Borneo J. Resour. Sci. Tech. (2011) 1: 14-23

CROSS-SPECIES AMPLIFICATION STUDY OF *Tor douronensis* AND *Tor tambroides* USING MICROSATELLITES FROM OTHER CYPRINIDS

YUZINE B. ESA[*1], SITI SHAPOR SIRAJ[3], KHAIRUL ADHA A. RAHIM[2], SITI KHALIJAH DAUD[4], HO GIM CHONG[4] & TAN SOON GUAN[5]

[1]Department of Zoology; [2]Department of Aquatic Sciences, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak; [3]Department of Aquaculture, Faculty of Agriculture; [4]Biology Department, Faculty of Science; [5]Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor DE, Malaysia

ABSTRACT

This study examined twenty six microsatellite primers developed from three cyprinid fishes (*Cyprinus carpio*, *Barbus barbus* and *Barbonymus gonionotus*) in two indigenous mahseer, *Tor douronensis* and *T. tambroides*. A total of 10 (38%) and 12 (46%) primers were successfully amplified producing four and five polymorphic loci in *T. douronensis* and *T. tambroides*, respectively. The number of alleles per locus ranging from 2 to 5 and 2 to 7 in *T. douronensis* and *T. tambroides*, respectively. A significant deviation from Hardy-Weinberg equilibrium (HWE) was observed at three loci (Barb37, Barb59 and Barb62) in one or more populations in *T. tambroides* while two loci (Barb37 and Barb62) were deviated in *T. douronensis* population of Batang Ai. Bayesian cluster analysis performed with STRUCTURE showed that the most likely K value identified was $K = 2$ with no evidence of population substructuring, similar to those identified by the UPGMA dendrogram. The low genetic distances among populations were also supported by low interpopulation genetic differences (F_{ST}) among pairwise populations in both mahseer. Overall, the identified microsatellite loci exhibit promise for use in fine scale population structure analysis of *T. douronensis* and *T. tambroides* natural populations.

Keywords**:** Cross-species study, microsatellites, mahseer, population structure

INTRODUCTION

Borneo holds high chiropteran diversity. There are at The mahseer from the genus *Tor* Gray such as *Tor tambroides* and *T. douronensis*, are among the most valuable and highly priced cyprinid fish in Malaysia (Litis *et al*. 1997; Ng 2004). The market price for mahseer is considered high due to their delicious flesh and unique scales, and they seldom reach the urban market. For example, the price of *T. douronensis* can reach above RM 100/kg while *T. tambroides* reaches above RM400/ kg in the open market in Kapit, Sarawak. Thus, the genus *Tor* has great potential for freshwater aquaculture industry (Ingram *et al*. 2005). In addition, the *Tor* fishes are also recognized as an excellent game fish, and have high demand in the ornamental fish industry due to their attractive coloration (Ng 2004). Realising the economic importance of the two mahseer, limted distributions and population sizes, studies on the population structure and level of genetic variations are required for effective management and and conservation strategy of this important freshwater resource.

Microsatellites or simple sequence repeats (SSRs) are short tandem repeat motifs (1-6 bases) with high levels of allelic polymorphism and co-dominant inheritance (Jarne & Lagorda 1996; DeWoody & Avise 2000; Zane *et al*. 2002). They are present in both coding and noncoding regions and are usually characterized by a high degree of length polymorphism (Zane *et al*. 2002), useful for direct assessment of pattern and distribution of genetic variability at the intraspecific level (O'Connell & Wright 1997; Primmer *et al*. 2006). The flanking sequences of microsatellites within related taxa are found to be highly conserved (Scribner *et al*. 1996) including in fish (Rico *et al*. 1996), allowing for cross-amplification from species that diverged as long as 470 million years ago (Ma) (Zane *et al*. 2002). Thus, the potential of developing microsatellite markers through cross- is enhanced when primers designed for one species amplify homologous loci in other species (Zheng *et al*. 1995; Zardoya *et al*. 1996), hence eliminated the tedious

***Corresponding author**: *eyuzine@frst.unimas.my*

procedures to isolate microsatellite markers in a novel species. A few cross-species amplification studies had identified polymorphic microsatellite loci from other fishes useful for population genetic structure analysis of the genus *Tor*, for example the *Tor putitora* from three other cyprinids (Mohindra *et al*. 2004) and *Tor tambroides* from a catfish, *Mystus nemurus* (Keong *et al*. 2008).

The present study examines cross-species amplification of primers, developed for three cyprinids (*Cyprinus carpio*, *Barbus barbus* and *Barbonymus gonionotus*), in two indigenous mahseers, *T. douronensis* and *T. tambroides*. The objective was to identify polymorphic microsatellite loci and evaluate the suitability of the identified loci in population structure analysis of both mahseer in Malaysia.

