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Phylogenetic evaluation of subfamily classification of the Cyprinidae focusing on Vietnamese species

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Abstract – The Cyprinidae is the largest freshwater fish family in Vietnam, with over 220 recognised species, many of which play an important role in aquaculture or are harvested from the wild. Despite numerous studies on the taxonomy of this family based on traditional morphological data, the relationships between major cyprinid groups is poorly understood and the taxonomic validity of a number of these groups is under debate. While an increasing number of molecular studies on cyprinid relationships have been conducted many have used restricted sampling and none have incorporated Vietnamese species. In this study, mitochondrial 16S rRNA, D-loop and cytochrome *b* gene sequences from 25 species of cyprinids collected from Vietnam were obtained and combined with sequences of cyprinids available in GenBank, in order to investigate the taxonomic validity of subfamilies within Cyprinidae and their phylogenetic relationships. The molecular data supported traditional division of the Cyprinidae into two major lineages: Cyprinines and Leuciscines. The placement of the Danioninae as the sister lineage to this grouping was not supported. Many of the subfamily boundaries were questioned and doubt was raised on some of the generic level classifications. The validity of species designation in *Cyprinus*, *Tor* and *Cyclocheilichthys* was also questioned. This study will need to be extended with greater taxon and gene sampling to further consolidate our understanding of cyprinid relationships and classification.

Résumé – Evaluation phylogénétique de la classification en sous-familles des Cyprinidae, en considérant les espèces du Vietnam en particulier. La plus grande famille de poissons d'eau douce, au Vietnam, est celle des Cyprinidae, avec plus de 220 espèces identifiées ; la plupart de ces espèces joue un rôle important en aquaculture ou bien sont pêchées. Bien que de nombreuses études sur la taxonomie de cette famille soient basées sur des données morphologiques traditionnelles, les relations entre les grands groupes de Cyprinidae sont peu connues, et la validité taxonomique de ces groupes reste à discuter. Pourtant, un nombre croissant d'études moléculaires sur les relations entre les groupes de Cyprinidae ont été conduites, mais souvent sur des échantillonnages réduits, et aucune n'a tenu compte des espèces du Vietnam. Dans cette étude, les gènes mitochondriaux codant pour l'ARNr 16S, *D-loop* (la région de contrôle) et le cytochrome *b* de 25 espèces de Cyprinidae collectés au Vietnam ont été obtenus et combinés avec ceux des Cyprinidae disponibles dans « GenBank », en vue d'analyser la validité taxonomique des sous-familles de Cyprinidae et leurs relations phylogénétiques. Les données moléculaires s'appuient sur la division traditionnelle des Cyprinidae en deux grandes lignées : Cyprininae et Leuciscinae. La place des Danioninae en tant que lignée soeur n'est pas soutenue. Les frontières entre de nombreuses sous-familles sont floues, et soulèvent quelques doutes sur le niveau de classification au niveau des genres. La validité des espèces désignées comme *Cyprinus*, *Tor* and *Cyclocheilichthys* pose question. Cette étude nécessite l'échantillonnage d'autres taxons et l'étude d'autres gènes afin de consolider notre compréhension des relations entre les différents Cyprinidae et leur classification.

Key words: Phylogeny / Mitochondrial RNA sequence / Cytochrome b / Cyprinid/ Asia

1 Introduction

The Cyprinidae is the largest freshwater fish family in the world with over 200 genera and 2000 species (Liu and Chen 2003). Approximately, 3400 species of the order of Cypriniformes occur all over the world (Saitoh et al. 2006). While the family has a relatively diverse fauna in Africa, Europe and North America, over 1200 species are recorded from Asia with the centre of diversity being China and South East Asia (Liu and Chen 2003). A large number of well known fish species belong to the Cyprinidae including the barbels, the common carp, goldfish, chubbs and roach. The family also contains many species important to aquaculture and inland fish

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production with an annual world production over 17 million tons (FAO 2003). The Cyprinidae is, thus, perhaps the most important taxonomic group of fish consumed by humans.

As with many Asian countries, Vietnam has an abundant cyprinid fauna with over 220 recognised species. Members of the family play an important role in aquaculture in Vietnam (Nguyen and Ngo 2001). There are 13 indigenous and five introduced species that contribute to about 75% of inland fish production in the country. Cyprinids are mainly cultured in polyculture systems, the main species being silver carp *Hypophthalmichthys molitrix* (Valenciennes 1844), grass carp *Ctenopharyngodon idella* (Valenciennes 1844), bighead carp *Aristichthys nobilis* (Richardson 1845), rohu C*tenopharyngodon idella* (Valenciennes 1844), *Labeo rohita* (Hamilton 1822), mrigala *Cirrhinus cirrhosus* (Bloch 1795) and local fish species such as common carp *Cyprinus carpio carpio* Linnaeus 1758. A number of Vietnamese cyprinids have restricted distributions and are threatened due to overfishing, interbreeding between indigenous and introduced exotic species or translocated native species (Nguyen and Ngo 2001; Nguyen et al. 2005), environmental degradation and anthropogenic changes such as construction of reservoirs and hydroelectric dams.

Taxonomically, the Cyprinidae have been divided into a greater or lesser number of subfamilies (Chen et al. 1984; Rainboth 1996; Nguyen and Ngo 2001). For example, Chen et al. (1984), based on a cladistic analysis divided the Cyprinidae into 10 subfamilies (Labeoninae + Cyprininae + Barbinae + Tincinae + Acheilognathinae + Gobioninae + Xenocyprinae + Cultrinae + Leuciscinae + Danioninae or Rasborinae). In contrast, Rainboth (1996) divided the Cyprinidae into just four subfamilies (Alburinae + Danioninae + Leuciscinae + Cyprininae).

