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

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Abstract: The vertebrate mitochondrial genome has been an important model system for studying molecular evolution, organismal phylogeny, and genome structure. Phylogenetic relatioships were inferred from analysis of 570 base pairs (bp) of mithocondrial 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.

Earlier classifications of cyprinids were proposed (*Banarescu, 1973, Chen et al., 1984, Howes, 1991, Cavender and Coburn, 1992, Berrebi et al., 1996, Zardoya and Meyer, 1996, Huanzhang Liu and Yiyu Chen, 2003*), but the relationship between the members of this family is still open.

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^{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 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 ^{TM} 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 *16SRNA* 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 *BLAST2sequence*.

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 erytrophtalmus* and *Rutilus rutilus* and for the *Hypophthalmichthys molitrix* and *Arischthys nobilis.*

Both the neigbhor-joinig (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 *16SRNA gene* NJ tree (fig.1) and the *16SRNA 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 erytrophtalmus, 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*).

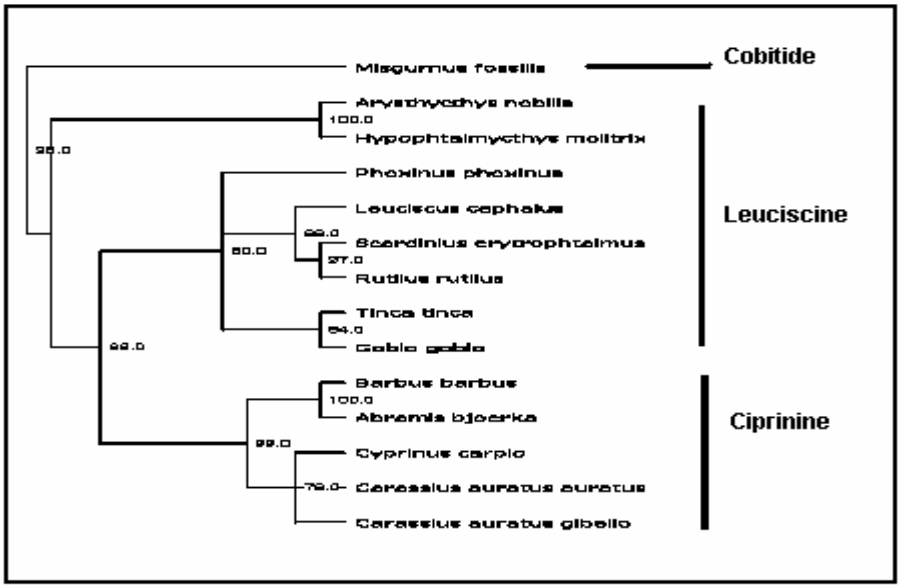

Figure 1. Neighbour-joining tree based on sequenced *16SRNA* gene fragment

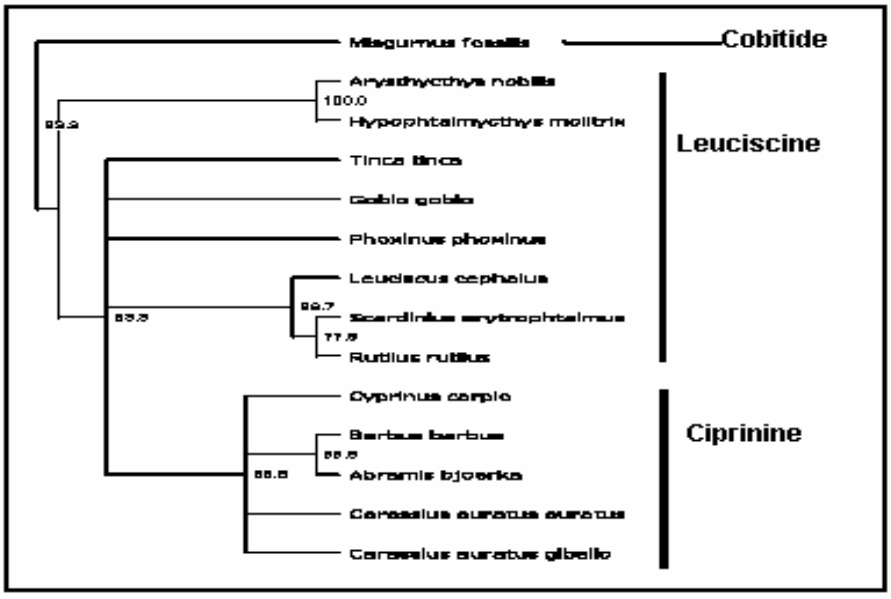

Figure 2. Maximum parsimony tree based on sequenced *16SRNA* gene fragment

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.

FILOGENEZA RUMUNSKIH VRSTA RIBA IZ FAMILIJE Cyprinidae ZASNOVANA NA 16S ARN MITOHONDRIJALNOM GENU

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Rezime

Evropske vrste riba iz familija *Cyprinidae* su od skora postali fokus molekularnih studija, istraživanja kojima se ispituju bilo kakve velike divergencije unutar familije ili, što je češći slučaj, odnosi između vrsta i populacija u specifičnim evropskim oblastima. Ispitivali smo filogenetske odnose među rumunskim vrstama *Cyprinidae* koristeći nizove/sekvence gena 16SRNA. Na osnovu poređenja nizova/sekvenci nukleotida vrsta riba iz familije *Cyprinidae* mithondrijalnih 16SRNA gena, utvrdili smo filogenetske odnose između analiziranih vrsta (*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 i spoljašnje grupe *Misgurnus fossilis*.) Naši rezultati ukazuju da vrste pripadaju dvema osnovnim kladama (grupe organizama koji su se razvili od istih predaka) koje su konzistentne u odnosu na one prethodno definisane iz morfoloških struktura. Jedna grupa je *ciprininae* grupa na osnovnoj poziciji stabla i uključuje: *Cyprinus carpio, Barbus barbus, Abramis bjoerka, Carassius auratus gibelio* i *Carassius auratus auratus* i druga klada/grupa: *leuciscinae* grupa koja uključuje *Hypophthalmichthys molitrix, Arischthys nobilis, Tinca tinca, Gobio gobio, Phoxinus phoxinus, Leuciscus cephalus, Scardinus erytrophtalmus, Rutilus rutilus*. Iako su dobijeni korišćenjem dve različite metode, stabla imaju približno istu topologiju sa vrednostima u nodama višim od 50 što ukazuje na stvarne veze između vrste riba iz familije *Cyprinidae* vrsta analiziranih u istraživanju. *Tincinae* je najkontroverznija grupa kod vrste ribe iz familije *Cyprinidae*, koja je svrstana u *Leuciscinae* grupu, mesto koje može ali i ne mora da bude potvrđeno studijom o filogenetskim odnosima/vezama između *Ciprinidae* na bazi njihovih mitohondrijalnih filogenetskih markera.

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