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MOLECULAR PHYLOGENETIC RELATIOSHIPS IN ROMANIAN CYPRINIDS BASED ON cox1 AND cox2 SEQUENCES

Cătălina LUCA*, Eduard CONDAC, Anca DINISCHIOTU, Elena IONICĂ, Călin TESIO, Marieta COSTACHE

University of Bucharest, Faculty of Biology, Molecular Biology Center, no. 91-95, Spl. Independentei, Bucharest 5, Romania

Corresponding authors: costache@bio.bio.unibuc.ro, catalina_noem@yahoo.com

ABSTRACT. Cyprinids (Teleostei:Cypriniformes:Cyprinidae) are the major component of Eurasian temperate freshwater fish fauna with respect to the number of both individuals and species (about 2010 reported species). Mitochondrial DNA (mtDNA) has proven to be useful in molecular phylogenetic studies because evolutionary relationships can be inferred among higher levels, between recently divergent groups, populations, species and even individuals. Phylogenetic relatioships were inferred from analysis of 302 base pairs (bp) of mithocondrial DNA (mtDNA), representing a fragment of the subunit I cythocrom c oxidase gene (*cox1*) and the 274 bp of mtDNA, representing a fragment of the subunit II cythocrom c oxidase gene (*cox2*). We sequenced 9 cyprinids species from Romania. Bootstrap analysis distingushed two principal lineages in cyprinids: 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 genera based on both *cox1* and *cox2* sequences.

KEYWORDS*:* cyprinids, molecular phylogeny, *cox1* gene, *cox2* gene

INTRODUCTION

Cyprinids are the major component of Eurasian temperate freshwater fish fauna with respect to the number both of individuals and of species (more than 2000 species; Banarescu & Coad, 1991). 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. A well-supported phylogeny is also required to address the question of hybridization: interspecific and even intergeneric cyprinid hybrids are common, and their taxonomic meaning is worth investigating. (Jerome Briolay, 1997).

In recent years, numerous efforts have been devoted to clarifying the relationships among cyprinids using molecular techniques (Briolay et al. 1998; Gilles et al. 1998, 2001; Zardoya and Doadrio 1998, 1999, Huanzhang Liu and Yiyu Chen, 2003). Mitochondrial DNA (mtDNA) has proven to be 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 appear useful because molecular characters are less likely related to adaptative evolution than are morphologic characters.

In Romania, cyprinids classification matter based on molecular analysis is still an open issue. The present study is the first attempt to realize a molecular-based phylogeny to clarify romanian cyprinid relationships. The species included in this study are: *Carrasius carrasius,Carassius auratus, Rutilus rutilus, Barbus meridionalis, Cyprinus carpio, Hypophthalmichthys molitrix, Arischthys nobilis, Ctenopharyngodon idellus, Leuciscus celensis*. The markers assigned by us to determine the phylogenetic relationships between cyprinids are mitochondrial genes coding for subunits I (*cox1*) and II (*cox2*) of citochrome oxidase.

MATERIAL AND METHODS

(a) DNA extration

The Nucet Fishery Research Centre provided us the 9 fish species analyzed: *Carrasius carrasius,Carassius auratus, Rutilus rutilus, Barbus meridionalis, Cyprinus carpio, Hypophthalmichthys molitrix, Arischthys nobilis, Ctenopharyngodon idellus, Leuciscus celensis*. Total DNA was extracted from the liver following the protocol Wizard Genomic DNA Purification Kit (Promega).

(b) PCR Amplification and Sequencing

The fragments containing mtDNA *cox1* gene (302pb) and mtDNA *cox2* gene (274pb) were obtained by polimerase chain reaction (PCR) amplification. According to complete *cox1* and *cox2* genes sequences of the common carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*), primer sets COX1-F (5`-AGCCTTTGTGCATTGATTCCC-3`) /COX1-R (5`AGAGCAAATCGCCGCTTCCGA-3`) and COX2-F (5`-AGGACACCAATGATACTGA AG-3`) /COX2-R (5`-GTTTAAAGTCTCGTAACAGGC-3`) were designed for this study. PCR amplification was performed at an initial denaturation 95^{o}C for 3 min, followed by 35 cycles at 95^{o}C for 45s, 55^{o}C for 60s and 72^{o}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. The sequences were processed with ABI PRISM DNA Sequencing Analysis Software.

(c) *Sequence alignement and phylogenetic analysis*

The nucleotide sequences were aligned with the CLUSTAL X multiple alignement 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) (Saitou and Nei, 1987) algoritm. Bootstrap analysis (Felstein 1985) was used to examine the confidence of nodes.