The taxonomic confusion and uncertainties within the Cyprinidae are evident by considering just the taxonomic treatment of Vietnamese cyprinids. Mai (1978) recognised 9 subfamilies (Cyprininae + Barbinae + Acheilognathinae + Gobioninae + Gobiobotinae + Xenocyprinae + Cultrinae + Leuciscinae + Hypophthalmichthyinae). In contrast, Truong and Tran (1993) and Mai et al. (1992) placed Vietnamese cyprinids just into four groups (Cyprininae + Abraminae + Rasborinae + Garrinae). Nguyen and Ngo (2001) divided Cyprinidae in Vietnam into 11 subfamilies (Labeoninae + Cyprininae + Barbinae + Acheilognathinae + Gobioninae + Gobiobotinae + Xenocyprinae + Cultrinae + Leuciscinae + Danioninae + Hypophthalmichthyinae). Such contrasting opinions on cyprinid classification hinder evolutionary, biogeographic and even comparative studies, and are clearly undesirable for such a widespread and important group of fishes.

Molecular phylogenetic studies are increasingly being used to investigate cyprinid classification and evolution at a variety of taxonomic levels including the validity of various families and their inter-relationships using nucleotide sequences from the mitochondrial DNA (mtDNA) Cytochrome b (Cyt b) (Briolay et al. 1998; Gilles et al. 1998; Zardoya and Doadrio 1998; Zardoya et al. 1999; Fuchs et al. 2000; Cunha et al. 2002; Durand et al. 2002) and D-loop (Gilles et al. 2001; Liu and Chen 2003) and more recently from nuclear DNA (Wang et al. 2007). While these studies bring important new insight into the evolutionary history of the family and its taxonomic classification, most studies have focused on European, Eurasian, North America and East Asian cyprinids with the sampling of species from South East Asia including Vietnam having been neglected so far.

In the present study, sequences of the mitochondrial DNA 16S rRNA (16S), D-loop and Cyt*b* fragments were used to evaluate taxonomic and phylogenetic relationships within the Cyprinidae. Using sequences obtained from previous studies and from a set of species obtained from Vietnam, subfamily groupings are critically examined and the relationships suggested by Chen et al. (1984) and Cavender and Coburn (1992) and Gilles et al. (2001) are evaluated using maximum likelihood based hypothesis testing procedure (Shimodaira and Hasegawa 1999).

2 Materials and methods

2.1 Sample collection

Vietnamese cyprinid species were identified using the taxonomies of Cavender and Coburn (1992) and Nguyen and Ngo (2001). Tissue samples of several cyprinid species were obtained from fish kept in the National Brood Stock Center of Research Institute for Aquaculture No 1, Hai Duong, Vietnam (RIA1). These fish were originally obtained by Fish Gene Conservation Programs in 2004 and 2005, a national conservation initiative by the Vietnam Government. Additional fish samples were collected from lakes, reservoirs and rivers in Vietnam using seine net and baited traps. Tissue samples were preserved in 95% ethanol and voucher specimens were preserved in 70% ethanol and deposited in the Fish Museum of RIA1. Details of sampled species, GenBank accession numbers and collection localities are given in Table 1.

2.2 DNA extraction and PCR amplification

Total DNA was extracted from fin-clip tissue, following the Crandall et al. (1999) method. One individual was first analysed by direct sequencing from each species. Twenty nine fish tissue samples were sequenced. The Cyt*b* gene was polymerase chain reaction amplified using the primers H15891 (5'GTT TGA TCC CGT TTC GTG TA 3') and L 15267 (5' AAT GAC TTG AAG AAC CAC CGT 3') (Briolay et al. 1998). The D-loop was amplified by using the primers Carp-Pro (5' AAC TCT CAC CCC TGG CTA CCA AAG 3'), and Carp-Phe (5' CTA GGA CTC ATC TTA GCA TCT TCA GTG 3') (Thai et al. 2004). The 16*S* region was amplified using the primers 16Sar (5' GCC TGT TTA ACA AAA ACA T 3') and 16Sbr (5' CCG GTCTGA ACT CAG ATC ATG T 3') (Simon et al. 1991). PCR was carried out in 50 μl reaction volumes (1 X reaction buffer, 2 mM dNTP, 1.5 mM $MgCl_2$, 0.5 μM of each primer, 0.5 units *Taq* polymerase, and approximately 200 ng DNA template). Thermal cycling comprised 95 °C for 3 min, followed by 34 cycles of 95 °C for 30 s, annealing at 55 °C (D-loop and Cyt *b*) and 58 °C (16S) for 30 s, and an extension temperature of 72 °C for 1 min. This was then followed by a final extension of 72 °C for 3 min. PCR products

Table 1. Species, sampling localities, GenBank accession, and numbers subfamily designation as proposed by previous studies for all samples used. (* samples from this study).

