Mitochondrial DNA diversity of broodstock of two indigenous mahseer species, *Tor tambroides* and *T. douronensis* (Cyprinidae) cultured in Sarawak, Malaysia

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Abstract

Tor tambroides and *T. douronensis*, locally referred to as empurau and semah, respectively, are high valued mahseer species, indigenous to Sarawak, East Malaysia, with an aquaculture potential and of conservational value. Direct sequencing of mitochondrial DNA (mtDNA) 16S rRNA gene region (542 bp) was used to investigate genetic variation of *T. tambroides* and *T. douronensis* broodstock collected from different geographic locations in Sarawak and maintained at the Indigenous Fish Research and Production Center (IFRPC), Tarat, Sarawak, Malaysia. A total of 11 unique haplotypes were identified, of which six were detected in *T. tambroides*, and five in *T. douronensis*. Overall, nucleotide diversity (π) was low, ranging from 0.000 to 0.006, and haplotype diversity (h) ranged from 0.000 to 0.599. Although the analysis failed to detect genetic variation amongst populations of *T. tambroides* (significant pairwise F_{ST} was found for only one test, but pairwise haplotype frequencies were not statistically significant), substantial inter-population divergence among *T. douronensis* was recognised, especially those originating from different river systems (pairwise $F_{ST}=0.754$ to 1.000, $P<0.05$). Fixed haplotype differences were found in one population of *T. douronensis*. Average nucleotide divergence between *T. tambroides* and *T. douronensis* was 0.018, similar to the amount recognised between *T. tambroides* and the outgroup *T. khudree* (0.017). In addition, phylogenetic analysis revealed that the *T. douronensis* mtDNA consisted of two highly divergent clusters (0.020), one of which is more closely related to *T. tambroides* rather than with the other group of haplotypes of the conspecifics. The findings from the present study have important implications for aquaculture, management and conservation of these two species. The data also raise some concerns regarding the taxonomic status of *T. douronensis*, which needs to be addressed.

Keywords: T. tambroides and *T. douronensis*; Mahseer; Broodstock; MtDNA; 16S tRNA; Conservation

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1. Introduction

Fish of the genus *Tor*, commonly referred to as the mahseers, are important to most nations in the Asian region for biodiversity reasons, and are also sought after as high-valued food and game fish (Ng, 2004). However, due to developments in the watersheds within the natural range of many *Tor* species, critical habitats, particularly spawning grounds, have been disturbed. Together with increased fishing pressure on *Tor* species, this has resulted in the depletion of natural stocks. Consequently some species have become rare and/or endangered, for example, *T. putitora* (Hamilton) and *T. tor* (Hamilton) are listed as vulnerable in West Bengal, India (Mijkherjee et al., 2002) and both are considered to be in danger of extinction in Nepal (Shrestha, 1990), while *T. yunnanensis* is listed as endangered in China (Baillie and Groombridge, 1996).

In view of the conservational value and the aquaculture potential of some *Tor* species, there has been a concerted effort to artificially propagate several *Tor* species, but with relatively little success to date (Petr and Swar, 2002; Ingram et al., 2005). Captive breeding of most *Tor* species are based on wild-caught, mature fish (Joshi, 1988; Nandeesha et al., 1993; Gurung et al., 2002), although some successes with pond reared broodstock of a few species have been reported (Ogale, 2002; Ingram et al., 2005).

The State of Sarawak, Malaysia, harbors *T. tambroides* (Bleeker) and *T. douronensis* (Valenciennes) which are commonly known as empurau and semah, respectively. These two indigenous species live in headwaters of most major river systems of this state. These two species also occur in Peninsular Malaysia and are distributed throughout southeast Asia from Indonesia to southern China (Kottelat et al., 1993; Zhou and Cui, 1996; Roberts, 1999). *Tor douronensis* is the State Fish of Sarawak, and both species currently fetch a very high market price (in 2004, 1 kg of either species ranged from Malaysian Ringit 160 to 240; personal observation; 3.80 Malaysian Ringit = 1 US$) and are of high cultural value. Juveniles of these two species are also increasingly sought after by the aquarium industry (Ng, 2004). The Government of Sarawak, recognising the importance of these two species, made a concerted attempt to evaluate their aquaculture potential, including captive breeding using long-term pond-reared broodstock, commencing in the 1990s. However, limited success was achieved until the period 2002–2004 when, in an international collaboration, researchers from Australia and Sarawak were able to captive breed both species using hormone induction techniques on long-term, pond-reared broodstock. Details of the hormonal treatments used, the hatching techniques, development and larval rearing of *T. tambroides* and *T. douronensis*, have been documented by Ingram et al. (2005).

