SOME PHYLOGENETIC ASPECTS OF DECORATIVE FISHES BASED ON MITOCHONDRIAL 12S AND 16S rDNA GENES

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În lucrare sunt prezentate unele aspecte de analiză filogenetică a unor specii de peşti decorativi în baza procentului de similaritate a genelor mitocondriale 12S şi 16S rADN, fiind utilizată metoda PCR cu primeri specifici şi informaţia din baza de date NCBI, inclusiv programul CLUSTAL W.

Analiza comparativă a pus în evidenţă cel mai înalt grad de omologie a genelor: 12S – 94% şi 16S – 92% la *Trichogaster trichopterus* şi *Trichogaster leerii* din ordinul *Perciformes*. În cazul speciei *Platydoras costatus*, ordinul *Silusiformes,* s-au constatat cele mai mari divergenţe pentru ambele gene studiate.

Introduction

Fishes show a remarkable level of diversity affecting their morphology, ecology, behavior and genomes as well as multiple other facets of their biology. This makes them extremely attractive for the study of many evolutionary questions related to diverse aspects of biology. Fish biodiversity is important to humans at the economical, ecological and cultural points of view, and its maintenance is an important challenge for the next generations. New insights from several fish species and sequencing projects have shed new light on the organization and evolution of fish genomes and now allow one to approach the evolutionary mechanisms possibly underlying biodiversity in the fish lineage.

Phylogenetic analysis of DNA has become an important tool for studying the evolutionary history of organisms from bacteria to humans. The reconstruction of phylogenetic relationships has increased dramatically since the advent of molecular techniques (as allozyme analysis, restriction fragment length polymorphism, random amplified polymorphic DNA). Mitochondrial DNA markers are used as tools for estimating the phylogenetic relationships of different kinds of organisms [1]. So, it is important to choose an appropriate genetic marker for phylogenetic analysis.

The animal mtDNA is haploid [2,3], generally considered as non-recombining [4,5] and the signal from genetic drift is therefore stronger than for nuclear loci [6]. The mitochondrial genome of fishes contains 13 proteins genes which code for subunits of enzymes (ATP synthesis or electron transport), 2 ribosomal RNA (rDNA) genes, and 22 transfer ARN (tRNA) genes (Figure 1).

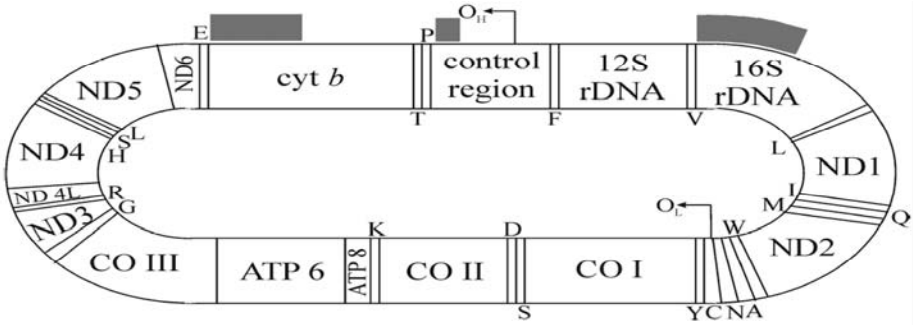

Fig.1. Mitochondrial functional map of fish:
O_H – origin of H-strand replication; O_L – origin of L-strand replication; The majority of tRNA-genes and coding sequences foe all proteins are on the H-strand (except ND6); tARN genes coded for by L-strand outside; grey bars indicate regions sequenced in this study [after 3,7].

Mitochondrial genes show different rates of evolution, which determine their applicability as molecular markers. Slow evolving sequences, such as 16S and 12S ribosomal RNA (rRNA) genes have been widely used in phylogenetic studies among vertebrates [8-11].

In this work, we present some molecular phylogeny aspects for six species of decorative fishes based on *neighbor-joining* by the percent of similarity for mitochondrial sequences (12S and 16S RNA genes) from GeneBank, using Clustal W Software.