MATERIALS & METHODS

Sample collections

The difficulties in obtaining the mahseer samples were due to their reduced numbers in most of the major rivers (Ng 2004) and their natural populations are currently confined only to the upper streams of rivers or protected areas such as national parks. *T. douronensis* samples chosen for the cross-species study were selected randomly from two locations in Sarawak; the Batang Ai River (N=37) and the Limbang River (N=15). The Batang Ai River was a tributary of the Batang Lupar River located in the southern part of Sarawak, while the Limbang River was located in northern part of Sarawak. Meanwhile, *T. tambroides* samples used in this study were obtained from three locations in Peninsular Malaysia; the Sia River, Pahang (N=17), the Kampung Esok River, Negeri Sembilan (N=20) and the Perak River, Perak (N=19). The Sia River, Pahang and the Kampung Esok River, Negeri Sembilan, both served as tributaries of the Pahang River that drained to the South China Sea while the Perak River flow west into the Straits of Malacca. The mahseer samples were morphologically identified by using the keys provided by Mohsin & Ambak (1983), Kottelat *et al*. (1993) and Inger & Chin (2002).

Cross-species amplification of microsatellite primers

Microsatellite primers from three freshwater cyprinids: *Cyprinus carpio* (Crooijmans *et al*. 1997), *Barbus barbus* (Chenuil *et al*. 1999) and *Barbonymus gonionotus* (McConnell *et al*. 2001; Kamonrat *et al*. 2002) were tested for amplification of homologous loci (Table 1). The initial cross-species amplification standardization including optimization of annealing temperature for each primer pair was carried out using eight random samples of *T. douronensis* and *T. tambroides* each. The primers yielding scoreable amplified products were further evaluated using larger sample sizes to assess their suitability in the population structure analysis of both mahseer.

The total DNA was extracted using the modified CTAB method (Grewe *et al*. 1993) in the presence of Proteinase K. Polymerase chain reaction (PCR) amplifications were performed in a final volume of 10 µl, containing 25–50 ng of genomic DNA, 1X PCR buffer (10 mM Tris-HCl, pH 9.0; 50 mM KCl; 0.01% gelatin), 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol of each primer and 1.5 units of *Taq* DNA polymerase. Amplification conditions were 94°C for 5 min followed by 25 cycles at 94°C for 30 s, T_a for 30 s and 72°C for 1 min, with a final extension of 72°C for 4 min. The optimum annealing temperature (T_a), were determined through experimental standardization for each primer pair.

After amplification, 10 µl of PCR products were electrophoresed on 4% high resolution MetaPhore agarose gels for 2 h at 78V/cm. The gels were stained using ethidium bromide (0.1µl/ml) and photographed under UV light using an Alpha Imager 2200. The alleles were designated according to PCR product size and calculated relative to a standard molecular marker (20 bp and 100 bp; Cambrex).

Statistical Analysis

Microsatellite genetic diversity was quantified as the number of alleles (A), the allelic richness A_R (the measure of the number of alleles per locus independent of the sample size), observed (H_o) and expected (H_e) heterozygosity values, and inbreeding coefficient (F_{IS} or f) as a measure of heterozygote deficiency or excess (Weir & Cockerham 1984) using FSTAT version 2.9.3.2 (Goudet 2001). GENEPOP version 3.3 (Raymond & Rousset 1995) was used to test genotypic distributions for conformance to Hardy–Weinberg expectations (HWE) and to test for genotypic disequilibria at each locus for each population using the Markov chain method (Guo & Thompson 1992). Genetic homogeneity tests of genotype frequency distribution

at each locus were determined through an exact G- test (Goudet *et al.* 1996 in order to test the null hypothesis of no genetic differentiation between populations, also using FSTAT. Sequential Bonferroni adjustments (Rice 1989) were applied to correct for the effect of multiple tests.

Genetic differentiation among populations was measured by the fixation index F_{ST}, calculated according to Weir & Cockerham (1984) using ARLEQUIN. Permutation tests (10,000 permutations) were performed in order to determine if estimates differed significantly from zero. The genetic distance between populations (rivers) in both mahseer was calculated based on an unbiased measure following Nei (1978). An Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) dendrogram was constructed to illustrate the relations among geographic samples using POPGENE 1.32 (Yeh & Boyle 1997). Finally, a Bayesian approach was used to infer the number of clusters (*K*) in the data set without prior information of the sampling locations, available in STRUCTURE version 2.0 (Pritchard *et al*. 2000). A model where the allele frequencies were correlated within populations was assumed (λ was set at 1, the default value). The software was run with the option of admixture, allowing for some mixed ancestry within individuals, and α was allowed to vary. Five independent runs were done for each value of *K* (K= 1 to 5) with a burn–in period of 25,000 iterations and 25,000 replications.