The recent successful hatchery production of *T. tambroides* and *T. douronensis* brought to the forefront problematic questions regarding genetic variation of the broodstock. Firstly, the taxonomic status of these two species remains highly contentious, as for the whole genus (Roberts, 1999); the descriptions provided by different authors and the related drawings in most instances are inconsistent and highly variable, leading to potential misidentifications. While some authors considered *T. tambroides* and *T. douronensis* as two valid species (Zhou and Cui, 1996; Ng, 2004), Roberts (1999) considered them to be a single species and a junior synonym of their congener, *T. tambra* (Valenciennes). Given such taxonomic confusion, however, hitherto no attempts have been made to resolve the relationship among these species.

Secondly, long-term management of aquaculture production and conservation of these two species would be compromised without an adequate assessment of the genetic diversity of newly established broodstock populations. From an aquaculture perspective, information on levels of genetic variability within and among broodstock populations permits fish breeders to avoid potential detrimental effects of inbreeding and other genetic changes from one generation to another (Gjedrem, 1992). Also, genetic markers can be used to assist establishment of base-line stock, including family lines for selection purposes (Cross et al., 2000).

There have been documented cases in many fish species of genetic changes and loss of genetic variability in hatchery-reared stocks, and also resulting in alteration of genetic diversity of their wild counterparts due to interbreeding with escapees of hatchery-reared stocks (Crozier, 1993; Clifford et al., 1998) or those used for restocking (Bentsen, 1991; Hindar et al., 1991;). Genetic variability is pivotal to maintaining the capability of restocked fish to adapt to a changing environment (Avise, 1994). For conservation purposes, a successful restocking program depends largely on a broodstock management strategy that ensures maintenance of a wide gene pool, including a greater degree of genetic diversity. This minimises adverse effects on the genetic diversity of wild populations once stock enhancement commences, thereby helping to maintain the genetic integrity of the species under consideration (Vrijenhoek et al., 1985). The lack of systematic genetic surveys to ascertain the genetic variability within and between *T. tambroides* and *T. douronensis* prompted the present

study, the results of which may help in the design of a broodstock management strategy and subsequent application to future aquaculture production and genetic conservation strategies (Ryman and Laikre, 1991).

Mitochondrial DNA (mtDNA) analysis has been successfully used as a molecular marker for species identification and for determination of population genetic structure in a wide variety of fish taxa (see reviews by Ovenden, 1990; Billington and Hebert, 1991; Meyer, 1993). MtDNA gives a better estimate of genetic differentiation than nuclear markers since it is approximately fourfold more sensitive (Birky et al., 1983). Also, in the context of the two species investigated, it is almost impossible that a nuclear marker such as allozymes be employed due to destructive sampling requirements of the technique, and microsatellite markers are not yet developed for these species per se. But admittedly, few microsatellites developed for other cyprinids have been cross-amplified for several *Tor* species (Mohindra et al., 2004).

The overall objective of the current study was to conduct a base-line survey on the genetic composition of broodstock populations of *T. tambroides* and *T. douronensis* maintained at the Indigenous Fish Research and Production Center (IFRPC), Tarat, Sarawak, Malaysia (Fig. 1), using nucleotide sequences of partial 16S rRNA gene of the mtDNA. The data are also used to discuss the implications for management of these two species, including aquaculture and conservation developments.

2. Materials and methods

2.1. Collection of samples

DNA was routinely sampled from individual fish in the present study via non-destructive finclip samples. Finclips were preserved in 75–95% ethanol for mtDNA analysis. Samples originated from two sources: (1) broodstock of *T. tambroides* and *T. douronensis* currently held at the IFRPC, and (2) young fish recently