Material and Methods

All the specimens including in this study represent three orders: *Perciformes* (family *Osphronemidae* with three species), *Cyprinodontiformes* (family *Poeciliidae* with two species), and *Siluriformes* (family *Doradidae* with one species), (table 1).

Table 1

The orders used in this study

	Species (accession numbers)	
Order *Perciformes* Family *Osphronemidae*	*Betta splendens (*AF519650*)*	
	*Trichogaster trichopterus (*AY763713*)*	
	*Trichogaster leerii (*AF519656*)*	
Order *Cyprinodontiformes* Family *Poeciliidae*	*Xiphophorus hellerii (*EF017497*)*	
	*Poecilia reticulata (*EF017485*)*	
Order *Siluriformes* Family *Doradidae*	*Platydoras costatus (*AY264078*)*	

Extraction of DNA. Genomic DNA was isolated from fresh muscle six species. About 300-500 mg of tissue was ground with nitrogen liquid in 500 µl homogenization buffer (133 mM Tris-OH; 0,95 mM NaCl; 6,7 mM EDTA; 1,33% Na Sarcosyl; 1,33% mercaptoethanol) with pH 7,8. The mixture was incubated at 55ºC for an hour. DNA was purified with chloroform:isoamyl alcohol (24:1) extractions, precipitated with 76% ethyl alcohol with sodium acetate (0,2 M) and cooled absolute ethanol, followed by washing with 70% ethanol and resuspended in sterile water. DNA quality was verified by 1% agarose gel electrophoresis.

PCR conditions. The polymerase chain reaction (PCR) was used to amplify 12S and 16S rRNA genes using following PCR primers (Table 2) [12]:

Table 2

Primers used in this study

Primer	**Sequence (5´to 3´)**	**T_m (ºC)**
12SA(F)	AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT	96,0
12SB(R)	TGACTGCAGAGGGTGACGGGGCGGTGTGT	96,0
16SA(F)	CGCCTGTTTATCAAAACAT	52,0
16SB(R)	CCGGTCTGAACTCAGATCACGT	68,0

Amplifications were performed in a final volume of 25 µl containing: 1X PCR tampon, 1 unit/reaction of Taq DNA polymerase (Germany), 200 µM of dNTP, 50 pM of each primer and 100 ng DNA template. The amplifications were carried out in a thermal cycler (Corbett, Australia) programmed for initial heat denaturation in one step of 2 minutes at 95ºC. Subsequent 35 cycles: 40 seconds at 92ºC, 40 seconds at 54ºC and 90 seconds at 72ºC; followed by one final step of primer extension at 72ºC for 2 minutes.

For the second step, 12S and 16S rRNA genes' sequences of all six studied fish's species from Gene Bank database were analyzed by NCBI-Blast and then compared using Clustal W (accession numbers are listed in Table 1).

Results and Discussion

The electrophoresis analysis of PCR amplicons, obtained by using the *12S rRNA* and *16S rRNA* genes universal primers, revealed the amplification of expected fragments having a size of 374 bp for the first and 544 bp for the second to all assays, according literature data (Figure 2) [13].

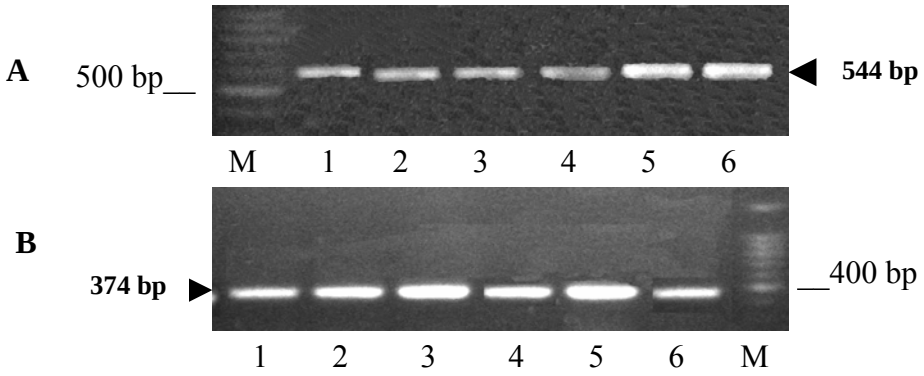

Fig.2. PCR products of 12S and 16S ribosomal genes in 1,4% agarose gel:
M – marker molecular (bp); **A –** PCR/primer universal 16S a rARN; **B** – PCR/primer universal 12S a rARN: 1 – *Trichogaster trichopterus*, 2 – *Poecilia reticulata*, 3 – *Betta splendens*, 4 – *Platidoras costatus* , 5 – *Xiphophorus hellerii*, 6 – *Trichogaster leerii.*