Species	Code	Locality	*Cyt b*	*16S*	*D-loop*	Chen et al. (1984)	Cavender & Coburn (1992)	Gilles et al. (2001)	Nguyen & Ngo (2001)
Ancherythroculter daovantieni	ANC*	Vietnam	DQ464975	DQ464929	DQ464940	Cultrinae	Cultrinae	Cultrinae	Cultrinae
Aristichthys nobilis	BHC*	Vietnam	DQ464976	DQ464908	DQ464949	Xenocyprinae	Xenocyprinae	Xenocyprinae	Hypophthalmichthyinae
Barbonymus gonionotus	BOB*	Vietnam			DQ464945	Barbinae	Barbinae	Barbinae	Barbinae
Barbus fluviatilis	BAF	Europe			AJ388415	Barbinae	Barbinae	Cyprininae	Cyprininae
Barbus meridionalis	BAM	Europe			AJ388417	Barbinae	Barbinae	Cyprininae	Cyprininae
Carassioides phongnhaensis	CHA*	Vietnam			DQ464946	Cyprininae	Cyprininae	Cyprininae	Cyprininae
Carassioides cantonensis	NHU*	Vietnam	DQ464980	DQ464930	DQ464962	Cyprininae	Cyprininae	Cyprininae	Cyprininae
Carassius auratus	CRU*	Vietnam	DQ464978	DQ464926	DQ464961	Cyprininae	Cyprininae	Cyprininae	Cyprininae
Chondrostoma nanus	CHO	Europe	AY026402	AJ247047	AJ388396	Leuciscinae	Leuciscinae	Leuciscinae	Leuciscinae
Cirrhinus cirrhosus	MRI*	Vietnam	DQ464981	DQ464904	DQ464952	Labeoninae	Labeoninae	Labeoninae	Labeoninae
Cirrhinus molitorella	MUD*	Vietnam	DQ464968	DQ464921	DQ464964	Labeoninae	Labeoninae	Labeoninae	Labeoninae
Crossostoma lacustre(outgroup)	CRO	Taiwan	M91245	M91245	M91245	Balitorinae	Balitorinae	Balitorinae	Balitorinae
Ctenopharyngodon idellus	GRC*	Vietnam	DQ464983	DQ464928	DQ464953	Leuciscinae	Xenocyprinae	Leuciscinae	Leuciscinae
Culter alburnus	CUT	China			AY095331	Cultrinae	Cultrinae	Cultrinae	Cultrinae
Culter mongolieus	CUM	China			AY095329	Cultrinae	Cultrinae	Cultrinae	Cultrinae
Culter oxycephaloides	CUO	China			AY095328	Cultrinae	Cultrinae	Cultrinae	Cultrinae
Cultrichthys erythropterus	THI*	Vietnam	DQ464977	DQ464934	DQ464954	Cultrinae	Cultrinae	Cultrinae	Cultrinae
Cyclocheilichthys repasson	CLO*	Vietnam			DQ464938	Barbinae	Barbinae	Barbinae	Barbinae
Cyclocheilichthys apogon	CYL*	Vietnam	DQ464989	DQ464918	DQ464955	Barbinae	Barbinae	Barbinae	Barbinae
Cyprinus carpio	XIN*	China	AY347282	DQ864655	DQ532110	Cyprininae	Cyprininae	Cyprininae	Cyprininae
Cyprinus carpio	BBC1	China			AY347303	Cyprininae	Cyprininae	Cyprininae	Cyprininae
Cyprinus carpio	LBW	Japan	AB158803	AP009047	AB158808	Cyprininae	Cyprininae	Cyprininae	Cyprininae
Cyprinus carpio	HUS*	Hungary	DQ532114	DQ864654	AY597981	Cyprininae	Cyprininae	Cyprininae	Cyprininae
Cyprinus carpio	CYC*	Vietnam	DQ464969	DQ464909	DQ464944	Cyprininae	Cyprininae	Cyprininae	Cyprininae
Cyprinus melanes	CYM*	Vietnam	DQ464970	DQ464910	DQ464943	Cyprininae	Cyprininae	Cyprininae	Cyprininae
Dangila lineatus	DAN*	Vietnam	DQ464991	DQ464907	DQ464939	Labeoninae	Labeoninae	Labeoninae	Labeoninae
Danio rerio	DAR	Europe	NC002333	NC002333	NC002333	Danioninae	Rasborinae	Rasborinae	Rasborinae
Discogobio tetrabarbatus	DIS	China			AY095326	Labeoninae	Labeoninae	Labeoninae	Labeoninae

Table 1. Continued.

Species	Code	Locality	*Cyt b*	*16S*	*D-loop*	Chen et al. (1984)	Cavender & Coburn (1992)	Gilles et al. (2001)	Nguyen & Ngo (2001)
Distoechodon tumirostros	DTT	China			AY014165	Xenocyprinae	Xenocyprina	e Xenocyprinae	Xenocyprinae
Gobio gobio 1	GOB	Europe			AJ388393	Gobioninae	Gobioninae	Gobioninae	Gobioninae
Gobio gobio 2	GOG	Europe	AJ388431	AJ247056	AJ388392	Gobioninae	Gobioninae	Gobioninae	Gobioninae
Gobiobotia filifer	GOF	China			AY095341	Gobiobotinae	Gobiobotinae	Gobiobotinae	Gobiobotinae
Hampala macrolepidota	HAN*	Vietnam	DQ464974	DQ464916	DQ464947	Barbinae	Barbinae	Barbinae	Barbinae
Hemicutter leucisculus	MXA*	Vietnam	DQ464973	DQ464923	DQ464957	Cultrinae	Cultrinae	Cultrinae	Cultrinae
Hypophthalmichthys molitrix	SIL*	Vietnam	DQ464966	DQ464936	DQ464958	Xenocyprinae	Xenocyprinae	Xenocyprinae	Hypophthalmichthyinae
Labeo bicolor	LBI	Europe			AJ388414	Cyprininae	Cyprininae	Cyprininae	Cyprininae
Labeo rohita	ROH*	Vietnam	DQ464965	DQ464935	DQ464950	Labeoninae	Labeoninae	Labeoninae	Labeoninae
Leuciscus cabeda	LEU	Europe			AJ388406	Leuciscinae	Leuciscinae	Leuciscinae	Leuciscinae
Leuciscus cephalus	LEC	Europe	AJ252805	AJ247054	AJ388407	Leuciscinae	Leuciscinae	Leuciscinae	Leuciscinae
Leuciscus sofia	LES	Europe			AJ388398	Leuciscinae	Leuciscinae	Leuciscinae	Leuciscinae
Lobocheilos melanotaenia	LOB*	Vietnam	DQ464990	DQ464917	DQ464948	Labeoninae	Labeoninae	Labeoninae	Labeoninae
Mylopharyngodon piceus	BLC*	Vietnam	DQ464971	DQ464905	DQ464937	Leuciscinae	Xenocyprinae	Leuciscinae	Leuciscinae
Paracheilognathus imberbis	PAR	China			AY017147	Acheilognathinae	Acheilognathinae	Acheilognathinae	Acheilognathinae
Puntius brevis	PUB	Vietnam	DQ464967	DQ464912	DQ464942	Barbinae	Barbinae	Barbinae	Barbinae
Rasbora trilineata	RAT	Europe			AJ388423	Danioninae	Rasborinae	Rasborinae	Rasborinae
Rhodeus amarus	RHA	Europe			AJ388412	Acheilognathinae	Acheilognathinae	Acheilognathinae	Acheilognathinae
Rutilus rubilio	RUT	Europe			AJ388400	Leuciscinae	Leuciscinae	Leuciscinae	Leuciscinae
Schizothorax chongi	SCH	China			AY095325	Schizothoracinae	Schizothoracinae	Schizothoracinae	Schizothoracinae
Semilabeo obscurus	AVU*	Vietnam	DQ464988	DQ464913	DQ464963	Labeoninae	Labeoninae	Labeoninae	Labeoninae
Sinibrama macrops	SIM	China			AY095332	Cultrinae	Cultrinae	Cultrinae	Cultrinae
Spinibarbus denticulatus	BON*	Vietnam	DQ464984	DQ464906	DQ464956	Barbinae	Barbinae	Barbinae	Barbinae
Tinca tinca	TIN	Europe	Y10451	AJ247053	AJ388411	Tincinae	Tincinae	Tincinae	Tincinae
Tor duronensis	TOD*	Vietnam	DQ464986	DQ464925	DQ464959	Barbinae	Barbinae	Barbinae	Barbinae
Tor stracheyi	TOS*	Vietnam	DQ464987	DQ464915	DQ464951	Barbinae	Barbinae	Barbinae	Barbinae
Tor tambroides	TOT*	Vietnam	DQ464985	DQ464914	DQ464960	Barbinae	Barbinae	Barbinae	Barbinae
Toxabramis houdemeri	TOX*	Vietnam	DQ464972	DQ464924	DQ464941	Cultrinae	Cultrinae	Cultrinae	Cultrinae
Xenocypris hupeiensis	XEH	China			AY014164	Xenocyprinae	Xenocyprinae	Xenocyprinae	Xenocyprinae