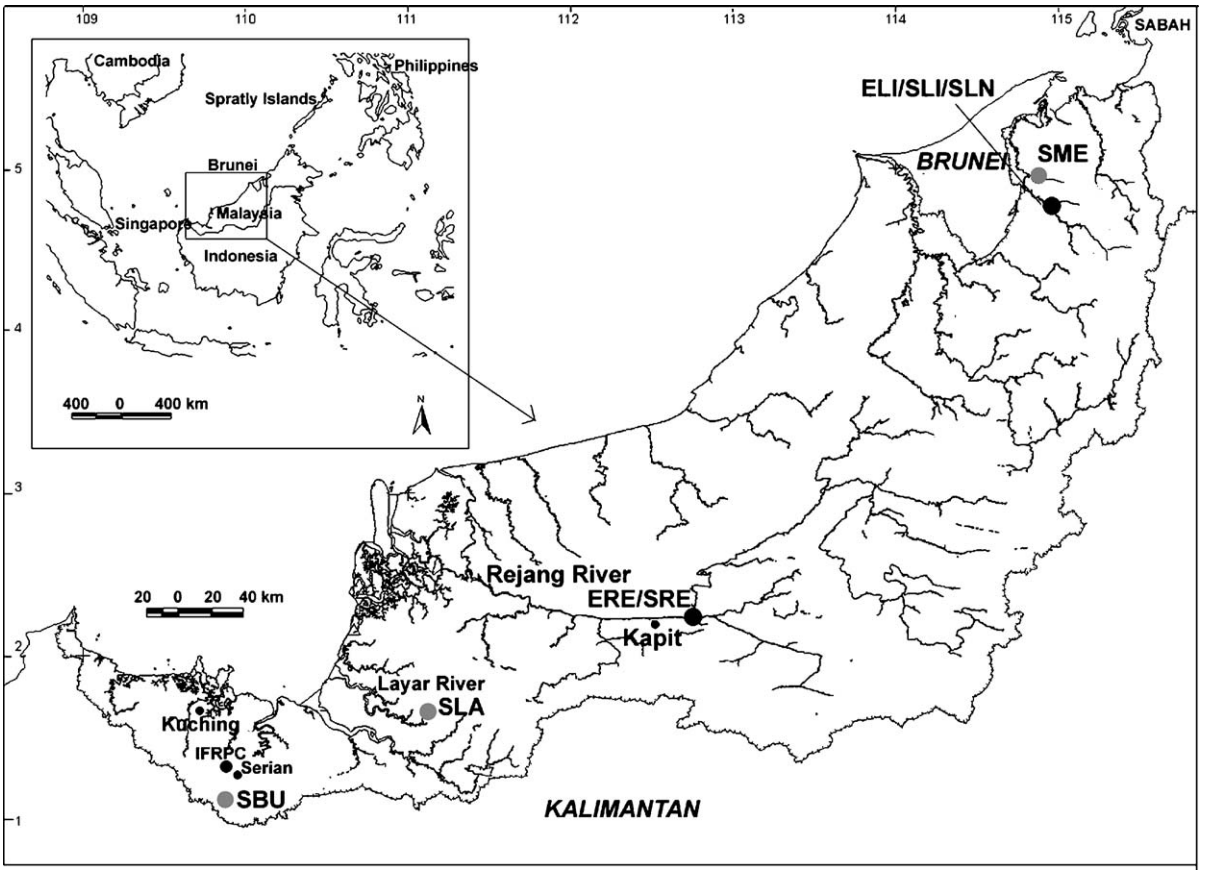

Fig. 1. Sample codes, sample sizes and original localities of *T. tambroides* and *T. douronensis* populations studied. The inset shows the areas of collection in relation to the Malaysian Peninsular and Sarawak.

collected from several rivers in the wild in Sarawak, as potential broodstock.

Sampled broodstock (mature and sub-adult) of *T. tambroides* and *T. douronensis* were collected between 1990 and 1997 from two major river systems in Sarawak, Malaysia—Limbang and Rejang rivers (Table 1 and Fig. 1). Species maintained at the IFRPC were initially separated based on morphological differences in scale size and colour, the length of the median lobe and the thickness of the lip (Table 2). Broodstock of each species, originating from different localities were maintained separately. Broodstock of *T. douronensis* collected a second time, in 2000, from the Limbang River were also maintained separately. In addition, specimens of *T. tambroides* from Indonesia were also obtained, but limited to nine individuals currently maintained in ponds at the IFRPC. Ingram et al. (2005) described the husbandry practices utilised for maintaining the *Tor* broodstock at the IFRPC. In 2004, samples of *T. douronensis* from the Menuang River, a tributary of the Limbang River were collected. In addition, samples of juvenile *T. douronensis* were also collected from the Layar River (Fig. 1), which is thought to be the territory of only *T. douronensis* (D. Tinggi, personal communication), and included in the analysis for comparative purposes. For taxonomic comparison, four individuals of *T. khudree* (Sykes) were obtained from Sri Lanka.