Alignment of nucleic acid or protein sequences is a critical early step in comparative molecular and evolutionary study. With increasing sequences divergence, it becomes harder to construct alignments that reliable reflect historical relationships.

PCR results demonstrated the presence of expected bands for both analyzed genes at all essays, but it is not enough to see differences between fish's species. In these situations, multiple-sequence alignment programs are often favored because of their more objective (mathematical) criteria.

For the follow step, 12S and 16S rRNA genes' sequences of all six studied fish's species from Gene Bank database were analyzed by NCBI-Blast and then compared using Clustal W.

Clustal W is a general purpose multiple sequence alignment program. It is use to align DNA or protein sequences in order to elucidate their relatedness as well as their evolutionary origin. It calculates the best match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen. Evolutionary relationships can be revealed via analyzing of phylograms that is assumed to be an estimate of phylogeny for all species included in study. The branch lengths are proportional to the amount of inferred evolutionary change.

On first step Clustal W determine all pair wise alignments between sequences and the degree of similarity between them. Then construct a similarity tree by calculating the distances based on neighbor-joining clustering method. It is based on the minimum-evolution criterion for phylogenetic trees. The main virtue of neighbor-joining is its efficiency. It can be used on very large data sets for which other means of phylogenetic analysis like minimum evolution, maximum parsimony and maximum likelihood.

After the sequences were aligned, the based compositions of species studied were analyzed separately for the 12S and 16S ribosomal genes.

The obtained results by comparison of these 6 species of decorative fish's sequences in Clustal W for 12S rRNA gene, show the following score of identity:

SeqA name	**Len (nt)**	**Start of Pairwise alignments**
		Aligning...
Sequence 1: betta.splendens-12.	501 bp	Sequences (1:2) Aligned. Score: 75
Sequence 2: platydoras.costatus-12.	950 bp	Sequences (1:3) Aligned. Score: 83
Sequence 3: poecilia.reticulata-12.	459 bp	Sequences (1:4) Aligned. Score: 85
Sequence 4: trichogaster.leerii-12.	495 bp	Sequences (1:5) Aligned. Score: 86
Sequence 5: trichogaster.trichopterus-12.	496 bp	Sequences (1:6) Aligned. Score: 79
Sequence 6: xiphophorus.hellerii-12.	458 bp	Sequences (2:3) Aligned. Score: 81
		Sequences (2:4) Aligned. Score: 83
		Sequences (2:5) Aligned. Score: 79
		Sequences (2:6) Aligned. Score: 80
		Sequences (3:4) Aligned. Score: 84
		Sequences (3:5) Aligned. Score: 84
		Sequences (3:6) Aligned. Score: 90
		Sequences (4:5) Aligned. Score: 94
		Sequences (4:6) Aligned. Score: 85
		Sequences (5:6) Aligned. Score: 85

Phylogram based on *neighbor-joining* using % identity for 12S ribosomal rRNA gene for fish species, shows that species *Poecila reticulata* and *Xiphophorus hellerii*, from *Cyprinodontiformes* order are more closely (Figure 3). Also, we can mention that species from order *Perciformes,* family *Osphronemidae (Trichogaster leerii*, *Trichogaster trichopterus* and *Betta plendens*) have common ancestry. This thing can be explained that these two orders are genetically and morphologically closed. At the same level with *Betta splendens* is another group with: *Trichogaster leerii* and *Trichogaster trichopterus*. These three species are from the *Perciformes* order.

