PHYLOGENETIC RELATIONSHIPS OF *CARASSIUS GIBELIO* BLOCH., 1782 AS INFERRED FROM MITOCHONDRIAL DNA VARIATION

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Keywords: mitochondrial DNA, D-loop, Cyt b, *Carassius gibelio* Bloch., 1782.
Abstact: The analysis of control region (D-loop) and nucleotide sequence variation of the cytochrome b (Cyt b) gene were used to study the mtDNA divergence for *Carassius gibelio* Bloch., 1782 individuals, and to examine the phylogenetic relationships within studied populations.

INTRODUCTION

The Cyprininae is the largest subfamily of the Order Cypriniformes, which is the most diverse group of freshwater fishes on the planet, widely distributed in Eurasia, East Indian Island, Africa, and North America (Nelson, 2006). 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. (Briolay, 1998).

In southern Europe, cyprinids are extremely rich in number of endemic species (about 77 species), especially when compared with the relatively uniform cyprinid fauna of central and northern Europe (Kottelat *et al*., 1997). Despite numerous publications concerning the species classification within this family, the relationships among the main lineages of cyprinids still remain unclear, and even the monophyly of the whole family is sometimes in doubt (Howes *et al*., 1987). Traditionally, cyprinids were grouped as different subfamilies according to their morphological characters (Cavender *et al*., 1992). Depending on the authors, the number of subfamilies was very different.

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 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.

MtDNA has an important advantage in all phylogenetic research, because it is inherited through the maternal line. This fact facilitates the monitoring of its transmission along the evolutive lines starting in the early evolution. In case that one individual is not available for a direct comparison with a biological sample, any sample which comes from the maternal genitor can be a usable one.

Another advantage is the one that mtDNA has a high level of variability and a high rate of mutation, comparatively with the nuclear DNA, in spite of the fact that it doesn’t encode the information for the synthesis of many proteins. This fact makes it ideal for the phylogenetic and phylogeographical studies, as described previously (Brown *et al*., 1979). The great number of polymorphism belonging to the nucleotide sequences from two hypervariable areas located in the region of noncoding mitochondrial control, can allow the discrimination between different individuals or different biological samples.

Another charasteristic of mtDNA, generally accepted as a real advantage for the population genetic studies, is the lack of recombination. The majority of the animals have a sexed reproduction. In this type of reproduction the genes are transmitted from both parents after the recombination process through crossing-over and the independent segregation of chromosomes during the first meiosis. But the genes of organelles coming from different lines of filiation connot ever recombine because the genome of the organelles (such as mtDNA) is uniparental transmitted. Also, if it has been biparental inherited, the organelles from the two genitors wouldn’t be able to merge, one cannot find the recombinant genomes at mitochondrial level (Birky, 2001).

MATERIALS AND METHODS

The research was carried out on 83 individuals of *Carassius gibelio* (Bloch, 1782) (Cyprinidae) from two different populations, Buzau River (Buzau County) (36 individuals) (Figure 1) and Sofronesti pond (Vaslui County) (47 individuals) (Figure 2), from which dorsal muscular tissue was sampled. The samples had approximately 1 cm long tissue from the dorsal muscle and they were kept in absolute ethanol at -20°C.

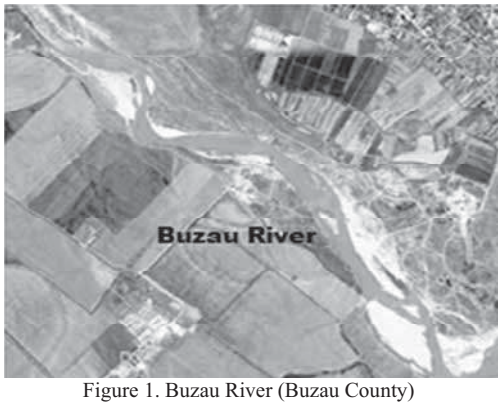

Figure 1. Buzau River (Buzau County)

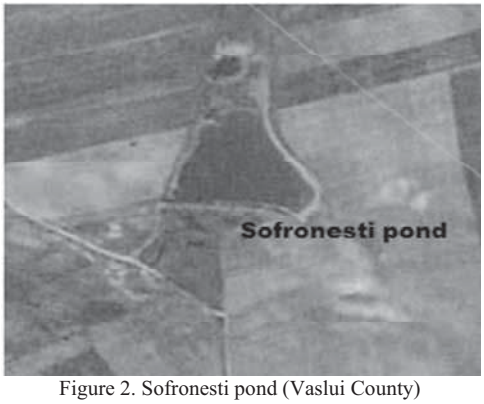

Figure 2. Sofronesti pond (Vaslui County)

DNA isolation and purification was performed with Wizard SV Genomic DNA Purification System (*Promega*).