RESULTS

Of the 26 heterologous primer pairs tested, only 10 (38%) and 12 (46%) primers produced successful amplification of homologous loci in *T. douronensis* and *T. tambroides*, respectively (Tables 1 and 2). In *T. douronensis*, four primers (Bgon13, Barb37, Barb62 and MFW7) were polymorphic exhibiting 2-5 alleles while the other six primers (Bgon22, Bgon69, Bgon75, MFW1, MFW5 and MFW11) were monomorphic when tested in all 52 individuals. In *T. tambroides*, five primers (Bgon13, Barb37, Barb59, Barb62 and MFW7) were polymorphic exhibiting 2-7 alleles while primers Bgon22, Bgon69, Bgon75, MFW1, MFW5, MFW11 and MFW17 produced a monomorphic band in all the 56 individuals tested. The characteristics of the polymorphic primers of both mahseer are summarised in Tables 2 and 3.

The observed heterozygosity (H_o) values over all loci in *T. tambroides* ranged from 0.0000 (locus MFW7 of N. Sembilan) to 0.8421 (locus Barb59 of Perak) (Table 4). In *T. douronensis*, the H_o values ranged from 0.0606 (Locus MFW7 of Batang Ai) to 0.3939 (locus Barb62 of Batang Ai) (Table 5). Test of linkage disequilibrium between loci found no significant disequilibrium among pairwise comparisons (data not shown).

Pairwise estimates of F_{ST} over all loci between samples in both species are presented in Table 6. Within *T. tambroides*, only one (between the Pahang and the Perak populations) pairwise estimate of F_{ST} showed significant genetic differentiation ($p< 0.05$) while pairwise estimates of F_{ST} showed no significant differentiation between *T. douronensis* populations from Batang Ai and Ulu Limbang. Pairwise estimates of genetic distances computed by Nei (1978) among populations (Table 6) showed that the highest genetic distance was between *T. tambroides* population from Pahang and *T. douronensis* population from Ulu Limbang (0.4187).

Table 1. Primers of microsatellite loci tested for cross-species amplification in *T. douronensis* and *T. tambroides*

Source species	Number of primer pairs tested	Locus	Successful amplification (n(%))	
			T. douronensis	*T. tambroides*
Barbonymus gonionotus	10	Bgon2, 8, 12, 13, 17, 19, 22, 69, 75, 79	4 (40)	4(40)
Barbus barbus	4	Barb37, 54, 59, 62	2 (50)	3(75)
Cyprinus carpio	12	MFW1, 2, 5, 7, 11, 15, 17, 18, 19, 24, 26, 28	4 (33)	5(42)
Total tested	26		10 (38)	12(46)

Table 2. List of amplified microsatellite primers with their sequence, repeat motif, annealing temperature (T_a) and references

Locus	Primer sequence (5' to 3')	Repeat motif	T_a (ºC)	Reference
Bgon13	F: CCCGTGCAATTCAATATG R: TAAGTAGCACAGATGTGAGG	GT	53	McConnell *et al*., 2001, Kamonrat *et al*., 2002
Bgon22	F: TCTTGTTGATCACACGGACG R: GTGACTGTATCAATGAGTCTG	TCC	49	McConnell *et al*., 2001, Kamonrat *et al*., 2002
Bgon69	F: GCAAAGGTTCTGTCAAGG R:GTATCCAGAAACATGTTCAG	TG	49	McConnell *et al*., 2001, Kamonrat *et al*., 2002
Bgon75	F: CTGGTAAAGACTTCAGATGC R: GCATGCAAAATGAGAAAGGCT	AC	53	McConnell *et al*., 2001, Kamonrat *et al*., 2002
Barb37	F: AAATACGCTCTCCTCATTAC R: TACAAAAGCAAAAATAAATTA	ATTT	50	Chenuil *et al*., 1999
Barb59	F: CTGTATCCATCACATAGGCT R: CATGATTTAATAGAACACACAC	GATA	56	Chenuil *et al*., 1999
Barb62	F: GGCACAAAAATGGATTCATATC R: GTACACGAGCATATGGACAA	ATTT	58	Chenuil *et al*., 1999
MFW1	F: GTCCAGATCGTTCATCAGGAG R: GAGGTGTACACTGAGTCACGC	CA	55	Crooijmans *et al*., 1997
MFW5	F: GAGATGCCTGGGGAAGTCAC R: AAAGAGAGCGGGGTAAAGGAG	CA	55	Crooijmans *et al*., 1997
MFW7	F: TACTTTGCTCAGGACGGATGC R: ATCACCTGCACATGGCCACTG	CA	55	Crooijmans *et al*., 1997
MFW11	F: GCATTTGCCTTGATGGTTGTG R: TCGTCTGGTTTAGAGTGCTGC	CA	55	Crooijmans *et al*., 1997
MFW17	F: CAACTACAGAGAAATTTCATG R: GAAATGGTACATGACCTCAAG	CA	55	Crooijmans *et al*., 1997

Bayesian cluster analysis performed with STRUCTURE showed that the most likely K value identified was $K = 2$, and results from other K values ($K= 3$ to $K= 5$) did not identify any formation of additional clusters (Fig. 1). The two identified cluster correspond to the two mahseer studied; Cluster 1 consisted of the three *T. tambroides* populations while Cluster 2 consisted of the two *T. douronensis* populations. No evidence of population substructuring was found in either species based on the STRUCTURE analysis. The UPGMA dendrogram also generated two clusters corresponding to the two species studied (Figure 2), similar to those identified by structure.

