Pertanika J. Trop. Agric. Sci. **31(2)**: 263 - 269 (2008)
ISSN: 1511-3701

Molecular Systematics of Mahseers (Cyprinidae) in Malaysia Inferred from Sequencing of a Mitochondrial Cytochrome C Oxidase I (COI) Gene

Yuzine B. Esa[1,2*], Siti Shapor Siraj[2], Siti Khalijah Daud[2], Japning Jeffrine Rovie Ryan[3], Khairul Adha A. Rahim[1] and Soon Guan, Tan[2]

[1]*Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia*

[2]*Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*

[3]*Institute of Biodiversity, Bukit Rengit, 28500 Lanchang, Pahang, Malaysia*

**E-mail: kelahzine@yahoo.com*

ABSTRACT

This study examined the molecular systematics among three Mahseers (*Tor douronensis, Tor tambroides* and *Neolissochilus stracheyi*) using partial sequencing of a Cytochrome C Oxidase I (COI) mitochondrial DNA segment (466bp). The phylogenetic results using the Neighbour-Joining (NJ) method supported the monophyletic status (hence the taxonomic status) among the three putative Mahseer species. The close genetic relationships (0.1-0.4%) found between *T. tambroides* samples from Peninsular Malaysia (kelah fish) and those from Sarawak (empurau fish) also supported their classification as belonging to the same species. The phylogenetic analysis also showed that the *T. douronensis* mtDNA consisted of three highly distinct lineages supported by high bootstrap values, with the Sabah samples forming its own cluster. Thus, this phylogenetic study, although based on a limited number of samples and only a single mtDNA gene managed to provide useful insights into the systematic status of the Mahseers found in Malaysia.

Keywords: Freshwater fish, Mahseers, COI, molecular systematics

INTRODUCTION

Freshwater fishes of the genus *Tor* Gray, commonly known as the Mahseers, belong to the family Cyprinidae (subfamily Cyprininae) (Inger and Chin, 1962; Mohsin and Ambak, 1983; Roberts, 1989; Kottelat *et al.*, 1993). There are currently 17 described species under the genus *Tor* from all across Asia (Ng, 2004) but only three species were reported in Malaysia: *Tor tambroides* Bleeker, *Tor tambra* Valenciennes, and *Tor douronensis* Valenciennes (Kottelat *et al.*, 1993; Kottelat and Whitten, 1996; Ng, 2004). The taxonomic status of *Tor soro* Valenciennes had been revised and it is currently re-classified as *Neolissochilus stracheyi* (Rainboth, 1996). Mahseers are important as food fish as well as ornamental and recreational fishes. However, recently the population sizes of their natural stocks are decreasing rapidly due to environmental degradation and increased fishing pressure (Ng, 2004).

So far, very little taxonomic work to systematically sort out Malaysian Mahseer has been documented. The most cited work was by Mohsin and Ambak (1983) who described *Tor tambroides* and *Tor soro* as two valid Mahseers found in Peninsular Malaysia while a more recent view by Ng (2004) suggested the occurrence of three species; *T. tambroides*, *T. tambra* and *T. douronensis.* Other taxonomic works recognized *T. douronensis* and *T. tambroides* as two valid species (Roberts, 1989; Kottelat *et al.*, 1993; Rainboth, 1996; Zhou and Chu, 1996), although Roberts (1999) classified them to be a single species, and a junior synonym to *T. tambra.* The presence of the median lobe has been characterized as a

Received: 12 July 2007
Accepted: 30 June 2008
* Corresponding Author

diagnostic morphological character distinguishing the genus *Tor* from the genus *Neolissochillus* (Rainboth, 1996; Ng, 2004), though it cannot be used consistently as a marker to discriminate between fishes of the genus *Tor*. Thus, the application of molecular techniques (such as DNA sequencing) can provide new and better insights into the unresolved taxonomy and phylogenetic relationships of all the putative Mahseers in Malaysia (Nguyen *et al.*, 2006).

