PHYLOGENETIC RELATIONSHIPS OF CYPRINIDAE (TELEOSTEI: CYPRINIFORMES) INFERRED FROM THE cox1 GENE SEQUENCES

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ABSTRACT. The family Cyprinidae is the largest freshwater fish group in the world, including over 200 genera and 2100 species. Mitochondrial DNA investigations have been largely used to explore phylogenetic cyprinid relationships. A fragment of (302bp) from the mitochondrial DNA (mtDNA) subunit I citochrome oxidase gene (cox1) of cyprinid species was sequenced and compared in order to study their phylogenetic relationships. The topologies of neighbor-joining, maximum parsimony and maximum likelihood trees based on cox1 sequences allowed us to identify two major lineages in cyprinids: *Cyprininae* and *Leuciscinae*. The *Cyprininae* group is represented by *Carassius auratus auratus* and forms a separate branch in the trees. The *Leuciscinae* group includs *Rutilus rutilus, Tinca tinca, Hypophthalmichthys molitrix, Arischthys nobilis, Ctenopharyngodon idella, Leuciscus borysthenicus celensis* and *Leuciscus cephalus. Misgurnus fossilis* was used as outgroup species.

Keywords: molecular phylogeny, cox1 gene, cyprinid

INTRODUCTION

Cyprinids (*Cyprinidae*) are a major component of Eurasian temperate freshwater fish fauna with respect to the number both of individuals and of species (about 2010 reported species, Nelson, 1994). The role of this family within freshwater ecosystems is therefore central. They have considerable morphological variability, which is likely related to their highly diversified habitat. The relationship between this variability and the phylogeny of the group is an open, interesting question, relevant for the study of evolutionary rates of adaptative traits and for discriminating between convergences and shared traits due to common ancestry, i.e., true homologies. Several molecular phylogenetic studies have already used this family to define phylogenetic links (Briolay *et al.*,1998; Gilles *et al.,* 1998) and for biogeographical inferences (Brito *et al.,* 1997; Zardoya and Doadrio,1998, 1999; Durand *et al.,* 2002; Tsigenopoulos and Berrebi, 2000).

Mitochondrial DNA (mtDNA) has proved useful in molecular phylogenetic studies because evolutionary relationships can be inferred among higher levels, between recently divergent groups, populations, species and even individuals (Avise, 1994). Such data are helpful because molecular characters are less likely related to adaptative evolution than are morphologic characters. The mitochondrial 12S rRNA and 16S rRNA genes were analyzed for North American cyprinids (Simos and Mayeden, 1998, 1999). Phylogenetic relationships of the Cyprinidae in East Asia were inferred from the mitochondrial cytochrome b by He et al., (2001, 2004). Phylogeny of the lower level cyprinids in East Asia was reconstructed by Wang et al. (2002, 2004) based on cytochrome b and 1st intron of the *S7* ribosome protein gene. These studies bring new insight into the evolution and history of this family, but the systematic status of these species and genera with their phylogenetic links are still unclear.

The aim of this study was to identify the phylogenetic relationships of nine Romanian cyprinid species using the genetic variability of the cox1 gene already used in our previous phylogenetic studies (Luca et al., 2005a, 2005b) and also to check out whether similarity exists between the topology tree obtained by us and those obtained by others (Zardoya and Doadrio,1998, 1999). Ten species were included in this study: *Carassius auratus auratus, Rutilus rutilus, Barbus meridionalis, Tinca tinca, Hypophthalmichthys molitrix, Arischthys nobilis, Ctenopharyngodon idella, Leuciscus borysthenicus celensis, Leuciscus cephalus* and a cobitid species *(Misgurnus fossilis)* as an outgroup one.

