upper reaches of clean unpolluted river systems with rocky beds through hilly terrain (Singh and Menon 1994).

Tor tambroides can morphologically be identified based on the presence of a long median lobe character that is shorter in the other 2 Mahseers described in Malaysia (*T. douronensis* Valenciennes and *T. tambra* Valenciennes) (Kottelat et al. 1993, Kottelat and Whitten 1996, Rainboth 1996). Nevertheless, *T. tambroides* exhibiting

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shorter or medium types of median lobes similar to its 2 congeners has been reported on several occasions. Furthermore, *T. tambroides* collected from Peninsular Malaysia tends to exhibit 2 color types of silver-bronze and reddish (Ng 2004). Thus, species identification strictly on the basis of morphological characters alone is quite unreliable, because of considerable geographical and ecological variability (Tsigenopoulos and Berrebi 2000, Siraj et al. 2007).

To date, very little taxonomic work to systematically sort out the Malaysian Mahseer has been documented. The most-often cited evidence is by Mohsin and Ambak (1983) who described *T. tambroides* and *T. soro* as 2 valid Mahseers found in Peninsular Malaysia while a more-recent opinion by Ng (2004) suggests the occurrence of 3 species; *T. tambroides*, *T. tambra*, and *T. douronensis*. Thus, identifying Mahseer samples collected from Peninsular Malaysia mostly relies on reference specimens from northern Borneo (Inger and Chin 2002) and western Kalimantan (including Sarawak State (Roberts 1989)) where *T. tambroides* and *T. douronensis* are 2 valid Mahseers described from the region. On a broader scale, other widely cited taxonomic references on Southeast Asian Mahseers include those by Kottelat et al. (1993), Rainboth (1996), Zhou and Chu (1996), and Roberts (1999). Environmental degradation (i.e., river pollution, deforestation, watershed erosion, etc.) of headland areas and upper streams has led to the rapid destruction of natural habitats of *T. tambroides*. In addition, excessive demand for the highly priced *T. tambroides* flesh (which can reach 450 Malaysian Ringgit per kg (≈US$150/kg) in Sarawak) has led to uncontrolled fish harvesting and destructive fishing practices by locals and illegal fish poachers. These practices have caused rapid reductions in its population size (Ng 2004). Thus, the distribution of *T. tambroides*, particularly in Peninsular Malaysia, is currently limited to less-disturbed or undisturbed upper stream reaches and protected areas (such as national parks) (Ng 2004).

Although currently not listed by the IUCN as a protected or endangered species, the drastic decline in natural populations of *T. tambroides* has increased awareness among relevant authorities (e.g., Fisheries Department, Malaysia and policy makers) of the importance of the conservation and proper management of this species. Recent success in the captive breeding of *T. tambroides* (Ingram et al. 2005) has created an opportunity for mass-producing this highly valued Mahseer for aquaculture and commercialization as well as for restocking natural water bodies for conservation purposes (Nguyen et al. 2006).

The application of molecular techniques (such as DNA sequencing) has provided new and better insights into the taxonomy, population structure, and conservation management of *T. tambroides* (Smith and Wayne 1996, Nguyen et al. 2006). Molecular markers can provide reliable and consistent results for rapid identification among species (Ryan and Esa 2006), levels of genetic variability, levels of gene flow and population subdivisions, and understanding factors contributing to fitness (Vrijenhoek 1998). A molecular study has the advantage over morphological and biochemical traits due to precision in detecting relationships among and within population of various organisms. It requires only a very small quantity of DNA from any tissue (scale, fin clip, muscle, etc.) for analysis using polymerase chain reaction (PCR) technology (Avise 1994). This advantage is crucial particularly for endangered, protected, or remnant populations or species where non-destructive sampling is required for genetic analysis (Ward 2000).

Analysis of DNA sequence polymorphism utilizing the existing "universal primers" for mitochondrial (mt)DNA (e.g., Palumbi et al. 1991) provides the highest resolution of genetic variability and has been widely applied in molecular systematic studies (Arnason et al. 2002, Liu and Chen 2003).

Nguyen et al. (2006) recently investigated broodstocks of *T. tambroides* (empurau) and *T. douronensis* (semah) maintained at the Indigenous Fisheries Research and Production Center (IFRPC), Tarat, Sarawak by sequencing the mtDNA 16S ribosomal (r)RNA gene (542 bp). Their results showed low genetic variation among *T. tambroides* populations but substantial inter-population divergence levels among *T. douronensis* populations.

