
Simultaneous analysis of two markers provides phylogenetic relationships for the cyprinid fish

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Abstract

The phylogenetic relationships among Romanian cyprinids were inferred using the complete nucleotide sequence of the cytochrome b gene (cit b) and a fragment from subunit 2 of cytochrome oxidase gene (cox 2). The phylogenetic utility of mitochondrial genes has been studied at different taxonomic levels among fish taxa.

*We sequenced nine cyprinids species from Romania. Bootstrap analysis distinguished two principal lineages in cyprinids family both in neighbour-joining tree based on sequenced cox 2 gene and in neighbour-joining tree based on sequenced cit b gene: Cyprinine and Leuciscine, with Cyprinine at the basal position. For the Leuciscine group *Hypophthalmichthys molitrix* and *Arischthys nobilis* were found to belong to the same subfamily *Hypophthalmichthyinae* based on both cox 2 sequences.*

Keywords: cyprinids, phylogeny, mitochondrial DNA, cit b gene, cox 2 gene

Introduction

The *Cyprinidae* family is an excellent group for biological studies consisting of 2010 species distributed across 210 genera in a large variety of habitats. In southern Europe, cyprinids are extremely rich in number of endemic species (about 77 species), especially when compared with the relatively uniform cyprinid fauna of central and northern Europe [11]. Despite numerous publications concerning the species classification within this family, the relationships among the main lineages of cyprinids still remain unclear, and even the monophyly of the whole family is sometimes in doubt [8]. Traditionally, cyprinids were grouped as different subfamilies according to their morphological characters [3; 2]. Depending on the authors, the number of subfamilies was very different.

To understand the phylogenetic relationships among representative cyprinid lineages, and their biogeographical origins, we have sequenced the complete mitochondrial cytochrome b gene (*cit b*) and the partial mitochondrial subunit 2 of cytochrome oxidase gene (*cox 2*) for nine cyprinid species. The sequence of the mitochondrial *cox 2* gene has been widely used to estimate phylogenetic relationships at different taxonomic levels across insects and monkeys [6; 1]. The phylogenetic utility of the *cyt b* gene has been studied at several taxonomic levels among vertebrate taxa [9; 16; 4; 7; 12; 15; 17] and particularly in fish taxa [18; 19; 14; 22; 13; 10]. Molecular phylogenetic studies using *cyt b* in cyprinid fishes included only few selected representatives from the lineages of the family *Cyprinidae*.

Materials and methods

(a) DNA extraction

Total cellular DNA was extracted from the muscle of the Romanian cyprinid species following the protocol Wizard Genomic DNA Purification Kit (Promega). We studied nine cyprinid species: *Carrasius carrasius*, *Carassius auratus gibelio*, *Cyprinus carpio*, *Leuciscus borysthenicus celensis*, *Leuciscus cephalus*, *Rutilus rutilus*, *Hypophthalmichthys molitrix*, *Arischthys nobilis*, *Barbus meridionalis*.

(b) PCR Amplification and Sequencing

The fragments containing mtDNA *cox 2* gene (274 base pairs- bp) and mtDNA *cit b* gene (one fragment of 660bp and other fragment of 521bp) were obtained by a polymerase chain reaction (PCR) amplification. According to complete *cox 2* genes sequences of the common carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*), primer set COX2-F (5'-AGG ACA CCA ATG ATA CTGA AG-3') /COX2-R (5'-GTT TAA AGT CTC GTA ACA GGC-3') were designed for the amplification of a fragment from *cox 2* gene. Other two sets of primers was designed for the amplification of two fragments from cytochrome b gene: Glu-F, 5'-GAA GAA CCA CCG TTG TTA TTC AA-3' / Cytb-R, 5'-TCT TTA TAT GAG AAR TAN GGG TG-3'; Cytb-F, 5'-CAC GAR ACR GGR TCN AAY AA-3'/Thr-R, 5'-ACC TCC RAT CTY CGG ATT ACA-3' [21]. They were used to amplify two contiguous and overlapping fragments (660bp and 521bp) that covered the entire cytochrome b gene.