MATERIALS AND METHODS

Sample Collection

The Nucet Fishery Research Centre provided the fish species required for analysis: *Carassius auratus auratus, Rutilus rutilus, Barbus meridionalis, Tinca tinca, Hypophthalmichthys molitrix, Arischthys nobilis, Ctenopharyngodon idella, Leuciscus borysthenicus celensis, Leuciscus cephalus*, *Misgurnus fossilis.*

Total genomic DNA was extracted with Wizard Genomic DNA Purification Kit (from Promega) according to the manufacturer's instructions (tissue protocol).

mtDNA Amplification and Sequencing

Conditions of amplification for the polymerase chain reaction (PCR) used in this study, as well as the sequencing protocol, were described in our previous studies (Luca *et al.,* 2005c). A primer set COX1-F (5`-AGC CTT TGT GCA TTG ATT CCC-3`) and COX1-R (5`AGA GCA AAT CGC CGC TTC CGA-3`) was used to amplify a fragment of 302pb from cox1 mitochondrial gene and to sequence. Double-stranded PCR products were purified with Wizard PCR Preps DNA Purification System (Promega). Fluorescently labeled dideoxy terminators were used for single-strand DNA sequencing reactions according to the manufacturer's recommendations (ABI PRISM ® BigDye ™ Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems). Labeled extension products were gel separated and analyzed with an automated DNA sequencer (ABI PRISM 3130, Applied Biosystems).

Statistical Analyses

Sequences were aligned using the CLUSTAL X multiple alignement program and refined manually. Genetic variability (empirical frequencies, transversion (Tv), transition (Ts) and parsimonious rates for all sites) was assessed with the PAUP v4.0 beta 10 program (Swofford, 1998). Relationships between species were determined through the distance method, parsimony method (MP) and the likelihood method (ML). Distance trees were constructed according to the neighbor-joining (NJ) method of Saitou and Nei (1987) with models that take into account the branch-swapping algorithm: tree-bisection-reconnection (TBR, Felsenstein, J. 2004) and Hasegawa-Kishino-Yano the 85 parameter model (HKY85). The parsimonious tree (Fitch, 1971) was constructed with the same branch-swapping algorithm: tree-bisection-reconnection (TBR.). The tree corresponding to maximum likelihood method was built by setting the HKY+Γ model (Hasegawa, Kishino, and Yano's model with a gamma distribution of substitution rate among different nucleotide sites). This model was used to determine the number of substitution types. The bootstrap resampling technique (1000 replicates) was used to assess the statistical significance of internal nodes (Felstein, 1985). *Misgurnus fossilis,* an outgroup species from the Cobitidae family close to the Cyprinidae one was used as a root of the phylogeny tree.

RESULTS AND DISCUSSIONS

The PCR reactions have led to the amplification of a 302bp ADN fragment from cox1 gene of all cyprinids species included in the study (*Carassius auratus auratus, Rutilus rutilus, Barbus meridionalis, Tinca tinca, Hypophthalmichthys molitrix, Arischthys nobilis, Ctenopharyngodon idella, Leuciscus borysthenicus celensis, Leuciscus cephalus*, *Misgurnus fossilis*). The lenghts of the fragments have been checked by agarose gel electrophoresis. The obtained sequences were compared with nucleotide retrieved from GenBank (Table 1). The resulted identity was greater than 85%, denoting a phylogenetic relationship between the analyzed species. The *Tinca tinca* sequence was introduced in the GenBank (www.ncbi.nlm.nih.gov.url/ GenBank) and received the accession number EF112527.

The sequences were aligned with the Clustal X program (see Figure 1) and the results were used to build the phylogenetic trees through three different methods: neighbor-joining method, parsimony method and likelihood method. A total of 284 characters were analyzed, of which 183 were constant, 43 were variable (parsimony-uninformative) and 58 were parsimony-informative. The combined dataset resulted in the best likelihood score

(-lnL=1192.63391) for the HKY+Γ model. The estimated nucleotide empirical frequencies were: A=0.27477, C=0.26193, G=0.16647 and T=0.29683. An overall Ts/Tv ratio of 4.46 was estimated for this dataset. Base composition was calculated across all taxa for 1st, 2nd, and 3rd codon positions and all codon positions combined.

The divergence between each two species was estimated based on the HKY85 test. Pairwise sequence divergence between taxa varied from 5 to 30% (Table 2).