Thus, the objectives of the present study were to 1) examine the genetic identity and phylogenetic relationships among *T. tambroides* samples, and 2) to characterize the genetic structure of *T. tambroides* from 4 populations of Peninsular Malaysia and 1 population from Sarawak by analyzing the cytochrome *c* oxidase I (COI) nucleotide sequences of mtDNA.

MATERIALS AND METHODS

Sample description and collection locations

Samples of *T. tambroides* used in this study were provided by the relevant government authorities, such as the Fisheries Department (DF), the Wildlife Department (WD), Universiti Putra Malaysia (UPM), and Universiti Malaysia Sarawak (UNIMAS). Fish were collected from 4 locations (rivers) in Peninsular Malaysia (20 from Negeri Sembilan, 23 from Pahang, 19 from Perak, and 27 from Kelantan) and 1 location in Sarawak (5 from Batang Ai) on Borneo I. (Fig. 1). Samples of *T. tambroides* (empurau) from Batang Ai, Sarawak were provided by the Indigenous Fisheries Research and Production Center (IFRPC), Tarat, Sarawak. In many cases, a nondestructive method was applied where only fin clips or scales were collected, and the fish were released back to their native habitat. Alternatively, some full specimens were retained for future studies and references with permission from the authorities (DF, WD, or the relevant state or national park). Morphological identification was done using keys provided by Inger and Chin (2002), Mohsin and Ambak (1983), and Kottelat et al. (1993). However, in some cases, only tissue samples were available with no information (or incomplete information) on

