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Genetic diversity of Malaysian indigenous Mahseer, *Tor douronensis* in Sarawak river basins as revealed by cytochrome *c* oxidase I gene sequences

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Malaysian Mahseer, *Tor douronensis*, locally known as "kelah" in Peninsular Malaysia or semah in Sarawak is one of the important Mahseers used for food as well as the aquarium industry and game fishing. This species is endemic to Malaysian Borneo river basins. The wild stocks of *T. douronensis* have declined substantially in recent years, due to over exploitation, pollution and illegal fishing practices. DNA sequencing based on a partial sequence of the cytochrome *c* oxidase I (COI) gene, was used to determine genetic variation in wild stocks of *T. douronensis* originated from eight different populations in Sarawak River Basins of Malaysian Borneo. The highest haplotype diversity was found in Entabai river population while the lowest were marked in four populations of Lundu, Terbat, Bau and Kg. Pa Puti. The population structure analyses based on ANOVA or found low level of intra and high level of inter population variations in samples of *T. douronensis* of Sarawak. The presence of unique haplotype in some populations, along with high F_{ST} values indicated that there has been restricted or no migration among the existing population which were separated by geographical barrier or river systems. The population structure of the three regions was also analysed using ANOVA and revealed that most of the variations occurred among regions and among subpopulations within a region. Population structure of *T. douronensis* showed high degree of genetic heterogeneity and appeared to be significantly structured into geographically discrete population.

Key words: *T. douronensis*, COI gene, Mahseer, mtDNA diversity

INTRODUCTION

Fish of the genus *Tor*, locally known as Mahseer or *kelah,* is an important indigenous cyprinid in most Asian region and inhabits clean, fast flowing and shallow rivers with rocky bottom especially at the upper streams. In Malaysia, they are most captured in places such as in Royal Belum in Perak, National Park in Pahang and Andang River in Sarawak (Ingram et al., 2005). It is one of the most fighting game fish and thus become a popular sport fish among anglers around the world. There are three *Tor* species that have been recognized in Malaysia as reported by Ng (2004), Eddy (1997) and Mohsin and Ambak (1983) namely *T. tambroides, T. duoronensis* and *T. tambra. Tor douronensis* is the most common, widespread and native Mahseer species in East Malaysia and it is the only Mahseer species that has been described from Sabah (Inger and Chin, 2002). This species has been used in

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captive breeding program in Sarawak to support aquaculture, capture fisheries and conservation purposes (Ingram et al., 2007).

Although *T. douronensis* occurs in number of river system in Sarawak, its natural population have been declined over the past few decades due to anthropogenic activities such as deforestation, over harvesting, watershed erosion, river pollution and logging. These factors have greatly reduced their population size in nature (Ng, 2004). Their distribution are now restricted to the upper streams and some protected areas in Peninsular Malaysia, Sarawak and Sabah (Litis et al., 1997; Nyanti et al., 1999; Ng, 2004). The lack of systematic genetic surveys to ascertain the genetic variability of *T. douronensis* in wild population prompted us to conduct this study, which may help in designing and application of genetic conservation strategies for restocking program (Ryman and Laikre, 1991).

A wide range of molecular marker systems have been used to study genetic diversity in aquaculture species (Chen et al., 2004; Diniz et al., 2005; Sotka et al., 2005; Thai et al., 2006). Mitochondrial DNA analysis has been successfully used as a molecular marker for the determination of stock structure in a wide variety of fish taxa (Billington and Hebert, 1991; Dowling and Naylor, 1997). The mtDNA gives a better estimation on genetic differentiation than other nuclear marker because it is approximately fourfold more sensitive to genetic drift and founder effects (Birky et al., 1983).

In Malaysia, artifical propagation of this species started more than 10 years back and were only successful very recently (Ingram et al., 2005). The success has inititated an opportunity for mass production of this valued species which able of serving both aquaculture for commercial production and restocking for conservation purposes. However, there were little works undertaken to assess the genetic status of Malaysian Mahseer the wild (Esa et al., 2006; Nguyen et al., 2006; Nguyen, 2008). The present study is exclusive in that it presents part of an extensive data on genetic status of *T. douronensis* in Sarawak.

