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# Cross Species Amplification of Ikan Kelah, *Tor tambroides* by Using *Mystus nemurus* Microsatellite Markers

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## ABSTRACT

Thirty eight microsatellite markers developed from a Random Hybridising Microsatellite (RAMs) enrichment protocol created from the DNA of the river catfish, ikan baung, *Mystus nemurus* were screened to cross amplify ikan kelah, *Tor tambroides*. Only five primers which amplified bands at the expected allele size regions were used for characterizing this species. The observed heterozygosity values were higher than the expected heterozygosity values for the bands amplified by primer pairs MnSC4-3B, MnLR2-1-52A and MnRmC3-1 across the three populations but the bands amplified by primer pairs MnSC4-1A and MnLR2-1-17B showed lower observed heterozygosity values than the expected heterozygosity values. The mean FIS value across the three populations was negative, indicating no deficit in heterozygosity. The mean value of FST was low indicating no gene was fixed within populations relative to the total population. The high value of Nm suggested high gene flow among the three populations. Both the ($x^2$) chi-square and the ($G^2$) likelihood ratio tests showed significant differences ($P<0.05$), indicating deviations from Hardy-Weinberg equilibrium in most loci except for one locus (MnSC4-1A) in the Negeri Sembilan population and two loci (MnLR2-1-52A and MnSC4-1A) in the Kelantan population. The genetic distance values generated ranged from 0.1053 to 0.1960. The UPGMA dendrograms constructed from the genetic distances based on the microsatellite markers showed that the Negeri Sembilan and Kelantan populations shared a similar cluster while the Pahang population was on its own.

**Keywords: *Tor tambroides*, DNA microsatellite, cross species amplification**

## INTRODUCTION

The Malaysian mahseer, *Tor tambroides* locally known as kelah, is one of the most sought-after local freshwater fishes, both for food as well as being a highly priced sport fish. Kelah is classified under the sub-family Cyprinidae and sub-tribe Tores. This sub-tribe includes fishes belonging to the genera *Neolissocheilus / Acrossocheilus hexagonolepis, Probarbus* and *Tor* (Rainboth, 1996). Although generally kelah only refers to the large scaled barbs of the sub-tribe i.e. *Neolissocheilus / Acrossocheilus*, it is also being referred to those belonging to the genus *Tor*. Differences in colour patterns and geographical locations have led to various vernacular names for this fish of probably the same species. In Pahang and Kelantan, the kelah, *Tor tambroides*, is also referred to as kelah padi, kecau, kelah emas. These names have been sometimes confused with tengas (*Neolissocheilus* or *Accrossocheilus hexagonolepis*), which inhabits the same habitats (Eddy, 1997). There is also the kelah merah, which probably refers to *Tor duoronensis*, but the species is difficult to locate according to the local tribes in Kelantan. The distribution of this fish includes clear and clean rivers in West and East Malaysia and spawn in the upper parts of the river system.

In fisheries, the use of biochemical and molecular genetic markers has increased over the past years particularly in identification of species or hybrids, and in population characterization. Later, with the discovery of various types of DNA level genetic markers such as Restriction Length Polymorphism (RFLP),

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Randomly Amplified Polymorphic DNA (RAPD) and microsatellites, they have become the favourite choice in aquaculture research (Kesher *et al.,* 1998).

Microsatellite DNA, or short sequence repeat (SSR), is a PCR-based and co-dominant DNA marker (Tautz, 1989). This highly polymorphic SSR has great potentials as genetic tag for use in aquaculture. It has been proven to be particularly valuable for parentage studies, stock discrimination, population genetics and genome mapping because of its high levels of polymorphisms (O'Connell and Wright, 1997). Presently, there are very few studies and documentation on the use of molecular markers for the classification of kelah. Molecular markers as a tool for facilitating confirmation of taxonomy, examination of genetic variation as well as for molecular breeding strategies have gained importance and popularity among fish geneticists, conservationists and taxonomists. Thus, the aim of this study was to investigate the genetic relationships among three populations of *Tor tambroides* using newly developed microsatellite markers of *M. nemurus*.