First we prepare the Digestion Solution for every tissue sample, combining the following reagents: Nuclei Lysis Solution 200µl, 0.5M EDTA (pH 8.0) 50µl, proteinase K, 20mg/ml 20µl, RNase A Solution 5µl**,** total volume 275µl per sample.

We cut up to 20mg of tissue sample, into two equally sized pieces and place them in a 2ml microcentrifuge tube then we add 275µl of the prepared Digestion Solution to each sample tube and incubate the sample tubes overnight (16–18 hours) in a 55°C water bath. After overnight proteinase K digestion, centrifuge samples at 2000 × *g* to pellet any undigested hair or cartilage. We transfer supernatant to a new 1.5ml microcentrifuge tube and in the next phase we add 250µl of Wizard SV Lysis Buffer to each sample and vortex to mix.

We prepare and labeled one Wizard SV Minicolumn assembly for each lysate and after we transfer the entire sample lysate from the 1.5ml microcentrifuge tube to a minicolumn assembly.

We placed the minicolumn assembly containing the sample lysate into a microcentrifuge and spin at 13000 × *g* for 3 minutes to bind the genomic DNA to the minicolumn.

Then we remove the minicolumn from the minicolumn assembly and discard the liquid in the Collection Tube. We add 650µl of Wizard SV Wash Solution to each minicolumn assembly and centrifuge at 13000 × *g* for 1 minute, after we discard the liquid in the Collection Tube and replace minicolumn into the empty Collection Tube.

We repeat the washing three times more so in the end they were four washes of the minicolumns. After the last wash, we empty the collection tube and we centrifuged the minicolumn assembly at 13000 × *g* for 2 minutes to dry the binding matrix.

We remove the minicolumn and placed in a new labeled 1.5ml microcentrifuge tube for elution. We add 150µl of room temperature Nuclease-Free Water to the minicolumns. Incubate for 2 minutes at room temperature and place the minicolumn/elution tube assembly into the centrifuge and spin at 13000 × *g* for 1 minute. In the same way we made a second elution with 100µl and a third one with 80µl. (Water was heated at 65°C before adding it to the column for elution). Total elution volume will be approximately 380µl.

We remove the minicolumns and discard and kept the elution tubes containing the purified genomic DNA stored at –20°C.

Polymerase chain reaction (PCR) was used to amplify the mitochondrial control region (D-loop) using two specific primers 16373F and 00992R and for cytochrome b the primers used were 15245F and 16490R.

16373F 5`-TTCGCACTGTTCCTTGTTCTCTTC-3` 15245F 5`-CCGAGACCAATGACTTGAAGAACAACCG-3`

00992R 5`-GTCGGGACCATGCCTTTGTG-3` 16490R 5`-CTGGGCGCTAGGGAGGAATTTAACC-3`

PCR conditions were as follows: a 25µl final reaction volume/sample containing (2,5µl 10X PCR buffer, 2µl $MgCl_2$, 0,8µl dNPPs, 0,2µl AmpliTaq DNA Polymerase (*Applied Biosystems*) 0,5µl F, R primers and 1µl DNA). Amplification cycles were the following: 3′ at 94°C; 35 cycles of denaturation at 94°C for 30″, annealing at 50°C for 30″, and 72°C for 30 ″, final extension 7′ at 72°C.

The PCR products were electrophoretically tested using a 1.5% agarose gel with 100ml TAE 1X volume and 1,5g of agarose and 0,8µl ethidium bromide and a 100 base pairs molecular marker. The electrophoresis were made at 80V for 30-40 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 16000× *g*, 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 16000× *g*. The wash was repeated with 500µl Membrane Wash Solution and then were centrifugated for 5 minutes at 16000× *g*.