MATERIAL AND METHODS

Hundred and thirty four samples were collected from eight rivers in Sarawak (Fig. 1). Muscle tissues were collected via non-destructive finclip samples. Finclip were preserved in 90% ethanol for DNA extraction. Total genomic DNA was isolated based on the method described by Taggart et al. (1992) with slight modifications. A segment of the COI mtDNA gene was amplified with the oligonucleotide primers COIf (5' CCTGCAGGAGGAGGAGAYCC 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 MgCl2, 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 60 s), annealing (at 45°C for 30 s), and extension (at 72°C for 60 s). The amplified products were visualized on 2% agarose gels containing ethidium bromide, run for approximately 30 min at 90 V, and photographed under UV light. The PCR products were further purified using a DNA purification kit (Vivantis, Kuala Lumpur, FT, Malaysia) according to the manufacturer's instructions. The PCR products were purified for sequencing and only partial sequences were aligned using MEGA 4.0 program (Tamura et al., 2007).

DNA Sequence polymorphism (dnaSP) program version 5 (Librado et al., 2009) was used to generate haplotype sequences. Genetic diversity in Sarawak river basins were measured as the number of haplotypes, haplotypes diversity (*h*) and nucleotide diversity (π). Population subdivision and structure were estimated using an analysis of molecular variance (AMOVA) (Excoffier et al., 1992). Pairwise population F_{ST} significance test (Cockerham and Weir, 1993) were calculated by Arlequin 3.5 (Excoffier et al., 2010).

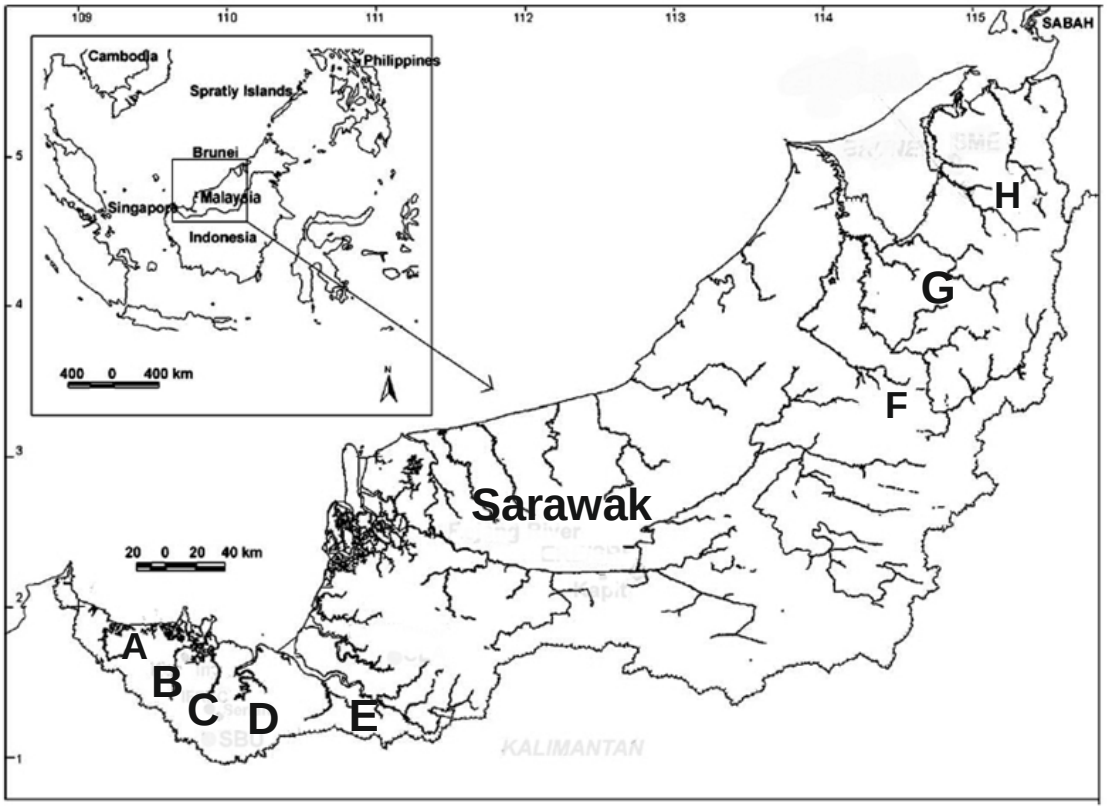

FIGURE 1. Map showing the sampling locations of *T. duoronensis* used in the mtDNA diversity study A: Lundu, B: Semadang, C:Bau, D:Terbat, E:Entabai River, F:Bakun Dam, G:Tuyo River, H:Kg. Pa Puti.

