THE ANALYSIS OF CYTOCHROME B SEQUENCE FOR *CARASSIUS GIBELIO* BLOCH., 1782 INDIVIDUALS FROM TAUTESTI LAKE (IASI COUNTY)

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Keywords: mitochondrial DNA, Cyt b, *Carassius gibelio* Bloch., 1782.
Abstact: In this experiment we investigated the genetic diversity of *Carassius gibelio* Bloch, individuals, from Tautesti Lake (Iasi County), by analysing the sequence of cytochrome b. The study of cytochrome b first part gene coding region has permitted the identification of two new haplotypes.

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 and 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.

The wide distribution of cyprinids raises very interesting biogeographical and evolutionary questions regarding the origin and further radiation of these fish. For instance, cyprinids within Europe show a particularly interesting distribution pattern with numerous endemic species in the Iberian Peninsula and southern Greece, and relatively small species genera in Central Europe (Bănărescu, 1973). This characteristic distribution has been explained in terms of an ancient isolation of the Iberian Peninsula and southern Greece from the rest of the continent, which would have limited (as they are primary freshwater fish) the number of cyprinid genera able to colonize both regions. However, the precise scenario that led to the actual biogeographical distribution remains unsettled. Although some of the oldest cyprinid fossils are found in the Oligocene strata of Central Europe, it is generally accepted that European cyprinids are of Asian origin (Bănărescu 1989, 1992).

It is difficult to build a comprehensive phylogeny of *Cyprinidae* due to the large number of genera and species. Previous systematic analyses have focused on morphology but in recent years, numerous efforts have been devoted to clarifying the relationships among cyprinids using molecular techniques, as described previously (Briolay *et al*., 1998; Gilles *et al*., 1998, 2001; Zardoya and Doadrio, 1998; Liu and 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, as described previously. Such data appear useful because molecular characters are less likely related to adaptative evolution than are morphologic characters.

Mitochondrial DNA (mtDNA) is DNA that is located in mitochondria. This is in contrast to most DNA of eukaryotic organisms, which is found in the nucleus. Nuclear and mtDNA are thought to be of separate evolutionary origin, with the mtDNA being derived from bacteria that were engulfed by early precursors of eukaryotic cells. Thus in cells in current organisms, the vast majority of proteins found in the mitochondria (~1500 in mammals) are encoded by nuclear DNA: some, if not most, are thought to have been originally of bacterial origin and have since been transferred to the nucleus during evolution.

Unlike nuclear DNA in which the genes are rearranged by ~50% each generation (due to the process called recombination), there is usually no change in mtDNA from parent to offspring by this mechanism. Because of this and the fact that its mutation rate is higher than nuclear DNA and easily measured, mtDNA is a powerful tool for tracking matrilineage, and has been used in this role for tracking many species back hundreds of generations.

Most animal mitochondrial genomes contain 37 genes, including 13 proteincoding genes, 2 ribosomal RNAs (rRNA) and 22 transfer RNAs (tRNA) necessary for translation of the proteins encoded by the mtDNA. They also possess a major noncoding control region that contains the initial sites for mtDNA replication and mtRNA transcription. The mitochondrial genome generally evolves at elevated rates (5–10 times) compared to single copy nuclear genes, however its genes order often remains unchanged over long periods of evolutionary time, with some exceptions. The genetic code of mitochondrial genomes is more degenerated and thus less constrained than the universal eukaryotic nuclear code.

Mitochondrial DNA derived markers have become popular for evolutionary studies, as the data obtained by their analysis may yield significant insights into the evolution of both the organisms and their genomes.

MATERIALS AND METHODS

The experimental material was represented by 40 samples of dorsal muscle, the equivalent of the same number of individuals of *Carassius gibelio* Bloch., 1782, from Tautesti (Iasi County) pond (Figure 1). The samples obtain had approximately 1 cm long tissue from the dorsal muscle and they were kept in absolute ethanol at -20ºC.

Figure 1. Tăuteşti Lake and the sampling point (market with the arrow)

DNA isolation and purification was performed with phenol : chloroform : isoamilic alcohol (25 : 24 : 1) (Ausubel *et al*., 1995).

Cellular lyses was done using 500µl lyses buffer, 10µl K proteinase and 20-200mg muscular tissue which were incubated at 37º C for 12 hours.

