



UNIVERSITI PUTRA MALAYSIA

**MOLECULAR PHYLOGENETICS AND POPULATION STRUCTURE
OF TWO MAHSEER SPECIES (*Tor tambroides* BLEEKER AND *Tor
douronensis* VALENCIENNES: CYPRINIDAE) IN MALAYSIA**

**YUZINE BIN ESA
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MAHSEER SPECIES (*Tor tambroides* BLEEKER AND *Tor douronensis*
VALENCIENNES: CYPRINIDAE) IN MALAYSIA**

By

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This study examines the phylogenetic relationships of mahseer (genus *Tor* and *Neolissochillus*) and their relationships with other selected cyprinids using sequence analysis of mitochondrial DNA *cytochrome c oxidase* I (COI) gene (464 base pairs). This study also describes the genetic structure of *Tor tambroides* and *Tor douronensis*, two important mahseer in Malaysia using both mitochondrial COI sequences and nuclear microsatellites DNA.

The findings of the phylogenetic study strongly support the reciprocally monophyletic status between genus *Tor* (*T. douronensis* and *T. tambroides*) and genus *Neolissochillus* (represented by *Neolissochillus stracheyi*.) thus, strengthen the taxonomic status for all the three indigenous mahseer. For the other indigenous cyprinids, an interesting finding was that



Barbonymus gonionotus was phylogenetically distinct from its morphologically similar species, *Barbonymus schwanefeldii* (K2P distance value = 15.1%), and did not group together in a single *Barbonymus* clade.

The population structure analysis inferred from mitochondrial COI sequences found high levels of intra and inter-population variations in *T. douronensis*. The presence of fixed haplotype differences among the populations, along with high F_{ST} values indicated that there has been little or no migration occur among the extant populations separated by large geographic distances, or river systems. For *T. tambroides*, low level of mitochondrial variations was found among the populations. The reason is probably due to the high proportion of the HKE1 haplotype found in most populations (0.736-1.000) studied except from Endau-Rompin (HKE4 is the dominant haplotype), or by the small number of samples used in the present study.

The large genetic differences (3.3% to 5.2%) between the *T. douronensis* population from Sabah with its congeners from Sarawak, and the presence of fixed haplotypes supported the long period of genetic isolation of the mahseer of North Borneo, possibly prior to the Pleistocene glaciation periods. The sharing of haplotype HKE4 between samples of *T. tambroides* from Sarawak (Batang Ai (80%), Batang Baleh (100%) and Ulu Limbang (60%)) and Peninsular Malaysia (Endau-Rompin (97.9%) and Perak (15.8%)) indicated the

historical interconnection of drainages of Borneo and Peninsular Malaysia during the Tertiary and Quaternary periods (10-5 Ma) via the North Sunda River.

The population structure analysis using microsatellites found low levels of genetic diversity in both mahseers. The mean expected heterozygosity (0.3184) at 15 microsatellite loci found in *T. tambroides* and (0.2674) 13 loci found in *T. douronensis* were lower than the mean heterozygosity (0.5400) reported in most freshwater fish species but were comparable with those found in other studies of mahseer population structure using microsatellites. The results of the microsatellite analyses revealed a higher degree of population differentiation among extant populations of *T. tambroides* (F_{ST} : 0.0011 to 0.6494, genetic distances: 0.2% to 17.1%) compared with the *T. douronensis* populations (F_{ST} : 0.0057 to 0.3533, genetic distances: 0.0% to 4.8%). The population structuring among the *T. tambroides* populations was evidently higher using mitochondrial dataset compared with microsatellite dataset except in the Kelantan population where both dataset showed very low level of genetic diversity. In *T. douronensis*, microsatellites found very little genetic differentiation between the Sabah population and the other *T. douronensis* populations from Sarawak (F_{ST} : 0.0057 to 0.0537, genetic distances: 0.0% to 4.2%), in contrast to the mitochondrial results. The lack of adequate sample sizes in most populations in this study might have affected the observed microsatellite variations in both mahseer.

The identification of a single haplotype (ER8*) consisted of four individuals from the Endau-Rompin population genetically closer to haplotypes of *T. douronensis* suggested that the haplotype ER8* might actually represents a rare or cryptic *T. douronensis* haplotype occurred in Peninsular Malaysia. Nevertheless, additional microsatellites results using Bayesian cluster analysis showed that the samples of haplotype ER8* were clustered together with the other *T. tambroides* samples with a high proportional membership of 92.8%. However, UPGMA dendrogram suggested a different viewpoint where samples of ER8* were clustered together within the *T. douronensis* clusters with a weak support since a tied trees was found in the cluster analysis. Thus, the inclusion of nuclear microsatellite datasets could not fully resolve the genetic identity of haplotype ER8* under the current study. Finally, the conservation unit criterion was assigned to the species (ESUs between *T. tambroides* and *T. douronensis*) and populations (MUs) levels of both mahseer.