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 38µl 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 16000× *g*, 1 minute.

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

Products quantification were electrophoretically tested using 1,5% agarose gel.

The sequencing reaction was made by Macrogen Inc. These sequences were analyzed using Chromas 233 and Lasergene v.7 softwares. 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 was established the haploptypes frequence in each population. To analysis the phylogenetic relationships a phylogenetic tree was realized based on similarity and divergence percents.

RESULTS AND DISCUSSIONS

We analysed the data for each population and for each gene. From the alignment of the 36 sequences of mitochondrial control region from Buzau population we identified the existence of 136 differences, most were found in the sequences C02BF, C11BF and C13BF, 20 differences from which 17 transitions, 2 transvertions and 1 deletion. Another important difference was observed within the sequences C01BF, C03BF, C06BF, C07BF, C12BF, C15BF, C17BV, C23BF, C24BF, C26BF, C27BF, C28BF, C29BF, C32BF, C33BF, C34BF, C35BF and C36BF the deletion of a 18pb fragment.

Eight new haplotypes were identified for mitochondrial control region (D-loop).

The similarity percents varies between 100% for C01BF, C21BF and 98,2 % for C02BF (Table 1).

Table 1. Similarity and divergence percents for the analyzed haplotypes in Buzau population, based on D-loop sequences

	Similarity percent								
Divergence percent		C01BF	C02BF	C04BF	C09BF	C10BF	C14BF	C21BF	C30BF	
	C01BF		98.2	99.9	99.8	99.8	99.7	100.0	99.6	C01BF
	C02BF	0.8		98.0	97.9	98.2	97.8	98.1	98.1	C02BF
	C04BF	0.1	2.0		99.9	99.7	99.8	99.9	99.5	C04BF
	C09BF	0.2	2.1	0.1		99.6	99.9	99.8	99.4	C09BF
	C10BF	0.2	1.8	0.3	0.4		99.5	99.8	99.8	C10BF
	C14BF	0.3	2.2	0.2	0.1	0.5		99.7	99.4	C14BF
	C21BF	0.0	2.0	0.1	0.2	0.2	0.3		99.6	C21BF
	C30BF	0.4	2.0	0.5	0.6	0.2	0.7	0.4		C30BF

		C01BF	C02BF	C04BF	C09BF	C10BF	C14BF	C21BF	C30BF	

From Figure 3 we can observe that C01BF is the most frequent haplotype in Buzau population (50%) while C14BF and C30BF have the lowest frequence (3%).

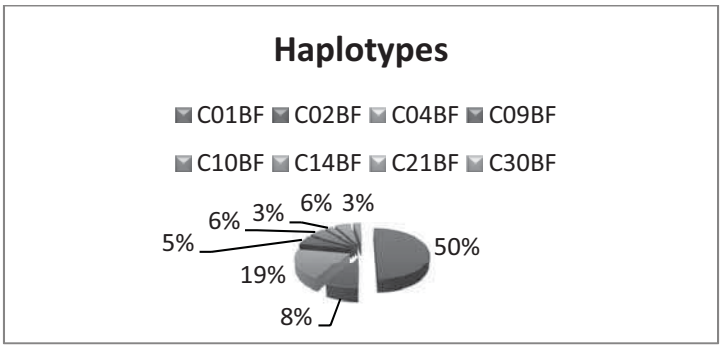

Figure 3. Haploptypes frequence in Buzau population based on D-loop sequences

In order to establish the phylogenetic relationships between the analyzed haplotypes a phylogenetic tree was made based on the similarity percents (Figure 4).