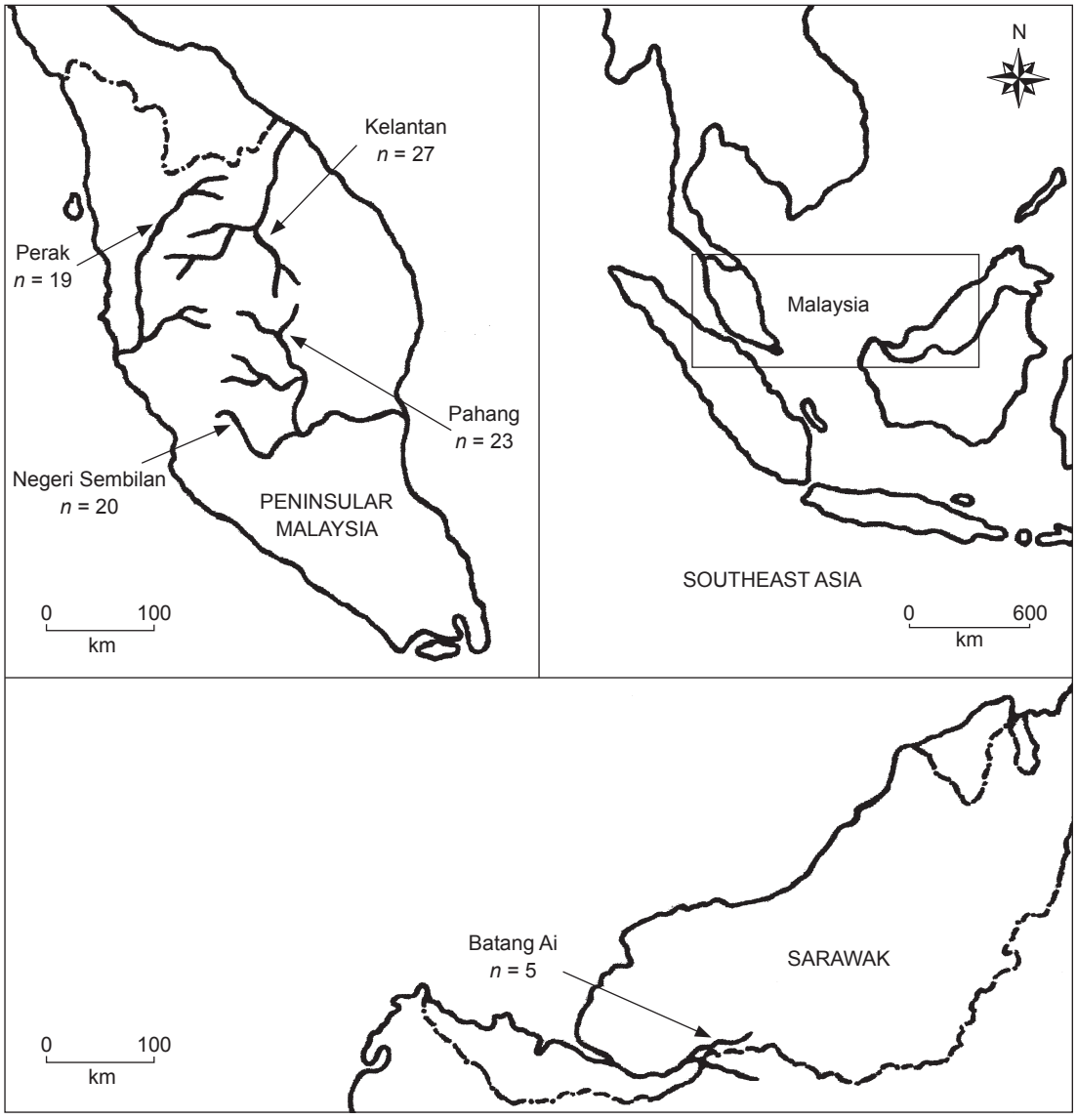

Fig. 1. Map showing sampling locations and sample sizes of *Tor tambroidess* collected for the present study. *n*, sample size.

the morphological identity. The fish samples (fin clipping, scale, muscle tissue, or whole fish) were preserved in 95% ethanol or kept on dry ice during field collection, and were subsequently stored at -20 or -80°C prior to the genetic analyses.

DNA extraction, polymerase chain reaction (PCR), and sequencing

Total DNA was extracted using the modified cetyl-trimethylammonium bromide (CTAB) method (Grewe et al. 1993) in the presence of proteinase K. Pelleted DNA was re-dissolved in 100 μl of sterilized distilled water. The DNA quality and approximate yield were determined by electrophoresis in a 1% agarose gel containing ethidium bromide at 90 V for 30 min. The isolated genomic DNA was used for the mtDNA analysis.

A 500-base pair (bp) segment of the *cytochrome c oxidase* I gene was amplified with the oligonucleotide primers COIf (5'-CCTGCAG GAGGAGGAGAYCC-3', forward) and COIe (5'-CCAGAGATTAGAGGGAATCAGTG-3', reverse) (Palumbi et al. 1991). Approximately, 50-100 ng of the template DNA was amplified in a 25 μl reaction mixture containing 50 mM 10x buffer, 2 mM $MgCl_2$, 0.2 μM of each dNTP (Promega), 0.1 μM of each primer, and 0.5 units of *Taq* DNA polymerase (Promega, Madison WI, USA). The cycle parameters consisted of 35 cycles of denaturation (at 95°C for 30 s), annealing (at 47°C for 30 s), and extension (at 72°C for 60 s). The amplified products were visualized on 1% agarose gels containing ethidium bromide, run for approximately 30 min at 90 V, and photographed under UV light. A digested lambda DNA ladder (GeneRuler™ 100-bp DNA Ladder) was used as a standard size marker (Promega). The PCR products were further purified using a DNA purification kit (Vivantis, Kuala Lumpur, FT, Malaysia) according to the manufacturer's instructions. All purified PCR products were directly sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequencing was carried out on an ABI 377 automated sequencer (Applied Biosystems) 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.

Data analysis

The CHROMAS (vers. 1.51) software (Technelysium Pte Ltd, http://www.technelysium.com.au/chromas.html) was used to display the fluorescence-based DNA sequencing results. The multiple sequence alignments for the forward reactions were done using the CLUSTAL X program (vers. 1.81, Thompson et al. 1997), and subsequently aligned by eye. The pairwise genetic distance between populations was calculated using the Tamura-Nei distance (Tamura and Nei 1993), based on unequal base frequencies and unequal ratios of transitions to transversions (Ti: Tv) implemented in MEGA (vers. 3.1, Kumar et al. 2004). The MEGA program was also used to construct a Neighbor-joining (NJ) (Saitou and Nei 1987) tree and a maximum-parsimony (MP) tree (applying the close-neighbor interchange (CNI) method with 1000 randomizations) using 2 indigenous cyprinids (*Barbonymus gonionotus* (GenBank accession no.: DQ532806) and *Barbonymus schwanenfeldii* (GenBank accession no.: DQ532805)) obtained from the Jempol River, Negeri Sembilan as outgroup species. Four haplotypes of *T. douronensis* (GenBank accession nos.: EF192444, EF192447, EF192448, and EF192449) were also included in the analysis to demonstrate the reciprocally monophyletic status between the 2 Mahseers. Phylogenetic confidence was estimated by bootstrapping (Felsenstein 1985) with 1000 replicate datasets.