PCR amplification was performed at an initial denaturation 95°C for 3 min, followed by 35 cycles at 95°C for 45s, 50-55°C for 60s and 72°C for 90s. The amplified fragments were purified with the Wizard PCR Preps DNA Purification System Kit (Promega). The purified fragments were sequenced by ABI PRISM 310 Genetic Analyzer, using the ABI PRISM ® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and the sequences were processed with ABI PRISM DNA Sequencing Analysis Software.

(c) Sequence alignment and phylogenetic analysis

The nucleotide sequences were aligned with the CLUSTAL X multiple alignment program and refined manually. The homology between two species was established with BLAST program using *BLAST 2 SEQUENCES* analysis. Phylogenetic analysis was performed with NJplot program using the neighbor-joining (NJ) [20] algorithm. Bootstrap analysis [5] was used to examine the confidence of nodes.

Results and discussions

The assigned primers for the *cyt b* and *cox 2* mitochondrial gene have led to the amplification of three fragments (660bp, 521bp from *cyt b* gene, respectively 274bp from *cox 2* gene), for all cyprinids species studied and have been verified by agarose gel electrophoresis.

The sequences were aligned (table 1 and table 2) and analyzed every two using the Blast 2 Sequences application. In Cyprinine group we identified a 95% sequence homology for *cox2* gene between *Carassius* species and between species *Arischthys nobilis* and *Hypophthalmichthys molitrix* was found 95%, which indicated that both species are included in the same *Hypophthalmichthyinae* subfamily. The high value of bootstrap analysis (97) from neighbor-joining (NJ) tree based on sequenced *cox 2* (Fig. 1) sustains the same hypothesis. For the species from the same genus, as *Leuciscus cephalus* and *Leuciscus borysthenicus celensis*, a high homology of *cit b* sequences 99% was found.

The neighbour-joining analysis arrived at a similar and congruent tree. The robustness of the NJ tree was confirmed by bootstrapping (Fig.1 and Fig. 2). According to our results, the family *Cyprinidae* is divided into two major groups, the *Cyprinae* and the *Leuciscinae*. These two major groups were also recognized by Chen et al. [3] and Cavender and Coburn [2] based on osteological characters, and ranked as subfamilies within the *Cyprinidae*.

Following Zardoya R, Doadrio I. [21] the *Barbus* lineage formed a monophyletic group with Ciprinine and a paraphyletic group with leuciscine. The *cox 2* phylogenetic tree (fig.1) was recognized to have the same topology, whereas in *cit b* phylogenetic tree the lineage *Barbus* appeared to be paraphyletic both with ciprinine and with leuciscine (fig.2). These results remains uncertain because no outgroup from another fish family than ciprinids were included in this study.

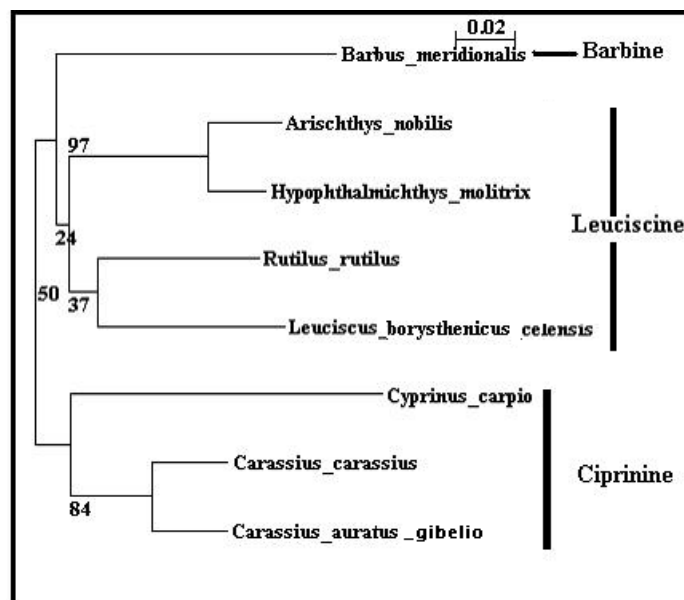


Figure 1. Phylogenetic relationships among Romanian Cyprinidae. Neighbour-joining tree based on sequenced *cox 2* fragments. In bootstrap consensus trees only values (numbers at nodes representing the percentage of 100 bootstrap replications) greater than 50 are significant, revealing a true phylogenetic relationship.