RESULTS AND DISCUSSION

The mitochondrial *cox1* and *cox2* genes were amplified by PCR and sequenced in both orientations in all cyprinid species tested. A 302bp fragment from *cox1* was aligned for 5 species (Fig.1), and a 274bp *cox2* fragment was aligned for 8 species (Fig.2).

The sequences were analysed every two using the Blast 2 Sequences application. In Cyprinine group we identified a 95% sequence homology for *cox2* gene between *Carassius* species. Both *cox1* and *cox2* fragment alignment for *Arischthys nobilis* and *Hypophthalmichthys molitrix* gave the same homology degree of 95%.

The neigbhor-joinig (NJ) analysis arrived at a similar and congruent tree. The robustness of the NJ tree was confirmed by bootstrapping (Fig.3 and Fig.4). Two major assemblages could be distinguished within the *Cyprinidae* based on the *cox2* NJ tree (Fig.4). One clade, the Cyprinine included the carp and the goldfish, whereas the other, the Leuciscine included *Leuciscus, Rutilus, Hypophthalmichthys* and *Arischthys*.

The barbin lineages formed a paraphyletic group with the leuciscine lineages both on *cox1* and *cox2* NJ trees. According to the results of Ignacio Doadrio, 1998 and Jerome Briolay, 1997, barbins apears as a monophyletic group within Cyprinine group. In bootstrap analysis for the node of Barbins and Leuciscine we obtained a value (Fig.4) smaller than 50, whitch may indicate hybrid species.

CONCLUSIONS

The present results are largely in agreement with other molecular phylogeny studies on cyprinids. The topologies of *cox1* and *cox2* based neighbor-joining trees allowed us to identify two major lineages in cyprinids: Cyprinine and Leuciscine. In Cyprinine group we identified a 95% sequence homology for *cox2* gene between *Carassius* species, *Cyprinus carpio* being mapped close to *Carrasius sp*. For the Leuciscine group *Hypophthalmichthys molitrix* and *Arischthys nobilis* were found to belong to the same genera based on both *cox 1* and *cox 2* sequences. Positioning of *Barbus meridionalis* as a different branch from Cyprinine and Leuciscine may indicate a hybrid species, as it should fall in the Leuciscine lineage.

The results obtained thus far clearly prove that the used methodology represents the technical support which will allow the evaluation of homology degree between different cyprinids from Romania and the analysis of a large number of species.

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FIGURE CAPTIONS

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cox1F A.nobilis          GTAATATTCA T-CGGCGTAA ATCTTACATT CTTCCCACAA CACTTCCTAG
cox1F C.idellus          GTAATGTTCA T-CGGTGTAA ACCTCACATT CTTCCCGCAA CACTTCCTAG
cox1F H.molitrix         GTAATGTTCA T-CGGCGTAA ATCTTACATT CTTCCCACAA CACTTCCTAG
cox1F R.rutilus          ATTATATTTA TTCGGTGTGA -TCTTACATT CTTCCCACAA CACTTCCTAG
cox1F B.meridionalis     GGAGTTCACT CTCGAGGTGA -TAGAAGATT CTTCCCTCA- CATTTCCTAG
Clustal Consensus              *       **  **  *       *  ***  ******  **   **  *******

cox1F A.nobilis          GTCTAGCAGG AATGC----C ACGACGATAC -TCTGACTAC CCAGATGCCT
cox1F C.idellus          GCCTAGCAGG AATGC----C ACGACGATAC -TCCGACTAT CCGGACGCCT
cox1F H.molitrix         GCCTAGCAGG AATGC----C ACGACGATAC -TCCGACTAC CCAGATGCCT
cox1F R.rutilus          GCCTAGCAGG AATAC----C ACGACGATAT -TCTGACTAC CCAGACGCTT
cox1F B.meridionalis     G-CTACGAGG AATACACAGC AGACAGATAC ATCTGACTAC CCCGACGCCT
Clustal Consensus        *  ***   ***  ***  *      *  *      ****    **  *****   **  **  **  *

cox1F A.nobilis          ACGCCCTGTG AAATACAGTA TCATCTATCG GATCTCTTAT TTCCCTGGTA
cox1F C.idellus          ACGCCCTATG AAATACAGTA TCATCTATCG GATCACTTAT CTCCTTAGTA
cox1F H.molitrix         ACGCCCTGTG AAATACAGTA TCATCTATCG GATCTCTTAT TTCCCTAGTA
cox1F R.rutilus          ATGCCCTATG AAATACAGTG TCGTCTATCG GCTCACTCAT CTCATTAGTG
cox1F B.meridionalis     ACGCCCTATG AAATACAGTG TCATCCATTG GATCACTCAT CTCCCTGGTC
Clustal Consensus        *  *****  **  **********  **  **  **  *  *  **  **  **   **   *  **

