ROMANIAN CYPRINIDS PHYLOGENY BASED ON 16S ARN MITOCHONDRIAL GENES**

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** Original scientific paper

Abstract: The vertebrate mitochondrial genome has been an important model system for studying molecular evolution, organismal phylogeny, and genome structure. Phylogenetic relationships were inferred from analysis of 570 base pairs (bp) of mitochondrial DNA (mtDNA), representing a conserved region of 16S rRNA. We sequenced 13 cyprinids species and one putative outgroup (Misgurnus fossilis) from Romania. Based upon nucleotide sequence comparisons of cyprinid mitochondrial 16SRNA genes, we established the phylogenetic relationships between analyzed species. The phylogenetic trees obtained by two different methods (neighbor-joining and maximum parsimony) have the same topology and show that most species examined have supported the traditional division of the Cyprinidae into two subfamilies: Cyprininae and Leuciscinae.

Keywords: cyprinids, molecular phylogeny, 16S rRNA mitochondrial gene

Introduction

The mitochondrial genome (mtDNA) represents a favored genetic source for evolutionary studies due to four valuable features: a) a faster evolutionary rate than nuclear genome, and this provides higher resolution in phylogenies of closely related species; b) a mechanism of maternal inheritance and lack of recombination, which introduces fewer errors into the phylogenetic reconstructions; c) a compact genome, which allows easier DNA sequence determination and computational analyses than nuclear genomes would; d) the presence of various protein-coding genes, which
provide an evolutionary context of the genome. For this study, we selected as mitochondrial marker the 16S RNA gene.


The species included in this study are: *Carrasius auratus Gibelio, C. auratus auratus, Barbus meridionalis, Cyprinus carpio, Abramis bjoerka, Hypophthalmichthys molitrix, Arischthys nobilis, Tinca tinca, Gobio gobio, Phoxinus phoxinus, Leuciscus cephalus, Scardinus erytrophtalmus, Rutilus rutilus* and the outgroup *Misgurnus fossilis*.

**Materials and Methods**

Total DNA was isolated from liver tissue using the protocol Wizard Genomic DNA Purification Kit (Promega) and then was amplified with one set of primers: 16S F (5’-AGA GTG GGA AGA GCT CCG GGT-3’) and 16S R (5’-CCG AAC ACA AAC GGC TCA AG -3’). PCR amplification was performed at an initial denaturation 95°C for 3 min, followed by 35 cycles at 95°C for 45s, 55°C for 60s and 72°C for 90s.

The fragments containing mtDNA 16SRNA gene (570pb) were electrophoretically separated and excised from agarose gels, followed by purification using the Wizard PCR Preps DNA Purification System Kit (Promega). Purified PCR products were sequenced with the ABI PRISM ® BigDye ™ Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems). The sequences were processed with ABI PRISM DNA Sequencing Analysis Software.

Sequences of 16SRNA genes were aligned using ClustalX followed by manual adjustment. For the species with high homology, sequences were aligned using BLAST2sequence program. The phylogeny used and presented here was inferred using the 16S RNA genes by maximum-parsimony (MP) and neighbor-joining (NJ) analysis in PAUP 4.0 beta Trial Version (Swofford 1997). This analysis incorporated the GTR+ Γ +I model of evolution, which was the best-fit model under all criteria in ModelTest (Posada and Crandall, 1998). Support for this topology was evaluated based on 1000 NJ bootstraps (in PAUP) with MP distances calculated under the same model as above.
Results and Discussion

The mitochondrial 16S RNA genes were amplified by PCR and sequenced in both orientations in all cyprinid species tested. A 570bp fragment sequence obtained was aligned only for the species with high homology using the program BLAST2 sequence.

For the Carassius species we identified 100% sequence homology, while for the Carassius auratus gibelio and Barbus barbus sequence homology was 86%. These three species belong to the same group of ciprinids: Ciprininae. In another group (Leuciscinae), the sequence homology was found 96% both for the Scardinus erythropthalmus and Rutilus rutilus and for the Hypophthalmichthys molitrix and Arischthys nobilis.

Both the neighbor-joining (NJ) and maximum parsimony (MP) analyses arrived at a similar and congruent tree. The robustness of the trees was confirmed by bootstrapping (fig.1 and fig.2). In bootstrap consensus trees only values (numbers at nodes representing the percentage of 1000 bootstrap replications) greater than 50 are significant, revealing a true phylogenetic relationship.

It may be observed that high bootstrap values in tree nodes coincide with a high homology between the species (obtained via Blast analysis), which supports a close kinship relation between the assayed species.

Two major assemblages could be distinguished within the Cyprinidae based on both the 16S RNA gene NJ tree (fig.1) and the 16S RNA gene MP tree (fig.2): i) the ciprininae group at the basal position of the tree included: Cyprinus carpio, Barbus barbus, Abramis bjoerka, Carassius auratus gibelio and Carassius auratus auratus and ii) the leuciscinae group with Hypophthalmichthys molitrix, Arischthys nobilis, Tinca tinca, Gobio gobio, Phoxinus phoxinus, Leuciscus cephalus, Scardinus erythropthalmus, Rutilus rutilus. This topology with two exception is in accord with the specialized literature (Zardoya, R. and Doadrio I., 1998, Howes, 1991, Cavender and Coburn, 1992). The exceptions were: Tinca whose positioning is controversial in the literature as it may be part of one of two groups or it may be a separate branch: the Tincninae group and Abramis, which in the literature about trees constructed based on a cytochrome b gene is seen as part of the leuciscinae group (Zardoya, R. and Doadrio I., 1998).
Figure 1. Neighbour-joining tree based on sequenced 16S RNA gene fragment

Figure 2. Maximum parsimony tree based on sequenced 16S RNA gene fragment
Conclusion

The present results are largely in agreement with other molecular phylogeny studies on cyprinids. The topologies of 16SRNA based neighbor-joining and maximum parsimony trees allowed us to identify two major lineages in cyprinids: Cyprinine and Leuciscine. The outgroup species, Misgurnus fossilis is identified separately in both trees, this species belonging to another fish family: Cobitidae.

Tincinae is the most disputed group in the cyprinids. It has been assigned to Cyprininae and Leuciscinae by different authors (Chen et al. 1984; Howes 1991; Cavender and Coburn 1992). The present study supports its position in the lineage Leuciscinae, but its exact position is unresolved. The present monophyly of the Cyprininae (sensu Howes) is congruent with the groupings of Chen et al.(1984) and the suggestion of Arai (1982) that the Barbinae and the Cyprininae are closely related.

Both the neighbor-joining (NJ) and maximum parsimony (MP) analysis arrived at a similar and congruent tree with the bootstrap values greater than 50. These great values were sustained by the Balst2Sequence analysis.

**References**