Nguyen *et al.* (2006) recently produced the first molecular work on Mahseers in Malaysia by examining the genetic diversity and phylogenetic relationships of broodstocks of *T. douronensis* and *T. tambroides* cultured in Sarawak (Borneo) through sequencing analysis of the mitochondrial DNA (mtDNA) 16S rRNA gene region. Thus, the present study also aimed to clarify the phylogenetic relationships among Mahseer fishes in Malaysia but with a few different approaches. First, we utilized direct sequencing of the *Cytochrome C Oxidase* I (COI) mtDNA gene region, a gene with a faster evolutionary rate compared to the 16S rRNA (Simon *et al.*, 1994), and thus capable of providing a better resolution at the interspecific level. Secondly, we included additional *Tor* samples from Peninsular Malaysia (kelah fish) and Sabah (pelian fish), to compare with the *T. douronensis* (semah fish) and *T. tambroides* (empurau fish) of Sarawak. Thirdly, *N. stracheyi* representing the genus *Neolissochilus* were included in the phylogenetic study to quantify the genetic differences between the two genera.

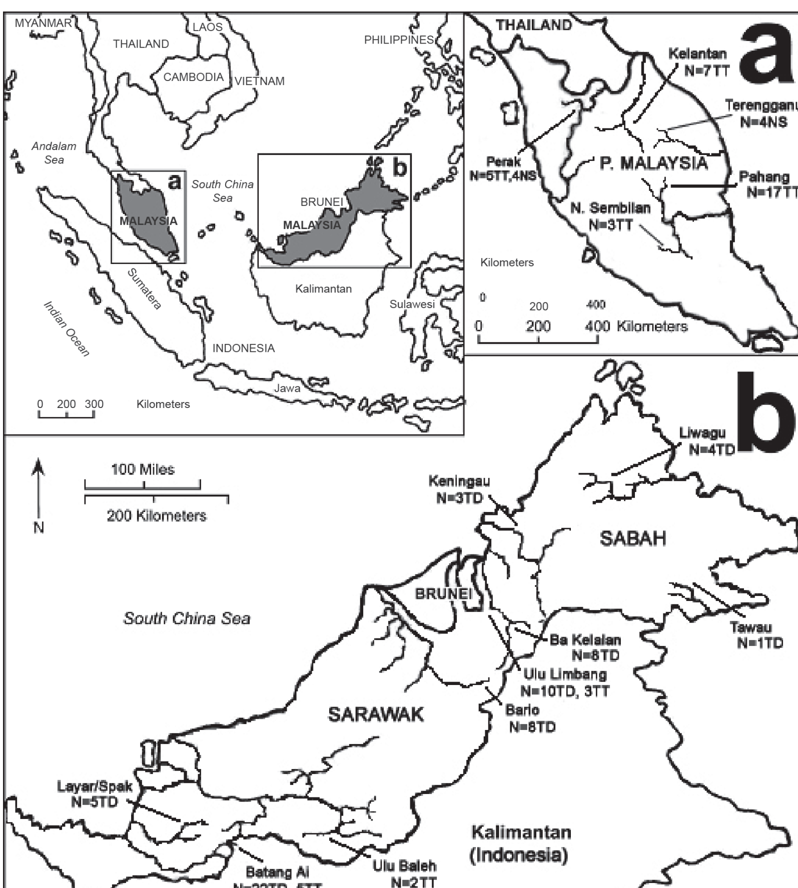

Fig. 1: Map showing sampling locations and sample size (N) in Peninsular Malaysia, Sarawak and Sabah. TD= T. douronensis, *TT=* T. tambroides, *NS=* N. stracheyi

MATERIALS AND METHODS

A total of 111 individuals of the three putative mahseers (*T. douronensis*, *T. tambroides* and *N. stracheyi*) were collected from several locations in Peninsular Malaysia, Sarawak and Sabah (*Fig. 1*). Total DNA was extracted using a modified CTAB method (Grewe *et al.*, 1993) in the presence of Proteinase K. The isolated genomic DNA was used for the mtDNA analysis.