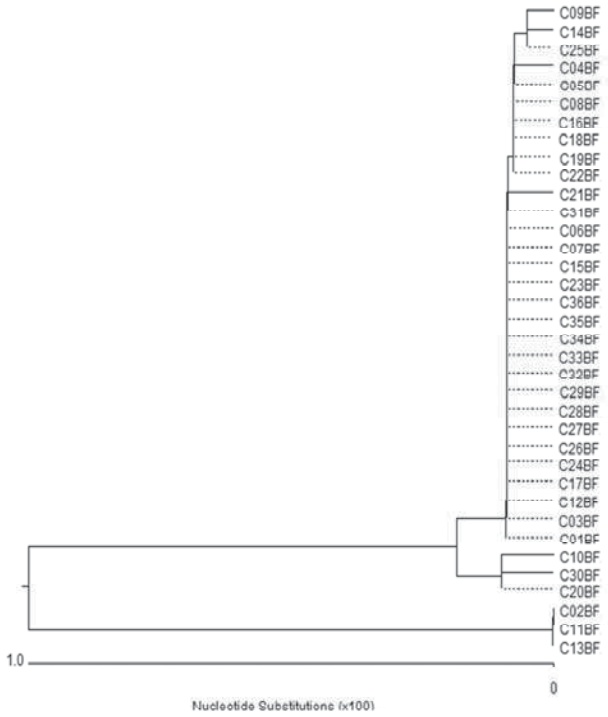

Figure 4. Phylogenetic tree based on the D-loop sequences, based on the degree of similarity, for individuals belonging to *Carassius* genera (Buzau population)

For the second population (Vaslui) we align 46 sequences of mitochondrial control region and we identified the existence of 52 differences, most were found in the sequences C03VF and C22VF, we identified 4 differences, 3 transitions and 1 deletion. Another important difference was observed within the sequences C02VF, C09VF, C25VF, C29VF, C30VF, C31VF, C32FV, C36VF, C37VF, C45VF and C46VF the deletion of a 18pb fragment.

For mitochondrial control region (D-loop) eleven new haplotypes were identified.

From Table 2 we can see that he similarity percents varies between 100% for C01VF and C02VF, and 99,7 % for C03VF.

Table 2. Similarity and divergence percents for the analyzed haplotypes in Vaslui population, based on D-loop sequences

	Similarity percent												
Divergence percent		C01 VF	C02 VF	C03 VF	C04 VF	C07 VF	C11 VF	C12 VF	C15 VF	C42 VF	C44 VF	C47 VF	
	C01 VF		100.0	99.7	99.8	99.9	99.8	99.8	99.8	99.8	99.7	99.9	C01 VF
	C02 VF	0.0		99.7	99.8	99.9	99.8	99.8	99.8	99.8	99.7	99.9	C02 VF
	C03 VF	0.3	0.3		99.5	99.6	99.5	99.7	99.7	99.5	99.6	99.6	C03 VF
	C04 VF	0.2	0.2	0.5		99.9	99.6	99.8	99.6	99.8	99.7	99.7	C04 VF
	C07 VF	0.1	0.1	0.4	0.1		99.7	99.9	99.7	99.9	99.8	99.8	C07 VF
	C11 VF	0.2	0.2	0.5	0.4	0.3		99.6	99.6	99.6	99.5	99.7	C11 VF
	C12 VF	0.2	0.2	0.3	0.2	0.1	0.4		99.6	99.8	99.9	99.7	C12 VF
	C15 VF	0.2	0.2	0.3	0.4	0.3	0.4	0.4		99.6	99.5	99.7	C15 VF
	C42 VF	0.2	0.2	0.5	0.2	0.1	0.4	0.2	0.4		99.7	99.7	C42 VF
	C44 VF	0.3	0.3	0.4	0.3	0.2	0.5	0.1	0.5	0.3		99.8	C44 VF
	C47 VF	0.1	0.1	0.4	0.3	0.2	0.3	0.3	0.3	0.3	0.2		C47 VF
		C01 VF	C02 VF	C03 VF	C04 VF	C07 VF	C11 VF	C12 VF	C15 VF	C42 VF	C44 VF	C47 VF	

In Figure 5 we can identified that C01VF, C02VF and C07VF (24%) are the haplotypes with the highest frequence in Vaslui population and that C04VF, C11VF, C42VF, C44VF and C47VF have the lowest frequence (2%).