## MATERIALS AND METHODS

Kelah samples were collected from three locations in Malaysia. The samples were taken from the upper reaches of streams by rods and hooks. As many samples as possible were taken due to difficulties in catching and restrictions in accessing the sampling areas. The geographical regions, sampling sites and number of individuals taken together with the range of weight, total length and standard length are shown in Table 1.

A total of 38 microsatellite primer pairs designed for *M. nemurus* by Chan (2003) and Usmani (2002) were screened for possible cross-species amplification and for use in the population structure analysis of *T. tambroides*. Of these, five primers were selected based on polymorphic bands which amplified at the expected allele size regions (Table 2) for the T. *tambroides* samples.

### *Microsatellite-PCR Amplification*

The PCR amplification mixture of 10 µl reaction volume consisted of 2mM $MgCl_2$, 10X buffer (Promega), 400µM of each dNTP, 0.5µM of each of the forward and reverse microsattelite primer, 30 ug of DNA samples, appropriate amount of $ddH_20$ and 3 units of *Taq* polymerase (Promega). The amplifications were carried out in a Peltier Thermal Cycler DNA Engine-DYAD™ (MJ Research) with an initial predenaturing step of 3 minutes at 96°C, denaturation step of 30 seconds at 95°C, annealing step of 30 seconds at varied temperatures, extension step of 40 seconds at 68°C followed by 39 repeated cycles of the previous steps, and a final extension of 5 minutes at 68°C. The final step was held at 4°C.

Optimization of the microsatellite was made on the adjustment of annealing temperatures which ranged from 45°C to 60°C. Optimization to omit stutter bands was made by increasing the annealing temperature. Bands that formed below 100bp were likely to be primer dimers and were

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 |
| TOTAL | | 63 | | | |

TABLE 2
Microsatellite primer pairs used in the population structure study of kelah

| Locus | Primer Sequence (5'-3') | Genebank Accession Number | Product Size | Annealing Temperature °C (based on primer list) | Optimized Annealing Temperature °C |
|---|---|---|---|---|---|
| *MnSC4-3B | F: GCCAAGGAGCTATGAACTGG | AF458324 | 208 bp | F: 59.84 | 50 |
| | R: GACGCAACTATGTCCACCAC | | | R: 59.01 | |
| *MnLR2-1-52A | F: TCCCCTTTTTATTGCCATTC | AF425680 | 189 bp | F: 58.87 | 60 |
| | R:GGAACGAGGAGGGCTCTCTCT | | | R: 63.53 | |
| £MnRmC3-1 | F: AGTGCGAGGTGTGTGTGTG | AF462254 | 251 bp | F: 59.30 | 45 |
| | R: GGTGGACCAGTGCCTCTAGT | | | R: 60.53 | |
| *MnSC4-1A | F: GCCAGCAACAAGGGGCCA | AF458322 | 173 bp | F: 67.93 | 55 |
| | R: CCTTGGATCGGAACTGGTC | | | R: 60.46 | |
| *MnLR2-1-17B | F: GCAGTTTCCTTCTCTTCACT | AF360982 | 132 bp | F: 55.82 | 45 |
| | R: GGGGGGCGCGCAACTCTCTC | | | R: 72.67 | |

* Microsatellite markers developed by Chan (2003), £ microsatellite markers developed by Usmani (2002) designed for *Mystus nemurus*.

omitted from scoring. Bands that amplified within the range of the expected allele size were further optimized by making adjustments on the concentrations of $MgCl_2$, DNA, dNTP's and Taq polymerase.

*Microsatellite-gel Electrophoresis*

The PCR products were loaded onto metaphore gels containing 4.0% metaphore, 1X TBE buffer, 0.1µl/ml ethidium bromide, together with standard DNA ladders (20bp ladder). The gels were electrophoresed in 1X TBE buffer for 4 hours at 78 V/cm and photographed under UV light using an Alpha Imager 2200.