A 500 bp segment of the COI gene was amplified with the oligonucleotide primers COIf (5' CCTGCAGGAGGAGGAGAYCC 3', forward) and COIe (5' CCAGAGATTAGA GGGAATC AG TG 3', reverse) (Palumbi *et al.* 1991). Approximately, 50-100 ng of the template DNA was amplified in a 25 ml reaction mixture containing 50 mM 10X buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP (Promega), 0.1 mM of each primer, and 0.5 units of *Taq* DNA Polymerase (Promega). The cycle parameters consisted of 35 cycles of denaturation (95^{o}C, 30 seconds), annealing (45^{o}C, 30 seconds), and extension (72^{o}C, 60 seconds). The amplified products were visualized on 1% agarose gel containing ethidium bromide, run for approximately 30 min at 90 V and photographed under UV light. The purified PCR products were directly sequenced using the BigDye® Terminator v3.0 Cycle Sequencing kit (ACGT) on an ABI 377 automated sequencer (PE Applied Biosystem) using only the forward primer (COIf). Sequencing reaction using the reverse primer (COIe) was subsequently carried out on some of the samples (haplotypes) to verify the polymorphism in the DNA sequence initially detected using the forward primer.

The CHROMAS (Version 1.45) program was used to display the fluorescence-based DNA sequencing analysis results. The multiple sequence alignments were done using the CLUSTAL X program version 1.81 (Thompson *et al.*, 1997), and subsequently aligned by eye. The Molecular Evolutionary Genetics Analysis (MEGA) version 3.1 (Kumar *et al.*, 2004) program was used to construct a neighbour-joining (NJ) (Saitou and Nei, 1987) tree using two indigenous cyprinids (*Barbonymus gonionotus* (Genbank accession number: DQ532806) and *Barbonymus schwanenfeldii* (Genbank accession number: DQ532805)) as outgroup species. The phylogenetic confidence was estimated by bootstrapping (Felsenstein, 1985) with 1000 replicate data sets. The pairwise genetic distance between populations was calculated using the Tamura-Nei distance (Tamura and Nei, 1993), based on unequal base frequencies and unequal ratios of transition to transversion (Ti:Tv) implemented in MEGA.

RESULTS AND DISCUSSION

The sequence analysis of the partial COI gene (466 base pairs) revealed a total of 24 haplotypes in the nucleotide data set: 14 haplotypes belonging to *T. douronensis*, six haplotypes belonging to *T. tambroides* and four haplotypes belonging to *N. stracheyi* (*Fig. 2*). The sequence of each of the haplotypes was deposited in the GenBank (GeneBank Reference Numbers: DQ532824-DQ532827 and EF192444-192463). In total, 74 (15.9%) variable sites were found, of which 56 (12.0%) were parsimony informative sites, while 392 (84.1%) were monomorphic sites. Transitional changes occurred more frequently than transversional changes as is typical of animal mitochondrial genomes (Briolay *et al.,* 1998).

The phylogenetic results obtained by using the NJ method supported the monophyletic status among the three mahseers (*Fig. 3*), although the bootstrap support between *T. tambroides* and *N. stracheyi* was low (58%). The high genetic divergence separating *T. douronensis* and *T. tambroides* confirmed their status as distinct species (Table 1). Likewise, the high genetic divergence separating the *N. stracheyi* lineage from the *Tor* lineages (7.7-8.7%) also supported its recent reclassification from the genus *Tor* into the genus *Neolissochilus* (Rainboth, 1996).

The close genetic relationships (0.1-0.4%) found between *T. tambroides* samples from Peninsular Malaysia (kelah fish) and those from Sarawak (empurau fish) supported their taxonomic status as belonging to the same species (Table 1). However, the overall very low level of genetic differentiation within and among *T. tambroides* populations may have resulted from the limited number of samples (2-17) analysed for each population, but was consistent with those found by Nguyen *et al.* (2006) in the Sarawak populations.