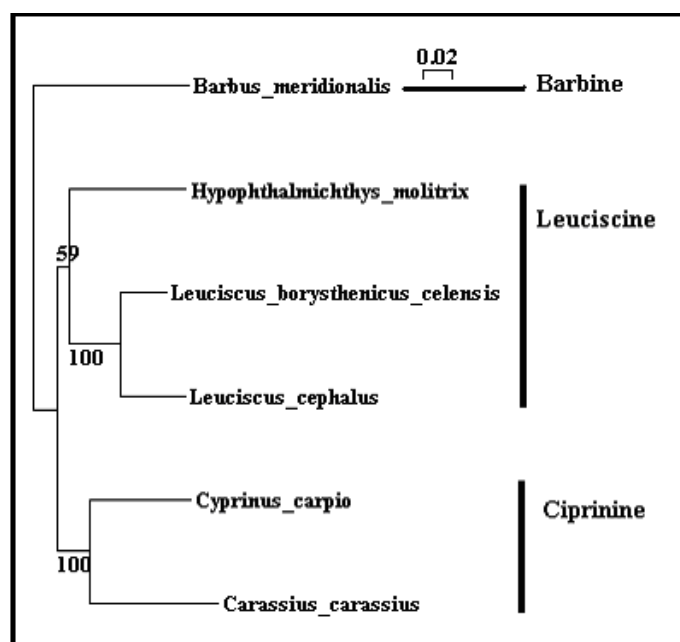


Figure 2. Phylogenetic relationships among Romanian *Cyprinidae*. Neighbour-joining tree based on sequenced *cit b* gene. In bootstrap consensus trees only values (numbers at nodes representing the percentage of 100 bootstrap replications) greater than 50 are significant, revealing a true phylogenetic relationship.