Table 1
Sample codes, sample origin and sample sizes of *T. tambroides* and *T. douronensis*, together with the outgroup *T. khudree* analysed in the present study

Species/ sample code	Locality of origin	Year of collection	Sample size
T. tambroides, Empurau,			
ELI	Adang River, tributary of Limbang River, Sarawak	1989–1995	47 (179)
EIN	Indonesia*	Uncertain	9 (9)
ERE	Rejang River, Sarawak	1990–1993	15 (18)
T. douronensis, Semah,			
SLI	Adang River, tributary of Limbang River, Sarawak	1990–1995	62 (220)
SRE	Rejang River, Sarawak	2000–2003	40 (45)
SLN	Adang River, tributary of Limbang River, Sarawak	2000	48 (482)
SME	Menuang River, tributary of Limbang River, Sarawak	2004	21
SBU	Bunan River, Sarawak	2004	29
SLA	Layer River, Sarawak	2004	35
T. khudree, Deccan mahseer,			
TKS	Bopath Ella, Sri Lanka	2004	4

Where relevant the total number of fish of each group maintained at the IFRPC, Tarat, is given in parentheses. Those collected in 2004 are potential broodstock only.
*Collection site of fish unknown.

Table 2
Characters used for identifying *T. tambroides* and *T. douronensis*

Characters	*T. tambroides*	*T. douronensis*
Scale size	Larger	Smaller
Scale colour	Reddish, white	Yellowish, gold, silvery
Length of median lobe	Short, not extending to a line connecting inner corners of mouth	Long, extending to a line connecting inner corners of mouth
Thickness of lip	Thicker and more fleshy	Thinner and less fleshy

2.2. Laboratory procedures

Extraction of genomic DNA was undertaken according to the protocols outlined by Fetzner (1999) with a slight modification. PCR amplifications of 16S rRNA mitochondrial gene fragments employed the oligonucleotide primers 16Sar and 16Sbr, flanking a region of the large subunit ribosomal RNA gene (16S rRNA, Palumbi et al., 1991). Amplifications were conducted in 25 μL volumes, containing reaction buffer and 0.5 unit of *Taq* DNA Polymerase (Promega), 200 μM dNTPs, 0.5 μM of each oligonucleotide primer, 2.5 mM $MgCl_2$, and 50 ng of genomic DNA. Thermal cycling conditions were 30 cycles of 94 °C/30 s, 55 °C/30 s, and 72 °C/30 s. An initial denaturation of 94 °C/3 min and a final extension of 72 °C/10 min were employed. For each sample, three μL of PCR product were electrophoresed through 1% agarose gels following ethidium bromide staining, and visualized under UV illumination. Successful PCR products were sent to MacroGen Inc. (D.R. Korea) for purification and sequencing. Both strands of PCR fragment were sequenced, enabling the identification of ambiguities.

2.3. Data analysis

Sequences were viewed, edited and aligned using MEGA software (Kumar et al., 2004). Sequence divergences between haplotypes were calculated using the Kimura 2-parameter model, and the phylogenetic relationships amongst mtDNA haplotypes were examined using both neighbour-joining (NJ) and maximum parsimony (MP) as implemented in the same software. Tree robustness was assessed by 1000 bootstrap replicates.

Levels of mtDNA 16S rRNA variability within samples of *T. tambroides* and *T. douronensis* were examined by computing the nucleotide and haplotype diversity indices using Arlequin version 2.000

Table 3
Distribution of 11 observed mtDNA 16S rRNA haplotypes, nucleotide diversity, number of haplotypes and number of polymorphic sites among populations of *T. tambroides* and *T. douronensis*

																								T. tambroides			*T. douronensis*					
	1 3	7 0	7 6	1 1 6	1 3 2	1 5 2	1 5 3	2 0 7	2 0 9	2 1 1	2 1 4	2 2 3	2 2 9	2 5 4	2 7 6	2 8 4	2 8 7	2 9 0	3 1 0	3 1 3	3 1 5	3 3 0	3 3 2	ELI	EIN	ERE	SLI	SRE	SLN	SME	SBU	SLA
Hap01	C	G	T	C	A	G	T	A	G	A	A	T	A	T	G	A	A	–	C	–	C	G	A	0.787	0.667	0.533						
Hap02	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A	.	–	.	.	.	0.021								
Hap03	.	.	.	T	.	.	.	.	.	C	G	.	.	.	.	.	.	A	.	–	.	.	.	0.128		0.067						
Hap04	.	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	A	.	–	.	.	.	0.064	0.222		0.032			0.048		
Hap05	.	.	.	.	.	.	.	.	.	C	G	C	.	.	.	G	.	A	.	–	.	.	.			0.200						
Hap06	.	.	.	.	.	.	.	.	.	C	G	C	.	.	.	G	.	A	T	–	.	.	.		0.111	0.200						
Hap07	.	.	.	.	G	.	.	.	.	C	G	.	G	C	.	.	.	A	.	T	T	A	.				0.758		0.791	0.809		
Hap08	.	.	.	.	G	.	C	.	.	C	G	.	G	C	.	.	.	A	.	T	T	A	.				0.145		0.208	0.142		
Hap09	T	A	C	.	.	A	.	.	A	C	G	.	G	C	.	.	T	A	.	T	.	.	.				0.016					
Hap10	T	A	C	.	.	A	.	G	A	C	G	.	G	C	.	.	T	A	.	T	.	.	T					1.000				
Hap11	T	A	C	.	.	A	.	G	A	C	G	.	G	C	A	.	T	A	.	T	.	.	.				0.048				1.000	1.000
Nucleotide diversity (π)																								0.002	0.005	0.006	0.003	0.000	0.001	0.001	0.000	0.000
Number of haplotypes																								4	3	4	4	1	2	2	1	1
Haplotype diversity (h)																								0.367	0.556	0.676	0.367	0.000	0.337	0.268	0.000	0.000
Number of polymorphic sites																								4	6	7	12	0	1	6	0	0

