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GENETIC CHARACTERIZATION OF THE TWO COLOUR-TYPE OF KELAH

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ABSTRACT

Randomly Amplified Polymorphic DNA (RAPD) marker was used to examine the genetic relationships among three populations of two different colour-types (silver-bronze and reddish) of ikan kelah (*Tor tambroides*). Sixty three individuals of the kelah were sampled from Sia River of Pahang and Kampung Esok River of Negeri Sembilan (silver-bronze) and Nenggiri River of Kelantan (reddish). Twelve RAPD primers generated a total of 226 scorable loci with 100% polymorphism across the sixty-three individuals. The RAPD banding patterns and sizes ranged from 4 to 17 and from 100bp to 1500bp, respectively. The intra-population UPGMA dendrogram produced two major clusters, with the Nenggiri River (Kelantan) samples formed a subcluster in both major clusters dominated by the Pahang samples (Cluster 1) and N. Sembilan (Cluster 2) samples, respectively. The inter-population UPGMA showed that the Kelantan samples were genetically closer to the N. Sembilan samples than to the Pahang samples. Thus, the results of this study did not support the hypothesis that the two colour-types of *T. tambroides* were genetically distinct from each other.

ABSTRAK

Penanda 'Randomly Amplified Polymorphic DNA (RAPD)' telah digunakan untuk melihat hubungan genetik di kalangan tiga populasi ikan kelah (*Tor tambroides)* yang mempunyai dua jenis warna berbeza (perak-gangsa dan kemerahan). Enam puluh tiga individu kelah telah disampel dari Sungai Sia, Pahang dan Sungai Kampung Esok, Negeri Sembilan (jenis warna perak-gangsa) dan Sungai Nenggiri, Kelantan (jenis warna kemerahan). Dua belas "primer" RAPD yang telah dipilih menghasilkan sejumlah 226 lokus yang 100% polimorfik daripada enam puluh tiga individu tersebut. Corak dan saiz lokus RAPD yang terhasil daripada ketiga-tiga populasi berjulat daripada 4 hingga 17 lokus, dan daripada 100bp hingga 1500bp, masing-masing. Keputusan dendrogram UPGMA intra-populasi menghasilkan dua kelompok utama; sampel-sampel dari Sungai Nenggiri (Kelantan) membentuk subkelompok di dalam kedua-dua kelompok utama yang didominasi oleh masing-masing sampel-sampel dari Pahang (Kelompok 1) dan N. Sembilan (Kelompok 2). Keputusan dendrogram UPGMA untuk inter-populasi menunjukkan sampel-sampel dari Kelantan adalah lebih dekat secara genetik dengan sampel-sampel dari N. Sembilan berbanding sampel-sampel dari Pahang. Justeru, keputusan daripada kajian ini tidak menyokong hipotesis bahawa kedua-dua jenis warna *T. tambroides* adalah berbeza secara genetik di antara satu sama lain.

Key words: Freshwater fish, *Tor tambroides*, colour-type, RAPD, genetic marker

INTRODUCTION

Tor tambroides or locally known as kelah or mahseer, belongs to the family Cyprinidae (Kottelat *et al*., 1993, Rainboth, 1996, Ng, 2004). It is one of the most sought-after of the local freshwater fishes, both for food as well as a highly priced sport fish (Ng, 2004). The species can be found in the upper reaches of the major rivers of the Peninsular Malaysia that drained both into the South China Sea (the Pahang and the Kelantan Rivers) and the Strait of Mallacca (the Perak and the Muda Rivers).

The taxonomic status of fishes of the genus *Tor* is still unclear (Rainboth, 1996; Ng, 2004; Esa *et al*., 2006; Nguyen *et al*., 2006). Several authors suggested that *T. tambroides* of the Peninsular Malaysia could be recognized into two different colour-type (silver-bronze and reddish) based on its colouration (Mohsin and Ambak, 1983; Eddy, 1997; Chang, 2000; Ng, 2004). However, the variation in colour could probably resulted from

environmental influences (such as local water conditions, type of diet consumed etc) as observed in many other freshwater fish species (Kottelat *et al*., 1993; Vrijenhoek, 1998).