DISCUSSION

The results of this study showed the potential of finding polymorphic microsatellites loci through a rapid non-cloning method. Although only a small proportion of polymorphic loci 10% (four out of 26) in *T. douronensis* and 12% (five out of 26 loci) in *T. tambroides*) were found, the genetic diversity parameters were comparable to the results found in other cross-species amplification studies of mahseer

This includes a study by Keong *et al*. (2008) on *T. tambroides* using five polymorphic microsatellites developed from a catfish (*Mystus nemurus*) in which the number of allele per locus ranged from1 to 7 and observed heterozygosities ranged from 0.2400 to 1.0000). Another study by Mohindra *et al*. (2004) in *Tor putitora* used seven (22%) polymorphic (out of a total of 32 primers tested) microsatellites developed from *Catla catla*, *C. carpio* and *B. barbus* in which the number of alleles per locus ranged from 1 to 10 and the corresponding observed heterozygosities ranged from 0.0000 to 0.9000

However, the current genetic diversity results

were lower than those found by Nguyen *et al*. (2007) in microsatellites developed for *T. tambroides* (number of alleles per locus ranged from 1 to 9 and observed heterozygosities ranged from 0.0390 to 0.7510) and subsequent cross-species study in *T. douronensis* by Nguyen (2008) (number of alleles per locus ranged from five to 21 and observed heterozygosities ranged from 0.0400 to 0.7850). Nevertheless, the results of this study supported the hypothesis that certain sequences flanking the microsatellite regions of the genome might be conserved among the cyprinids (Zane *et al*. 2002), thus potentially allowing primers to be used interspecifically among cyprinids (Yue & Orban 2002; Lal *et al*. 2004; Mohindra *et al*. 2004; Nguyen 2008).

The results of this study also showed that the optimum annealing temperature (T_a ºC) observed in both mahseer differed from that reported in the source species for the respective primer pair, except in Barb37 (Table 2) similar to the findings by Mohindra *et al*. (2004). The fact that eight out of 12 (67%) of the successfully amplified primers in this study exhibited annealing temperature lower that those found in the source species supported the general assumption that cross-species amplification tends to have lower annealing temperature as compared with the species where the primer(s) were originally developed (Zane *et al*. 2002).

Two and three out of the five polymorphic loci were not in Hardy-Weinberg equilibrium (HWE) in *T. douronensis* and *T. tambroides* in one or more population, respectively. Departure from HWE may result from one or more of the followings reasons: (i) Sampling error because only a small sample size was studied, thus did not have a true representation of the population allele frequencies (Mohindra *et al*. 2004). (ii) Wahlund effect, i.e. presence of fewer heterozygotes in a population than predicted on account of a population subdivision (Kumar *et al*. 2006). (iii) presence of null alleles as suggested by excess of homozygotes for most allele size classes (Nguyen 2008) and (iv) Reduction in the effective breeding population size in *T. tambroides* as a result of overexploitation and/or anthropogenic disturbances (i.e. river pollution, deforestation, watershed erosion etc).

The microsatellite analysis done in this study showed low levels of genetic differentiation among the three *T. tambroides* populations of Peninsular Malaysia (N. Sembilan, Pahang and Perak) similar to the results found using mitochondrial *cytochrome c oxidase* I sequences (Esa *et al*. 2008). In addition, the two *T. douronensis* populations (Batang Ai and Ulu Limbang) separated by high geographical distance (around 800 km) also showed very low genetic substructuring between them and was not concordant with the high population structuring results found by Nguyen (2008). Thus, the inclusion of more polymorphic microsatellite loci and of sample sizes in each population might provide a better resolution of the population genetic structure of the two mahseer species.