*Data Analysis*

Data collected were analyzed using the Popgene 1.31 (Yeh and Boyle 1997) computer software. The allelic frequencies were estimated from the genotypes assuming codominance. The measurement of genetic variability calculated for all the populations included mean number of allele per locus and the mean heterozygosity. Chi-square goodness of fit tests and the G log-likelihood ratio test were used to determine whether the observed genotypic numbers were consistent with the Hardy-Weinberg expectations for each population. The F-statistics was calculated according to Wright (1978). An unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal 1973) dendrogram based on Nei's (1978) genetic distance estimates was constructed using Pop Gene 1.31 and the multivariate analysis software (NT-SYS) of Rohlf (1989).

## RESULTS AND DISCUSSION

Of the 38 *M. nemurus* microsatellite primer pairs screened for possible cross-species amplification and population studies of kelah, five were selected based on band amplifications at the expected allele size regions. These selected primer pairs produced clear and distinct bands that were polymorphic at the expected allele sizes.

The microsatellite primer pair MnSC4-3B with an expected allele size of 208 bp, produced the bands at 160 bp, 170 bp, 180 bp, 190 bp, 200 bp and 210 bp and 220 bp. The microsatellite primer pair MnLR2-1-52A with an expected allele size of 189 bp, produced the bands at 220 bp and 230 bp. The microsatellite primer pairs MnRmC3-1a and MnRmC3-1b, with the expected allele size of 250 bp, produced two consistent bands at 280 bp and 320 bp, which were homozygous across the three populations (Table 3). The microsatellite primer pair MnSC4-1A, with an expected allele size of 173 bp, produced bands ranging from 160 bp to 220 bp (*Fig. 1*) with a common band shared among all individuals from the three populations at 180 bp. The microsatellite primer pair MnLR2-1-17B, with an expected allele size of 132 bp, produced clear bands at the expected regions ranging from 150 bp to 180 bp. However, clear distinct bands were also shown at higher base

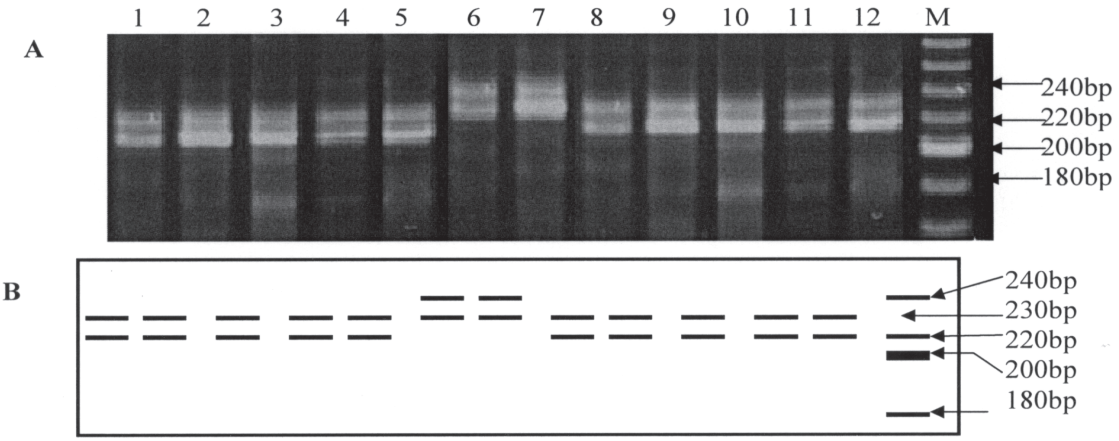


*Fig. 1: Microsatellite banding profile of kelah samples from Pahang using primer pair MnLR2-1-52A. (B): A diagrammatic representation of the microsatellite bands in (A). Lane M: 20bp ladder. Lane 1-12: Individuals from Pahang*

pairs but they were not considered for band scoring (*Fig. 2*).