Table 2. Major hypotheses for phylogenetic relationships of cyprinid species.

Source	Topology
Chen et al. (1984)	(((Ba, Cy), La), Ti), (Da, (Go, ((Xe, Cu), Le)))
Cavender and Coburn (1992)	(((((((Ba, Cy), La), Ti), (Xe, Cu)), Le), Go), Da)
Gilles et al. (2001)	(((((((Ba (Cy, La), Ti), (Xe, Cu)), Go), Le), Da)

Ba: Barbinae; Cy: Cyprininae, La: Labeoninae; Ti: Tincinae; Da: Danioninae; Go: Gobioninae; Xe: Xenocyprinae; Cu: Cultrinae; Le: Leuciscinae.

were purified using the Qiagen (Hiden Germany) QIA quick PCR purification kit, following ABI PRISM BigDye Terminator (Foster city, CA, USA) protocols. For each individual, sequencing reactions were performed using both primers.

2.3 Data analysis

According to Gilles et al. (2001), and Liu et al. (2002), both morphology and molecular genetic data supports a monophyletic Cyprinidae. Following Liu and Chen (2003), sequences of *Crossostoma lacustre* from the Balitoridae (GenBank access number: M91245) were used as the outgroup. Two data sets were assembled for the analysis of cyprinid relationships. For the first data set, the 29 D-loop sequences generated in this study were combined with 27 D-loop sequences of the same length available for cyprinid species from GenBank. The second data set consisted of D-loop, 16S and Cyt *b* sequences obtained in this study from 23 species and were combined with 9 additional cyprinid species for which sequences for these same mtDNA regions and length are available from GenBank.

Sequences were aligned using CLUSTAL X (Thompson et al. 1997). The tree length frequency distribution skewness statistic ($g1$) was calculated by exhaustive search to test for presence of a significant phylogenetic signal in each data set (Hillis and Huesenbeck 1992). Four tree building methods were used to reconstruct phylogenetic relationships. Maximum-likelihood (ML), neighbour-joining (NJ) and maximum parsimony (MP) methods were implemented using PAUP* version 4.0b10 (Swofford 2001); and the Bayesian method was carried out using MrBayes 3.0 (Huelsenbeck and Ronquist 2001). The appropriate model of evolution for ML, NJ and Bayesian analyses was obtained via testing alternative models of evolution using Modeltest (Posada and Crandall 1998). Heuristic searches used for ML analyses consisted of 100 replicates of random sequence additions, while non-parametric bootstrapping consisted of 100 replications with 10 random sequence additions. MP analyses were performed with gaps treated as missing data, heuristic searches as per maximum-likelihood analyses, but with 1000 non-parametric bootstrap replicates. The NJ tree was constructed with distances calculated under the same model of evolution as the ML analysis, with bootstrapping performed using 1000 replicates. Bayesian analyses were performed using the same general model identified by Modeltest. Analyses were initiated with random starting trees and run for 1.0×10^6 generations, sampling the four Markov chains every 100 generations resulting in 10 000 trees. The likelihood scores of the sampled trees were plotted against generation time to ensure that stationarity was reached, trees generated prior to stationarity being reached were discarded as "burn-in" (1500 trees in this case). Bayesian posterior probabilities of each bipartition, representing the percentage of times each node was recovered were calculated from a 50% majority rule consensus of the remaining trees.

Table 3. Summary of results of phylogenetic analysis in three mitochondrial DNA gene regions of cyprinid species.

Characters	*16S*	*Cyt b*	*D-loop*
Number of base pairs	446	582	758
% Variable sites	13.2	7.0	17.3
% Parsimony informative	10.5	4.0	46.4
Transition /Transversion	3.1	2.1	1.4
Sequence divergence (%)	0.5–17.2	0.53–26	0.62–38
Skeweness ($g1$)*	–0.4	–0.5	–0.5
Model of evolution	HKY + I + G	GTR + I	HKY + I + G

* Significance level ($p < 0.01$)

2.4 Phylogenetic hypothesis testing

To test taxonomic and phylogenetic hypotheses proposed by other authors, comparisons were made between trees derived from these hypotheses and the optimal trees recovered by our analysis, using the SH test (Shimodaira and Hasegawa 1999). Due to incomplete or limited taxonomic sampling Gobiobotinae, Acheilognathinae, Phoxininae, Alburninae and Schizothoracinae were excluded from hypothesis testing. Three hypotheses for taxonomic and phylogenetic relationships of Vietnamese cyprinid species, as proposed by Chen et al. (1984), Cavender and Coburn (1992) and Gilles et al. (2001) studies, were tested (Table 2).

3 Results

3.1 Sequence variation

All sequences obtained in this study have been submitted to GenBank (accession numbers DQ464904-464992; DQ864654-864655). A summary of the characteristics of each mitochondrial region is presented in Table 3. It can be seen from Table 3 that all three fragments show significant phylogenetic signal (based on $g1$ values). The *D-loop* sequences show the most variation and the *16S* region the least.