The vertical numbers indicate the position of variable nucleotides within the 542-bp sequence. Dots indicate that the same nucleotide is present as in Hap01 and a dash (–) indicates a deletion. Numbers under each population indicate the frequencies of individuals with that haplotype in each population.

(Schneider et al., 2000). Statistical testing for population structuring involved an exact test (Raymond and Rousset, 1995) of a contingency table based on haplotype frequencies and pairwise comparisons of the F_{ST} using analysis of molecular variance (Excoffier et al., 1992) based on 1000 permutations of the data matrix. Multiple tests of the same null hypothesis were subjected to table-wide sequential Bonferroni correction to avoid elevated Type I error rates.

3. Results

3.1. Sequence variation

A total of 542 base pairs of the 16S rRNA gene fragment were successfully sequenced for 71 individuals from three broodstock populations of *T. tambroides*; 150 individuals from three broodstock populations and 85 individuals from three wild juvenile populations of *T. douronensis* and four individuals of *T. khudree* from Sri Lanka. In total, 12 unique haplotypes with twenty-eight variable sites (5.17%) were identified among all samples, including one haplotype representing *T. khudree*. Sequences representing each haplotype were submitted to the GenBank (GenBank Reference Numbers: AY973157-AY973168). The mean total nucleotide composition was A=32.1%, T=21.7%, C=24.1% and G=22.1%.

3.2. Distribution of haplotypes and genetic diversity

An alignment of variable sites of 11 haplotypes found among *T. tambroides* and *T. douronensis* samples and the distribution of these haplotypes among populations are presented in Table 3. In total, 23 substitutions were found among haplotypes, of which there were 18 transitions, three transversions and two insertions/deletions. In general, Hap01 (53.3–78.7%) was dominant in *T. tambroides* populations, while Hap07 was found to be abundant among populations SLI, SLN and SME of *T. douronensis* (75.8–80.9%). Hap05 was unique to population ERE at a proportion of 20%, and all individuals of SRE shared Hap10, which is also unique. Two juvenile populations SBU and SLA shared the same haplotype (Hap11), which was also present in SLI at a proportion of 4.8%.

One surprising finding was that one haplotype of *T. tambroides* (Hap04) was found in one population of *T. douronensis*. Sequences of two individuals from SLI and one individual from SME populations showed Hap04, which is thought to be a haplotype of *T. tambroides* (see section on Phylogenetic analysis below). As such, these individuals were excluded from subsequent analyses.

Values of within-population nucleotide diversity (π), number of haplotypes, haplotype diversity (h), and number of polymorphic sites within each population are also presented in Table 3. Nucleotide diversity was low overall (0.000–0.006). With regard to *T. tambroides*, although EIN and ERE are relatively small in terms of census population size, these populations showed a similar number of haplotypes to ELI, higher number of polymorphic sites, much greater nucleotide diversity as well as haplotype diversity. Among *T. douronensis* populations, within-population variation was only found in SLI, SLN and SME that were originally from the

Table 4
Population divergence between samples (F_{ST}) based on mtDNA 16S rRNA sequence data for (a) *T. tambroides*, and (b) *T. douronensis*, significant level ($P<0.05$) of F_{ST} is indicated by the asterisk *

(a)

	ELI	EIN
EIN	0.037	
ERE	0.115 *	−0.030

(b)

	SLI	SRE	SLN	SME	SBU
SRE	(0.787) *				
SLN	−0.023	(0.819) *			
SME	−0.025	(0.908) *	−0.025		
SBU	(0.754) *	(1.000) *	(0.797) *	(0.889) *	
SLA	(0.766) *	(1.000) *	(0.809) *	(0.900) *	0.000

Significant probabilities ($P<0.05$) based on 1000 permutations of haplotype frequencies (after sequential Bonferroni corrections) among samples are indicated in parentheses.
* Significant population differentiation, $P<0.05$.