Presently, there are very few molecular studies on the taxonomy and phylogenetic relationships of *Tor* fishes in Malaysia (Esa *et al*., 2006; Nguyen *et al.*, 2006). Molecular markers such as Random Amplified Polymorphic DNA (RAPD) have regularly been employed to examine relationships at the intra and inter-populations of various organisms (Hadrys *et al.,* 1992) because it has been shown to have a high power of resolution, especially in detecting cryptic pairs of species and in confirming close relationships between species (Lehmann *et al*. 2000; Bartish *et al*. 2000, Kumar *et al*., 2003). Other studies using RAPD such as on the *Prochilodus marggravii* (Terumi *et al*., 2002) and *Silurus asotus* (Jong and Gye, 2001) have shown the usefulness of this molecular analysis in the genetic studies of freshwater fishes. Thus, RAPD could serve as a useful marker to investigate the genetic relationships among various colour-types of *T. tambroides*, hence provide insight into the validity of their current taxonomic status.

Therefore, this study employed RAPD technique to test the hypothesis that the two colour-types of *T. tambroides* (the silver-bronze and the reddish) are genetically distinct from each other, thus could represent a different taxon (species or subspecies).

MATERIALS AND METHODS

Sample collection

The silver-bronze *T. tambroides* samples were obtained from the Sia River, Pahang and the Kampung Esok River, Negeri Sembilan, where both of them served as tributaries of the Pahang River (Table 1). The reddish type was obtained from the Nenggiri River, a tributary of the Kelantan River. Samples were taken from the upper reaches of streams by rod and hook. The number of samples were taken as maximum as possible due to difficulties in catching and restriction in accessing the sampling areas. The samples were stored at -20ºC freezer until used for DNA analysis.

DNA extraction and RAPD procedures

DNA was extracted from caudal fins using a modified method by Taggart *et al.,* (1992), in the presence of proteinase-K. The pelleted DNA was redissolved in 100 µl sterilized distilled water. Quality and approximate yield was determined by electrophoresis in a 0.8% agarose gel containing ethidium bromide at 80 V for 45 min. The isolated genomic DNA was used for RAPD-PCR analysis.

A total of twenty primers from RAPD kit A (Operon) with more than 60% of GC content were screened and twelve primers (OPA1, OPA2, OPA3, OPA4, OPA6, OPA7, OPA8, OPA10, OPA11, OPA13, OPA 14 and OPA16) were selected for this study due to their reproducibility and consistency of banding patterns observed during amplification (Table 2).

The Polymerase Chain Reaction (PCR) reactions were carried out in 10µl reaction volumes based on primer list with modifications containing 2mM $MgCl_2$, 10X buffer (Promega), 400µM of each dNTP, 0.5µM of primer, 30ng of DNA samples, appropriate amount of ddH_20 and 3 units of *Taq* polymerase (Promega). The amplifications were carried out in a Programmable Thermal Cycler (PTC-200) with an initial predenaturing step of 3 minutes at 95ºC, denaturation step of 20 seconds at 94ºC, annealing step of 20 seconds at 30ºC, extension step of 25 seconds at 72ºC followed by 39 repeat cycles of the previous steps, and a final extension of 5 minutes at 72ºC. The final step was held at 4ºC. The PCR products were loaded onto agarose gel containing 2.0% agarose, 1X TBE buffer, 0.1µl/ml ethidium bromide, together with standard DNA ladders (100bp ladder). Gels were electrophoresed in 1X TBE buffer for 2 hours at 60 V/cm and photographed under UV light using Alpha Imager 2200.

Table 1. Geographical region, location and sample size of kelah used in the study

Geographical Region	Location	Sample Size (n)	Range of Weight (g)	Range of Total Length (cm)	Range of Standard Length (cm)
Central Part of Peninsular Malaysia	Pahang (Sungai Sia)	26	35.8–148.2	10.9–18.9	8.8–13.8
Western Part of Peninsular Malaysia	Negeri Sembilan (Sungai Kampung Esok)	26	3.1–5.7	5.5–8.8	4.2–7.0
Eastern Part of Peninsular Malaysia	Kelantan (Sungai Nenggiri)	11	25.0–1800	14.5–50.7	11.0–40.0

Table 2. Primer codes, sequences and annealing temperature of the 12 RAPD primers used in this study

Primer Codes	Primer Sequence 5' To 3'	Annealing Temperature (Based on Primer List)	Optimized Annealing Temperature
OPA01	5' CAG GCC CTT C 3'	34	32
OPA02	5' TGC CGA GCT G 3'	34	32
OPA03	5' AGT CAG CCA C 3'	32	34
OPA04	5' AAT CGG GCT G 3'	32	34
OPA06	5' GGT CCC TGA C 3'	34	34
OPA07	5' GAA ACG GGT G 3'	32	34
OPA08	5' GTG ACG TAG G 3'	32	34
OPA10	5' GTG ATC GCA G 3'	32	32
OPA11	5' CAA TCG CCG T 3'	32	34
OPA13	5' CAG CAC CCA C 3'	34	32
OPA14	5' TCT GTG CTG G 3'	32	36
OPA16	5' AGC CAG CGA A 3'	32	34