The phylogenetic tree by analyzing 12S rRNA gene presents that *Platydoras costatus*, order *Silusiformes*, is more distant from another clusters.

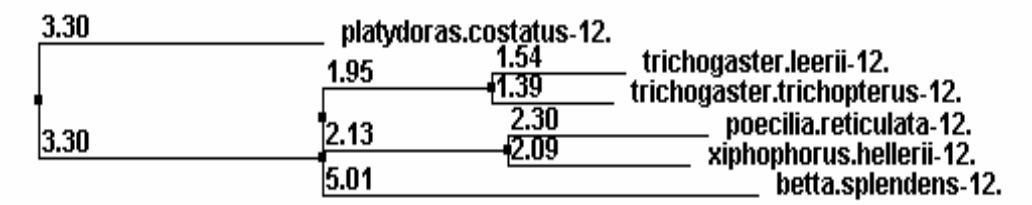

Fig.3. Phylogram based on neighbor joining using % identity for 12S ribosomal rRNA gene.

The comparison of 16S rRNA gene sequences for studied fish species show the following score of identity:

SeqA name	**Len (nt)**	**Start of Pairwise alignments**
		Aligning...
Sequence 1: betta.splendens-16.	1513 bp	Sequences (1:2) Aligned. Score: 1
Sequence 2: platydoras.costatus-16.	1726 bp	Sequences (1:3) Aligned. Score: 6
Sequence 3: poecilia.reticulata-16.	549 bp	Sequences (1:4) Aligned. Score: 2
Sequence 4: trichogaster.leerii-16.	2071 bp	Sequences (1:5) Aligned. Score: 2
Sequence 5: trichogaster.trichopterus-16	2074 bp	Sequences (1:6) Aligned. Score: 7
Sequence 6: xiphophorus.hellerii-16.	549 bp	Sequences (2:3) Aligned. Score: 6
		Sequences (2:4) Aligned. Score: 69
		Sequences (2:5) Aligned. Score: 69
		Sequences (2:6) Aligned. Score: 7
		Sequences (3:4) Aligned. Score: 82
		Sequences (3:5) Aligned. Score: 82
		Sequences (3:6) Aligned. Score: 88
		Sequences (4:5) Aligned. Score: 92
		Sequences (4:6) Aligned. Score: 83
		Sequences (5:6) Aligned. Score: 84

The phylogenetic tree based on *neighbor-joining* by analizing 16S rRNA gene demonstrated that Trichogaster *trichopterus* and *Trichogaster leerii* (order *Perciformes*) shows the similar results like in study of 12S rRNA gene (Figure 4). Nearly to this group is *Xiphophorus hellerii*, from order *Cyprinodontiformes* which are closed to order *Perciformes*. Concomitantly it shows that *Betta splendens* and *Platydoras costatus* are closely even if they bellow to different families (*Osphronemidae* and *Doradidae* respectively).

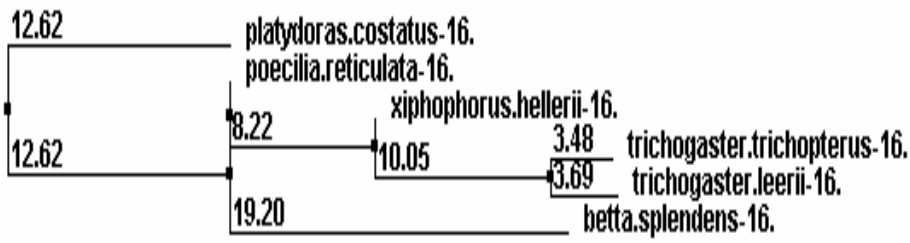

Fig.4. Phylogram based on neighbour joining using % identity for 16S ribosomal rRNA gene.

In conclusion, the comparative analyses of six species of decorative fishes based on percent identity for 12S and 16S ribosomal genes using NCBI database and Clustal W Software, demonstrated that *Trichogaster trichopterus* and *Trichogaster leerii,* order *Perciformes* are the most similar: 12S rRNA – 94% and 16S rRNA – 92% homology. While, *Platydoras costatus*, order *Silusiformes* highlighted more differences in both cases.

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