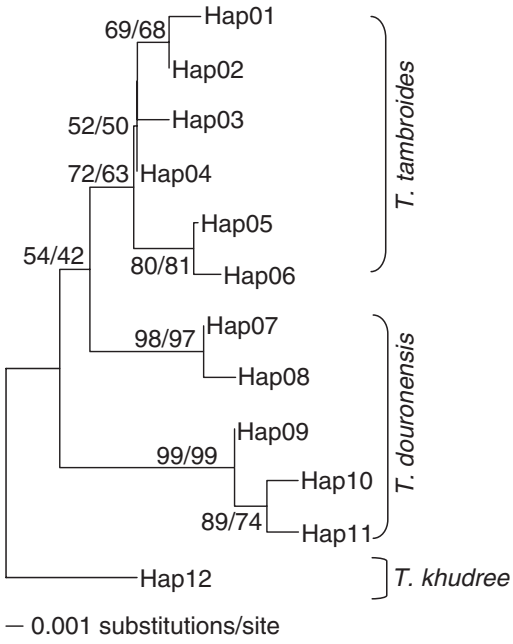

Fig. 2. Neighbour-joining phylogram showing the relationship between 16S rRNA haplotypes from *Tor* species analysed in the present study. The numbers at each node present bootstrap proportions (%) based on 1000 pseudoreplications for NJ/MP analyses.

same river system. Haplotype diversity varied, ranging from 0.000 to 0.599.

Pairwise F_{ST} and significant levels after sequential Bonferroni corrections, and results of exact tests for population differentiation are presented in Table 4. In a total of three pairwise comparisons among *T. tambroides* populations, no significant pairwise F_{ST} was observed, but significant population differentiation was found between ELI and ERE via exact test. In contrast, significant pairwise F_{ST} values and significant levels of genetic differentiation were found in all comparisons among *T. douronensis* populations, except for between SBU and SLA or those from the same river system, e.g., SLI, SLN and SME.

3.3. Phylogenetic analysis

A summary of the relationship amongst haplotypes from all samples is given in Fig. 2. Kimura-2 parameter genetic distances among haplotypes are presented in Table 5. Both MP and NJ analyses showed an identical tree topology with only minor differences in bootstrap confident levels.

The tree was bifurcated into two main clusters with moderate bootstrap support (42–54%). The first cluster included all *T. tambroides* haplotypes (Hap01 to Hap06) and two haplotypes (Hap07 and Hap08) of *T. douronensis*, with an average of 1.30% nucleotide divergence. The second cluster grouped the rest of *T. douronensis* haplotypes, which showed the similar degree of average nucleotide differences (2.10%) to *T. tambroides* haplotypes and the other *T. douronensis* haplotypes. As such, *T. douronensis* appeared to be separated into two different groups with a high level of divergence, which is even higher than what was observed between species, such as between *T. tambroides* and the outgroup *T. khudree* (1.73% nucleotide differences).

4. Discussion

The goal of this study was to obtain a general view of the population genetics of broodstock and/or potential broodstock of *T. tambroides* and *T. douronensis*, two indigenous species of high commercial and cultural significance currently held at IFRPC, with the intent to facilitate further developments of the captive breeding and restocking programs for aquaculture and conservation. Attempts at captive breeding of these species started more than 10 years back and were only

Table 5
Pairwise Kimura 2-parameter genetic distance among the different haplotypes of the 16S rRNA gene sequences detected in the present study

Haplotype	1	2	3	4	5	6	7	8	9	10	11
2	0.002										
3	0.006	0.004									
4	0.004	0.002	0.002								
5	0.007	0.006	0.006	0.004							
6	0.009	0.007	0.007	0.006	0.002						
7	0.013	0.011	0.011	0.009	0.013	0.015					
8	0.015	0.013	0.013	0.011	0.015	0.017	0.002				
9	0.019	0.017	0.017	0.015	0.019	0.021	0.017	0.019			
10	0.023	0.021	0.021	0.019	0.023	0.025	0.021	0.023	0.004		
11	0.023	0.021	0.021	0.019	0.023	0.025	0.021	0.023	0.004	0.004	
12	0.019	0.017	0.017	0.015	0.019	0.017	0.021	0.023	0.021	0.025	0.025

successful very recently (Ingram et al., 2005). This success has opened an opportunity for mass production of these valued species potentially capable of serving both aquaculture for commercial production and re-stocking for conservation purposes. However, no attempts have been undertaken to assess the genetic status of these species, both in captivity and in the wild. The present study is unusual in that it presents one of a few instances whereby a genetic survey is undertaken prior to commencement of commercial aquaculture production and restocking.