The combined 16S/Cyt*b*/D-loop data set consisted of 1786 aligned nucleotide positions. Of these, 861 were variable and 636 parsimony informative. The partition homogeneity test did not reject phylogenetic congruence between these

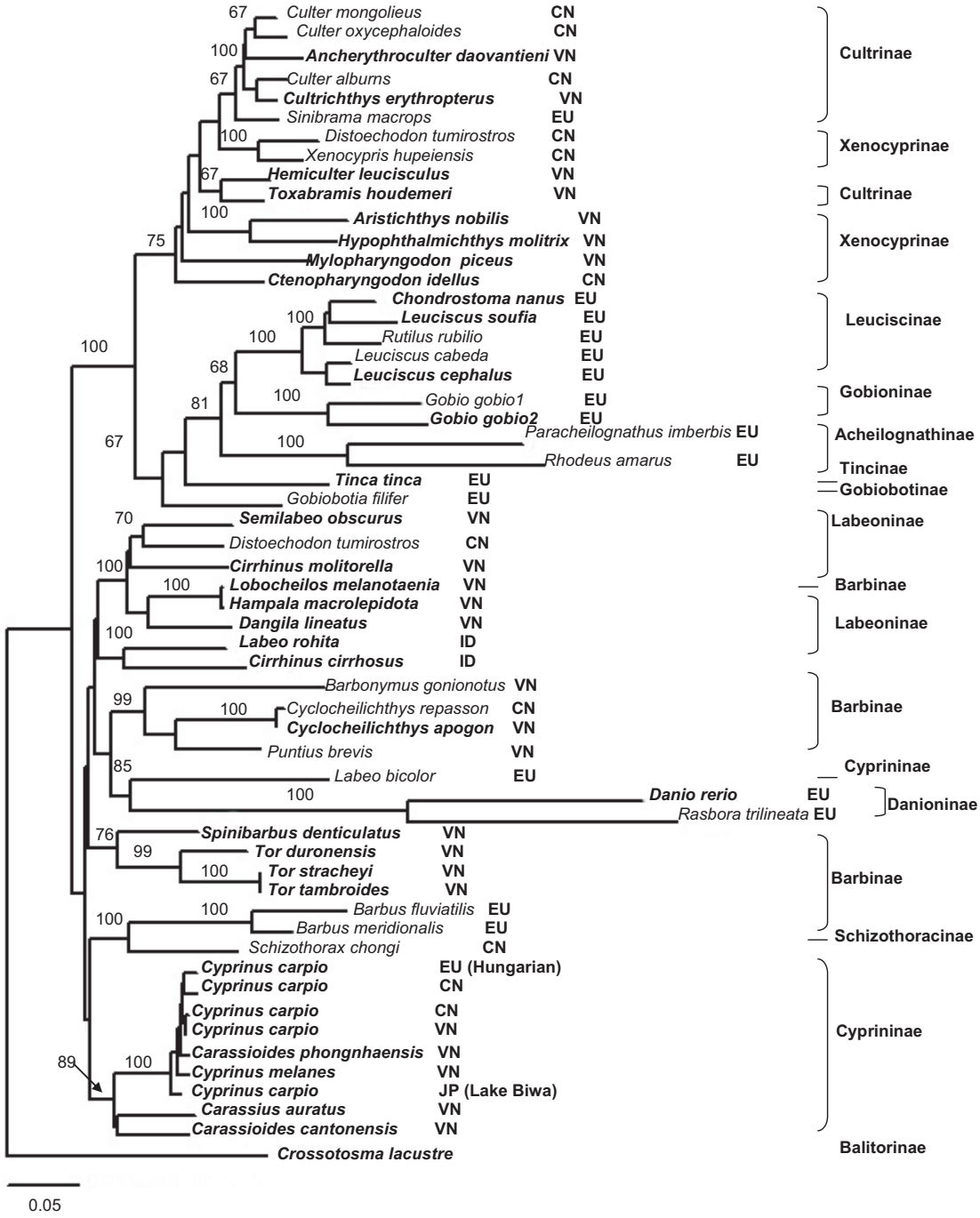

Fig. 1. Phylogenetic tree resulting from neighbour-joining analysis of mitochondrial DNA *D-loop* of 51 cyprinid species. Numbers on each branch represent bootstrap support value. The subfamily groups are based on Cavender and Coburn (1992).VN: Vietnam, CN: China, ID: India, JP: Japan, EU: Europe. Bold indicates samples used in combined three region data sets.

mtDNA fragments ($p > 0.05$), allowing their combination for phylogenetic analyses. Tree length frequency distributions were significantly skewed for all taxa ($g1 = -1.25$; $p < 0.05$), suggesting the presence of phylogenetic signal. The model selection for the NJ and ML analysis was Hasegawa-Kishino-Yano (HKY) + invariant sites (I) + gamma distribution shape parameter (G) which accommodates differing transition/ transversion mutation rates (Hasegawa et al. 1985). Percentage sequence divergence among taxa ranged from 0% (*Tor tambroides* and *Tor stracheyi*) to 31.6% (*Danio rerio* and *Hampala macrolepidota*).

3.2 Phylogenetic analysis

Following the classification of Cavender and Coburn (1992), the relationship among 51 cyprinid species