Table 1

THE COMPARISON BETWEEN COX1 SEQUENCE OBTAINED BY US AND GENBANK SEQUENCES

Species	Identities (%)	Database species and accession number
Arischthys nobilis	99	*Hypophthalmichthys nobilis/* EU343733
Ctenopharyngodon idella	98	*Ctenopharyngodon idella/* EU391390
Hypophthalmichthys molitrix	98	*Hypophthalmichthys molitrix/* EU315941
Rutilus rutilus	85	*Tinca tinca/* EF112527
Barbus meridionalis	91	*Gobio gobio/* AB239596
Tinca tinca	100	*Tinca tinca/* EF112527
Misgurnus fossilis	86	*Misgurnus nikolskyi/* AB242171
Carassius auratus auratus	97	*Carassius auratus auratus/* AB111951

Leuciscus borysthenicus celensis	88	*Tinca tinca*/ EF112527	
Leuciscus cephalus	90	*Tinca tinca*/ EF112527	

Table 2

LEVELS OF NUCLEOTIDE DIVERGENCE WITHIN AND BETWEEN 9 CYPRINID SPECIES, TOGETHER WITH THOSE FOR THE OUTGROUP *M. FOSSILS.* THE ESTIMATES WERE BASED ON HKY85 MODEL.

		1	2	3	4	5	6	7	8	9	10
1	M. fossilis	-									
2	A. nobilis	0.250	-								
3	H. Molitrix	0.250	0.060	-							
4	C. idella	0.248	0.106	0.082	-						
5	B. meridionalis	0.309	0.171	0.180	0.169	-					
6	L. boryst. celensis	0.293	0.180	0.172	0.185	0.189	-				
7	L. cephalus	0.288	0.174	0.176	0.145	0.195	0.052	-			
8	T. tinca	0.253	0.150	0.149	0.145	0.172	0.118	0.110	-		
9	R. rutilus	0.263	0.160	0.193	0.166	0.194	0.143	0.120	0.175	-	
10	C. auratus auratus	0.214	0.191	0.227	0.194	0.193	0.194	0.170	0.173	0.177	-

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Arischthys_nobilis                -----------------------CTCTTGAAC--GANTACTTTAAACGA 24
Ctenopharyngodon_idella           -------------------------TTGTGGTCCGGNTTACTTTAAACGA 25
Hypophthalmichthys_molitrix       -------------------------TTCTTTTCCGGTATACATTAAACGA 25
Misgurnus_fossilis                -AGCCTTTGTGC-ATTGATTCCCCCTATTTACGGGGTATACTTTACACAG 48
Carassius_auratus_auratus         TAGCCTTTGTGCCATTGATTCCCCCTACTAACAGGGTACACTCTGCATAG 50
Leuciscus_borysthenicus_celens    --AGCCTTTGTGCATTGATTCCCACTCTTCTCAGGATACACCTTAAATGA 48
Leuciscus_cephalus                ------------------------------CAGGATACACCCTAAATGA 19
Tinca_tinca                       -TAGCCTTTGTGCATTGATTCCCGCTATTCTCAGGATATACTCTAAACGA 49
Rutilus_rutilus                   ----------------------------------------AAACCTTAA 9
Barbus_meridionalis               ------------------------------------------------

Arischthys_nobilis                CACCTGAACAAAAATCC-CTTCGGGGTAATATTCATCGGCGTAAA-TCTT 72
Ctenopharyngodon_idella           CACCTGAACAAAAATCC-CTTTGGAGTAATGTTCATCGGTGTAAA-CCTC 73
Hypophthalmichthys_molitrix       CACCTGAACAAAAATCCATTTCGGAGTAATGTTCATCGGCGTAAA-TCTT 74
Misgurnus_fossilis                CACTTGACTAGA--TCCACTTTGGGGT-ATGTTCTTAGGTGTTAA-TCTC 94
Carassius_auratus_auratus         CGCTTGAACAAAAATCCACTTTGGGGTTATATTTATCGGAGTTAAACCTC 100
Leuciscus_borysthenicus_celens    TACTTGAACAAAA-TCCACTTTGCA-TCATATTCCTTGGCGTAAA-CCTT 95
Leuciscus_cephalus                TACTTGAACAAAAATCCACTTTGCAATCATATTCATTGGTGTAAA-CCTT 68
Tinca_tinca                       TACTTGAACAAAAATTCACTTCGGAGTTATGTTCATTGGCGTAAA-CCTC 98
Rutilus_rutilus                   TGATACTTGACAAATCCACTTTGGGATTATATTTATTCGGTGTGA-TCTT 58
Barbus_meridionalis               --------GGGGAGTTCACTCT---------------CGAGGTGA-TAGA 26
                                              * *  *                 *     *