*Allele Frequency, Levels of Heterozygosity and F-statistics*

The highest allele frequency among the populations studied was found in primer pair MnSC4-1A of the Negeri Sembilan population with a value of 0.788. The lowest allele frequency was found in primer pair MnLR2-17B of the Negeri Sembilan population with a value of 0.04. The highest allele frequency value for all the three populations was found in primer pair MnSC4-1A with a value of 0.718. The lowest allele frequency was found in the same primer pair with a value of 0.032 (Table 4). The common alleles for primer pair MnSC4-3B in the three kelah populations were found at 170 bp, 180 bp and 210 bp. The diagnostic alleles were found at 160 bp, 190 bp and 200 bp shown only in the Pahang population. The common alleles for primer pair MnLR2-1-52A in the three kelah populations were found at 220 bp and 230 bp. Primer pair MnRmC3-1 was homozygous for all the 63 samples and being considered as two separate loci. The most common allele for primer pair MnSC4-1A in the three kelah populations was found at 180 bp. The diagnostic alleles were found at 160 bp and 170 bp shown only in the Pahang population. The common alleles for primer pair MnLR2-17B in the three kelah populations were found at 160 bp and 170 bp. The diagnostic alleles were found at 150 bp and 180 bp shown only in the Pahang population while 155 bp was shown in the Negeri Sembilan population.

The number of observed and effective alleles per locus ranged from 2 to 7 (an average of 5) and 1.9 to 5.4 (an average of 3.1), respectively.

TABLE 3
Locus, allele size and allele frequency in the three kelah populations determined by using five microsatellite loci

| Locus | Allele | Allele size (bp) | Allele frequency | | | |
|---|---|---|---|---|---|---|
| | | | Pahang | Negeri Sembilan | Kelantan | Overall |
| MnSC4-3B | A | 160 | 0.115 | 0 | 0 | 0.048 |
| | B | 170 | 0.308 | 0.25 | 0.364 | 0.294 |
| | C | 180 | 0.039 | 0.231 | 0.136 | 0.135 |
| | D | 190 | 0.115 | 0 | 0 | 0.048 |
| | E | 200 | 0.192 | 0 | 0 | 0.079 |
| | F | 210 | 0.192 | 0.519 | 0.364 | 0.357 |
| | G | 220 | 0.039 | 0 | 0.136 | 0.039 |
| MnLR2-1-52A | A | 220 | 0.5 | 0.46 | 0.7 | 0.517 |
| | B | 230 | 0.391 | 0.46 | 0.3 | 0.405 |
| | C | 240 | 0.109 | 0.08 | 0 | 0.078 |
| *MnRmC3-1a | A | 280 | 1.0 | 1.0 | 1.0 | 1.0 |
| *MnRmC3-1b | A | 320 | 1.0 | 1.0 | 1.0 | 1.0 |
| MnSC4-1A | A | 160 | 0.08 | 0 | 0 | 0.032 |
| | B | 170 | 0.12 | 0 | 0 | 0.048 |
| | C | 180 | 0.64 | 0.788 | 0.727 | 0.718 |
| | D | 200 | 0.1 | 0.058 | 0 | 0.065 |
| | E | 210 | 0.06 | 0.077 | 0 | 0.057 |
| | F | 220 | 0 | 0.077 | 0.273 | 0.08 |
| MnLR2-17B | A | 150 | 0.083 | 0 | 0 | 0.033 |
| | B | 155 | 0 | 0.1 | 0 | 0.042 |
| | C | 160 | 0.25 | 0.24 | 0.273 | 0.25 |
| | D | 165 | 0.063 | 0.4 | 0 | 0.192 |
| | E | 170 | 0.208 | 0.04 | 0.091 | 0.117 |
| | F | 175 | 0 | 0.22 | 0.636 | 0.208 |
| | G | 180 | 0.396 | 0 | 0 | 0.158 |