Levels of mtDNA COI variation within the *T. tambroides* population were examined by computing the nucleotide (with the Jukes-Cantor correction (Jukes and Cantor 1969)) and haplotype diversity indices implemented in the DnaSP (vers. 4.0) program (Rozas et al. 2003). The level of population subdivision (F_{ST}) (Hudson et al. 1992) between populations and the Chi-square probability test for population differentiation using 1000 permutations of the datasets were also estimated using the DnaSP program. The nucleotide sequences were later translated into amino acid sequences using the genetic code for vertebrate mtDNA. Finally, sequences of each of the haplotypes were deposited into GenBank under accession nos. EF660856-EF660861, EF192458, EF192460, DQ532827, and DQ532856 (see also Table 1).

RESULTS

Ninety-two partial sequences of 464 bp each of the mtDNA COI gene were obtained, representing 5 populations of *T. tambroides*. We observed 11 (2.4%) variable/polymorphic sites including 1 parsimoniously informative site, while 453 sites (97.6%) were conserved. In total, 9 haplotypes were distinguished in the nucleotide dataset, with 7 unique haplotypes and 2 haplotypes being shared among the 5 populations (Table 1). In total, 11 substitutions were found among the haplotypes, including 8 transitions and 3 transversions. The mean total nucleotide composition was 25.9% A, 32.4% T, 22.4%, C, and 19.3% G. The protein translation of the 464-bp fragment retained only 7 haplotypes in the amino acid sequences comprising 154 amino acid residues, of which 8 (5.2%) were variable sites. The translation result indicated no pseudogene in the amino acid sequences.

HKE1 was the most common haplotype found in all populations from Peninsular Malaysia, but it was not detected in the Batang Ai population of Sarawak. On the other hand, HKE4 was the most common haplotype found in the latter population, and it was also found at a lower frequency in the Perak population. The Pahang and N. Sembilan populations had 2 unique haplotypes, respectively, while the Perak and the Batang Ai populations had a single unique haplotype each (Table 1). The Perak population harbored the highest number of haplotypes (n = 4), while Kelantan was the only population that possessed a single haplotype (HKE1) in all samples (n = 27), indicating a lack of mtDNA differentiation among samples.

Overall, the nucleotide diversity was very low (0-0.001) in all populations. The haplotype diversity varied, ranging from 0.186 (Pahang) to 0.450 (Perak) (Table 1). Pairwise F_{ST} values and results of the Chi-square tests for genetic differentiation among the populations are presented in table 2. Significant levels of genetic differentiation were found in all comparisons between *T. tambroides* populations from Peninsular Malaysia and the Batang Ai population of Sarawak ($p < 0.001$). However, there were no significant genetic differences in most comparisons among Peninsular Malaysia populations except between the Perak and the Kelantan populations ($p < 0.05$).

A summary of the relationships among

Table 1. Summary of variable sites and the distribution of 9 observed mitochondrial (mt)DNA cytochrome *c* oxidase I (COI) haplotypes, nucleotide diversity, number of haplotypes, and number of polymorphic sites among populations of *Tor tambroides*. Dots indicate identity with the HKE1 haplotype sequence. *n*, sample size

Haplotypes	Variable Sites											GenBank Accession Numbers	Population				
													Pahang *n* = 23	Perak *n* = 19	Negeri Sembilan *n* = 20	Kelantan *n* = 27	Batang Ai *n* = 5
			2	4	4	4	4	4	4	4	4						
	2	2	8	0	1	2	4	4	4	5	6						
	1	5	5	0	9	1	4	6	8	0	2						
HKE1	G	T	C	C	C	A	A	C	G	C	A	DQ532827	0.904	0.736	0.900	1.000	-
HKE2	.	C	G	.	.	.	.	.	.	.	.	EF192458	0.048	-	-	-	-
HKE4	A	.	.	G	.	.	.	.	.	.	.	DQ532856	-	0.158	-	-	0.800
HKE5	A	.	.	.	.	.	.	.	.	.	.	EF192460	-	-	-	-	0.200
HKE7	.	.	.	.	.	.	T	.	.	.	.	EF660857	-	0.053	-	-	-
HKE8	.	.	.	.	G	T	.	.	.	.	.	EF660858	-	-	0.050	-	-
HKE9	.	.	.	.	.	.	.	G	C	.	.	EF660859	-	-	0.050	-	-
HKE10	.	.	.	.	.	.	.	.	.	T	.	EF660860	0.048	-	-	-	-
HKE11	.	.	.	.	.	.	.	.	.	.	T	EF660861	-	0.053	-	-	-
Nucleotide diversity (Pi JC)													0.001	0.001	0.001	0.000	0.001
Number of haplotypes													3	4	3	1	2
Haplotype diversity (Hd)													0.186	0.450	0.195	0.000	0.400
Number of polymorphic sites													2	3	4	0	1