Arischthys_nobilis                ACATTCTTCCCACAACACTTCCTAGGTCTA-GCAGGAATGCCACGACGAT 121
Ctenopharyngodon_idella           ACATTCTTCCCGCAACACTTCCTAGGCCTA-GCAGGAATGCCACGACGAT 122
Hypophthalmichthys_molitrix       ACATTCTTCCCACAACACTTCCTAGGCCTA-GCAGGAATGCCACGACGAT 123
Misgurnus_fossilis                ACCTTCTTCCCACAACACTTCCTCGGCCTT-GCGGGCATGCCCCGACGGT 143
Carassius_auratus_auratus         ACATTCTTCCCACAACACTTCCTGGGTCTCAGCAGGAATACCACGACGGT 150
Leuciscus_borysthenicus_celens    ACATTTTTCCCACAACATTTCCTAGGATTG-GCAGGAATACCACGACGAT 144
Leuciscus_cephalus                ACATTCTTCCCACAACATTTCCTAGGATTG-GCAGGAATACCACGACGAT 117
Tinca_tinca                       ACATTCTTCCCACAACATTTCCTTGGATTA-GCAGGAATACCACGACGAT 147
Rutilus_rutilus                   ACATTCTTCCCACAACACTTCCTAGGCCTA-GCAGGAATACCACGACGAT 107
Barbus_meridionalis               AGATTCTTCCCTCA-CATTTCCTAGGC-TA-CGAGGAATACACAGCAGAC 73
                                  *  ** ***** ** ** ***** **  *     ** ** *   *  *

Arischthys_nobilis                AC-----TCTGACTACCCAGATGCCTACGCCCTGTGAAATACAGTATCAT 166
Ctenopharyngodon_idella           AC-----TCCGACTATCCGGACGCCTACGCCCTATGAAATACAGTATCAT 167
Hypophthalmichthys_molitrix       AC-----TCCGACTACCCAGATGCCTACGCCCTGTGAAATACAGTATCAT 168
Misgurnus_fossilis                AC-----TCAGATTACCCAGATGCCTATACACTATGAAACACAGTTTCAT 188
Carassius_auratus_auratus         AT-----TCTGATTATCCAGACGCTTATGCCCTATGAAATACAGTATCAT 195
Leuciscus_borysthenicus_celens    AT-----TCTGACTACCCAGACGCCTATGCCCTGTGAAATACGGTATCAT 189
Leuciscus_cephalus                AT-----TCTGACTACCCAGACGCCTATGCCCTATGAAATACAGTATCAT 162
Tinca_tinca                       AC-----TCTGACTACCCAGACGCCTATGCTCTATGAAATACAGTATCCT 192
Rutilus_rutilus                   AT-----TCTGACTACCCAGACGCTTATGCCCTATGAAATACAGTGTCGT 152
Barbus_meridionalis               AGATACATCTGACTACCCCGACGCCTACGCCCTATGAAATACAGTGTCAT 123
                                  *      ** ** ** ** ** ** **  * ** ***** ** ** ** *

Arischthys_nobilis                CTATCGGATCTCTTATTTCCCTGGTAGCAGTAATTATGTTCCTATTTATC 216
Ctenopharyngodon_idella           CTATCGGATCACTTATCTCCTTAGTAGCAGTAATTATATTCCTATTTATC 217
Hypophthalmichthys_molitrix       CTATCGGATCTCTTATTTCCCTAGTAGCAGTAATTATATTCCTATTTATT 218
Misgurnus_fossilis                CTATTGGATCTATAATCTCATTAGTGGCTGTAATTATGTTCTTGTTTATT 238
Carassius_auratus_auratus         CTATCGGATCCCTAATCTCCCTAGTAGCGGTAATTATGTTCCTATTTATT 245
Leuciscus_borysthenicus_celens    CTATCGGGTCACTCATCTCACTAGTGGCAGTAATCATATTCCTATTTATT 239
Leuciscus_cephalus                CTATTGGGTCACTTATCTCGTTAGTAGCAGTAATCATGTTCCTATTTATT 212
Tinca_tinca                       CTATTGGGTCGCTAATTTCCCTAGTAGCAGTAATTATATTCCTATTTATC 242
Rutilus_rutilus                   CTATCGGCTCACTCATCTCATTAGTGGCAGTAATTATGTTCCTATTTATC 202
Barbus_meridionalis               CCATTGGATCACTCATCTCCCTGGTCGCAGTAATTATATTCCTATTTATT 173
                                  * ** ** **  * ** **  * ** ** ***** ** *** * *****