cox1F A.nobilis          GCAGTAATTA TGTTCCTATT TATCCTATGA GAAGCCTTCG CCGCTAAAAC
cox1F C.idellus          GCAGTAATTA TATTCCTATT TATCCTATGA GAAGCCTTCG CCGCTAAAAC
cox1F H.molitrix         GCAGTAATTA TATTCCTATT TATTCTATGA GAAGCCTTCG CCGCTAAA-C
cox1F R.rutilus          GCAGTAATTA TGTTCCTATT TATCCTCTGA GAAGCCTTCG CCGCTAA--C
cox1F B.meridionalis     GCAGTAATTA TATTCCTATT TATTCTGTGA GAAGCCTTCG CCGCTCAA-C
Clustal Consensus        **********  *  *********  ***  **  ***  **********  *****  *   *

cox1F A.nobilis          GAGAA
cox1F C.idellus          GAGAA
cox1F H.molitrix         GAGAA
cox1F R.rutilus          GAGAA
cox1F B.meridionalis     GAGAA
Clustal Consensus        *****
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Figure 1. *Clustal X fragment alignment of mitochondrial cox1 gene*

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cox2F C.auratus          TAGTTGTCCC AATAGAGTCC CCAGTCCGTG TCTTAGTATC CGCTGAAGAC GTACTACACT
cox2F C.carassius        TAGTTGTCCC AATGGAGTCC CCAGTCCGTG TTTTAGTATC CGCTGAAGAC GTACTACACT
cox2F C.carpio           TAGTTGTTCC AATAGAATCC CCAGTCCGTG TCCTAGTATC TGCTGAAGAC GTGCTACATT
cox2F H.molitrix         TAGTAGTCCC CATAGAATCG CCAGTTCGTG TTCTAGTATC CGCCGAAGAT GTATTACACT
cox2F B.barbus           TAGTTGTACC AATAGAATCA CCTATTCGTG TGCTGGTATC CGCTGAAGAC GTTTTGCACT
cox2F L.celensis         TAGTAGTCCC AATAGAATCA CCAGTTCGTG TTTTAGTATC CGCAGAAGAC GTGTTACACT
cox2F R.rutilus          TAGTAGTTCC GATAGAGTCA CCAGTTCGTG TTTTAGTATC CGCAGAAGAC GTATTACACT
cox2F A.nobilis          TAGTAGTCCC CATAGAATCG CCAGTTCGTG TTCTAGTATC CGCCGAAGAT GTATTACACT
Clustal Consensus        ****  **  **   **  **  **   **   *  ****  *   *  *****   **  *****   **   *  **  *

cox2F C.auratus          CCTGAGCCGT TCCATCCTTA GGTGTAAAAA TAGACGCAGT CCCAGGCCGA CTAAATCAAA
cox2F C.carassius        CCTGAGCTGT TCCATCTTTA GGTGTAAAAA TAGACGCAGT CCCCGGACGA CTAAATCAAA
cox2F C.carpio           CTTGAGCTGT TCCATCCCTT GGCGTAAAAA TGGACGCAGT CCCAGGACGA CTGAATCAAG
cox2F H.molitrix         CTTGAGCCGT TCCATCCCTA GGCGTAAAAA TGGACGCAGT ACCAGGACGA CTTAACCAAA
cox2F B.barbus           CATGAGCCGT CCCATCTCTA GGTGTAAAAA TAGACGCAGT ACCAGGACGA CTAAACCAAA
cox2F L.celensis         CCTGAGCCGT TCCATCTTTA GGCGTAAAAA TAGACGCAGT GCCCGGCCGA CTAAACCAAA
cox2F R.rutilus          CTTGAGCAGT CCCATCTTTG GGCGTAAAAA TAGACGCAGT ACCAGGACGA TTAAATCAAA
cox2F A.nobilis          CCTGAGCCGT TCCATCCCTG GGCGTAAAAA TGGACGCAGT ACCAGGACGA TTAAACCAAA
Clustal Consensus        *  *****  **   *****   *   **  *******  *  ********   **  **  ***   *  **  ***