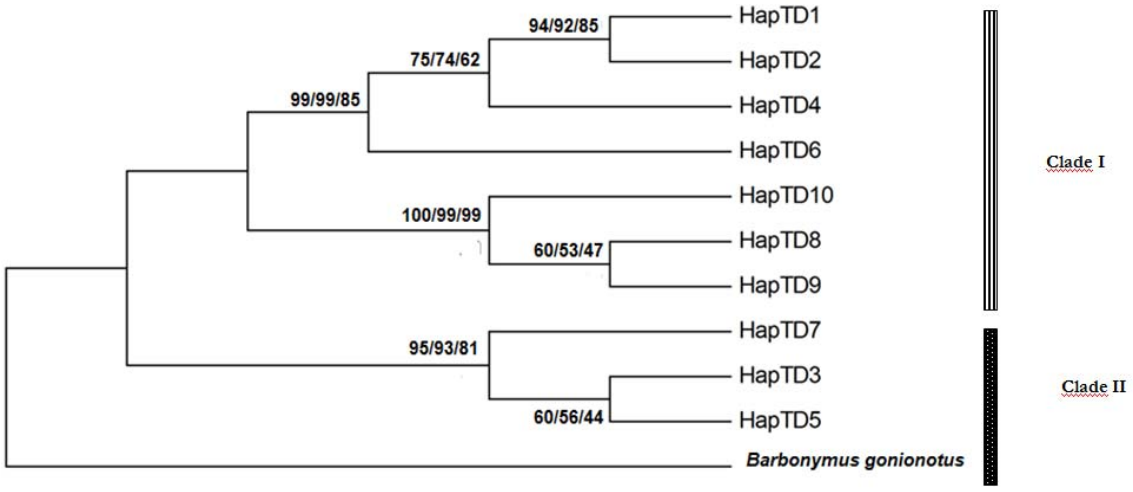

FIGURE 2. Maximum likelihood, Maximum Parsimony/Neighbour Joining dendogram showing the relationship between COI haplotypes. The number at each node represents the bootstrap proportions (%) based on 1000 pseudo replications for Maximum likelihood/Maximum Parsimony/Neighbour Joining analyses.

TABLE 1- Distribution of 10 observed COI gene haplotypes and Genbank accession of population of *T. duoronensis* in Sarawak. N: Number of individual in different populations.

Haplotype	Genbank Accession No.	Semadang N=23	Bakun Dam N=16	Lundu N=14	Kg. Pa Puti N=15	Tuyo River N=16	Bau N=22	Entabai River N=15	Terbat N=13
HapTD1	JF597591	0.913(21)	0.250(4)	-	-	-	0.864(19)	0.067(1)	1.000(13)
HapTD2	JF597592	0.044(1)	-	-	-	-	-	0.067(1)	-
HapTD3	JF597593	-	0.063(1)	-	-	-	-	-	-
HapTD4	JF597594	0.044(1)	-	-	-	-	-	-	-
HapTD5	JF597595	-	0.688(11)	-	-	-	0.045(1)	0.067(1)	-
HapTD6	JF597596	-	-	1.000(14)	-	-	-	-	-
HapTD7	JF597597	-	-	-	1.000 (15)	1.000 (16)	-	-	-
HapTD8	JF597598	-	-	-	-	-	0.045(1)	-	-
HapTD9	JF597599	-	-	-	-	-	0.045(1)	-	-
HapTD10	JF597600	-	-	-	-	-	-	0.800(12)	-

TABLE 2- Nucleotide diversity, number of haplotypes and number of polymorphic sites among population of *T. duoronensis* in Sarawak.

Haplotype	Semadang	Bakun Dam	Lundu	Kg. Pa Puti	Tuyo River	Bau	Entabai River	Terbat
Nucleotide diversity Pi, JC(π)	0.001	0.003	0.000	0.000	0.000	0.007	0.014	0.000
No. of haplotypes	3	3	1	1	1	4	4	1
Haplotypes diversity	0.139	0.268	0.000	0.000	0.000	0.214	0.291	0.000
No. polymorphic sites	4	6	0	0	0	18	27	0

The genetic relationship among haplotypes were also determined by constructing phylogenetic trees through Neighbour Joining (NJ) and Maximum Parsimony (MP) and Maximum liklihood (ML) methods which implemented in Paup software (Swofford, 2009). The tree was rooted by *Barbonymus gonionotus* sequence as an outgroup taxon.

The confidence limits were assessed using bootstrap procedure (Felsenstein, 1985) with 1000 pseudoreplicates for NJ, MP and ML , respectively.). Evaluation of the data for appropriate models to be used ML analysis was done using Akaike Information Criterion (Akaike, 1973) as implemented in the model Test 3.7 (Posada and Crandall, 1998); the best model used was GTR+C+I.