After the incubation period in each tube we added 600µl phenol-clorophorm-isoamylalcohol (25 : 24 : 1) and we shaked the tubes for 30-60 seconds, then the samples were centrifuged at 8000rpm, 4 minutes. After the centrifugation we separated 1 layer from liquid column in new labeled tubes. After we added 550µl chloroform, a second centrifugation at 8000rpm, 3 minutes and we shaked the tubes for 30-60 seconds with the separation of the first layer from liquid column in new labeled tubes. After the final separation, in the Eppendorf tubes which contained the purified DNA we added 1ml of absolute ethanol kept at -20º C, shaked the tubes 30-60 minutes and kept them 30-60 minutes at -20º C during which DNA precipitated.

In the next phase the tubes were centrifuged at 10000rpm, 5 minutes for pellet obtaining. After the ethanol removal the tubes were dryed in a centrifuge with vacuum for 10 minutes. All pellets were resuspended in TE buffer (pH=8.0) and kept at -20º C.

Polymerase chain reaction (PCR) was used to amplify the cytochrome b first part gene coding region sequence (400pb) and the two specific primers used were L14724 (Pääbo, 1990) and H15149 (Kocher *et. al*., 1989).

L14724 5`-CGAAGCTTGATATGAAAAACCATCGTTG-3`

H15149 5`-AAACTGCAGCCCTCAGAATGATATTTGTCCTCA-3`,

PCR conditions were as follows: a 25µl final reaction volume/sample containing (12,5µl Go Taq Flexi Mix (*Promega*), 1µl H, L primers and 2µl DNA). Amplification cycles were the following: 5′ at 95°C; 40 cycles of denaturation at 94°C for 1′, annealing at 45°C for 1′, and 72°C for 1′, final extension 10′ at 72°C.

The PCR products were electrophoretically tested using a 1.5% agarose gel with 30ml TBE 1X volume and 0,45g of agarose and a 100 base pairs molecular marker. The electrophoresis were made at 90V, 50-60mA for 40-50 minutes.

All amplicons were purified through Wizard SV Gel and PCR Clean-up System (*Promega*) following the manufacturer's instructions.

The PCR products were transferred in SV minicolomns assembly and incubated for 1 minute at room temperature. The SV minicolomns assembly were centrifugated at 14000rpm, 1 minute, after the liquid in the collection tubes were removed and the SV columns were put back.

The colomns were washed with 700µl Membrane Wash Solution (previously eluded with 95% ethanol) and then were centrifugated for 1 minute at 14000rpm. The wash was repeated with 500µl Membrane Wash Solution and then were centrifugated for 5 minutes at 14000rpm.

The liquid from the collection tubes was removed and then were centrifugated for 1 minute to eliminate the ethanol residues. Carefully the SV columns were transferred in new 1,5ml tubes and in the center of the colomns were added 50µl nuclease free water without reaching the membrane with the pipette. The tubes are incubated for 1 minute at room temperature and then are centrifugated at 14000rpm, 1 minute.

The SV columns were removed and the eluded DNA was stored at 4º C or -20º C.

Products quantification was made using a spectrophotometer.

The Sequencing reaction kit contains: 10X buffer, dNTP mix, ddATP, ddGTP, ddCTP, ddTTp, primer, DNA polymerases.

The final reaction volume/sample is 20µl, containing (12,5µl reaction Mix and 7,5µl DNA and H_2OmiliQ).

The next step was the precipitation of samples in ethanol. We prepared the Stop/glycogen solution (2µl sodium acetate 3M (pH 5,2), 2µl 100mM

Na_2-EDTA (pH 8,0) and 1µl 20mg/ml glycogen.

In each labelled 0,5ml tube we added 5µl Stop/glycogen solution and then we transferred the content of sequencing reaction and mix.

We added 65µl etanol 95% kept at -20°C, shake them and centrifúgated immediately at 4°C, 14000rpm, for 15 minutes. After the centrifugation the liquid was removed (the pellet should be visible).

After we added 200µl ethanol 70% kept at -20°C, in each tube, immediately centrifugated at 4°C, 14000rpm, for 2 minutes. The liquid was removed without eliminate the pellet and this last step was repeated.

After, the tubes were dryed in a vacuum centrifuge for 10 minutes and the samples were resuspended with 40µl SLS (Sample Loading Solution).

The resuspended samples were transferred in the instrument plate and covered with one drop of mineral oil. For this reaction we used an 8 capillaries Beckman Coulter 8000 sequencer, 120 minutes.

These sequences were analyzed using CEQ 8000 and Lasergene v.7 softwares and were registered the similarity and divergence percents.