Conclusions

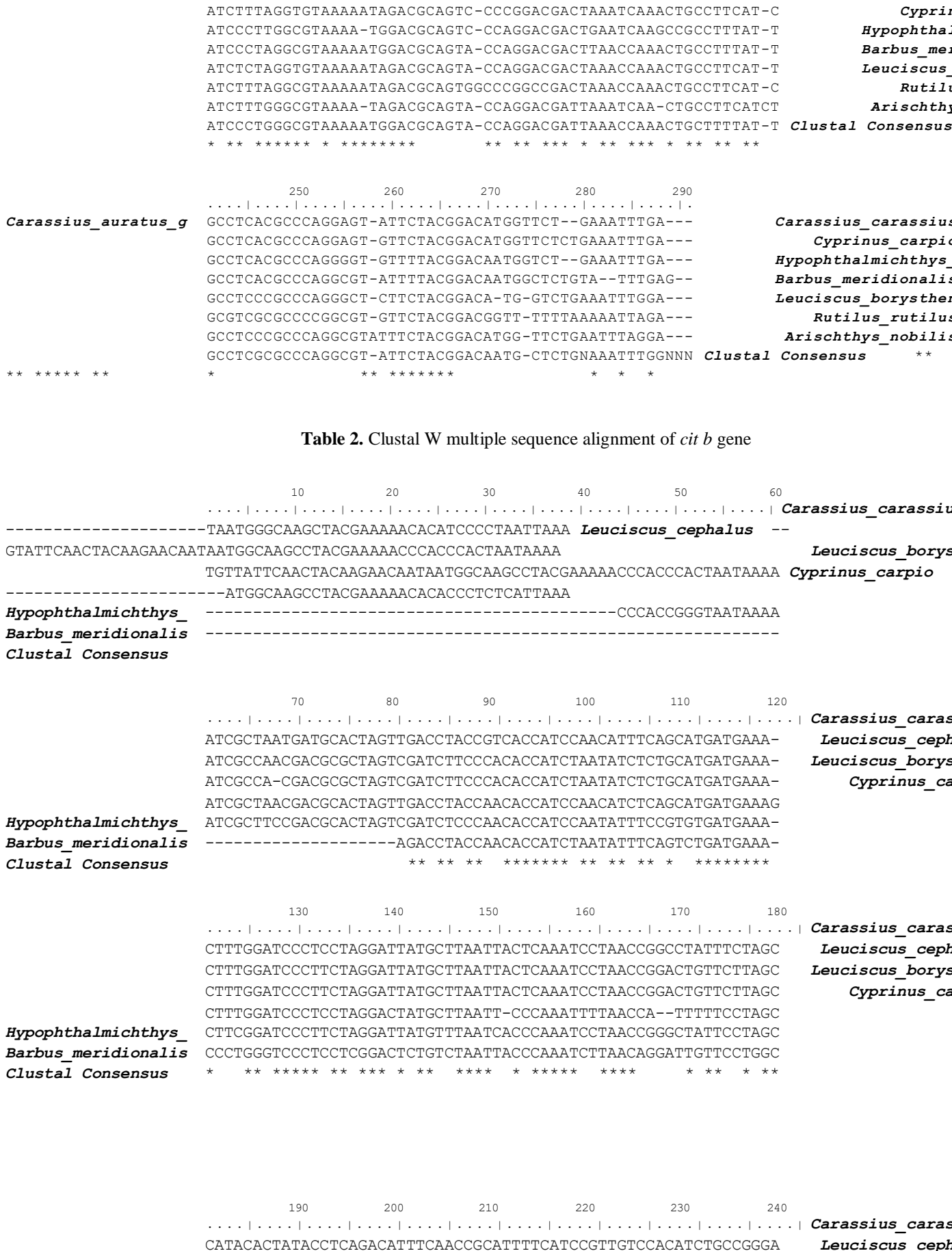
The high degree of identity between species sequences among the leuciscin group showed that *Hypophthalmichthys molitrix* and *Arischthys nobilis* belong to the same subfamily, namely *Hypophthalmichthyinae*.

The phylogenetic relationships among the main lineages of Romanian cyprinids based on cytochrome b and subunit 2 of cytochrome oxidase sequences data supported the traditional division of the Cyprinidae into two subfamilies: *Cyprinae* and *Leuciscinae*.

The phylogenetic position of the genus *Barbus* has not become resolved satisfactorily. However, the inclusion of *Barbus* into the *Leuciscinae* can be possible. Whether *Barbus* represents a separate lineage both by comparing with leuciscine group and by comparing with cyprinine group or should be assigned to the leuciscine group is still not clear.