* These two loci MnRmC3-1a and MnRmC3-1b were homozygous for all 63 samples

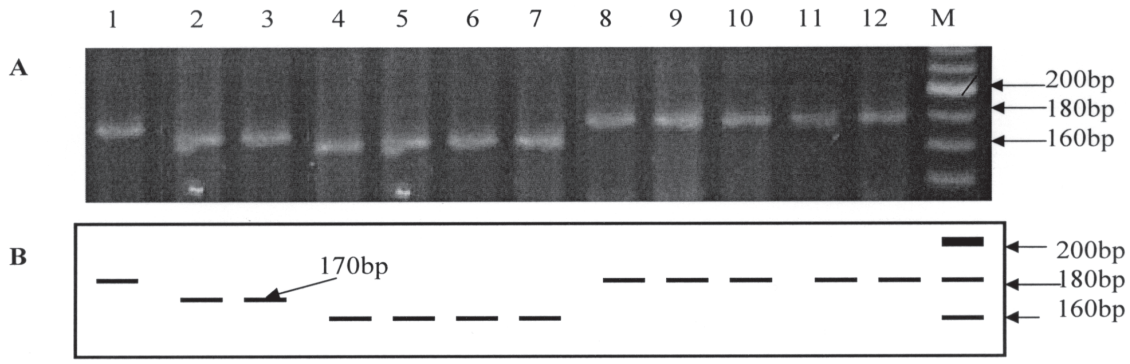


*Fig. 2: Microsatellite banding profile of kelah samples from Pahang using primer pair MnLR2-1-17B. (B): A diagrammatic representation of the microsatellite bands in (A). Lane M: 20bp ladder. Lane 1-12: Individuals from Pahang*

Whilst, the highest mean number of observed and effective alleles were found for the Pahang population with a value of 4.4 and 3.1, respectively. This was followed by the Negeri Sembilan and Kelantan populations with values of 3.4 and 2. 4, 2.6 and 2.1, respectively (Table 4).

Across the three populations, higher observed than expected heterozygosity values were observed for primer pairs MnSC4-3B, MnLR2-1-52A and MnRmC3-1, indicating excess of heterozygosity (Table 4). A check on the $F_{IS}$ (Wright, 1978) values of the three primer pairs showed negative values of -0.328, -0.6 and -1.0, respectively indicating no deficit in overall heterozygosity and no inbreeding across the three populations (Table 4). However, the primer pairs MnSC4-1A and MnLR2-17B showed lower observed than the expected heterozygosity values with positive $F_{IS}$ values of 0.082 and 0.688, respectively indicating deficit in heterozygosity across the three populations.

The mean value of $F_{ST}$ or fixation index was low (0.075), indicating little genetic differentiation among the sample populations (Lowe *et al.*, 2004). The $N_m$ estimate was 3.0627, a value indicating relatively high ongoing gene flow (Table 5).

*Hardy-Weinberg Equilibrium*

Both the ($x^2$) chi-square and the ($G^2$) likelihood ratio tests showed the presence of significant differences ($P<0.05$), indicating deviation from Hardy-Weinberg equilibrium for most loci except for one locus (MnSC4-1A) in the Negeri Sembilan population and two loci (MnLR2-1-52A and MnSC4-1A) in the Kelantan population.

*Genetic Distance and Cluster Analysis*

The values of genetic distance ranged from 0.1053 to 0.1960. The highest genetic distance was found between the Pahang and Kelantan populations with a value of 0.1960 followed by Pahang and Negeri Sembilan with a value of 0.1500. The lowest genetic distance was found between the Negeri Sembilan and Kelantan populations with a value of 0.1053 (Table 5). The UPGMA dendrogram constructed showed that the Negeri Sembilan and Kelantan populations were clustered into the same group while the Pahang population was away by itself (*Fig. 3*). The newly designed DNA microsatellites primer pairs for *Mystus nemurus* by Chan (2003) and Usmani (2002) were screened and only five of these primer pairs were selected on the basis of reproducibility and locus specificity. The ability of this newly designed microsatellite primer pairs to cross amplify on kelah suggested that certain sequences flanking the tandem repeats are highly conserved in Cypriniformes. Several studies have shown that flanking sequences of microsatellites may be conserved well enough throughout evolution to serve as primer-annealing sites for closely related species (Primmer *et al.*, 1996; Tong *et al.*, 2002). Reports of cross-species amplification of microsatellite primers designed from common carp, *Cyprinus carpio*, being used for the population studies of *Tor putitora* (Mohindra *et al.*, 2004), *Hypophthalmichthys molitrix* (silver carp) and *Aristichthys nobilis* (big head carp) (Tong *et al.*, 2002) are common and have produced reliable and satisfactory results. According to O'Connell and Wright (1997), microsatellite loci having numbers of allele