Arischthys_nobilis                CTATGAGAAGCCTTCGCCGCTAAAACGAGAA 247
Ctenopharyngodon_idella           CTATGAGAAGCCTTCGCCGCTAAAACGAGAA 248
Hypophthalmichthys_molitrix       CTATGAGAAGCCTTCGCCGCTAAA-CGAGAA 248
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Misgurnus_fossilis                TTATGAGAAGCCTTCGCCGCT---------- 259
Carassius_auratus_auratus         CTATGAGAAGCCTTCGCCGGCTAAACGAGAA 276
Leuciscus_borysthenicus_celens    CTCTGAGAAGCCTTCGCCGCTAAACGAGAA- 269
Leuciscus_cephalus                CTCTGAGAAGCCTTCGCCGCTAAACGAGAA- 242
Tinca_tinca                       CTCTGAGAAGCCTTCGCCGCTAAACGAGAA- 272
Rutilus_rutilus                   CTCTGAGAAGCCTTCGCCGCT-AACGAGAA- 231
Barbus_meridionalis               CTGTGAGAAGCCTTCGCCGCTCAACGAGAA- 203
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Fig. 1 CLUSTAL X multiple cox1 sequence alignment

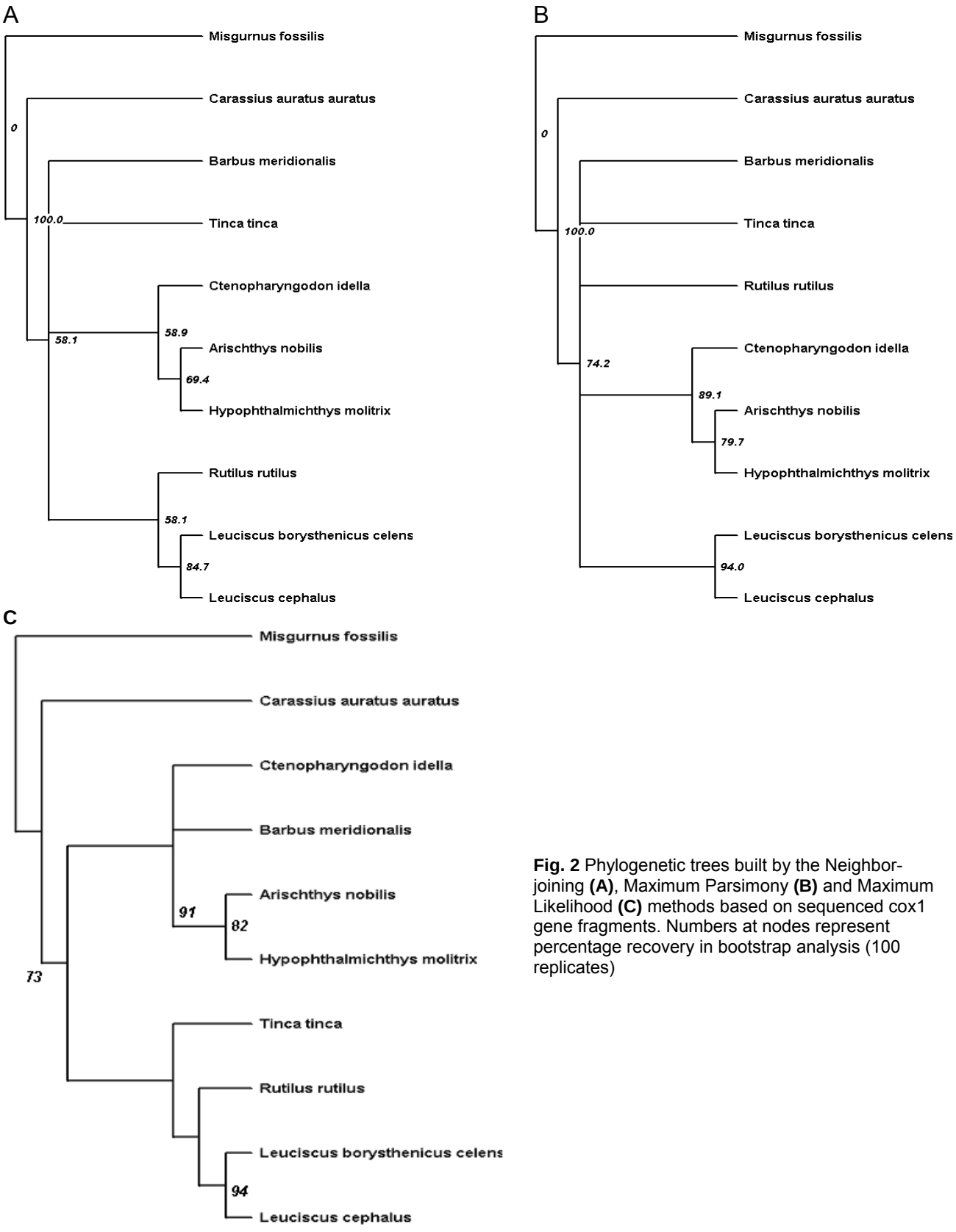

Fig. 2 Phylogenetic trees built by the Neighbor-joining **(A)**, Maximum Parsimony **(B)** and Maximum Likelihood **(C)** methods based on sequenced cox1 gene fragments. Numbers at nodes represent percentage recovery in bootstrap analysis (100 replicates)

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All the trees obtained by us presented almost the same topology (Figure 2). The robustness of the tree was corroborated with bootstrap analyses. A gamma-corrected ML (α= 0.24) analysis also yielded a congruent tree. The cyprinid species were grouped into two assemblages: *Cyprininae* and *Leuciscinae.