The phylogenetic analysis also revealed that the *T. douronensis* mtDNA consisted of three highly distinct clusters (Cluster I to III, *Fig. 3*) with the Sabah samples forming its own cluster (Cluster III) with strong bootstrap support. The genetic differentiations between the Sabah

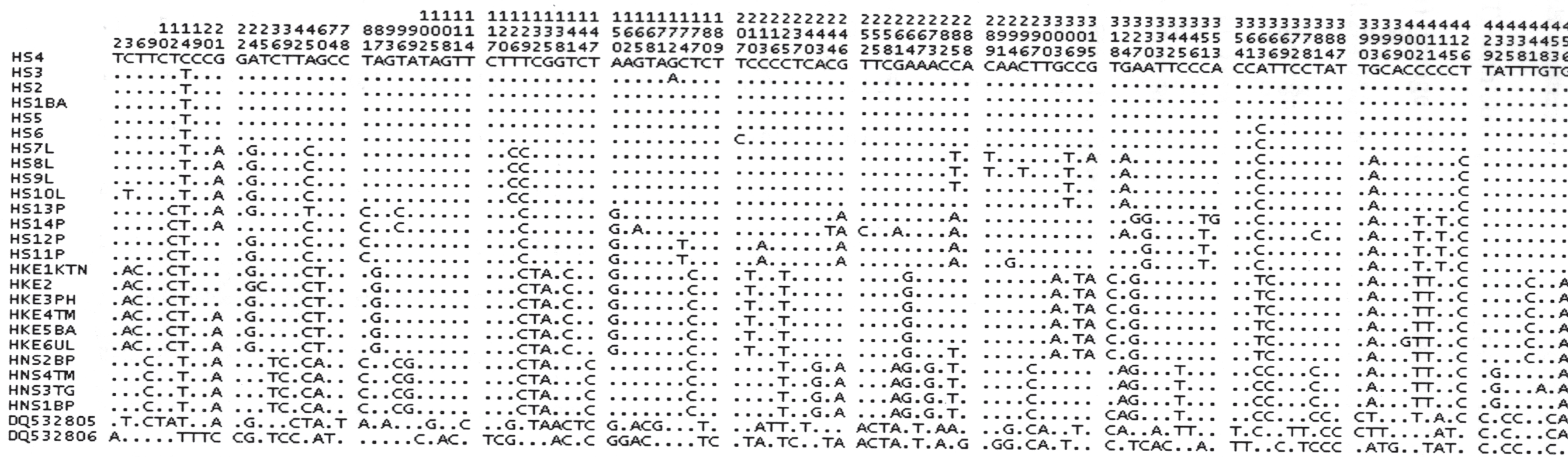

Fig. 2: Summary of nucleotide variations found in the three Mahseers. Only variable sites are shown. Haplotypes are named referring to the species and a number. HS= T. douronensis *haplotype, HKE=* T. tambroides *haplotype, HNS=* N. stracheyi *haplotype. Dots indicate identity with the HS4 haplotype sequence. DQ532805 and DQ532806 are* B. schwanenfeldii *and* B. gonionotus *haplotype sequences, respectively.*

(pelian fish) lineage (Cluster III) and both the Sarawak lineages (Cluster I and II) are relatively high (4.2-4.7%) for a conspecific group, and definitely higher than the two divergent *T. douronensis* lineages from Sarawak (2.0%) found by Nguyen *et al.* (2006) using 16s rRNA (Table 1). However, our phylogenetic analysis did not find any *T. douronensis* lineage genetically more similar to *T. tambroides* (6.8-8.2%) than to each other as was found by Nguyen *et al.* (2006). Thus, we suggest that the *T. douronensis* lineages from Sabah could represent a cryptic species.

Overall, the current study managed to provide insights into the phylogenetic relationships among the three putative species of the important mahseers of Malaysia. Nevertheless, the shortcomings of our results were clearly recognized and the data should be treated with great caution, since it was based on a limited number of samples (especially in *T. tambroides*) and a single maternally inherited gene (COI). Indeed, further studies on their taxonomy, population structures and phylogeography are required based on larger sample sizes per population, samples from other areas of their geographical distributions, a more variable mtDNA region such as the control region (D-Loop) to reveal more variations at the inter and intra population levels, and data from nuclear markers such as single locus microsatellite markers to complement the mtDNA findings.