TABLE 4
Microsatellite variations, ($\chi^2$) chi-square and ($G^2$) likelihood ratio tests among the three populations of kelah

| Location | Locus | Observed number of alleles | Effective number of alleles | Observed heterozygosity | Expected heterozygosity | Degrees of freedom | Chi-square test | | Likelihood ratio test | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | $\chi^2$ value | Probability | $G^2$value | Probability |
| Pahang | MnSC4-3B | 7.0 | 5.0 | 0.846 | 0.818 | 6 | 49.95 | 0.00* | 65.39 | 0.00* |
| | MnLR2-1-52A | 3.0 | 2.4 | 1.0 | 0.598 | 2 | 22.0 | 0.00* | 30.87 | 0.00* |
| | MnRmC3-1 | 2.0 | 2.0 | 1.0 | 0.51 | 1 | 25.0 | 0.00* | 35.03 | 0.00* |
| | MnSC4-1A | 5.0 | 2.3 | 0.24 | 0.567 | 4 | 56.43 | 0.00* | 35.86 | 0.00* |
| | MnLR2-17B | 5.0 | 3.7 | 0.292 | 0.742 | 4 | 72.44 | 0.00* | 47.24 | 0.00* |
| Mean | | 4.4 | 3.1 | 0.676 | 0.647 | | | | | |
| Standard Deviation | | 1.9 | 1.3 | 0.38 | 0.128 | | | | | |
| Negeri Sembilan | MnSC4-3B | 3.0 | 2.6 | 0.962 | 0.627 | 2 | 21.36 | 0.00* | 27.94 | 0.00* |
| | MnLR2-1-52A | 3.0 | 2.3 | 0.92 | 0.582 | 2 | 17.73 | 0.00* | 23.73 | 0.00* |
| | MnRmC3-1 | 2.0 | 2.0 | 1.0 | 0.510 | 1 | 25.0 | 0.00* | 35.03 | 0.00* |
| | MnSC4-1A | 4.0 | 1.6 | 0.423 | 0.370 | 3 | 1.67 | 0.9468 | 2.71 | 0.8432 |
| | MnLR2-17B | 5.0 | 3.6 | 0.32 | 0.737 | 4 | 48.04 | 0.00* | 41.69 | 0.00* |
| Mean | | 3.4 | 2.4 | 0.725 | 0.565 | | | | | |
| Standard Deviation | | 1.1 | 0.8 | 0.326 | 0.137 | | | | | |

Effective number of alleles [Kimura and Crow (1964)], Expected heterozygosity as computed using Levene (1949), *significant at 0.05 level.

Table 4 (continued)