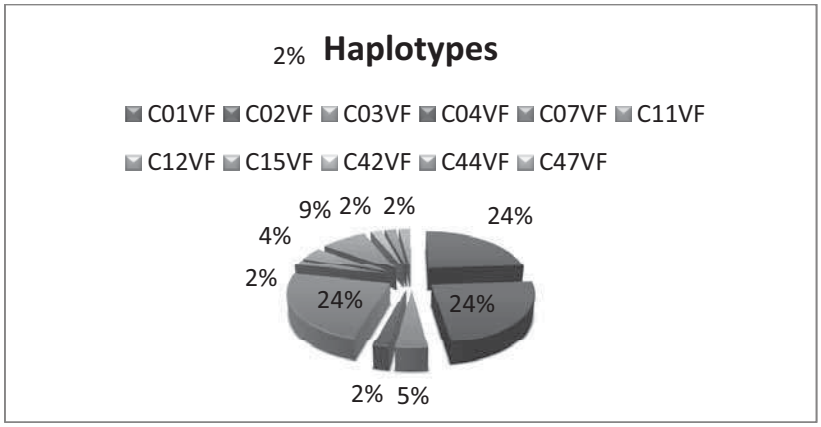

Figure 5. Haploptypes frequence in Vaslui population based on D-loop sequences

To examine the phylogenetic relationships between the analyzed haplotypes for Vaslui population, a phylogenetic tree was made based on the similarity percents (Figure 6).

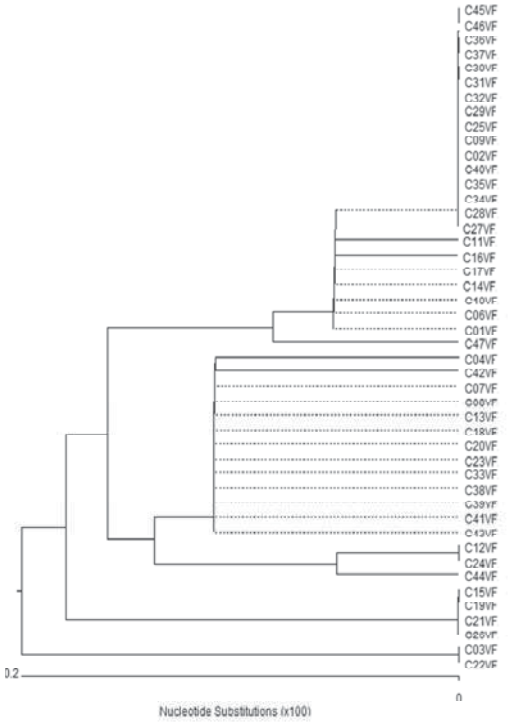

Figure 6. Phylogenetic tree based on D-loop sequences, based on the degree of similarity, for individuals belonging to *Carassius* genera (Vaslui population)

In Buzau population we identified the existence of 71 differences from the alignment of the 36 cytochrome b sequences. Most differences were found in the sequences C02B, C11B and C13B, 14 differences from which 13 transitions and 1 transvertion. Sequences C10B, C20B and C30B presented 3 differences, all of them transvertion.

Four new haplotypes were identified for cytochrome b (Cyt b).

From Table 3 we can observe that the similarity percents varies between 100% for C01B and 98,8 % for C02B.

Table 3 Similarity and divergence percents for the analyzed haplotypes in Buzau population, based on Cyt b sequences

	Similarity percent					
Divergence percent		C01B	C02B	C04B	C10B	
	C01B		98.8	99.8	99.7	C01B
	C02B	1.2		98.6	98.9	C02B
	C04B	0.2	1.4		99.6	C04B
	C10B	0.3	1.2	0.4		C10B
		C01B	C02B	C04B	C10B	

From Figure 7 we can see that C01B is the general haplotype in Buzau population (56%) while C02BF and C10BF has only a 8% frequence.

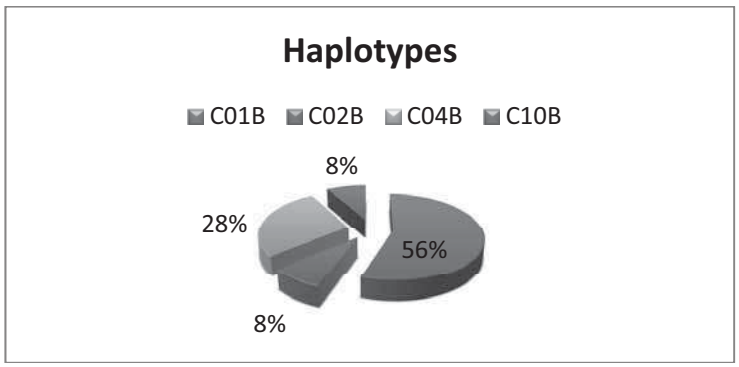

Figure 7. Haploptypes frequence in Buzau population based on Cyt b sequences

To establish the phylogenetic relationships between the analyzed haplotypes a phylogenetic tree was made based on the similarity percents (Figure 8).