Table 1. Clustal W fragment alignment of sequence *cox 2* gene

| | | | | | | | |
|----------------------------|---|-----|-----|-----|-----|-----|------------------------------------|
| | 10 | 20 | 30 | 40 | 50 | 60 | |
| <i>Carassius_auratus_g</i> | | | | | | | |
| | -----GAGG | | | | | | <i>Carassius_auratus_g</i> |
| | CACGAGATACGGGATTACGGAAAACCTAGGGATGTGACTCTCTATAAGTACCTACCCAAG | | | | | | <i>Cyprinus_carpio</i> |
| | -----TGAGGG | | | | | | <i>Hypophthalmichthys_molitrix</i> |
| | GGCTTCGACTCCTACATATCCA-CCCAG-- | | | | | | <i>Barbus_meridionalis</i> |
| | ATTCCTCAG-ACAAAAC-CCGAATTCG-AGACCTA-GGATTTGACTCCTATATGATCCCCTCAG- | | | | | | <i>Leuciscus_borysthen</i> |
| | ---AAAATACCCGACTACGCAGGCTA-GGATTTGACTCCTACATAATCCACCCAAGA | | | | | | <i>Rutilus_rutilus</i> |
| | ANTNNGNGTNCAGCCTTTNNTGNCT-G-GGCTCCGACTCCTACTAATCCAACCCAAG- | | | | | | <i>Arischthys_nobilis</i> |
| | | | | | | | Clustal Consensus |
| | 70 | 80 | 90 | 100 | 110 | 120 | |
| <i>Carassius_auratus_g</i> | | | | | | | |
| | CTTCAGATCGCAGGACAATGGCCGACTCTTAGAAACAGACCATCGATTAGTTGTCCCAAT | | | | | | <i>Carassius_auratus_g</i> |
| | TCTCTCTCTCCAGGGAGAGAGAAGACACTTAGAAACAGACCATCTCTCTTGTCCCAAT | | | | | | <i>Cyprinus_carpio</i> |
| | TCCAGTTCGCCGAGACAGGTACCGACTTCTGAAACAGACCACCGAATAGTTGTTCCAAT | | | | | | <i>Hypophthalmichthys_molitrix</i> |
| | ACCCTCCTCCAGGCCA-TTCCGGCTCCTAGAAACAGACCACCGAATAGTAGTCCCAT | | | | | | <i>Barbus_meridionalis</i> |
| | CCTTATCACCAGGTCAGTTCCGACTACTAGAAACGGACCATCGAATAGTTGTACCAAT | | | | | | <i>Leuciscus_borysthen</i> |
| | AATTCCTGGTCAGTTCCGACTCCTAGAGACAGACCACCGAATAGTAGTCCCAAT | | | | | | <i>Rutilus_rutilus</i> |
| | TTCCGACTCCTGGAGACAGACCACCGAATAGTAGTCCGAT | | | | | | <i>Arischthys_nobilis</i> |
| | TACCTGACACCAGGCCAATTCGGCTCCTAGAAACAGACCACCGAATAGTAGTCCCAT | | | | | | Clustal Consensus |
| | * * * * * | | | | | | * * * |
| | 130 | 140 | 150 | 160 | 170 | 180 | |
| <i>Carassius_auratus_g</i> | | | | | | | |
| | AGAGTCCCCAGTCCGTGTCTTAGTATCCGCTGAAGACGTACTACACTCCTGAGCCGTTCC | | | | | | <i>Carassius_auratus_g</i> |
| | GGAGTCCCCAGTCCGTGTTTTAGTATCCGCTGAAGACGTACTACACTCCTGAGCTGTCC | | | | | | <i>Cyprinus_carpio</i> |
| | AGAATCCCCAGTCCGTGTCTTAGTATCTGCTGAAGACGTACTACACTCCTGAGCTGTCC | | | | | | <i>Hypophthalmichthys_molitrix</i> |
| | AGAATCGCCAGTTCGTGTTCTAGTATCCGCCGAAGATGTATTACACTCCTGAGCCGTTCC | | | | | | <i>Barbus_meridionalis</i> |
| | AGAATCACCTATTCGTGTGCTGGTATCCGCTGAAGACGTTTTGCACTCATGAGCCGTTCC | | | | | | <i>Leuciscus_borysthen</i> |
| | AGAATCACAGTTCGTGTTTTAGTATCCGCAGAAGACGTATTACACTCCTGAGCCGTTCC | | | | | | <i>Rutilus_rutilus</i> |
| | AGAGTACCAGTTCGTGTTTTAGTATCCGCAGAAGACGTATTACACTCCTGAGCAGTCCC | | | | | | <i>Arischthys_nobilis</i> |
| | AGAATCGCCAGTTCGTGTTCTAGTATCCGCCGAAGATGTATTACACTCCTGAGCCGTTCC | | | | | | Clustal Consensus |
| | * * * * * | | | | | | * * * * * |
| | 190 | 200 | 210 | 220 | 230 | 240 | |
| <i>Carassius_auratus_g</i> | | | | | | | |
| | ATCCTTAGGTGTAATAATAGACGCAGTC-CCAGCCGACTAAATCAAACCTGCTTTTCAT-C | | | | | | <i>Carassius_auratus_g</i> |