The aligning of all sequences was made by Clustal W (Thompson *et al*., 1994) using the MegAlign module within the programme DNA STAR 5, Lasergene. The new haplotypes were identified and for each of them was established the base composition, and was modulated the secondary structure of RNAm. To analysis the phylogenetic relationships a phylogenetic tree was realized based on similarity and divergence percents.

RESULTS AND DISCUSSIONS

From the alignment of the 36 sequences of mitochondrial cyt b gene we identified the existence of two differences within the sequences C12TL and respectively C31TL, in both cases the differences were transvertions.

Two new haplotypes were identified for the gene that determines the synthesis of cytochrome b, the general haplotype which includes sequences with no differences (C01TL, C02TL, C03TL, C04TL, C05TL, C06TL, C07TL, C09TL, C10TL, C11TL, C13TL, C14TL, C15TL, C16TL, C17TL, C19TL, C20TL, C21TL, C22TL, C24TL, C25TL, C26TL, C27TL, C28TL, C29TL, C30TL, C32TL, C33TL, C34TL, C35TL, C36TL, C37TL, C38TL, C40TL) and a second haplotype which includes two sequences (C12TL, C31TL) that differ from the general haplotype by the existence of a transvertion in positon 379.

Table 1. Similarity and divergence percents for the analyzed haplotypes

	Similarity percent					
Divergence percent		C01TL	C02TL	C12TL	C31TL	
	C01TL		100.0	99.7	99.7	C01TL
	C02TL	0.0		99.7	99.7	C02TL
	C12TL	0.3	0.3		100.0	C12TL
	C31TL	0.3	0.3	0.0		C31TL
		C01TL	C02TL	C12TL	C31TL	

From Table 1 we can observe that the similarity percents varies between 100% for C01TL, C02TL and 99,7 % for C12TL and C31TL.

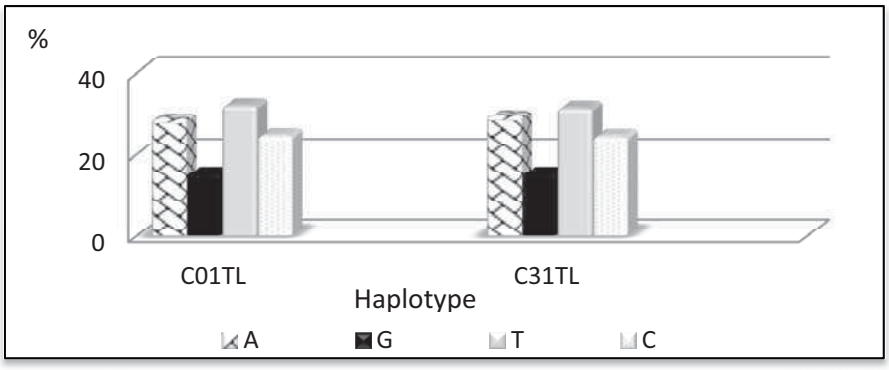

Figure 2. Nucleotides frequency chart within the analysed haplotypes

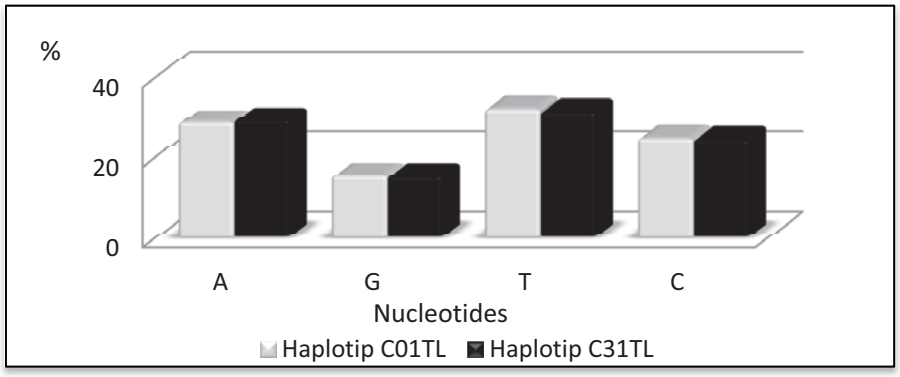

Figure 3. Comparative chart for the nucleotides frequences in the analysed

As we can see in Table 2 and Figure 2 and 3 there are no major differences between the 2 analysed haplotypes. Regarding the amount of adenine (A), this is 28.4% for the haplotype C01TL and 29,1%. for the haplotype C31TL. The amount of guanine is in proportion of 15.2% for both haplotypes. Thymine varies between 31.9% for haplotype C01TL and 31.3% and for haplotype C31TL, and cytosine between 24.6% for the haplotype C01TL and 24.4% for the haplotype C31TL.