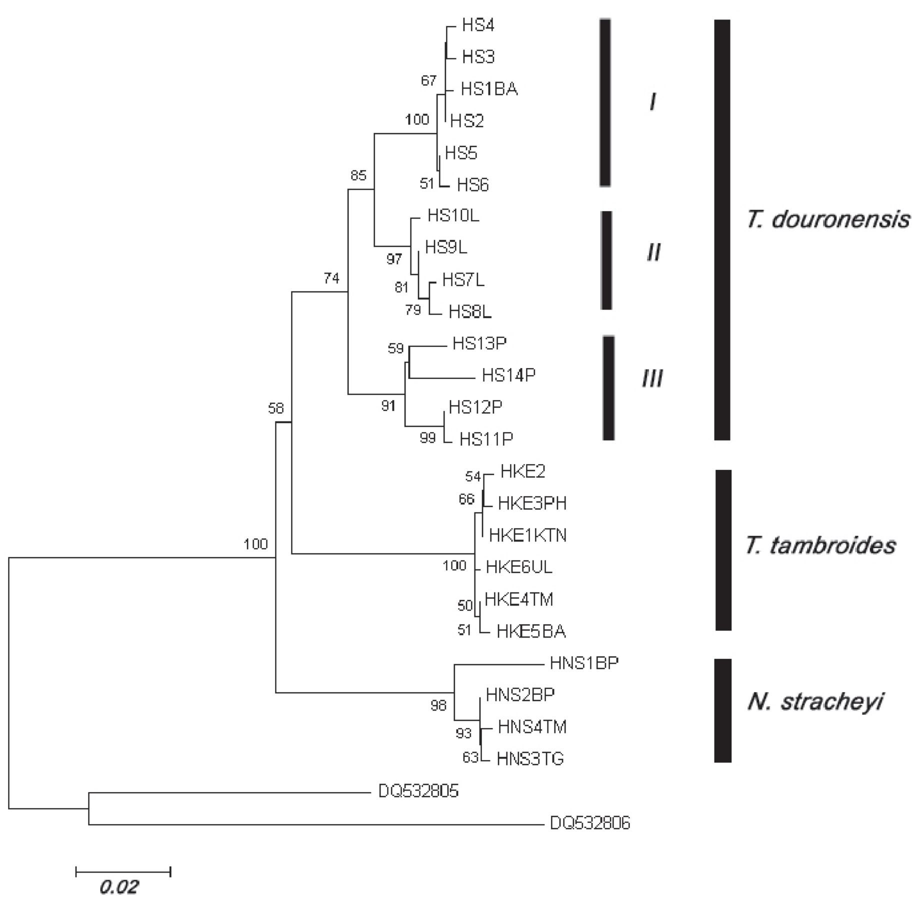

Fig. 3: Neighbour-joining (NJ) phylogram (consensus tree) showing the relationships among COI mtDNA haplotypes of the Mahseers. Haplotypes are named referring to the species and a number. HS= T. douronensis *haplotype, HKE=* T. tambroides *haplotype, HNS=* N. stracheyi *haplotype. Number at each node represents the bootstrap value (%) based on 1000 pseudoreplications for NJ analysis.*

TABLE 1
Pairwise Tamura-Nei genetic distance among the different populations of the three Mahseer species used in this study. Population 1-7 represents *T.tambroides* while population 8-13 represents *T.douromensis*

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
T. tambroides	1. Pahang	-													
	2. N. Sembilan	0.000	-												
	3. Kelantan	0.000	0.000	-											
	4. Perak	0.002	0.001	0.001	-										
	5. Batang Ai	0.002	0.002	0.002	0.001	-									
	6. Ulu Limbang	0.004	0.004	0.004	0.002	0.003	-								
	7. Ulu Baleh	0.002	0.002	0.002	0.001	0.001	0.001	-							
T. douronensis	8. Sabah	0.082	0.082	0.082	0.081	0.081	0.081	0.081	-						
	9. Layar/Spak	0.071	0.071	0.071	0.069	0.070	0.068	0.068	0.042	-					
	10. Batang Ai	0.072	0.071	0.071	0.073	0.073	0.075	0.073	0.046	0.024	-				
	11. Ba Kelalan	0.072	0.071	0.071	0.073	0.073	0.076	0.074	0.047	0.027	0.006	-			
	12. Ulu Limbang	0.071	0.071	0.071	0.072	0.073	0.075	0.073	0.046	0.026	0.005	0.001	-		
	13. Bario	0.074	0.073	0.073	0.075	0.075	0.078	0.076	0.047	0.027	0.006	0.005	0.004	-	
	14. *N. stracheyi*	0.083	0.083	0.083	0.081	0.081	0.078	0.080	0.079	0.077	0.085	0.087	0.086	0.086	-

ACKNOWLEDGEMENTS

We thank Dr Stephen Sungan and officers from the Indigenous Fish Production Research Centre (IFPRC), Department of Agriculture, Sarawak, for their assistances in sample collections. We also thank the Fisheries Department of Malaysia for their full support throughout the project, members of the Molecular Ecology Laboratory, Universiti Malaysia Sarawak, members of the Genetics Laboratory, Universiti Putra Malaysia, and all the people involved in the sample collection and laboratory work.