| Location | Locus | Observed number of alleles | Effective number of alleles | Observed heterozygosity | Expected heterozygosity | Degrees of freedom | Chi-square test | | Likelihood ratio test | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | $\chi^2$ value | Probability | $G^2$value | Probability |
| Kelantan | MnSC4-3B | 4.0 | 3.3 | 1.0 | 0.731 | 3 | 31.0 | 0.00* | 27.11 | 0.00* |
| | MnLR2-1-52A | 2.0 | 1.7 | 0.6 | 0.442 | 1 | 1.48 | 0.2232 | 2.22 | 0.1359 |
| | MnRmC3-1 | 2.0 | 2.0 | 1.0 | 0.524 | 1 | 10.0 | 0.00* | 14.22 | 0.00* |
| | MnSC4-1A | 2.0 | 1.7 | 0.546 | 0.416 | 1 | 1.25 | 0.2635 | 1.92 | 0.1649 |
| | MnLR2-17B | 3.0 | 2.0 | 0 | 0.537 | 2 | 33.9 | 0.00* | 21.41 | 0.00* |
| Mean | | 2.6 | 2.1 | 0.629 | 0.53 | | | | | |
| Standard Deviation | | 0.9 | 0.7 | 0.412 | 0.124 | | | | | |
| Overall | MnSC4-3B | 7.0 | 4.1 | 0.921 | 0.762 | 6 | 146.61 | 0.00* | 119.7 | 0.00* |
| | MnLR2-1-52A | 3.0 | 2.3 | 0.896 | 0.567 | 2 | 36.11 | 0.00* | 43.04 | 0.00* |
| | MnRmC3-1 | 2.0 | 2.0 | 1.0 | 0.504 | 1 | 62.0 | 0.00* | 86.32 | 0.00* |
| | MnSC4-1A | 6.0 | 1.9 | 0.371 | 0.471 | 5 | 130.01 | 0.00* | 48.05 | 0.00* |
| | MnLR2-17B | 7.0 | 5.4 | 0.25 | 0.823 | 6 | 250.86 | 0.00* | 146.49 | 0.00* |
| Mean | | 5.0 | 3.1 | 0.688 | 0.625 | | | | | |
| Standard Deviation | | 2.3 | 1.6 | 0.349 | 0.158 | | | | | |

Effective number of alleles [Kimura and Crow (1964)], Expected heterozygosity as computed using Levene (1949), *significant at 0.05 level.

TABLE 5
The value of F-statistics for all the loci across the three populations based on data generated by utilizing five microsatellite loci

| Locus | $F_{IS}$ | $F_{ST}$ | $N_m$ |
|---|---|---|---|
| MnSC4-3B | -0.328 | 0.058 | 4.044 |
| MnLR2-1-52A | -0.6 | 0.032 | 7.531 |
| MnRmC3-1 | -1.0 | 0.0 | ***** |
| MnSC4-1A | 0.082 | 0.053 | 4.515 |
| MnLR2-1-17B | 0.688 | 0.181 | 1.133 |
| Mean | -0.196 | 0.075 | 3.063 |

$N_m$ = Gene flow estimated from $F_{ST} = 0.25(1 - F_{ST})/F_{ST}$

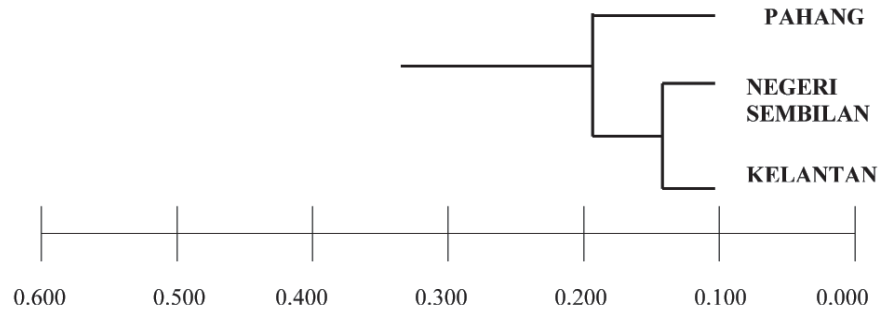


*Fig. 3: Dendrogram constructed based on Nei's (1978) genetic distance values clustered by UPGMA for the three populations of kelah utilizing data from five microsatellite loci*

ranging from 5 to 10 were sufficient to provide reliable results in a population study.