haplotypes from all samples is presented by the NJ tree in figure 2. Both the NJ and MP analyses showed an identical tree topology with only minor differences in bootstrap confidence levels. The phylogenetic analysis strongly supported the reciprocal monophyletic status between *T. tambroides* and *T. douronensis* with high bootstrap support (100%). Within the *T. tambroides* cluster, haplotypes HKE4 and HKE5, which were dominant in Batang Ai samples (except for the inclusion of 3 samples from Perak with HKE4, data not shown) formed a small subcluster but with only moderate bootstrap support (Fig. 2).

Pairwise genetic distances (number of nucleotide substitutions per site) calculated using the Tamura-Nei model among *T. tambroides* populations are shown in table 2. Overall, genetic distance values were low and similar (0.1%) among the 4 Peninsular Malaysian populations. The Batang Ai population of Sarawak genetically differed from all 4 Peninsular Malaysian populations with a genetic distance of 0.3%.

DISCUSSION

Results of the mtDNA analysis in the present study shed light on the genetic makeup of *T. tambroides*, particularly of Peninsular Malaysia populations. The phylogenetic analysis of the COI gene confirmed the reciprocally monophyletic status between all *T. tambroides*

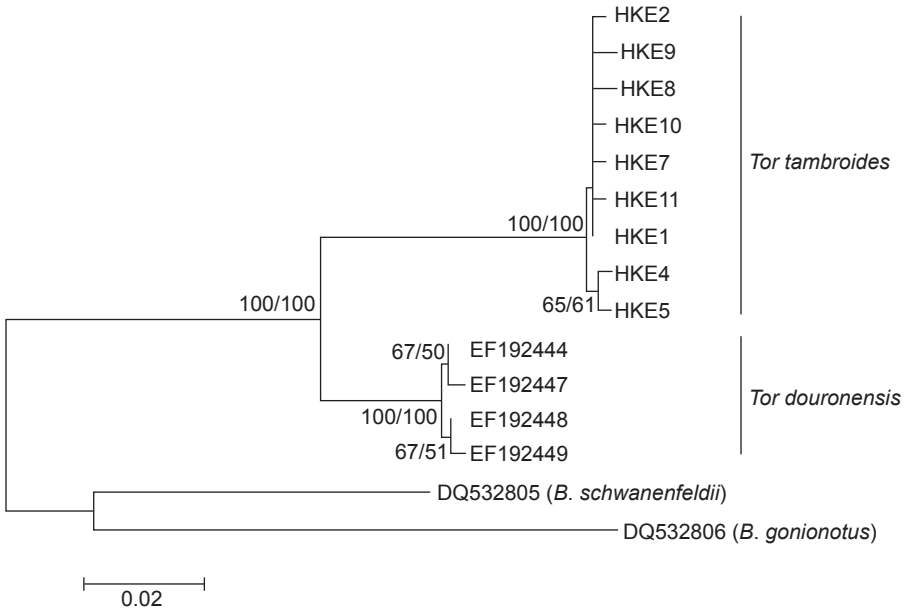

Fig. 2. Neighbor-joining (NJ) phylogram showing the relationships among cytochrome c oxidase I (COI) haplotypes of *Tor tambroides*, *T. douronensis*, and 2 outgroups analyzed in the present study. The number at each node represents the bootstrap percentage value based on 1000 pseudoreplications for the Neighbor-joining/maximum parsimony (NJ/MP) analyses.