REFERENCES

Briolay, J., Galtier, N., Brito, R.M. and Bouvet, Y. (1998). Molecular phylogeny of Cyprinidae inferred from cytochrome *b* DNA sequences. *Molecular Phylogenetics and Evolution, 9,* 100-108.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution, 7,* 1193-1204.

Grewe, P.M., Kruger, C.C., Aquadro, C.F., Bermingham, E., Kincaid, H.L. and May, B. (1993). Mitochondrial variation among lake trout (*Salvelinus namaycush*) strains stocked into Lake Ontorio. *Canadian Journal of Fish and Aquatic Science, 50,* 2397-2403.

Inger, F.R. and Chin, P.K. (1962). The freshwater fishes of North Borneo. Chicago: Chicago Natural History Museum.

Kottelat, M. and Whitten, A.J. (1996). *Freshwater fishes of Western Indonesia and Sulawesi: Additions and Corrections.* Hong Kong: Periplus Editions.

Kottelat, M., Whitten, A.J., Kartokasari, S.N. and Wirjoratmodjo, S. (1993). *Freshwater Fishes of Western Indonesia and Sulawesi.* Singapore: Berkeley Book Pte Ltd.

Kumar, S., Tamura, K. and Nei, M. (2004). MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings in Bioinformatics, 5,* 150-163.

Mohsin, A.K.M. and Ambak, M.A. (1983). *Freshwater Fishes of Peninsular Malaysia*. Serdang, Selangor: Universiti Pertanian Malaysia Publication.

Ng, C.K. (2004). *Kings of the Rivers: Mahseer in Malaysia and the Region.* Selangor: Inter Sea Fishery (M) Pte Ltd.

Nguyen, T.T.T., Ingram, B., Sungan, S., Gooley, G., Sim, S.Y., Tinggi, D. and De Silva, S.S. (2006). Mitochondrial DNA diversity of broodstock of two indigenous mahseer species, *Tor tambroides* and *Tor douronensis* (Cyprinidae) cultured in Sarawak. *Aquaculture, 253,* 259-269.

Palumbi, S., Martin, A., Romano, S., McMilan, W.O., Stice, L. and Grabowski, G. (1991). The simple fool's guide to PCR. Department of Zoology & Kewalo Marine Laboratories, University of Hawaii, Honolulu.

Rainboth, W.J. (1996). *Fishes of the Cambodian Mekong. FAO Species Identification Field Guide for Fishery Purposes.* Food and Agriculture Organization (FAO) Publication, Rome.

Roberts, T.R. (1989). *The Freshwater Fishes of Western Borneo (Kalimantan Barat, Indonesia).* California Academy of Sciences, California.

Roberts, T.Y. (1999). Fishes of the cyprinid genus *Tor* in the Nam Theun watershed (Mekong Basin) of Laos, with description of new species. *Raffles Bulletin of Zoology, 47,* 235-236.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4,* 406-425.

Simon, C., Frati, F., Beckhenbach, A., Crespi, B., Liu, H. and Flook, P. (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America, 87*(6), 651-701.

Tamura, K. and Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution, 10,* 512-526.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmourgin, F. and Higgins, D.G. (1997). The CulstalX windows interface: flexible stratergies for multiple sequence alignment aided by the quality analysis tools. *Nucleic Acids Research, 24,* 4876-4882.

Zhou, W. and Chu, G-H. (1996). A review of *Tor* species from the Lancangjiang River (Upper Mekong River), China (Teleostei: Cyprinidae). *Ichthyological Exploration of Freshwater, 7,* 131-142.