Table 3. Characteristics of amplified microsatellite loci in details of *Tor douronensis* and *T. tambroides*

Resource species	Locus	*T. douronensis*		*T. tambroides*	
		T_a(ºC)	No of alleles	T_a(ºC)	No of alleles
Barbonymus gonionotus	Bgon13	50	2	50	2
	Bgon22	50	2	50	2
	Bgon69	46	1	46	1
	Bgon75	46	1	46	2
Barbus barbus	Barb37	50	3	50	3
	Barb59	-	-	50	7
	Barb62	50	5	50	4
Cyprinus carpio	MFW1	50	1	50	1
	MFW5	65	1	65	1
	MFW7	65	2	65	2
	MFW11	50	1	50	1
	MFW17	-	-	50	1

Table 4. Parameters of genetic variability for polymorphic microsatellite locus in *T. tambroides* samples from three rivers. Given are number of alleles (A), allelic richness (A_R), inbreeding coefficient (F_{IS}), observed (H_o) and expected (H_e) heterozygosity values, the probability of Hardy-Weinberg equilibrium (HWE) and the probability of genotype homogeneity between samples

Locus	Population	A	A_R	H_o	H_e	F_{IS}	HWE	Genotype
MFW7	Pahang	2	2.0000	0.3529	0.2907	-0.2143	0.4256	0.0100*
	N. Sembilan	1	1.0000	0.0000	0.0000	0.0000	-	
	Perak	2	2.0000	0.3158	0.2659	-0.1875	0.4606	
Barb37	Pahang	3	3.0000	0.2353	0.4792	0.5090	0.0011**	0.3620
	N. Sembilan	3	2.8000	0.5500	0.4712	-0.1671	0.4371	
	Perak	3	3.0000	0.2632	0.4806	0.4525	0.0000*	
Barb59	Pahang	7	7.0000	1.0000	0.6816	-0.4670	0.0009*	0.0000**
	N. Sembilan	4	3.7940	0.5000	0.5775	0.1342	0.0278*	
	Perak	7	6.526	0.8421	0.7604	-0.1705	0.0003*	
Barb62	Pahang	3	3.0000	0.5294	0.4862	0.1530	0.0001*	0.0000**
	N. Sembilan	4	3.6000	0.1500	0.3362	0.5539	0.0134*	
	Perak	3	2.9790	0.5263	0.4598	-0.1446	0.0000**	
Bgon13	Pahang	2	1.9980	0.4235	0.1107	-0.0625	0.8575	0.8100
	N. Sembilan	2	1.9640	0.1000	0.0950	-0.0526	0.8694	
	Perak	2	1.9980	0.1579	0.1454	-0.0857	0.7632	

(*P*-value)[1] **$P<0.001$ and *$P<0.01$
(*P*-value)[2] **$P<0.001$

Table 5. Parameters of genetic variability for polymorphic microsatellite locus in *T. douronensis* samples from three rivers. Given are number of alleles (A), allelic richness (A_R), inbreeding coefficient (F_{IS}), observed (H_o) and expected (H_e) heterozygosity values, the probability of Hardy-Weinberg equilibrium (HWE) and the probability of genotype homogeneity between samples

Locus	Population	A	A_R	H_o	H_e	F_{IS}	HWE (*p*-value)[1]	Genotype homogeneity (*p*-value)[2]
MFW7	Batang Ai	2	1.6360	0.0606	0.0588	-0.0313	0.8997	0.5850
	Ulu Limbang	2	2.0000	0.0769	0.0740	-0.0400	1.0000	
Barb37	Batang Ai	3	2.9790	0.2424	0.4155	0.4166	0.0000**	0.0180*
	Ulu Limbang	2	2.0000	0.0769	0.0740	-0.0400	1.0000	
Barb62	Batang Ai	4	3.3810	0.3939	0.6028	0.3465	0.0012**	0.1980
	Ulu Limbang	3	3.0000	0.3846	0.5178	0.2571	0.5153	
Bgon13	Batang Ai	2	1.9260	0.1515	0.1400	-0.0820	0.6758	0.3640
	Ulu Limbang	2	2.0000	0.0769	0.0740	-0.0400	1.0000	

(*P*-value)[1] **$P<0.001$ and *$P<0.01$
(*P*-value)[2] **$P<0.001$

Table 6. Estimates of pairwise genetic distances (Nei 1978; below diagonal) and F_{ST} (Weir & Cockerham 1984; upper diagonal) among populations of *T. tambroides* and *T. douronensis*

		T. tambroides			*T. douronensis*	
		Pahang	N. Sembilan	Perak	Batang Ai	Ulu Limbang
T. tambroides	Pahang	-	0.0409	0.0499*	0.3170*	0.4187*
	N. Sembilan	0.0134	-	0.0000	0.2238*	0.2724*
	Perak	0.0181	0.0000	-	0.2163*	0.2581*
T. douronensis	Batang Ai	0.1952	0.1490	0.1412	-	0.0361
	Ulu Limbang	0.2201	0.1526	0.1416	0.0128	-

*P-value, significant level ($P< 0.05$)