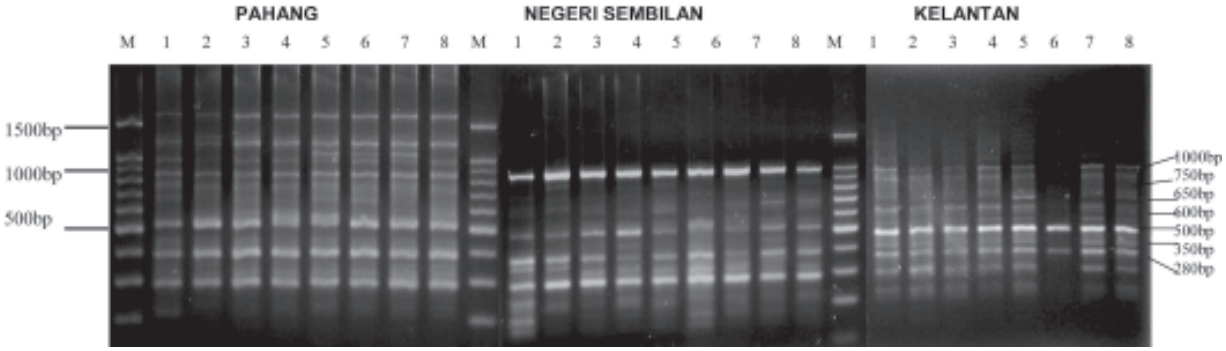

Fig. 1. The RAPD banding profile among the three populations of the two colour-types *T. tambroides*. Lane M: 100bp ladder. The RAPD banding profiles were generated from primer OPA10.

Data Interpretation and Analysis

Although a large number of fragments were generated from each primer, only clearly distinguishable and reproducible bands were selected for analysis. Bands were scored by counting the number of bands present or absent in each sample. All fragments were translated into a binary 0/1-matrix (0 for absence, 1 for presence of a specific DNA marker).

The genetic distances (Nei and Li's, 1979) between and within populations were calculated using the RAPD-distance package version 1.04 software (Armstrong *et al.,* 1998) followed by NTSYS-PC version 1.60 by Rohlf (1989) to construct a dendrogram based on the unweighted pair group method with arithmetic averaging (UPGMA; Sneath and Sokal, 1973) employing the SAHN (sequential, agglomerative, hierarchal and nested clustering) program.

RESULTS AND DISCUSSION

The twelve selected RAPD primers produced a total of 226 scorable bands (100% polymorphic) ranging from 100 base pairs (bp) to 1500bp across the three populations (Fig. 1). The complexity of the banding patterns varied among the primers. The highest number of amplified bands was observed in the Pahang samples followed by the Negeri Sembilan samples and lowest in the Kelantan samples (Table 3). The study did not find any RAPD primers producing specific allelic banding patterns interpreted as diagnostic bands, neither between the two varieties nor between the three populations of *T. tambroides*. Thus, the sharing of certain bands observed in the *T. tambroides* samples suggests that RAPD primers used in this study is of little use to discriminate between the two colour-types. The RAPD results

Table 3. The observed number of monomorphic, polymorphic and total RAPD bands found among the three populations of *T. tambroide*

	Location								
	Pahang			Negeri Sembilan			Kelantan		
Locus	M	P	T	M	P	T	M	P	T
OPA1	1	16	17	0	10	10	4	7	11
OPA2	1	12	13	0	9	9	0	9	9
OPA3	0	9	9	0	4	4	1	3	4
OPA4	0	13	13	0	11	11	2	11	13
OPA6	0	10	10	2	12	14	2	9	11
OPA7	0	13	13	2	5	7	1	8	9
OPA8	0	13	13	2	7	9	1	10	11
OPA10	2	9	11	0	16	16	4	7	11
OPA11	0	12	12	0	12	12	0	8	8
OPA13	0	13	13	0	12	12	2	5	7
OPA14	0	14	14	0	10	10	1	10	11
OPA16	1	10	11	1	12	13	3	8	11
Total	5	144	149	7	120	127	21	95	116
Total number of bands produced across the three populations : 226									
Total % Polymorphism : 100%									

M = monomorphic; P = polymorphic; T = total.