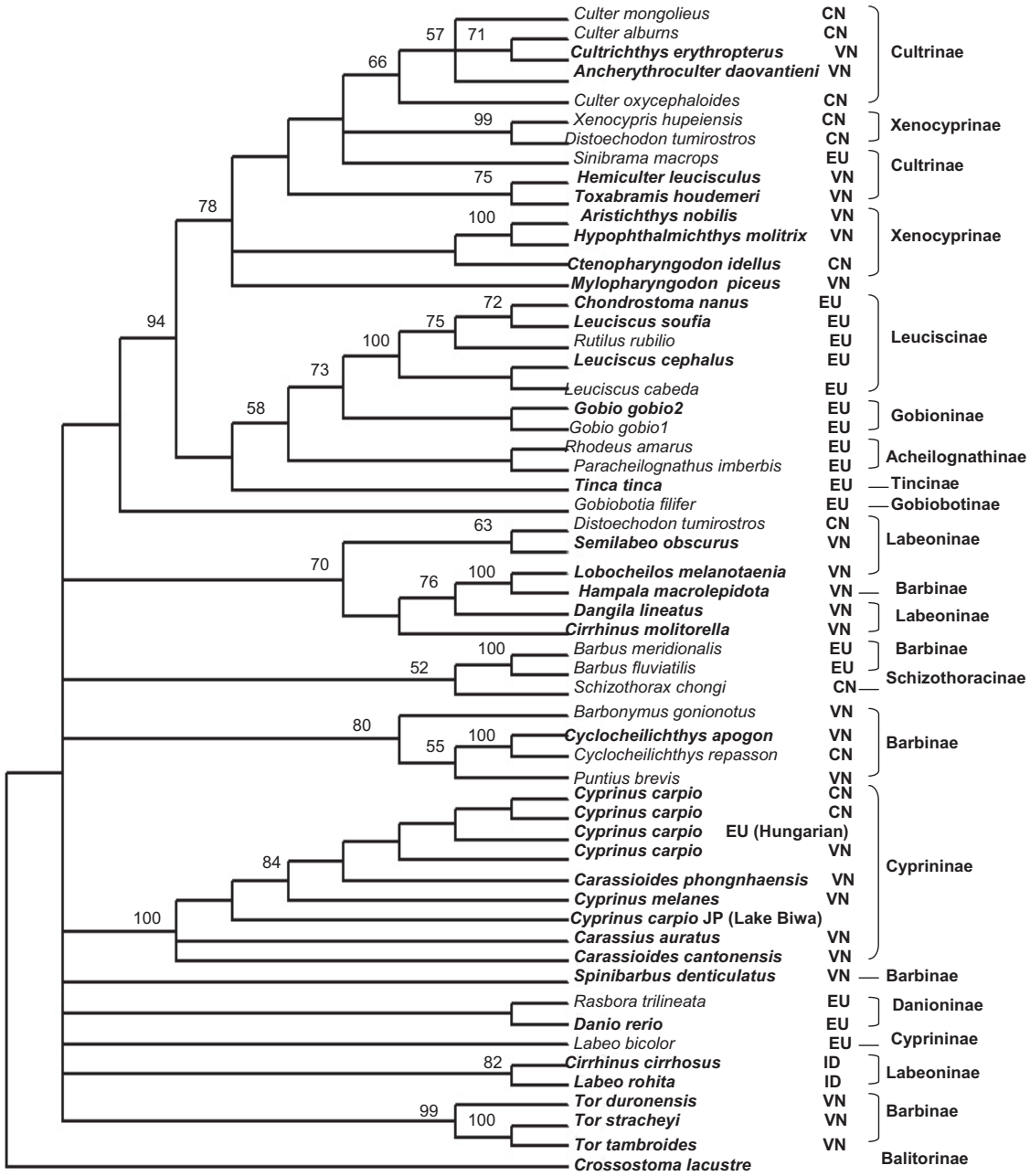

Fig. 2. Phylogenetic tree resulting from maximum parsimony analysis of mitochondrial DNA D-loop of 51 cyprinid species. Numbers on each branch represent bootstrap support value. The subfamily groups are based on Cavender and Coburn (1992).VN: Vietnam, CN: China, ID: India, JP: Japan, EU: Europe. Bold indicates samples used in combined three region data sets.

representing 41 genera and 12 subfamilies were evaluated using the *D-loop* sequences. In general, shallower relationships were resolvable to a much greater extent than at the deeper levels, which is consistent with the known rapid rate of evolution of this mtDNA region (Fig. 1). The NJ, ML and Bayesian methods of analysis generated almost identical relationships. Many of the relationships were unresolved using MP but those that were, mostly similar to the other analyses (Fig. 2). The tree indicated many inconsistencies with the current classification. At the family level, species of Cultrinae and Xenocyprinae did not form the two anticipated monophyletic groups, although together these species form a well supported monophyletic lineage. Sister to this lineage is a well supported group containing representatives of the Leuciscinae, Gobininae, Achelognathinae, Tincinae and Gobiotinae, entirely consistent with the current classification, although only the Leuciscinae is represented by more than two species.

The remaining species form a sister group to the two previously discussed lineages that failed to clarify deeper