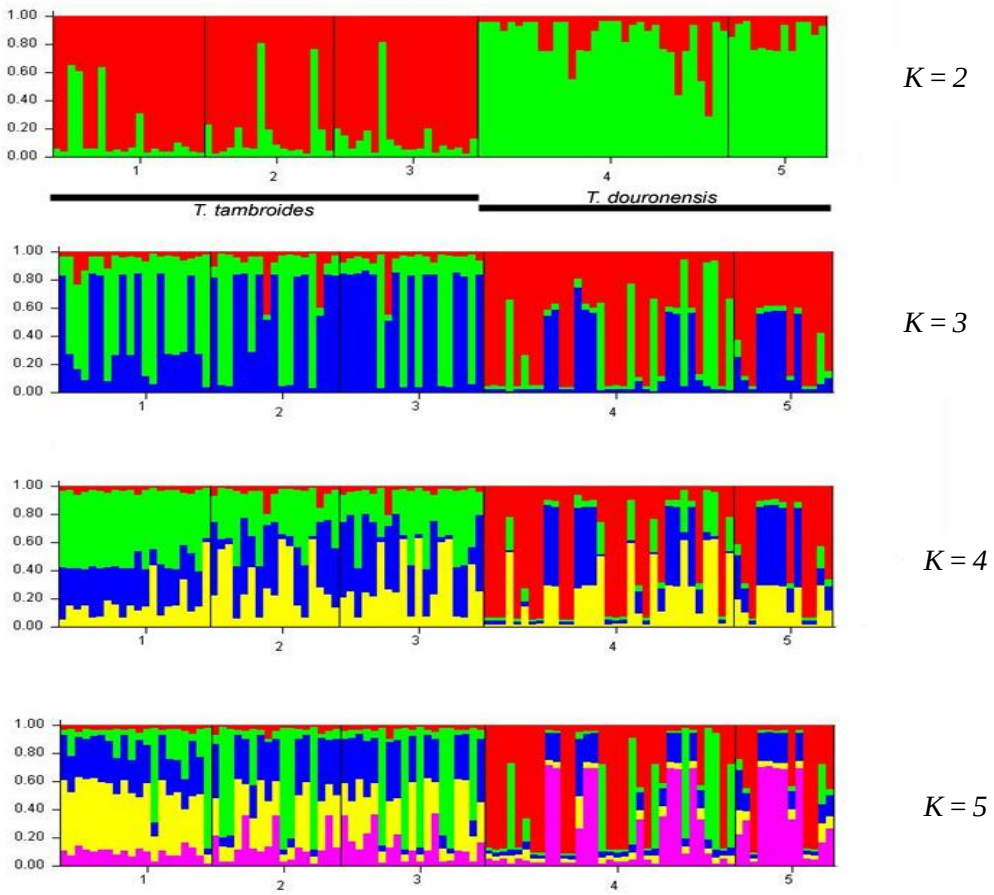

Figure 1. Proportional membership (Q) of each individual of *T. tambroides* and *T. douronensis* identified by STRUCTURE at K=2 to K=5. The numbers in the X-axis correspond to a specific population: 1-Negeri Sembilan, 2-Pahang, 3-Perak, 4-Batang Ai, 5-Ulu Limbang..