Table 2. Below the diagonal: pairwise Tamura-Nei genetic distances among 5 populations of *Tor tambroides*. Above the diagonal: population subdivision (F_{ST}) values and probability test (Chi-squared) for population differentiation based on 1000 permutations of the sequence dataset

	1. Batang Ai	2. Pahang	3. Perak	4. N. Sembilan	5. Kelantan
1	-	0.772***	0.600***	0.714***	0.833***
2	0.003	-	0.052^{ns}	0.000^{ns}	0.000^{ns}
3	0.003	0.001	-	0.041^{ns}	0.071*
4	0.003	0.001	0.001	-	0.000^{ns}
5	0.003	0.000	0.001	0.000	-

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

and *T. douronensis* haplotypes. However, with limited data on *T. douronensis*, the taxonomic status between these 2 Mahseers cannot be verified, but the present mtDNA data suggested the possibility that the 2 species genetically differ. The clustering of all *T. tambroides* haplotypes into a single clade with no sharing or overlap with *T. douronensis* haplotypes, and the low genetic distances among haplotypes (0.1%-0.3%) suggest that all *T. tambroides* samples used in the present study possibly belong to a single species (*T. tambroides*). Thus, the genetic identity of all *T. tambroides* samples could be elucidated, although some samples were provided to us only in the form of tissues (fin clips or scales) with no complete information on their morphological identity.

Another interesting finding of the present study is the sharing of haplotype HKE4 between samples of Batang Ai, Sarawak (n = 4) and Perak, Peninsular Malaysia (n = 3). Geological evidence suggests that the river systems of the southern parts of Sarawak were historically interconnected with most major river systems of Peninsular Malaysia during the Tertiary and Quaternary periods (10-5 Ma) via the North Sunda River (Inger and Chin 2002), thus allowing gene flow among these drainages. However, the limited number of samples obtained from each population (particularly only 5 samples analyzed from Batang Ai, Sarawak) might possibly have underestimated the actual haplotype distribution among *T. tambroides* in Malaysia.

Nevertheless, the higher genetic differences and higher F_{ST} values (0.600-0.833) between the *T. tambroides* population from Batang Ai of Sarawak with its congeners from Peninsular Malaysia, and the presence of a fixed haplotype (HKE5) in the former, support the absence of any contemporary migration/gene flow between *T. tambroides* of Borneo I. and their conspecific of mainland Asia (Peninsular Malaysia) since the rise of sea levels separated the land masses into the present situation in the late Pleistocene (~10,000 yr ago) (Inger and Chin 2002). A similar scenario of geographical structuring of haplotype distributions was also demonstrated by other indigenous freshwater fish species such as *Hampala macrolepidota* (Ryan and Esa 2006).

Within the peninsular population, low mtDNA variations were found among all *T. tambroides* populations. This was reflected by the high number of haplotypes within each population (3-4), high haplotype diversity (0.186-0.450), and high number of polymorphic loci (2-4), but low F_{ST} values among populations. The low level of mtDNA differences is probably due to the high proportion of the HKE1 haplotype found in all populations (0.736-1.000) studied, or by the small number of samples used in the present study. Nucleotide and haplotype diversity values detected in this study were slightly lower but the total number of haplotypes was higher than those reported by Nguyen et al. (2006). However, a comparison of genetic diversity between different mtDNA genes (COI and 16S) was not compatible since they have different evolutionary rates (Meyer 1993).

The occurrence of only a single haplotype in the Kelantan population might be explained by 3 possibilities, but these could not be confirmed based on the present study alone. First, habitat destruction and overexploitation in the upper reaches/headwaters of the Kelantan River that originates in the Titiwangsa Range (e.g., the Nenggiri River) might have resulted in a mass reduction in the effective population size (female parental stock) of *T. tambroides* in the area that led to large reductions in mtDNA variations. Second, *T. tambroides* samples from Kelantan might have originated from a single female parent, thus sharing a single common haplotype (HKE1), since mtDNA is maternally inherited (Avise 1994). Third, the limited number of samples (n = 23) analyzed might have underestimated the actual mtDNA variations harbored by the Kelantan population. The inclusion of highly variable nuclear markers such as microsatellites is needed to further elucidate the low mtDNA variations found in the present study.

In conclusion, in this study we were able to provide some useful insights into phylogenetic relationships, genetic identity, and population structure of *T. tambroides*, particularly those from Peninsular Malaysian drainages. However, further studies using larger sample sizes per population, samples from other areas of its geographical distribution, sequence data from other mtDNA regions, and information based on nuclear DNA (e.g., single-locus microsatellites) markers are required before any appropriate conservation management strategies or breeding programs of *T. tambroides* are implemented.

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