*Hypophthalmichthys
Barbus meridionalis*
Clustal Consensus

CATGCATTACACTTCTGACATCTCAACCGCATTTCTCATCAGTAGTCCACATTTGCCGGGA
CATGCATTACACTTCTGACATCTCAACCGCATTTCTCATCAGTAGTCCACATTTGCCGGGA
CATACTAGGCCCTCAGACATCTCAACC--CTTCTCATCTGTTNCCCACATCTGCCGAGA
CATGCACTATACCTCTGATATCTCAACCGCATTTTCATCAGTAGTCCACATCTGCCGAGA
TATGCACTATACCTCGGACATTTCAACTGCATTTTCATCAGTTGCCACATCTGCCGGGA
* *

*Leuciscus borys
Cyprinus ca*

*Hypophthalmichthys
Barbus meridionalis*
Clustal Consensus

250 260 270 280 290 300
TGTAATTTATGGCTGACTAATCCGTAATATTACGCCAACGGGCGCATCATTCTTCTTCAT
CGTCAATTACGGCTGACTTATCCGCAACCTACAGCCAACGGGGCATCATTCTTTTTTAT
CGTCAATTACGGCTGACTTATCCGCAACCTACAGCCAACGGGGCATCATTCTTTTTTAT
CGTAAATTACGGCTGACTAATCCGTAATGTACAGCCAACGGAGCATCATTCTTCTTCAT
TGTAAACTATGGCTGGCTTATTCGCAACCTACAGCTAACGGAGCATCATTCTTTTTTCAT
CGTAAACTACGGCTGATTTATTCGCAACATGCAGCCAACGGAGCCTCATTCTTTTTTCAT
* *

*Carassius caras
Leuciscus ceph
Leuciscus borys
Cyprinus ca*

*Carassius carassius
Leuciscus cephalus
Leuciscus borysthen*
Clustal Consensus

310 320 330 340 350 360
CTGTATTTATATACACATTTGCCGAGGCCATATTATGGATCTTACCTTTACAAAGAAAC
CTGTATTTATATACACATTTGCTCGCGGCCATACTATGGATCCTACCTTTACAAAGAAAC
CTGTATTTATATACACATTTGCTCGCGGCCATACTATGGATCCTACCTTTACAAAGAAAC
TTGCATTTACATACACATCGCCCGAGGCCATACTACGGATCATACCTTTACAAAGAAAC
CTGTATTTATATACACATCGCCCGTGGCTTATACTATGGATCTTACCTTACAAAGAGAC
CTGTATTTATATGCACGTAGCCGAGGACTCTATTACGGTCTTACCTTACAAAGAAAC
* *

*Cyprin
Hypophthal
Barbus mer
Clustal Consensus*

*Hypophthalmichthys
Barbus meridionalis*
Clustal Consensus

370 380 390 400 410 420
CTGAAACATCGGAGTAATCTTATTCTCTAGT-TATAATAACAGCCTTTGTTGGTTATG
CTGAAATATTGGAGTAGTCCTACTCCTATTAGT-TATAATAACGGCCTTCGTTGGCTACG
CTGAAATATTGGAGTAGTCCTACTCCTATTAGT-TATAATAACGGCCTTCGTTGGCTACG
CTGAAACATTTGGTGAATCCTTCTACTACTAGT-CATGATAACAGCCTTCGTTGGCTATG
CTGAAACATTTGGAGTAGTCCTACTCCTGCTAGT-CATAATAACAGCCTTCGTTGGCTACG
CTGAAACATTTGGGTAGTTCTGCTGCTACTAGAATATAATAACGGCCTTCGTTGGCTATG
* *