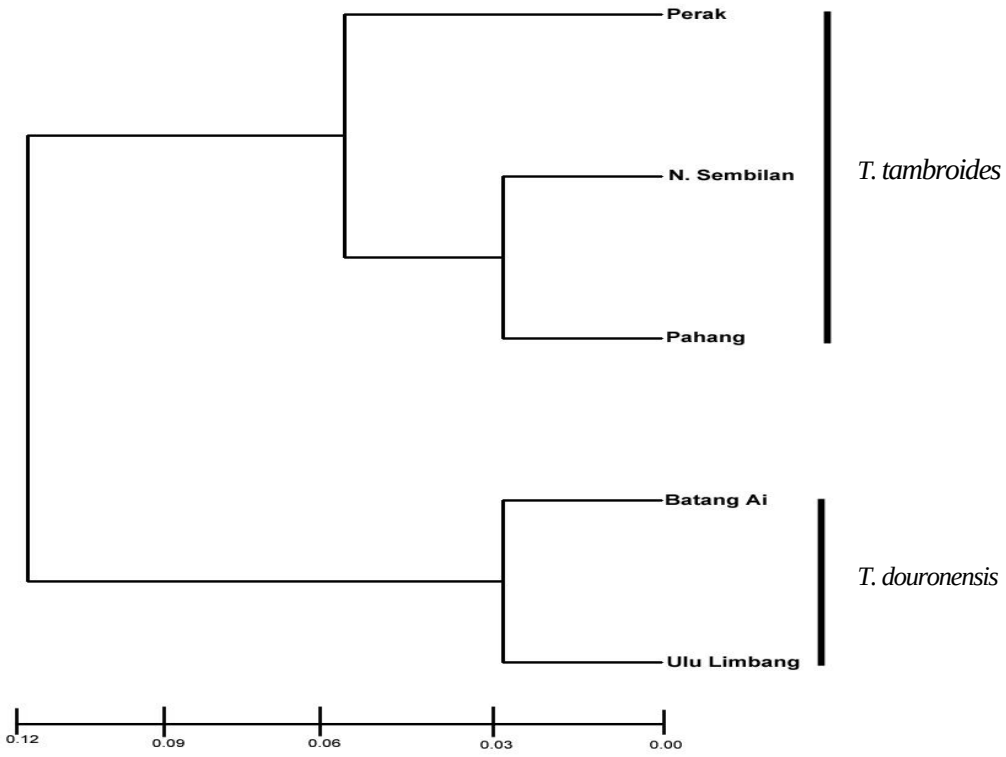

Figure 2. UPGMA dendrogram showing the genetic relationships among populations of *T. tambroides* and *T. douronensis.*

Overall, the cross-species amplification study identifies five polymorphic microsatellite loci with considerable genetic variation potentially useful in the fine scale population structure analysis of *T. douronensis* and *T. tambroides* natural populations.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Stephen Sungan and staffs of the IFPRC, Tarat, Sarawak and the Fisheries Department of Malaysia for their assistance. This project was funded by the National Biotechnology Directorate (MOSTI) grant no: 01-02-04-008/BTK/ER/33.

REFERENCES

Chenuil, A., Galtier, N., & Berrebi, P. (1999). A test of the hypothesis of an autopolyploid vs allopolyploid origin of a tetraploid lineage. Application to the genus *Barbus* (Cyprinidae). *Heredity*, 82: 373-380.

Crooijmans, R.P.M.A., Bierbooms, V.A.F., Komer, J., Van der Poel, J.J., & Groenen, M.E.M. (1997). Microsatellite markers in common carp (*Cyprinus carpio*). *Animal Genetics*, 28: 129-134.

DeWoody, J.A., & Avise, J.C. (2000). Microsatellite variation in marine, freshwater and anadromous fishes compared with other animals. *Journal of Fish Biology*, 56: 461-473.

Esa, Y.B., Siraj, S.S., Daud, S.K., Khairul Adha, A.R., Ryan, J.R.J. & Tan, S.G. (2008). Mitochondrial DNA diversity of *Tor tambroides* Valenciennes (Cyprinidae) from five natural populations in Malaysia. *Zoological Studies*, 47(3): 360-367.

Goudet, J. (2001). FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3) http://www.unil.ch/izea/softwares/fstat.html.

Goudet, J., Raymond, M., Demeeus, T. & Rousset, F. (1996). Testing differentiation in diploid populations. *Genetics*, 144: 1933-1940.

Grewe, P.M., Krueger, C.C., Aquadro, C.F., Bermingham, E., Kincaid, H.L., & May, B. (1993). Mitochondrial variation among lake trout (*Salvenilus namaycush*) strains stocked into Lake Ontario. *Canadian Journal of Fisheries and Aquatic Science*, 50: 2397-2403.

Guo, S.W., & Thompson, E.A. (1992). Performing the exact test of Hardy-Weinberg proportions for multiple alleles. *Biometrics*, 48: 361-372.

Inger, F.R., & Chin, P.K. (2002). *The Freshwater Fishes of North Borneo*. (Revised edition with supplementary chapter by PK Chin). Kota Kinabalu: Natural History Publications (Borneo).

Ingram, B.A., Sungan, S., Gooley, G.J., Sim, Y.S., Tinggi, D., & De Silva, S.S. (2005). Induced spawning, larval development and rearing of two indigenous Malaysian Mahseer, *Tor tambroides* and *Tor douronensis*. *Aquatic Research*, 36: 1001-1014.

Jarne, P., & Lagoda, P.J. (1996), Microsatellites, from molecules to populations and back. *Trends in Ecology and Evolution*, 11(10): 424-429.

Kamonrat, W., McConnell, S.K.J., & Cook, D.I. (2002). Polymorphic microsatellite loci from the Southeast Asian cyprinid, *Barbodes gonionotus* (Bleeker). *Molecular Ecology Notes*, 2: 89-90.

Keong, R.B.P., Siraj, S.S., & Daud, S.K. (2008). Cross species amplification of ikan kelah, *Tor tambroides* by using *Mystus nemurus* microsatellite markers. *Pertanika Journal of Tropical Agricultural Science*, 31(2): 179-189.

Kottelat, M., Whitten, A.J., Kartikasari, S.N., & Wirjoatmodjo, S. (1993). *Freshwater fishes of Western Indonesia and Sulawesi*. Singapore: Periplus Edition.