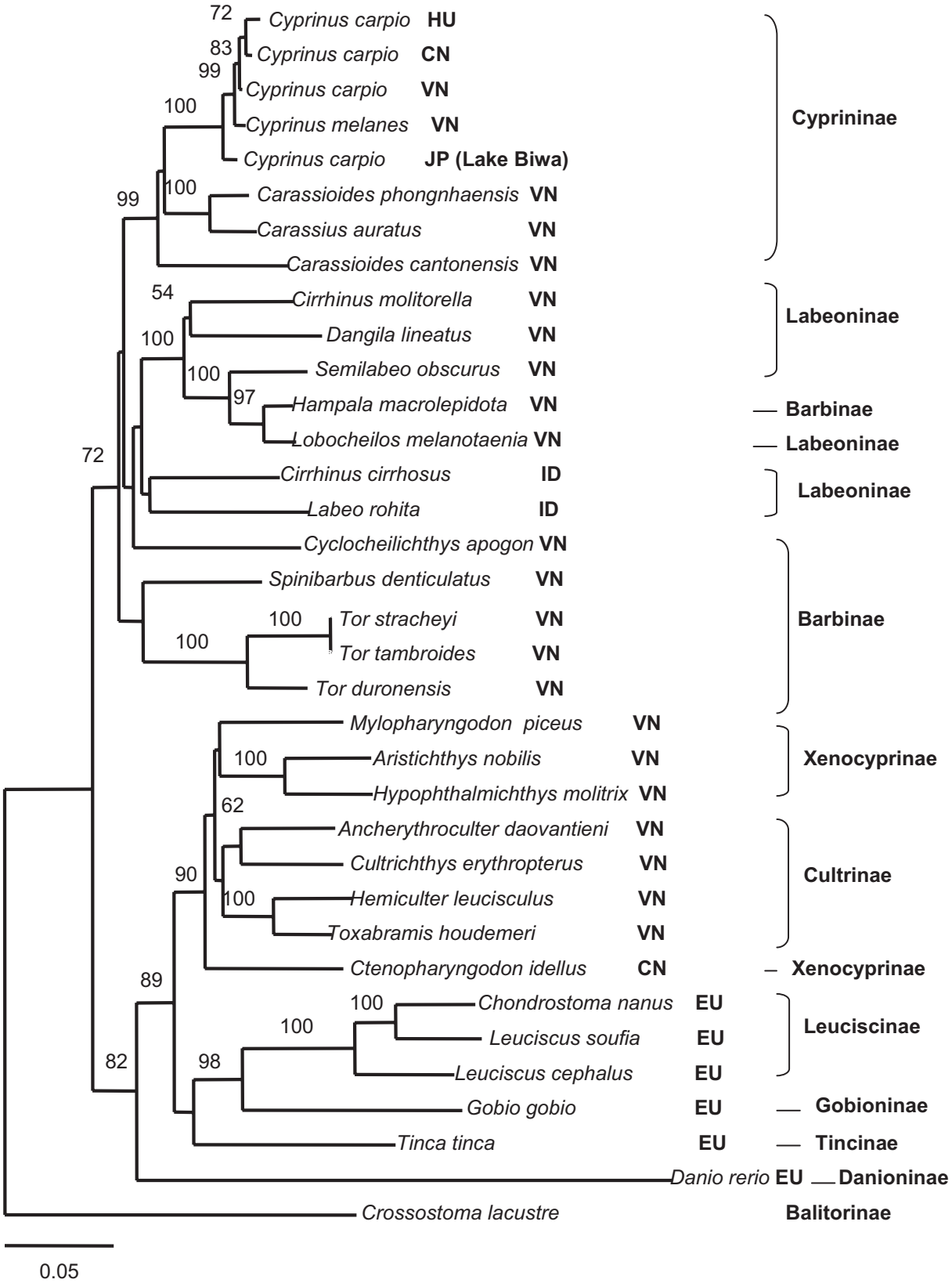

Fig. 3. Neighbour-joining tree from combined 16*S* , *Cyt b* and *D-loop* mtDNA data. Number on branches indicate bootstrap value. The subfamily groups are based on Cavender and Coburn (1992). VN: Vietnam, CN: China, ID: India, HU: Hungary, JP: Japan, EU: Europe.

level relationships. Further, the analyses do not support a monophyletic Barbinae with species of this subfamily distributed across four divergent lineages with varying levels of support. The Labeoninea is also non-monophyletic on the basis of the placement of *Hampala macrolepidota* (Barbinae) as sister to *Lobocheilos melamotaenia*. Otherwise there is some support for the Labeoninae as a natural group. In contrast the Cypriniae receives significant support as a monophyletic group based on five species representing three genera.

There are number of genera in the data set that are represented by two or more species and it is apparent that the morphologically based classification of cyprinids fails in many cases at this level as well. While the genera *Tor* (three species), *Barbus* (two species), *Cyclocheilichthys* (two species) and *Gobio* (two species) are monophyletic, the genera *Culter* (three species), *Leuciscus* (three species), *Cirrhinus* (two species), *Cyprinus* (two species) and *Carassioides* (two species) are all non-monophyletic.

An examination of divergence levels between species within genera, and species belonging to different genera, also indicates inconsistencies in the morphologically based cyprinid taxonomy. Divergence levels between monophyletic congeneric species supported by the phylogenetic analyses, range from 0.9% to 16.4% which overlaps broadly with divergence levels for monophyletic species pair placed in different genera which ranges from 0.5% to 38%. There was also no support for *Cyprinus melanes* as a distinct taxon as this sample clustered within the *C. carpio* sample with very low divergence levels (1–1.9%). Other species pairs that show very low levels of divergence are *Tor tambroides* and *Tor stracheyi* (0%) and *Cyclocheilichthys repasson* and *Cyclocheilichthys apogon* (1.3%), and are therefore of questionable status as valid species.

The data set consisting of the concatenated 16S, D-loop, and Cyt*b* sequences successfully clarified deeper level relationships, despite more limited taxon sampling. The different methods of analysis recovered similar results with the exception that the maximum likelihood analysis placed *Carassioides cantonensis*, rather than *Carassioides phongnhaensis* as a sister to *Carassius auratus* and the parsimony analysis placed *Danio rerio* in an alternative position. The cyprinids were divided into the same two major clades by each analysis, with the exception that *Danio rerio*, which was placed in a more basal clade either as sister to a clade containing (Tincinae, Gobioninae, Leuciscinae, Xenocyprinae and Cultrinae) (Fig. 3) or as sister to all other taxa (parsimony analysis tree not shown, see Thai 2007). Similarly to the D-loop analysis, all the European subfamilies (excluding Danioninae) form a monophyletic group which is sister to a clade containing representatives of the Xenocyprinae and Cultrinae. Also consistent with the D-loop only analysis, Xenocyprinae is polyphyletic and the monophyly of the Cultrinae is only weakly supported.

The other major lineage which is well supported by the concatenated data contains the Cypriniae, the Labeoninae, and the Barbinae. While the Cypriniae is supported as monophyletic the Barbinae and Labeoninae are polyphyletic which is also consistent with the *D-loop* analysis. All members of this lineage are from Asia with the exception of the sample of *C. carpio* from Hungary. The 16S, Cyt*b*, and D-loop analysis also fails to support several generic level groupings including *Leuciscus* (Leuciscinae), *Cirrhinus* (Labaoninae) and *Carassioides* (Cypriniae). Lastly, the analysis also fails to provide support for the recognition of *Cyprinus melanes* as a distinct species from common carp.

Table 4. Tests of alternate phylogenetic hypotheses using combine 16S, *D-loop*, *Cyt b* regions, Shimodaira-Hasegawa (SH) test.

Tree	lnL[1]	Diff-lnL	*p*
Optimal	16 787	(best)	
Chen et al. (1984)	16 888	97.3	0.000*
Cavender and Coburn (1992)	16 794	7.1	0.400
Gilles et al. (2001)	16 792	7.1	0.562
Cypriniae	16 799	21.0	0.530
Barbinae	16 737	47.3	0.120
Labeoninae	16 792	2.4	0.710
Xenocyprinae	16 892	72.0	0.001*
Cultrinae	16 873	81.0	0.035*
Leuciscinae	16 706	15.9	0.560

[1] log-likelihood.
* significant difference between optimal and alternate topologies, $p < 0.05$.