4.1. MtDNA variability within and amongst populations of T. tambroides and T. douronensis

With regard to *T. tambroides*, all populations showed relatively high levels of within population variation, but limited variation amongst populations. This was reflected by a high number of haplotypes within each population (3–4), a high number of polymorphic sites (4–7), high haplotype diversity (0.367–0.676), but a lack of significant differences in all pairwise F_{ST} tests. The low level of genetic differentiation between *T. tambroides* populations is probably due to the high proportion of Hap01 in the three populations (0.54–0.78), or the limited number of individuals available from two populations i.e., Indonesia (EIN) (9) and Rejang River (ERE) (15). Although attempts have been made to maintain stocks from different origins separately, it is possible that over the long rearing period exceeding 10 years or so, that some mixing of the stocks occurred, especially those from Indonesia and Rejang River which may have been inadvertently mixed as they were maintained in adjacent ponds, but well separated from those holding the Limbang River (ELI) stock.

In contrast, low levels of intra- and high levels of inter-population variation were found in *T. douronensis*. Within population variations were only found in three populations— SLI, SLN and SME, all of which originated from the Limbang River system. It should be noted that the genetic composition of the population SLN, collected from the same locality as the SLI population after a lapse of five years, still remained similar (Hap07 and Hap08 in similar proportion, insignificant pairwise F_{ST}, and insignificant population differentiation), except for the fact that Hap11 was found in SLI population but not in SLN.

The large differences amongst *T. douronensis* populations were rather unexpected, especially since this species and its congeners are morphologically conservative (Zhou and Cui, 1996; Roberts, 1999). These can be explained by one or several different factors including small population sizes, past bottleneck events, or the presence of physical barriers that limit migration among populations. The presence of fixed haplotype differences among populations, along with high F_{ST} values among populations of *T. douronensis*, support the conclusion that little or no migration occurs among extant populations separated by large geographic distances, or river systems.

It is noted that there are 23 major river systems in Sarawak (Leh, 2000) and there has been no systematic assessment of the natural distribution of *T. tambroides* and *T. douronensis* amongst these rivers. Available indigenous and anecdotal evidence suggests that these species are likely to have been widely distributed naturally, with the possible exception of *T. tambroides,* which has not been recorded to date in small river systems (e.g., the Layar and the Bunan) (D. Tinggi, personal communication). The natural extension of the present study is to examine the finer scale phylogeography and population genetics of these two species with more intensive sampling. This approach will help to explain the historical processes that are responsible for the distribution and population structure of these two species. Furthermore, the information will also be useful to determine evolutionary significant units (ESUs) and management units (MU) for conservation purposes.

4.2. Implications for aquaculture, management and conservation

Genetic variability is an important attribute of species domestication, since those with higher levels of variation are most likely to exhibit a high additive genetic variance for productive traits (Tave, 1993), although it is acknowledged that estimation of heritability is desired prior to selective breeding program. From an aquaculture perspective, populations with high intra-population variation (e.g., all three populations of *T. tambroides*, and *T. douronensis* from Limbang River) may be more effectively used as base-line stocks for selective breeding in preference to the others (e.g., SRE, SBU and SLA). However, it is also important to note that the isolation of small populations such as EIN and ERE may lead to subsequent effects of genetic drift and inbreeding, and it is recommended that effective breeding numbers should be from 50 to 500 individuals (FAO/UNEP, 1981). In addition, fixed haplotype differences observed among populations (e.g., SRE, SBU, SLA) are potentially useful for communal rearing experiments, for monitoring the genetic effects of selection during selective breeding programs, broodstock management, and for developing markers to assist

selection (markers-assisted selection, MAS) (Carvalho and Pitcher, 1995; Liu et al., 1999; Cross et al., 2000).

Genetic variation is pivotal for populations to adapt to a changing environment or demographic events. The efficacy of an aquaculture operation or a restocking program is influenced by the genetic variation of the broodstock and associated propagation practices (Allendorf and Ryman, 1987; Ferguson et al., 1991). More often than not, the variability in hatchery broodstock is found to be lower than in wild populations due to founder effects and inbreeding accumulated over generations, as observed in closed breeding systems where progeny become future broodstock. Genetic changes in captivity may lead to an alteration in genetic composition of the wild counterparts via restocking programs (Hindar et al., 1991; Crozier, 1993; Carr et al., 1997). As such, concerted attempts should be made to maintain large effective population sizes for captive breeding, including an increase in the number of breeding individuals held in captivity, with 1 : 1 sex ratio (Tave, 1993), as well as regular turn-over of broodstock with new fish from wild populations.