Generally, the observed heterozygosity was more than the expected heterozygosity across the three populations for primer pairs MnSC4-3B, MnLR2-1-52A and MnRmC3-1. A check on the $F_{IS}$ (Wright 1978) values of the three primer pairs showed negative values of -0.328, -0.6 and -1.0, respectively indicating no deficit in heterozygosity across the populations. The observed heterozygosity was more than the expected heterozygosity across the three populations in the present study. This was expected as outcrossing occurred naturally in the wild (Chan, 2003). However, the primer pairs MnSC4-1A and MnLR2-1-17B showed lower observed heterozygosities than the expected heterozygosities across the three populations. A check on the $F_{IS}$ (Wright 1978) values of the two primer pairs showed positive values of 0.082 and 0.688, respectively indicating deficit in heterozygosity. Similar situations were observed by Mohindra *et al.* (2004), Lal *et al.* (2004) and Mei *et al.* (2003) in their population studies of *Tor putitora*, *Cirrhinus mrigala* and *Zacco pachycephalus*, respectively. This could probably be due to methodological bias called stutter-related scoring errors. However, stutter bands were avoided during preliminary experiments. Secondly, such results could come from null alleles, leading to the scoring of heterozygous bands as homozygotes. Jarne and Lagoda (1996) reported that null alleles in fish might be quite common, and Pyatskowit *et al.* (2001) and Rodzen and May (2002) also reported the influence of null alleles in the inheritance of microsatellite loci in lake and white sturgeons. Founder effect during introduction might also be one of the reasons (Mei *et al.*, 2003) for the above observation.

The $N_m$ measuring the movement of individuals per generation between populations obtained in this study was high (3.062), suggesting high genetic flow between the

TABLE 6
Genetic distance (below diagonal) and identity (above diagonal) based on data generated from five microsatellite loci

| Populations | Pahang | Negeri Sembilan | Kelantan |
|---|---|---|---|
| Pahang | ****** | 0.8607 | 0. 8220 |
| Negeri Sembilan | 0.1500 | ****** | 0.9001 |
| Kelantan | 0.1960 | 0.1053 | ****** |

Nei's (1978)

populations studied. As indicated by Lowe *et al.* (2004), Nm values greater than 1 suggest that populations are expected to retain genetic connectivity. This may have been due to human intervention as there is no connection between the rivers system where the fishes were obtained. However, only through an understanding of how organisms disperse their genes and the ecological requirements for propagate establishment, can one predict the likely effects of contemporary environmental change on genetic diversity.

Both the ($x^2$) chi-square and ($G^2$) likelihood ratio tests showed significant differences ($P<0.05$), indicating deviation from Hardy-Weinberg equilibrium in most loci except for one locus (MnSC4-1A) in the Negeri Sembilan population and two loci (MnLR2-1-52A and MnSC4-1A) in the Kelantan population. Many hypotheses could explain deviation from Hardy-Weinberg equilibrium. This phenomenon could probably be due to Wahlund effects (Silverstein *et al.*, 2004), founder effects (Mei *et al.*, 2003) and small sample sizes (Das *et al.*, 2005; Mohindra *et al.*, 2004). A sample size of 50 is large enough to disregard the effect of population size for deviation from Hardy-Weinberg equilibrium (O'Connell and Wright, 1997). Another reason could probably be the effect of the lower observed heterozygosity than the expected heterozygosity (Mei *et al.*, 2003). A deficit in heterozygosity ($F_{IS}$ statistics) may cause deviation from Hardy-Weinberg equilibrium (Zhao *et al.*, 2005) but this is unlikely to be the reason as a negative $F_{IS}$ value was observed across the three populations in this study suggesting no deficit in heterozygosity. In this study, the deviations from Hardy-Weinberg equilibrium were most likely caused by small sample sizes. Hence, in terms of population studies, these results should only be considered as preliminary since the small sample sizes may be insufficient to observe the whole range of alleles.

## CONCLUSIONS

The newly developed primer pairs of *M. nemurus* were able to amplify the DNA of a fish from a different family and were useful for characterizing and differentiating the population structure of *T. tambroides*. The low range of genetic distances indicated that the *T. tambroides* from the three locations were genetically similar and were of the same species.

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