Table 4. A matrix of pair-wise genetic distance (Nei and Li, 1979) derived from similarity index (above diagonal) and distance (below diagonal) based on RAPD data

Populations	Pahang	Negeri Sembilan	Kelantan
Pahang	******	0.2395	0. 2357
Negeri Sembilan	0.7605	******	0.2452
Kelantan	0.7643	0.7548	******

also supported the finding based on mitochondrial DNA (mtDNA) sequences of *cytochrome c oxidase* I (COI) that the putative *T. tambroides* samples from Kelantan, Pahang and Negeri Sembilan formed a single monophyletic group, thus further reinforced their taxonomic status as belonging to a single species (Esa *et al.*, 2006).

The RAPD profiles of all *T. tambroides* samples were used to calculate the pair-wise genetic distance at the inter-population (between) and intra-population (within) levels. The highest inter-population genetic distances was found between the Pahang and Kelantan samples (0.7643), while the lowest genetic distances was between the Negeri Sembilan and Kelantan samples (0.7548) (Table 4). The intra-population genetic distances ranged from 0.25 to 0.9169.

The cluster analysis using the unweighted pair-group method with arithmetic averaging clustering (UPGMA) method for the inter-population study showed that the Negeri Sembilan and the Kelantan samples were closely related to each other than both of them with the Pahang samples (figure not shown). Meanwhile, the intra-populations UPGMA dendogram showed two major clusters; the first cluster consists of three samples of Kelantan (reddish variety) and twenty-six samples of Pahang (silver-bronze variety), while the second clustered consists of eight samples of Kelantan (reddish variety) and twenty-six samples of Negeri Sembilan (silver-bronze variety) (Fig. 2). However, all the Kelantan samples formed separate sub-clusters within the two major clusters dominated by the Pahang samples (Cluster 1) and the Negeri Sembilan samples (Cluster 2).

The high genetic differences observed among the three *T. tambroides* populations (rivers) suggests that the pattern of genetic differentiation/ subdivision of the species might be influenced by the biogeographical history of the region (known as Sundaland), particularly at the end of the Pleistocene glaciation period (estimated to be around 10,000 to 20,000 years ago) (Mohsin and Ambak, 1983). During the period, the sea level rose (presently known as the South China Sea) and Sundaland was submerged forming the Sunda shelf of no more than 100 meters in depth (Dodson *et al.*, 1995). As a result, a geographical barrier exists among the faunas particularly freshwater fishes of Peninsular Malaysia, Sumatra