RESULTS

Aligned sequences of COI gene were identified ten haplotypes from 134 individuals originated from eight rivers in Sarawak. All sequences were submitted to the Genbank under accession number of JF597591-JF597600. Number of haplotypes among population of *T. duoronensis* in Sarawak is shown in Table 1. Among all haplotypes, HapTD1 is the most dominant which was distribute abundantly in Semadang, Bakun Dam, Entabai River and Terbat populations. All individual of Tuyo River and Kg. Pa Puti shared the same haplotype (HapTD7) with haplotype proportion 76.2% and 23.8% respectively. This haplotype may probably be recognised as diagnostic haplotype for the northern population (Tuyo River and Kg. Pa Puti population). There was only single unique haplotyle (Hap TD6) identified for Lundu population. In addition, *Tor douronensis* samples from Bakun Dam harbored the majority of haplotype, HapTD5 which was also shared by Bau (0.8%) and Entabai River (0.8%) samples. The populations of Kg. Pa Puti, Tuyo river and Terbat possessed only single haplotype, indicating the lack of mtDNA divergence among samples. It was probably due to low samples size that represents each population in this study (Table 1and Table 2).

TABLE 3- Pairwise Tamura Nei genetic distances (below the diagonal) and population divergence between populations (F_{ST}) and Chi square probability test for population divergence among samples based on COI gene sequences (above the diagonal) asterisk (*) indicate significant F_{ST} values after Bonferonni correction ($p< 0.05$).

	SouthernSarawak					Central Sarawak	Northern Sarawak	
	Semadang	Lundu	Terbat	Bau	Entabai River	Bakun Dam	Tuyo River	Kg. Pa Puti
Semadang		0.896*	0.001	-0.015	0.730*	0.617*	0.901*	0.899*
Lundu	0.020		0.990*	0.844*	0.910*	0.764*	0.976*	0.987*
Terbat	0.001	0.013		0.013	0.790*	0.678*	0.958*	0.976*
Bau	0.001	0.016	0.003		0.663*	0.542*	0.851*	0.847*
Entabai	0.019	0.027	0.014	0.017		0.558*	0.820*	0.815*
Bakun Dam	0.012	0.018	0.005	0.007	0.014		0.777*	0.771*
Tuyo River	0.014	0.019	0.006	0.010	0.021	0.012		0.000
Kg. Pa Puti	0.014	0.019	0.006	0.010	0.021	0.012	0.000	

TABLE 4- ANOVA results for the hierarchical genetic subdivision for sum of squares, percentage of variation, F statistics and P value of partial mtDNA COI gene among three regions namely southern Sarawak (Terbat, Semadang, Lundu, Bau and Entabai), central Sarawak (Bakun Dam) and northern Sarawak (Tuyo River and Kg. Pa Puti).

Source of Variation	Sum of squares	Percentage of Variation	F statistics	P value
Among group (region)	83.205	41.82	$F_{CT} = 0.41823$	0.0137*
Among populations within group (region)	69.734	41.21	$F_{SC} = 0.70835$	0.0000*
Within population/Between population relative to the total variance	42.009	16.97	$F_{ST} = 0.83033$ (between population)	0.0000*

* Significant level, P<0.05

From the results obtained here, population of *T. duoronensis* in Sarawak showed haplotype diversity (0.000-0291) and nucleotide diversity, Pi (0.000-0.014). The populations of Lundu, Kg Pa Puti, Tuyo River and Terbat River showed the least genetic variation presenting a single haplotype. The Entabai River, with moderate sample size, appeared to be the most divergent with high number of polymorphic site, much greater nucleotide diversity ($\pi = 0.014$) and haplotype diversity ($h = 0.291$).
In order to do interpopulation analysis of eight populations of *T. douronensis* , pairwise F_{ST} and Chi square test were performed (Table 3). The findings showed high genetic differentiation among all populations. The P value after Bonferonni correction revealed that only F_{ST} values of the Terbat-Semadang,Bau-Semadang, Bau-Terbat and Tuyo River-Kg Pa Puti were not significant. The finding indicated that the populations were relatively homogenous to each other.
The populations divided into three different regions namely southern Sarawak (Terbat, Semadang, Lundu, Bau and Entabai), central Sarawak (Bakun Dam) and northern Sarawak (Tuyo River and Kg. Pa Puti). Pairwise F_{ST} analysis was undertaken to compare the genetic differentiation among different regions (Table 4). The results showed that the central Sarawak and northern Sarawak populations were the most divergent from the southern population.