*Carassius caras
Leuciscus ceph
Leuciscus borys
Cyprinus ca*

*Hypophthalmichthys
Barbus meridionalis*
Clustal Consensus

430 440 450 460 470 480
TTCTCCCATGAGGA-CAAATATCCTTTTGAGGCGCTACAGTAATTACAAACCTCCTATCC
TCCTCCCATGAGGA-CAAATATCTTTTGAGGCGCTACCGTAATTACAAACCTACTATCA
TCCTCCCATGAGGA-CAAATATCTTTTGAGGCGCTACCGTAATTACAAACCTACTATCA
TTCTTCCATGAGGA-CAAATATCCTTTTGAGGCGCCACAGTAATCACAAACCTCCTATCT
TCCTTCCATGAGGA-CAAATATCCTTCTGAGGTGCCACAGTAATTACAAACCTATTATCA
TTCTCCCATGAGGCTCAAATATCCTTCTGAGGTGCCACAGTAATTACAAACCTTTTATCA
* *

*Carassius caras
Leuciscus ceph
Leuciscus borys
Cyprinus ca*

*Hypophthalmichthys
Barbus meridionalis*
Clustal Consensus

490 500 510 520 530 540
GCTGTACCATATA-TGGGAGATAT-ATTAGTTC-AATGAATTTGAGGAGGCTTCT-CCGT
GCAGTCCCCTACA-TAGGAGATAC-TCTCGTCC-AATGAATTTGAGGTGGCTTCT-CAGT
GCAGTCCCCTACA-TAGGAGATAC-TCTCGTCC-AATGAATTTGAGGTGGCTTCT-CAGT
GCCGTACCATACA-TGGGAGACAT-GTTAGTCC-AATGAATCTGAGGTGGGTTCT-CGGT
GCAGTCCCCTACA-TAGGAGACAC-CCTCGTCC-AATGAATCTGAGGCGGTTTTTTCAGT
GCAGTACCTTATAATAGGAGACACATCTTGTCCAATGAATTTGGGCGGGTTTT-CAGT
* *

*Carassius caras
Leuciscus ceph
Leuciscus borys
Cyprinus ca*

Carassius caras

Hypophthalmichthys_
Barbus_meridionalis
Clustal Consensus
AACAAACTGGAGGAGTTCTTGCATTATTATCTCTATCCTTGACTAATGGTAGTACCC
AACAAACTAGGAGGGTACTTGCCTACTATTTCAGCATCCTAGTGCTAATAGTTGTGCCA
AACAAACTAGGAGGGTCTTGCCTACTATTATTTCCATCCTAGTACTAATAGTAGTGCCA
AACAAACTGGAGGTGTCCTTGCCTACTTCTCCATTCTAGTATTAATAGTAGTACCA
AACAAACTGGGAGGGTCTTGCCTACTATTATCTCCATTCTAGTACTAATAGTAGTGCCA
AACAAACTAGGAGGAGTCTTGCCTGCTGTTGTTTCTATTCTAATTCTCATGGTAGTCCCA
***** ** ** ***** * * ** ** * * ** * * ** *

Leuciscus_ceph
Leuciscus_borys
Cyprinus_ca

970 980 990 1000 1010 1020
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
CTACTACACACCTCAAAGCAACGAGGACTAACATTCCGCCAATCACTCAATTCCTATTC
ATCTTACACACCTCTAAACAACGAGGACTAACTTTCCGCCCCGTAATCAATTCCTATTC
ATCTTACACACCTCAAAAACAACGAGGACTAACATTCCGCCAATCACCCAATTCCTATTC
CTACTACACACCTCAAAAACAACGAGGGCTAACATTCCGCCCCATCACCCAATTCCTATTC
ATCTTACACACCTCAAAAACAACGAGGATTAACATTCCGCCAATCACCCAATTCCTATTC
ATTTTACACACCTCGAAAACAACGAGGACTAACCTTCCGCCCCCTAACACAATTCCTATTC
* ***** ** ***** ** * ***** * * * ***** *****