The data presented demonstrate that populations within each species are highly heterogeneous in terms of haplotype composition, especially among *T. douronensis* populations. This may reflect a genetic adaptation to specific climatic or environmental conditions, prolonged isolation of populations, or possibly repeated extinction and recolonization events by small founding populations. Based on the present results, it seems intuitive that broodstocks and wild populations of the two species under study should be managed separately in biosecure facilities. It is advisable to utilise the progeny of broodstock from the same river system for restocking purposes, or that broodstock are sourced from the respective river systems to be stocked to maintain genetic integrity. These strategies will ensure that the original stock will not be contaminated from genetic materials from elsewhere, thereby avoiding any possibilities of hybridisation and dilution of the gene pool which could possibly lead to extinction of the native stock (Hughes et al., 2002).

If aquaculture of *T. tambroides* and *T. douronensis* is expanded spatially in the next few years, care must be taken when selecting broodstock for an aquaculture program. It is recommended that selection of broodstocks should be from populations residing in the same areas in which aquaculture production is to occur. This will avoid many of the concerns relating to the likelihood of exotic cultured stocks escaping from aquaculture operations into local natural habitats. Aquaculture escapees have in many instances disrupted local adaptation, contributed to a long decline in productivity, and reduced genetic diversity of native populations (Hindar et al., 1991; Crozier, 1993; Carr et al., 1997; Fleming et al., 2000). These outcomes are undesirable from a conservation perspective, but need to be balanced with the growing interests in the species from commercial and recreational sectors, as well as the associated socio-economic benefits that arise from a productive long-term fishery.

Although the two species are relatively rare and difficult to collect, it will be beneficial to make all attempts to obtain additional samples from other areas of their range of distribution. Finer scale sampling will allow a thorough assessment of population structure and determination of a management strategy. With regard to *T. tambroides*, we recommend replacement of Rejang River stock (ERE), and stock from Indonesia (EIN) need not be maintained.

4.3. Taxonomic considerations

The results of mtDNA analysis in the present study raise several concerns regarding the taxonomic status of *T. tambroides* and *T. douronensis*. First, the finding of a *T. tambroides* haplotype (Hap04) in two populations of *T. douronensis*, especially in the adult population of SLI, suggests several possibilities: (a) inadvertent mixing between ponds, (b) the identification of the two species using morphological characters is relatively unreliable, and (c) that the two species have hybridized and morphological characteristics of the hybrids are more similar to *T. douronensis*. Further investigation using nuclear markers such as microsatellites is warranted in order to address these issues.

Second, *T. douronensis* consists of two highly differentiated groups, one of which is more closely related to *T. tambroides*. In fact, the more abundant form of *T. douronensis* found in the Limbang River was also similar to *T. tambroides* both in the wild and in captivity, in term of scale colour (e.g., more reddish), while the other form of the same species appeared to be more blue. Although the bootstrap supports were rather low at major nodes, and 16S rRNA sequence data may not reflect the true species tree, this finding indicates that the high level of genetic divergence among samples of *T. douronensis* is rather cryptic. In fact, the two groups of haplotypes of *T. douronensis* differ from each other by a larger degree compared to the difference between *T. tambroides* and the outgroup, *T. khudree*. A plausible explanation for this is that one group of *T. douronensis* may be a cryptic species. It is clear that further taxonomic examination on *T. douronensis* is required,

and related questions can be addressed using additional sequence data from other closely related species within the genus *Tor*, data from additional mtDNA and nuclear gene regions, morphological characters and reproductive experiments.

In view of the increasing emphasis and interest on the captive breeding and culture of selected *Tor* species in the Asian region (Petr and Swar, 2002) it is relevant to consider in some detail the contentious taxonomic status of these two species which will have relevance to the group as a whole in general. With regard to *T. douronensis*, there is a need to address this problem before mass production is carried out, as it is found in the present study that the major populations that are now used to produce offspring is SLI, which consists of both distinct genetic forms. If these forms are inadvertently crossbred and used for restocking purposes, it could lead to adverse genetic consequences.

This study perhaps highlights problems that may be associated in developing strategies for captive breeding for aquaculture and restocking of hitherto relatively little studied species. It emphasises the need to resolve taxonomic issues beforehand and most of all it highlights the importance of genetic studies at the commencement of a program for aquaculture and conservation, a strategy that is relatively rarely practiced. Accordingly, the current program of captive breeding and potential strategies for conserving of these two valued species are perhaps a good example for similar initiatives on other species/species groups, particularly in view of the fact that in Asian aquaculture there is an increasing realization to shift from alien species to indigenous species (Bakos, 1997; Jensen, 1999; Sverdrup-Jensen, 2002).

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