The percentage of complementary nucleotides is 60.25% for A + T for the haplotype C01TL and 60.45% for haplotype the C31TL.

The percentage of G + C is 39.75% for the haplotype C01TL and 39.55% for the haplotype C31TL.

Using DNA Star program GeneQuest module was modeled RNA structure for the two haplotypes. For the general haplotype the RNA structure was modeled based on a minimum energy of - 49.48 (Figure 4) and for the haplotype C31TL using a a minimum energy of -51,66 (Figure 5).

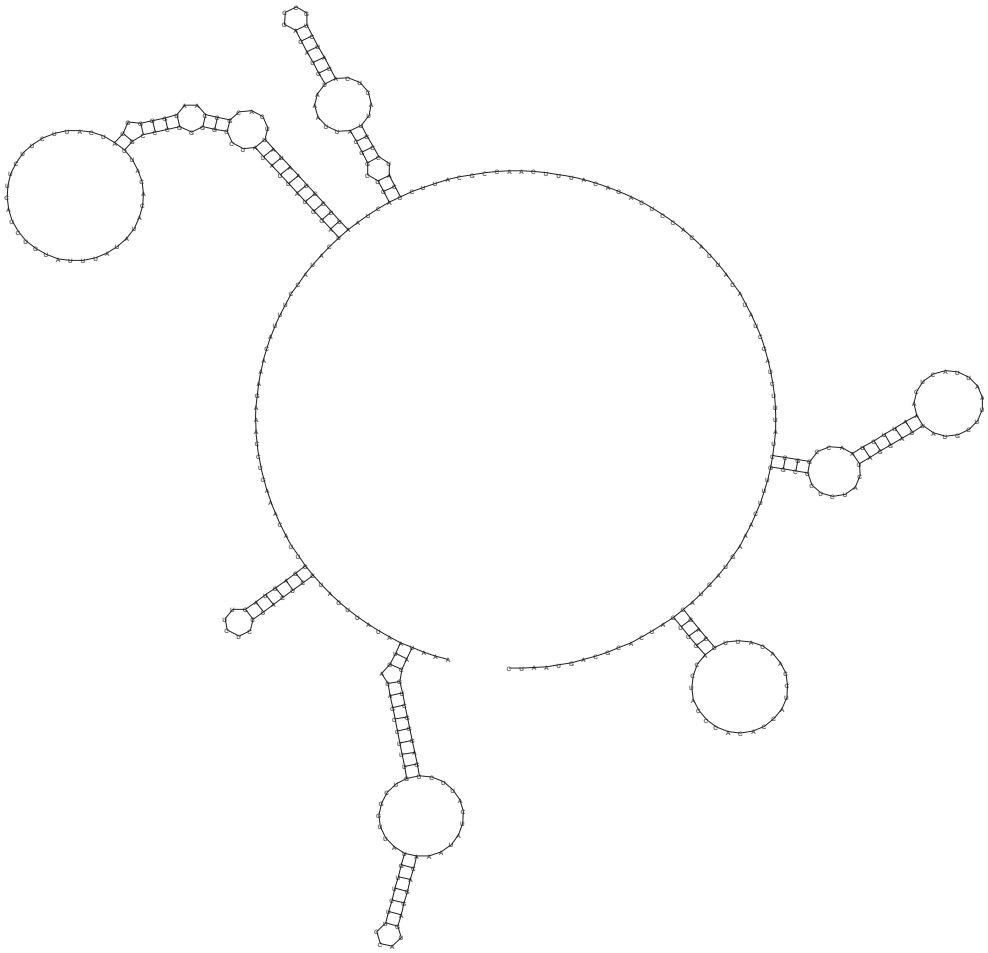

Figure 4. Modeling the secondary structure of mRNA for cytochrome b haplotype C01TL, at the species *Carassius gibelio* Bloch.

From Figure 4, we find the existence of 15 single-stranded regions and 14 doubled-stranded regions.