* The *Cyprininae* group is represented by *Carassius auratus auratus* and forms a separate branch in the trees with a high boostrap value presented in Figure 2C. The *Leuciscinae* group includs *Rutilus rutilus, Tinca tinca, Hypophthalmichthys molitrix, Arischthys nobilis, Ctenopharyngodon idella, Leuciscus borysthenicus celensis, and Leuciscus cephalus. Ctenopharyngodon, Arischthys* and *Hypophthalmichthys* genera (East Asian cyprinid) formed a monophyletic clade with strong support according boostrap value of 91 in the ML tree.

The NJ tree (Figure 2A) was identical to the MP tree (Figure 2B), with the exception of the phylogenetic position of *Rutilus rutilus.* In previous studies (Gilles A. et al., 1998; Zardoya R. and Doadrio I., 1998; Xuzhen Wang et al., 2007.) it was demonstrated that this species had close phylogenetic relationships with the *Leuciscus* group which is in accordance with the results of our NJ tree. The NJ and MP trees from figures 2A and 2B placed *Barbus meridionalis* as paraphyletic group to the cyprinin one. For *Misgurnus foossilis* the position of outgroup species in our phylogenetic trees (Figure 2A, 2B, 2C) was confirmed.

CONCLUSIONS

Our data are mostly in agreement with previous results concerning the phylogenetic relationships of *Cyprinidae* family. High percentage of identity of our sequence (>85%) denotes a close phylogenetic relationship between the analyzed species. The topologies of neighbor-joining, maximum parsimony and maximum likelihood trees based *on cox1* sequences have enabled us to identify two major lineages in cyprinids: *Cyprininae* and *Leuciscinae*. The NJ tree highlighted two monophyletic clades: one for East Asian cyprinids (*Ctenopharyngodon, Arischthys* and *Hypophthalmichthys*) and one for the *Leuciscus* species and *Rutilus* species using *Misgurnus fossilis* as an outgroup species.

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