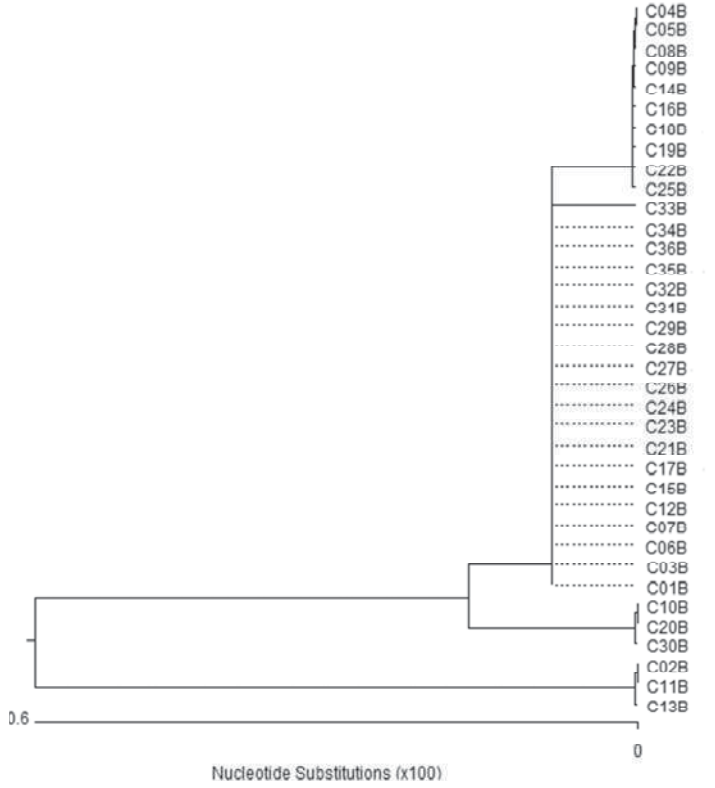

Figure 8. Phylogenetic tree based on the Cyt b sequences, based on the degree of similarity, for individuals belonging to *Carassius* genera (Buzau population)

For Vaslui population we align 47 sequences of cytochrome b and we identified the existence of 52 differences, most were found in the sequences C03VF and C22VF respectively C15V, C19V, C21V and C26V, in both we identified 3 differences, all of them transitions.

Four new haplotypes were identified for cytochrome b (Cyt b).

The similarity percents varies between 100% for C01V and 99,7% for C03VF and C15V (Table 4).

Table 4. Similarity and divergence percents for the analyzed haplotypes in Vaslui population, based on Cyt b sequences

		Similarity percent				
Divergence percent		C01V	C03V	C04V	C15V	
			99.7	99.8	99.7	C01V
C03V	0.3			99.6	99.6	C03V
C04V	0.2	0.4			99.6	C04V
C15V	0.3	0.4	0.4			C15V
		C01V	C03V	C04V	C15V	

In Figure 9 we can identified that C01V is the haplotype with the highest frequence (51%), in Vaslui population and that C03V has the lowest frequence (4%).