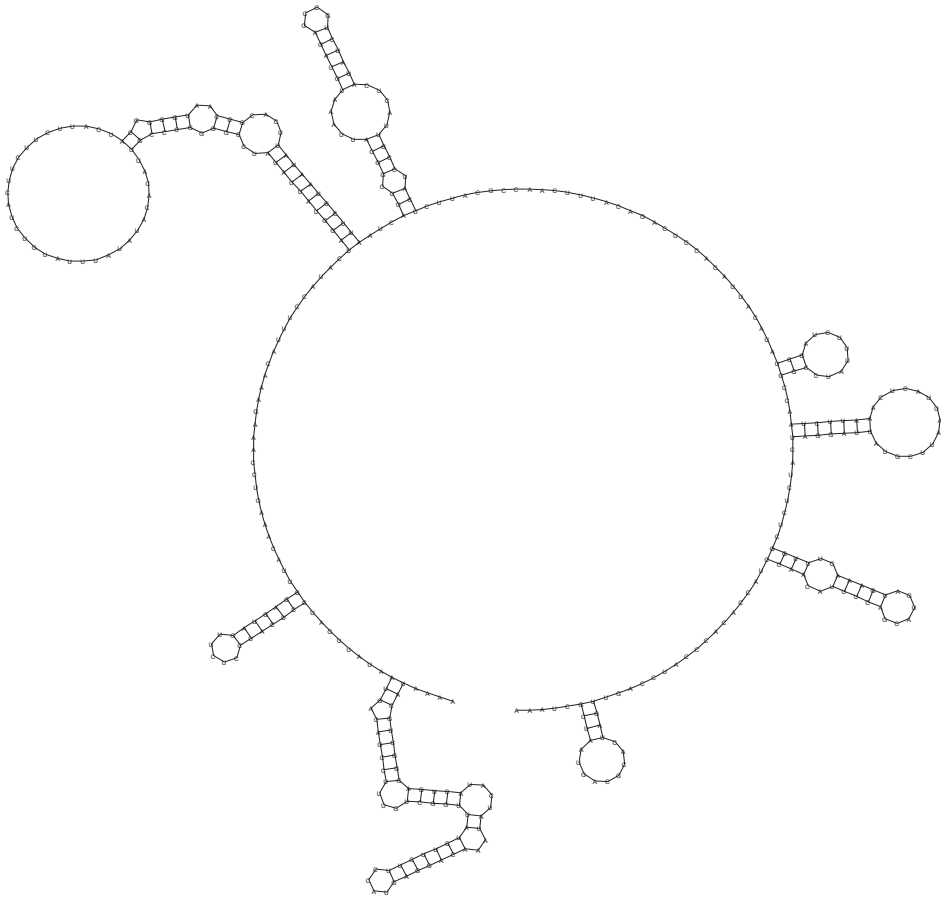

Figure 5. Modeling the secondary structure of mRNA for cytochrome b haplotype C31TL, at the species *Carassius gibelio* Bloch.

From Figure 5, we find the existence of 19 single-stranded regions and 18 doubled-stranded regions.

In order to establish the phylogenetic relationships between the analysed haplotypes a phylogenetic tree was made based on the similarity percents (Figure 6).

Figure 6. Phylogenetic tree based on the cytochrome b sequences, based on the degree of similarity, for individuals belonging to *Carassius* genera.

CONCLUSIONS

From the alignment of 36 sequences, 2 differences were recorded. The comparison of the sequences from all individuals of the analyzed population shows the existence of 2 differences, both transvertions in 379 position (where thimine was substituted with adenine), within C12TL and C31TL sequences.

Two new haplotypes for cytochome b were found: one general haplotype and a specific haplotype for sequences C12TL and C31TL with a mutation in 379 position.

The similarity percents for the 2 studied haplotypes are of 99.7% because the difference consists of one nucleotide.

We can conclude that for both new haplotypes from the analyzed population, the adenine concentration is 28.4% for the general haplotype (C01TL) and 29.1% for the second one (C31TL).

Guanine quantity is the same for both haplotypes 15.2%.

Thymine quantity varies between 31,9% for the haplotype C01TL and 31,3% for the haplotype C31TL.

Cytosine has values between 24,6% for the general haplotype and 24,4% for the second haplotype.

A+T percent is 60.25% for the first haplotype and 60.45% for the second one, while, C+G concentration is 39.75% for general haplotype and
39.55% for the second.

Modelling the mRNA secondary structure has highlighted 15 single-stranded regions and 14 doubled-stranded regions for the haplotype C01TL and 19 single-stranded regions and 18 doubled-stranded regions for the second haplotype.

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Acknowledgements: This work was supported by the European Social Fund in Romania, under the responsibility of the Managing Authority for the Sectorial Operational Programme for Human Resourses Development 2007-2013 [grant POSDRU/88/1.5/S/47646.