cox2F C.auratus          CTGCTTTCAT CGCCTCACGC CCAGGAGTAT T-CTACGGAC ATG
cox2F C.carassius        CTGCCTTCAT CGCCTCACGC CCAGGAGTGT T-CTACGGAC ATG
cox2F C.carpio           CCGCCTTTAT TGCCTCACGC CCAGGGGTGT T-TTACGGAC ATG
cox2F H.molitrix         CTGCCTTTAT TGCCTCACGC CCAGGCGTAT T-TTACGGAC ATG
cox2F B.barbus           CTGCCTTCAT TGCCTCCCGC CCAGGGCTCT T-CTACGGAC ATG
cox2F L.celensis         CTGCCTTCAT CGCGTCGCGC CCCGGCGTGT T-CTACGGAC ---
cox2F R.rutilus          CTGCCTTCAT CGCCTCCCGC CCAGGCGTAT TTCTACGGAC ATG
cox2F A.nobilis          CTGCTTTTAT TGCCTCGCGC CCAGGCGTAT T-CTACGGAC ATG
Clustal Consensus        *  **  **  **   **  **  ***  **  **   *  *  *   *******
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Figure 2. *Clustal X fragment alignment of mitochondrial cox2 gene*

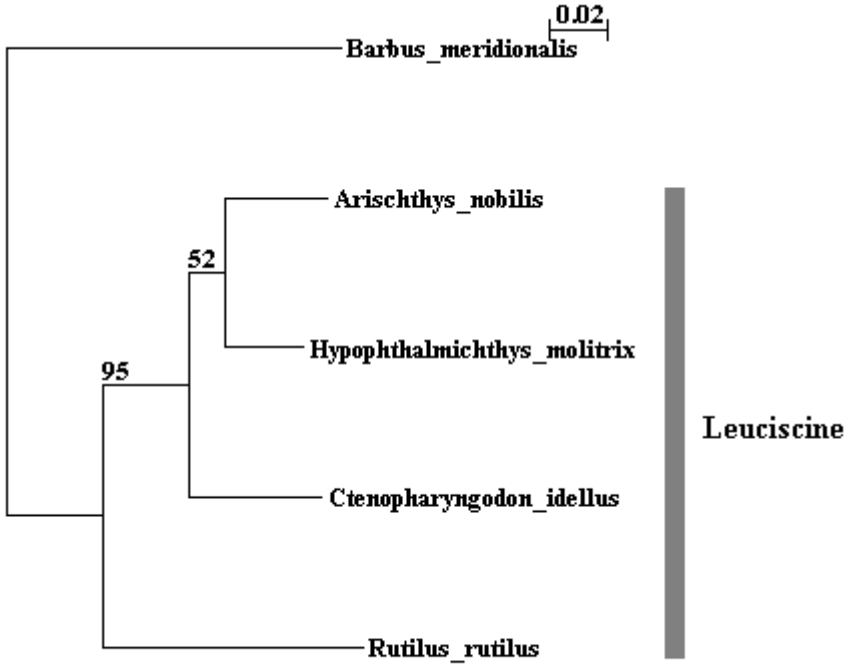

Figure 3. *Neighbor-joining tree based on sequenced cox1 fragment*

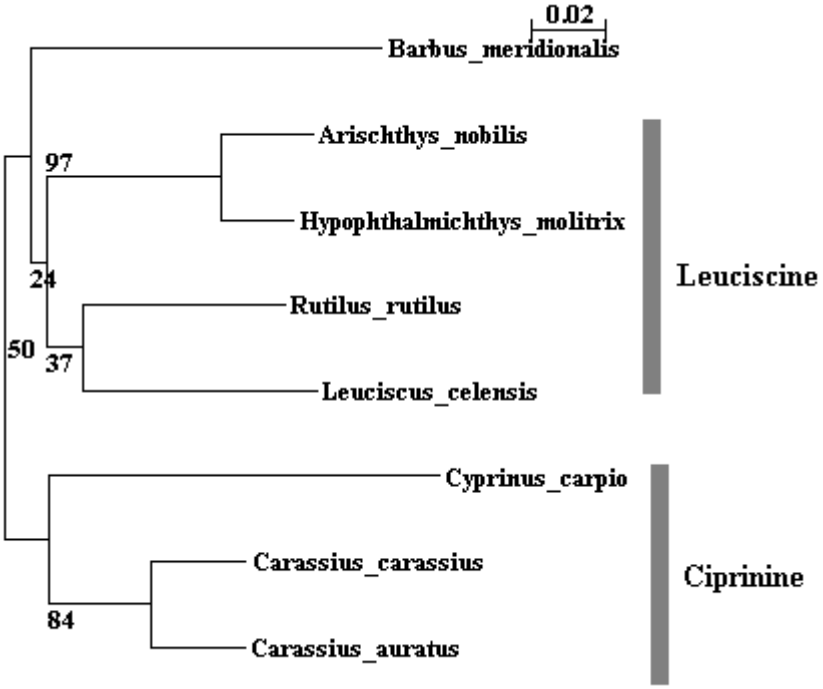

Figure 4. *Neighbor-joining tree based on sequenced cox2 fragment*