Overall, the phylogenetic analysis of mitochondrial COI sequences has managed to provide new insights into the evolutionary relationship and classification of the cyprinids under study including the mahseer. The phylogenetic results are generally in good agreement and concordant with the current classification of the cyprinids based on morphology and meristic characters. The microsatellite markers in conjunction with mitochondrial COI markers demonstrated in this study have shown to be a better approach than a single molecular marker analysis in examining natural population of the mahseer.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**FILOGENETIK MOLEKUL DAN STRUKTUR POPULASI DUA SPESIES IKAN
KELAH (*Tor tambroides* BLEEKER DAN *Tor douronensis* VALENCIENNES:
CYPRINIDAE) DI MALAYSIA**

Oleh

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Oktober 2009

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Kajian ini telah dijalankan untuk memeriksa perhubungan filogenetik ikan kelah (genus *Tor* dan *Neolissochillus*) serta perhubungan dengan ikan siprinid terpilih yang lain menggunakan analisis penjujukan DNA mitokondria gen *sitokrom c oksides I* (COI) (464 pasangan bes). Kajian ini juga menghuraikan struktur populasi *Tor tambroides* dan *Tor douronensis*, dua spesies ikan kelah yang penting di Malaysia menggunakan kedua-dua kaedah penjujukan mitokondria COI dan DNA mikrosatelit nukleus.

Keputusan kajian filogenetik menyokong kuat status saling monofiletik di antara genus *Tor* (*T. douronensis* dan *T. tambroides*) dan genus *Neolissochillus* (diwakili oleh *Neolissochillus stracheyi*) seterusnya menyokong status taksonomi untuk ketiga-tiga ikan kelah indigenus tersebut. Untuk ikan siprinid indigenus yang lain, satu penemuan menarik iaitu *Barbonymus*



gonionotus yang secara secara filogenetikanya sangat berbeza daripada *Barbonymus schwanenfeldii*, spesies yang secara morfologi hampir sama (nilai jarak genetik K2P = 15.1%) dan tidak tergolong bersama di dalam satu kumpulan *Barbonymus*.

Analisis struktur populasi menggunakan penjujukan COI mitokondria mendapati variasi di peringkat intra dan inter-populasi yang tinggi dalam *T. douronensis*. Kehadiran perbezaan haplotip tetap antara populasi, disusuli dengan nilai F_{ST} yang tinggi antara populasi menunjukkan bahawa tiada atau terlalu sedikit proses migrasi yang berlaku antara populasi yang dipisahkan oleh jarak geografi atau sistem sungai yang jauh. Untuk *T. tambroides*, tahap variasi COI mitokondria yang rendah telah dijumpai di kalangan populasinya. Ini kemungkinan disebabkan oleh perkadaran tinggi haplotip HKE1 dikesan di dalam kebanyakan populasi (0.736 hingga 1.000) yang dikaji kecuali dari Endau-Rompin (HKE4 adalah haplotip yang dominan), atau kerana jumlah sampel ikan yang terlalu kecil digunakan di dalam kajian ini.

Perbezaan genetik yang besar (3.3% hingga 5.2%) di antara populasi *T. douronensis* dari Sabah dengan populasi dari Sarawak; dan kehadiran haplotip tetap menyokong jangka masa pemencilan genetik yang panjang untuk ikan kelah dari Borneo Utara, berkemungkinan sebelum era “Pleistocene” lagi. Perkongsian haplotip HKE4 di kalangan sampel *T. tambroides* dari Sarawak (Batang Ai (80%), Batang Baleh (100%) dan Ulu Limbang (60%)) dan Semenanjung Malaysia (Endau-Rompin (97.9%) dan Perak (15.8%)) menunjukkan,

secara sejarahnya, terdapat perhubungan di antara sungai di Borneo dan Semenanjung Malaysia semasa zaman Tertiar and Quaternar (10 - 5 Mya) melalui Sungai Sunda Utara.