The phylogenetic relationships among haplotypes for 134 samples is given in Figure 2. The generated MP and ML phylogenetic trees were almost identical with the NJ tree with slight difference in bootstrap confidence level at each node. Cluster I grouped all the *T. douronenesis* haplotypes from the southern Sarawak, while Cluster II consisted of *T. douronensis* haplotypes from the northern Sarawak and the central Sarawak (Bakun Dam) populations which consisted of one unique haplotype from the Bakun Dam (HapTD3) and one haplotype (HapTD5) that was shared with Bau and Entabai River populations. HapTD1 which was dominant in the *T. douronenesis* of Sarawak was clustered in Clade I and this haplotype can be found in most populations of southern Sarawak.

DISCUSSION

The current study revealed low level of intra and high level of inter-population variations among *T. douronensis* populations of Sarawak. Similar result was also observed by Nguyen et al. (2006) on the genetic variation of this species from Limbang River tributaries, Bunan River and Layar River of Sarawak. According to Wei and Cui (1996) and Roberts (1999), the large difference for inter-population variations in *T. douronensis* is rather unexpected, especially since this species and its congeners, *T. tambroides* are morphologically conservative.

The mtDNA analyses also suggested the occurence of population substructuring among *T. douronensis* populations in Sarawak which were previously identified by microsatellite markers (Nguyen, 2008). The present analysis suggests that the presence of two major lineages of *T. douronensis*, namely southern (Clade I) and central-northern (Clade II) Sarawak. This pattern of population subdivisions observed in this study may be related to the independent drainages that separately enter the sea, which was formed when the sea levels were lowered in the past. This had restricted the movement of fish between the river basins via great river connection (e.g North Sunda River) and eventually favoured the establishment of the discrete lineages.

The mtDNA data reported in this study indicated the isolation of some populations, for example northern Sarawak populations (Kg. Pa Puti and Tuyo River). The same populations isolation evidence was detected in previous study (Nguyen et al., 2006). Large genetic differences between *T. douronensis* populations from the northern part with its congeners from the central and southern parts, plus the presence of fixed haplotype supported the hypothesis of historical isolation of the northern Sarawak and this region might be the most isolated region and probably had no connection with the other regions of Sarawak prior to the Pleistocene glaciation period.

The genetic data obtained here also suggested that some populations may probably have experienced immigration that results in gene influx. For examples, fish from the Semadang, Bakun Dam, Bau and Entabai exhibited the highest haplotype admixture, probably appeared to have received quite significant number of immigrant from other populations. Long distance dispersal of *T. douronesis* is unlikely to occur naturally, as not all of the water systems are interconnected. Therefore, human mediated translocation is probably responsible for the gene migration that has occured between these populations.

Avise (1998) proposed that large population sizes may lead to extraordinarily high level of genetic diversity. Although *Tor douronensis* or Semah occurs in numbers of river in Sarawak, the haplotype diversity observed in this study was quite low. The low haplotype diversity within population might be due to the environmental homogeneity and life history traits that favour slow population increase (Nei, 1987). The low level of haplotype diversity observed in the *T. douronensis* populations was supported by a relatively low nucleotide diversity that might probably be due to a possible bottleneck occured during its earlier colonization which was also found in previous study by Franck et al. (1998)

Despite the significant differentiation among populations from different geographical regions, overall, mtDNA data did not distinguish populations within the same geographical regions, as indicated by low percentage of variation (within population). In Borneo Island, due to rugged landscape, most rivers are separated from each other by mountain ridges, thus, neigbouring rivers are not connected to each other and genetic differentiation would be expected between populations. However, some rarely occurring floods may create opportunities for fish migration across drainage systems. Wang et al. (2000) explained about the inter-stream migration pattern of *Arossocheilus paradoxus* that may led to the heterogenous composition of populations and the paraphyly and polyphyly of the mtDNA alleles.

Another interesting finding of the current study is the sharing of haplotype HapTD1 between samples of *T. douronensis* from Bakun Dam (central Sarawak) and southern Sarawak populations. These populations belong to different river systems that are not interconnected at present time. It is posibble that Sunda Shelf connecting the islands of Sumatera and Borneo to the Southeast Asia mainland might provide a historical connection between central Sarawak and southern Sarawak river systems. As *T. douronensis* has been recognized as one of the ecologically threatened species, restocking programmes have been extensively practiced in most of the river systems in Sarawak.

The goal of this study was to obtain a general view of population status of potential broodstock of *T. douronensis*, an indigenous Mahseer species of high commercial and cultural significance currently held in several hatcheries in Malaysia, with the intent to fascilitate further developments of the captive breeding and restocking programs for aquaculture and conservation.

The haplotype composition surveyed in the present study may provide baseline for future comparisons to monitor the temporal variability of haplotype frequency and poplutaion structure of *T. douronensis*. Finally, the understanding of the population structure is also important in management of the wild population of this species and the identification of potentially broodstocks for commercial propagation of the species.

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