Kumar, S., Gupta, J., Kumar, N., Dikshit, K., Navani, N., Jain, P., & Nagarajan, M. (2006). Genetic variation and relationships among eight Indian riverine buffalo breed. *Molecular Ecology*, 15: 593-600.

Lal, K.K., Chauhan, T., Mandal, A., Singh, R.K., Khulbe, L., Ponniah, A.G., & Mohindra, V. (2004). Identification of microsatellite DNA markers for population structure analysis in Indian major carp, *Cirrhinus mrigala* (Hamilton-Buchanan, 1882). *Journal of Applied Ichthyology*, 20: 87–97.

Litis, B.A., Sungan, S., Jugang, K., Ibrahim, M., & Bin, H.A. (1997). *Features of indigenous fish species having potential for aquaculture*. Kuching: Inland Fisheries Division, Department of Agriculture, Sarawak.

McConnell, S.K.J., Skibinski, D.O.F., & Mair, G.C. (2001). Microsatelite markers from a commercially important South-east Asian cyprinid, the silver barb (*Barbodes gonionotus* Bleeker). *Molecular Ecology Notes*, 1: 135-136.

Mohindra, V., Ranjuna., Khulbe, L., Ponnial, A.G., & Lal, K.K. (2004). Microsatellite loci to access genetic variations in *Tor putitora*. *Journal of Applied Icthtyology*, 20: 466-469.

Mohsin, A.K.M., & Ambak, M.A. (1983). *Freshwater fishes of Peninsular Malaysia*. Serdang: Universiti Pertanian Malaysia Press, 744 pp.

Nei, M. (1978). Estimation of heterozygosity and genetic distance from a small number of individuals. *Genetics*, 89: 583-590.

Ng, C.K. (2004). *Kings of the rivers: Mahseer in Malaysia and the region*. Kuala Lumpur: Inter Sea Fishery (M).

Nguyen, T., Baranski, M., Rourke, M., & McPartlan, H. (2006). Characterization of microsatellite DNA markers for a mahseer species, *Tor tambroides* (Cyprinidae) and cross-species amplification in four congeners. *Molecular Ecology Notes*, 7: 109-112.

Nguyen, T.T.T. (2008). Population structure in the highly fragmented range of *Tor douronensis* (Cyprinidae) in Sarawak, Malaysia revealed by microsatellite markers. *Freshwater Biology*, 53: 924-934.

O'Connell, M., & Wright, J.M. (1997). Microsatellite DNA in fish. *Reviews in Fish Biology*, 7: 331–363.

Primmer, C.R., Veselov, A.J., Zubchenko, A., Poututkin, A., Bakhmet, I., & Koskinen, M.T. (2006). Isolation by distance within a river system: genetic population structuring of Atlantic salmon, *Salmo salar*, in tributaries of the Varzuga River in northwest Russia. *Molecular Ecology*, 15: 653-666.

Pritchard, J.K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, 155: 945-959.

Raymond, M., & Rousset, F. (1995). GENEPOP: population genetic software for exact test ecumenicism. *Journal of Heredity*, 86: 248-249.

Rice, W.R. (1989). Analysing tables of statistical tests. *Evolution*, 43: 223-225.

Rico, C., Rico, I., & Hewitt, G. (1996). 470 million years of conservation evolution-evidence for directionality and variation in rate between species. *Nature Genetics*, 10: 337-343.

Scribner, K.T., Gust, J.R., & Field, R.L. (1996). Isolation and characterisation of novel salmon microsatellite loci: cross species amplification and population genetics application. *Canadian Journal of Fisheries and Aquatic Science*, 53: 833–841.

Weir, B.S., & Cockerham, C.C. (1984). Estimating F-statistics from the analysis of population structure. *Evolution*, 38: 1358-1370.

Yeh, F.C., & Boyle, T.H.B. (1997). Population genetic analysis of codominant and dominant markers and quantitative traits. *Journal of Botany*, 129: 157.

Yue, G.H., & Orban, L. (2002). Polymorphic microsatellites from silver crucian carp (*Carassiusauratus gibelio* Bloch) and cross amplification in common carp (*Cyprinus carpio* L.). *Molecular Ecology Notes*, 2:534-536.

Zane, L., Bargelloni, L., & Patarnello, T. (2002). Strategies for microsatellite isolation: a review. *Molecular Ecology*, 11: 1-16.

Zardoya, R., Vollmer, D.M., Craddock, C., Streetman, J.T., Karl, S., & Meyer, A. (1996). Evolutionary conservation of microsatellite flanking regions and their use in resolving the phylogeny of cichlid fishes (Pisces: Perciformes). *Proceedings of the Royal Society of London B*, 263: 1589–1598.

Zheng, W., Stacey, N.E., Coffin, J., & Strobeck, C. (1995). Isolation and characterization of microsatellite loci in goldfish *Carassius auratus*. *Molecular Ecology*, 4: 791–792.