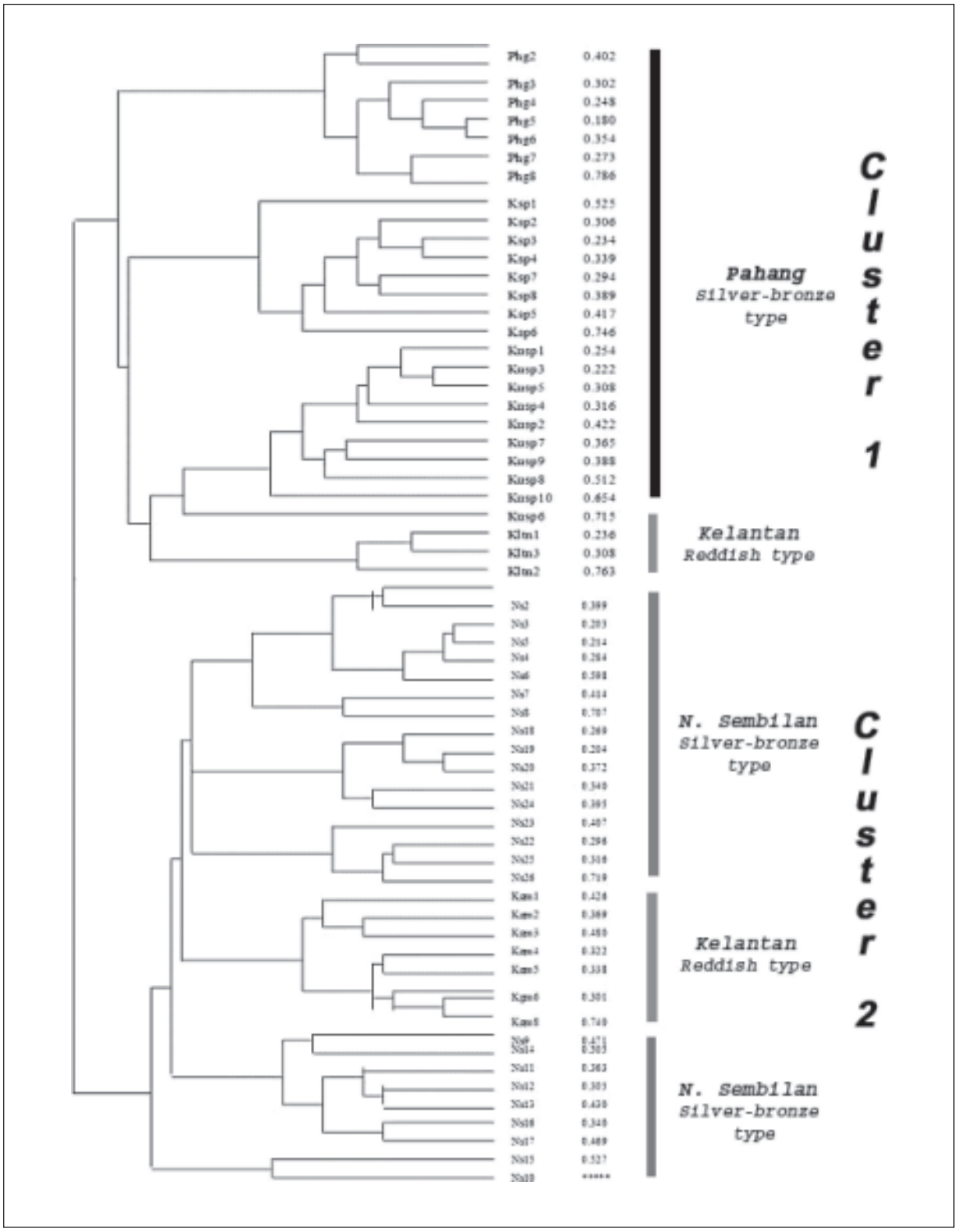

Fig. 2. Dendrogram of UPGMA of the two colour-types *T. tambroides* individuals from the three populations.

and western Borneo, which may have favoured the evolution of geographical races (Esa *et al.*, 2006).

Alternatively, the observed high genetic differences among the *T. tambroides* populations could result from more recent scenarios that had restricted gene flows between drainages (rivers and tributaries). This includes environmental degradation (i.e. river pollution, deforestration, watershed erosion) which has led to the rapid destruction of *Tor* natural habitat, and

uncontrolled fish harvest (overfishing) which had greatly reduced their population size (Esa *et al*., 2006; Nguyen *et al*., 2006).

The observed genetic distance values in *T. tambroides* are high compared to other studies such as in tilapia, *Oreochromis* sp (0.04 to 0.34; Bardakci and Skibinski, 1994); hilsa shad, *Tenualosa* populations (0.08 to 0.16; Dahle *et al.,* 1997) and discus, *Symphysodon* sp (0.07 to 0.18; Koh *et al.,* 1999). However, the value was theoretically expected as high genetic variability was common in wild compared to cultured populations (Vrijenhoek, 1998).

The current results based on the RAPD should be treated with great caution because of several factors. First, the statistical analysis could be affected by large sampling errors (such as the Wahlund effect), since a limited number of samples and populations were analysed. Thus, the RAPD profiles found in this study might not reflect the actual genetic variability of *T. tambroides* populations. Secondly, RAPD follows a dominant marker/pattern where a homozygote allele cannot be distinguished from a heterozygote allele, thus greatly underestimated the actual genetic variations of a particular population or species (Harris, 1999). In addition, it is unable to assign bands to specific loci unless a previous pedigree analysis is performed. In applying this method, it is assumed that populations are under the Hardy-Weinberg equilibrium, which polymorphic bands segregate in the Mendelian way, and that marker alleles from different loci do not co-migrate to the same position in the gel (D'Amato *et al*. 1996). Thus, the high degree of polymorphic bands (100%) and high genetic distances among the populations could simply result from technical errors during band scoring or gel interpretation (Jones *et al*., 1997).

Overall, the RAPD marker employed in this study managed to provide insights into the genetic relationships among the populations and between the two colour-types of *T. tambroides*, although it did not support the hypothesis that the two colour-types of *T. tambroides* (the silver-bronze of (N. Sembilan and Pahang) and the reddish of Kelantan are genetically different. Further studies using more powerful genetic markers such as microsatellites, bigger individuals, larger sample sizes and more populations will provide a better picture regarding the population structure and the taxonomic status of *T. tambroides*.

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