Analisis struktur populasi menggunakan mikrosatelit mendapati tahap variasi genetik yang rendah dalam kedua-dua spesies ikan kelah tersebut. Min heterozigositi jangkaan (0.3184) pada 15 lokus DNA mikrosatelit yang dijumpai dalam *T. tambroides* dan (0.2674) 13 lokus mikrosatelit dalam *T. douronensis* adalah lebih rendah daripada min heterozigositi (0.5400) yang dilaporkan dalam kebanyakan kajian ikan air tawar, tetapi masih boleh dibandingkan dengan nilai yang dijumpai di dalam kajian struktur populasi ikan kelah lain dengan menggunakan mikrosatelit. Keputusan daripada analisis mikrosatelit menunjukkan darjah perbezaan genetik yang lebih besar di antara populasi *T. tambroides* (F_{ST} : 0.0011 hingga 0.6494, jarak genetik: 0.2% hingga 17.1%) berbanding dengan populasi *T. douronensis* (F_{ST} : 0.0057 hingga 0.3533, jarak genetik: 0.0% hingga 4.8%). Corak penstrukturan di kalangan populasi *T. tambroides* adalah lebih tinggi dengan menggunakan set data mitokondria COI berbanding dengan set data mikrosatelit, kecuali di dalam kes populasi dari Kelantan di mana keputusan daripada kedua-dua set data menunjukkan kadar variasi genetik yang sangat rendah. Dalam *T. douronensis*, analisis mikrosatelit mendapati perbezaan genetik yang sangat rendah di antara populasi dari Sabah dengan lain-lain populasi *T. douronensis* dari Sarawak (F_{ST} : 0.0057 hingga 0.0537, jarak genetik: 0.0% hingga 4.2%), namun berbeza daripada keputusan menggunakan mitokondria COI. Kekurangan saiz sampel bagi kebanyakan populasi dalam kajian ini berkemungkinan besar telah mempengaruhi tahap variasi mikrosatelit yang dijumpai bagi kedua-dua spesies ikan kelah tersebut.

Pengenalpastian satu haplotip (ER8^{*}) daripada empat individu ikan kelah dari populasi Endau-Rompin yang mana jarak genetiknya adalah lebih dekat dengan haplotip *T. douronensis* menunjukkan bahawa haplotip tersebut mungkin merupakan satu haplotip *T. douronensis* yang nadir atau bersifat kriptik yang pertama kali dikesan di Semenanjung Malaysia. Walau bagaimanapun, keputusan tambahan mikrosatelit menggunakan kaedah analisis kluster Bayesian menunjukkan sampel haplotip ER8^{*} tergolong bersama-sama dengan sampel *T. tambroides* pada kadar keahlian 92.8%. Namun, dendrogram UPGMA menunjukkan pendapat yang berlainan di mana sampel ER8^{*} tergolong bersama-sama di dalam kumpulan *T. douronensis* yang disokong secara lemah, kerana pokok yang dihasilkan adalah terikat bersama. Justeru, kemasukan set data mikrosatelit nukleus tidak dapat menyelesaikan identiti genetik haplotip ER8^{*}. Kriteria unit pemuliharaan ditentukan pada aras spesies (ESUs di antara *T. tambroides* dan *T. douronensis*) dan populasi (MUs) untuk kedua-dua ikan kelah tersebut.

Secara keseluruhannya, analisis filogenetik penjujukan mitokondria COI telah berjaya memberikan pandangan yang baru terhadap perhubungan evolusi dan pengelasan ikan siprinid di dalam kajian ini termasuk ikan kelah. Keputusan filogenetik secara amnya saling menyetujui dan selari dengan pengelasan semasa ikan siprinid berdasarkan ciri-ciri morfologi dan meristik. Penanda mikrosatelit dengan gabungan penanda mitokondria COI seperti yang ditunjukkan di dalam kajian ini, telah terbukti merupakan satu pendekatan yang lebih baik berbanding dengan analisis yang menggunakan satu penanda genetik sahaja di dalam kajian populasi semulajadi ikan kelah.

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I certify that an Examination Committee has met on **26 October 2009** to conduct the final examination of **Yuzine bin Esa** on his **Doctor of Philosophy** thesis entitled “**Molecular phylogenetics and population structure of two mahseer (*Tor tambroides* and *Tor douronensis* Valenciennes: Cyprinidae) in Malaysia**” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions

YUZINE BIN ESA

Date: 24 December 2009



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LIST OF ABBREVIATIONS

µg	microgram
µl	microlitre
5X	five times
10X	ten times
bp	base pair
AMOVA	analysis of molecular variance
CA	correct assignment
COI	cytochrome c oxidase I
CTAB	cetyl-trimethylammonium bromide
ddH₂O	double distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediamine tetraacetic acid
ESU	evolutionary significant unit
g	gram
h	hour
HWE	hardy-weinberg equilibrium
IUCN	international union for conservation nature
K2P	kimura-2-parameter
kg	kilogram
LD	linkage disequilibrium
m	metre