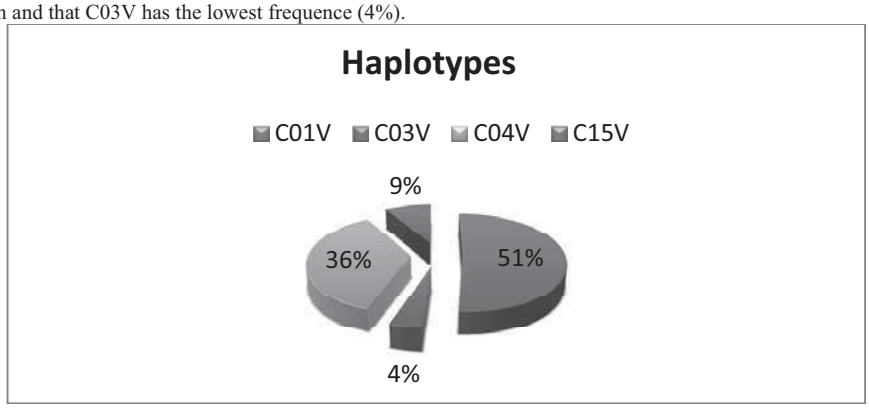

Figure 9. Haploptypes frequence in Vaslui population based on Cyt b sequences

In order to establish the phylogenetic relationships between the analyzed haplotypes for Vaslui population, a phylogenetic tree was made based on the similarity percents (Figure 10).

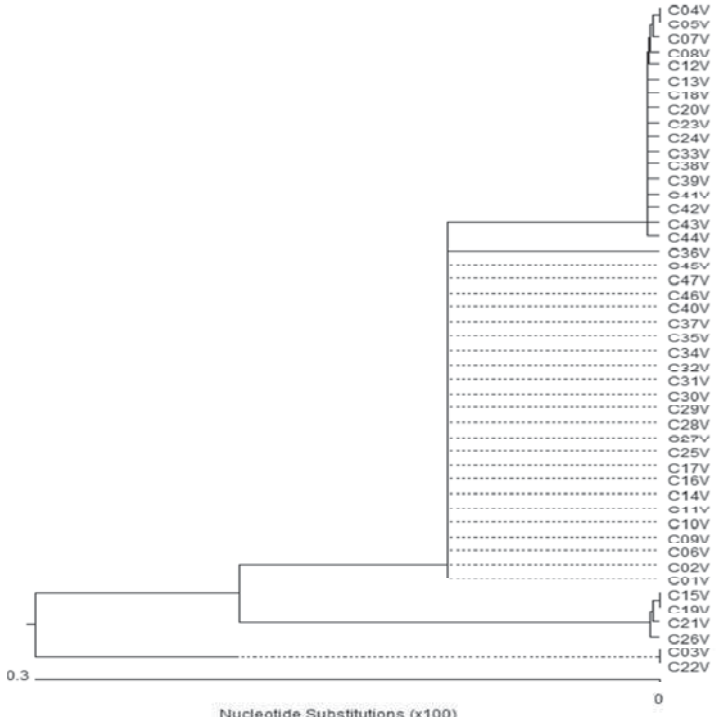

Figure 10. Phylogenetic tree based on the Cyt b sequences, based on the degree of similarity, for individuals belonging to *Carassius* genera (Vaslui population)

CONCLUSIONS

From the alignment of 36 sequences of mitochondrial control region from Buzau population we recorded 136 differences, and for Vaslui population, from the alignment of 46 sequences, 52 differences were identified.

In both population we observed the deletion of a 18bp fragment to more sequences.

For d-loop (mitochondrial control region) eight new haplotypes were identified for Buzau population, while for Vaslui population were identified eleven new haplotypes.

The similarity percents based on D-loop sequences, for Buzau population haplotypes varies between 100% for C01BF, C21BF and 98,2 % for C02BF and in Vaslui population case it varies between 100% for C01VF and C02VF, and 99,7 % for C03VF.

From the alignment of 36 sequences of cytochrome b from Buzau population were recorded 71 differences, and for Vaslui population from the alignment of 47 sequences 52 differences were identified.

In each of the studied population four new haplotypes were identified for cytochrome b.

Based on Cyt b sequences the similarity percents for Buzau population haplotypes varies between 100% for C01B, and 98,8 % for C02B and for Vaslui population it varies between 100% for C01V and 99,7 % for C03V and C15V.

Regarding the frequence of the new haplotypes in the two population we can say that, for D-loop in Buzau population C01BF is the most frequent (50%) while C14BF and C30BF have the lowest frequence (3%), while in Vaslui population we have 3 haplotypes with a frequence of 24% and C04VF, C11VF, C42VF, C44VF and C47VF have the lowest frequence (2%).

For Cyt b in Buzau population C01B is the general haplotype (56%) while C02BF and C10BF have only a 8% frequence and in Vaslui population C01V is the haplotype with the highest frequence (51%) while C03V is the haplotype with the lowest frequence (4%).

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