The testing of specific taxonomic hypotheses using the Shimodaira and Hasegawa (1999) procedure rejects several of them. The hypothesis of Chen et al. (1984) was rejected as significantly inferior to the optimal tree, as was also the monophyly of the Xenocyprinae and the Cutrinae. Based on the data set utilized in this study, none of the alternative taxonomic hypotheses could be statistically rejected, even though subfamilies such as Barbinae are non-monophyletic based on the reconstructed trees (Table 4).

4 Discussion

Comparison of the results of this study with the literature on Cyprinidae systematics is complicated because of the diversity of the family and the many and varied data sets with respects to kinds of data (molecular and morphological) and taxa sampled and methods of analysis that have been used (Gosline 1978; Chen et al. 1984; Howes 1991; Cavender and Coburn 1992; Nelson 2006; Gilles et al. 2001). Nevertheless some key points of agreement emerge between this and other studies.

At the deepest taxonomic level the results from the combined 16S/ Cyt*b*/ D-loop data supports a fundamental division between the Cyprinine (Cypriniae + Barbinae + Labeoninae + Schizothoracinae) and the Leuciscine (Leuciscinae + Acheilognathinae + Gobioninae + Gobiobotinae + Tincinae), with the Rasborinae or Danioninae joining the Leuciscine lineage at the most basal position in all analyses other than parsimony. This association of the Danioninae with the Leuciscine lineage, rather than as the sister group to (Leuciscine + Cyprinine) is consistent with the morphological based analysis of Cavender and Coburn (1992) and the molecular study of Liu and Chen (2003). However, with respect to the placement Danioninae, our results are contrary to both the morphologically based study of Chen et al. (1984) and Gilles et al. (2001) and Wang et al. (2007) molecular studies. The weight of evidence

would seem to favour the Danioninae as the sister lineage to the Leuciscinae as it is supported by two molecular studies using different tree building methods and does not require the independent evolution of a complex morphological trait associated with the pleural rib in two separate lineages (Gilles et al. 2001). However, it should be noted that the parsimony-based analysis in this study supported both Gilles et al. (2001) and Wang et al. (2007) studies, which may be a result of inherent limitations to this phylogenetic method known as "long-branch" attraction (Felsenstein 1978) because the *Danio rerio* samples are highly divergent from the other cyprinid samples. Further, while Chen's hypothesis could be rejected at very low level of significance, Gilles et al. (2001) hypothesis could not. Thus, this hypothesis requires further testing through greater taxon and gene sampling before it can be categorically refuted.

The close relationship of the Tincine to the Leuciscine taxa is supported by three previous molecular studies (Zardoya and Doadrio 1998; Gilles et al. 2001; Liu and Chen 2003) and by the Cavender and Coburn (1992) morphological study. This contradicts the morphological data of Chen et al. (1984). However, the precise relationships of the Tincines is uncertain as it is variously placed by different analyses as basal to the other Leuciscine taxa, as part of a polyphyletic node, or associated with a clade containing representative of the Gobioninae, Leuciscinae and Acheilognathinae.

Some strongly supported relationships such as between species of Cultrinae and Xenocyprinae is entirely consistent with both morphological (Cavender and Coburn 1992) and molecular studies (Liu and Chen 2003), although support for the monophyly of each subfamily is inconsistent based on this study and that of Liu and Chen (2003). Another strongly supported relationships which is consistent with Liu and Chen but is contrary to the morphologically based analyses of Cavender and Coburn (1992), is that between species of Leuciscinae and Gobioninae.

The other major lineage contains the Barbines and Labeonines, which together make up the Cyprinidae, a group that has been recovered by the major morphological analyses and all major molecular analyses (Zardoya and Doadrio 1999; Durand et al. 2002; Liu and Chen 2003). While this study suggested the Cyprinines may be monophyletic, it is still only based on relatively limited taxon sampling. In contrast, the data suggest that Labeoninae and Barbinae are non-monophyletic. Other studies sampling different species have concluded that the Barbinae is polyphyletic including the genus *Barbus* itself. In addition, Durand et al. (2002) commented that morphological characters are "sometimes irrelevant" in phylogenetic inference in the cyprinids. This comment is also seen to be at least partially true for several other genera that were found to be non-monophyletic in this study and the study of Gilles et al. (2001) including *Culter*, *Leuciscus*, *Labeo*, *Cirrhinus*, *Carassioides*, to which can be added *Rutilus* and *Chondrostoma*, based on Zardoya and Doadrio (1999).

The data from this study indicates that deficiencies in morphological information extend to the lowest taxonomic level. Thus, re-examination of species boundaries in several genera, including *Tor*, *Cyclocheilichthys* and *Cyprinus* are required. Base on morphological data, six species were identified in *Cyprinus*: *C. carpio* Linnaeus 1758; *C. melanes* Yen 1978; *C. multitaeniata* Pellegrin and Chevey 1936, *C. hyperdorsalis* Nguyen 1991, *C. exophthalmus* Mai 1978, and probably *C. quidatensis* Tu et al. 1999 (Nguyen and Ngo 2001). In this study, *C. carpio* and *C. melanes* are not differentiated at the molecular level. In fact, from the phylogenetic analysis, it is very clear that the levels of divergence within the genus *Cyprinus* are very modest compared with other genera and this analysis does not support the division of the genus into more than one species. Furthermore, *Carassioides phongnhaensis* may be more closely related to *Cyprinus* than to *Carassioides cantonensis*. Thus, *Carassioides phongnhaensis* may be a more appropriate outgroup for phylogenetic studies of *Cyprinus* and that the species could be placed in the genus *Cyprinus* rather than *Carassioides*.

In summary, this study confirms and contradicts elements of both morphological and molecular studies of the Cyprinidae at various taxonomic levels. Nevertheless, it is clear that there are two principal lineages within the Cyprinidae: Cyprinines and Leuciscines and that further molecular studies are required to define well supported monophyletic groups within each of these lineages that can be associated with existing named groups and morphological information. Such studies will need substantial taxon sampling to ensure the generality of the results and that stable taxonomic classification can be constructed.

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