Carassius_caras
Leuciscus_ceph
Leuciscus_borys
Cyprinus_ca

Hypophthalmichthys_
Barbus_meridionalis
Clustal Consensus
1030 1040 1050 1060 1070 1080
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
TGAACCTTAGTTGCAGATATGGCTATCCTAACATGGATTGGAGGCATACCCGTAGAACAC
TGAACCTTAGTTGCAGATATATTTATCCTGACATGAATTGGGGGCATACCTGTAGAACAC
TGAACCTTAGTAGCAGACATGATTATCTGACATGAATTGGAGGCATACCCGTAGAACAT
TGAACCTTAGTAGCAGACATAATTATCCTAACATGAATTGGAGGCATACCCGTAGAACAT
TGAACCTTAGTAGCAGACATAATTATCTGACATGAATTGGAGGTATACCTGTAGAACAT
TGAACCTCGTAGCAGACATACTAATCCTAACGTGAATTGGAGGCATGCCCGTGAACAT
***** * ** ***** ** * ***** ** * * * ***** *****

Carassius_caras
Leuciscus_ceph
Leuciscus_borys
Cyprinus_ca

Hypophthalmichthys_
Barbus_meridionalis
Clustal Consensus
1090 1100 1110 1120 1130 1140
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
CCATTCATTATTATGGACAAATTCATCCATTCTATATTTTACATTATTCCTTGCTCCTC
CCATACATTATCATTGGCCAAGTCGCATCCATTCTATATTTTGCCTCTCCTCATCCTT
CCATATGTCATTATTTGGACAAATTCATCAGTCCCTTTATTTGCTCATTATTCCTCATTTTA
CCCTTCATCATTATTTGGACAAATTCATCCGTCCTATACCTTCGCACTATTCCTCATTTT
CCATACATCATCATCGGACAAATTCGCATCAGTCCCTTTACTTCGCACTATTCCTGATTCTT
CCATATGTTGTCATCGGCAAGTCGCTTCAATTCTGTATTTTGCCTTTTCTTGCTCCTT
** * * * ** * * * ** * * * ** * * * ** * * * ** * * * ** * * *

Carassius_caras
Leuciscus_ceph
Leuciscus_borys
Cyprinus_ca

Hypophthalmichthys_
Barbus_meridionalis
Clustal Consensus
1150 1160 1170 1180 1190 1200
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
TTTCCACTAGCAGGATGACTAGAAAACAAGCACTGAAATGAGCTTGCCCTAG-TAGCTT
GTCCCACTAGCAGGATGAGTGGAAAATAAAGCATTAAAATGAGCCTGCCCTAG-TAGCTT
ACCCCACTAGCAGGATGATTAGAAAATAAAGCACTAAAATGAGCTTGCCCTAG-TAGCTT
ATGCCACTAGCAGGATGGTTAGAAAATAAAGCACTAAAATGAGCTTGCCCTAGGTAGCTT
ACCCCACTGGCAGGATGACTAGAAAATAAAGCACTAAAATGAGCTTGCCCTAG-TAGCTT
GTCCCACTGGCAGGATGACTAGAAAATAAAGCATTAAAATGAGCTTGCCCTAG-TAGCTT
*** * ***** ** * ***** ***** * ***** ***** *****

Carassius_caras
Leuciscus_ceph
Leuciscus_borys
Cyprinus_ca

Hypophthalmichthys_
Barbus_meridionalis
Clustal Consensus
1210 1220
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
AGTATAAAAGCATCGGTCTTGTAATCCG
AGTCTAAAAGCATCGGTCTTGTAATC--
AGCCTAAAAGCATCGGTCTTGTAATC--
AGCCTAAAAGCATCGGTCTTGTAATC--
AGTCTAAAAGCATCGGTCTTGTAATC--
AGTCTAAAAGCATCGGTCTTGTAATC--
** * ***** *****

Carassius_carassius
Leuciscus_cephalus
Leuciscus_borysthen
Cyprinus_carpio

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