THE STUDY OF GENETIC DIVERSITY WITHIN *Carassius* GENERA, BASED ON SEQUENCING SOME MITOCHONDRIAL MARKERS

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Abstract. In this study we investigated the genetic diversity within *Carassius* genera, studying individuals from isolated aquatic populations in Romania, by analysing the sequences of three mitochondrial DNA genes: cytochrome b (Cyt b), mitochondrial control region (D-loop) and cytochrome c oxidase I (COX I). The nucleotide sequence variation of the three genes were used to study the mtDNA divergence for *Carassius* genera individuals and to examine the phylogenetic relationships within analyzed populations.

Based on the alignment of cytochrome b gene sequences from individuals belonging to *Carassius* genera from analyzed populations, 21 haplotypes have been identified: two of them were found in four of the six analyzed populations and one in two of studied populations. Regarding the D-loop sequences there were identified 20 haplotypes: four of them were found in two or more populations. Following COX I sequence alignment, from individuals of the *Carassius* genera, in the six populations were identified 22 haplotypes, but only one was found in four of the analyzed populations.

Phylogeographic aspects of the D-loop showed that there are common haplotypes between Buzău (Buzău River, Buzău County, Romania), Sofroneşti (Sofroneşti Lake, Vaslui County, Romania) Delta (Fortuna Lake, Danube Delta, Romania) and Băile Felix (Bihor County, Romania) populations, and for COX I between Buzău (Buzău River, Buzău County, Romania), Tăuteşti (Tăuteşti Lake, Iaşi County, Romania), Delta (Fortuna Lake, Danube Delta, Romania) and Băile Felix (Bihor County, Romania) populations.

From the analysis of all sequences, it was found that the rate of occurrence of transitions is greater than the occurrence of transversions.

Keywords: D-loop; Cyt b; COX I (CO I); *Carassius*.

INTRODUCTION

Molecular phylogenetics applies a combination of molecular and statistical techniques to infer evolutionary relationships among organisms or genes.

The similarity of biological functions and molecular mechanisms in living organisms strongly suggests that species descended from a common ancestor. Molecular phylogenetics uses the structure and function of molecules and how they change over time to infer these evolutionary relationships. This branch of study emerged in the early 20th century but didn't begin in earnest until the 1960s, with the advent of protein sequencing, PCR, electrophoresis and other molecular biology techniques.

The primary objective of molecular phylogenetic studies is to recover the order of evolutionary events and represent them in evolutionary trees that graphically depict relationships among species or genes over time. This is an extremely complex process, further complicated by the fact that there is no one right way to approach all phylogenetic problems. Phylogenetic data sets can consist of hundreds of different species, each of which may have varying mutation rates and patterns that influence evolutionary change.

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 [6]. 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.

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 [8, 14, 15, 37].

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 [3]. 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 [4, 5].

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 [20].

The first classification of ciprinids relied on external characters, but in recent years, molecular biology methods have become essential in establishing the exact phylogenetic relationships within different groups of cyprinids.

In the last three decades, mitochondrial DNA has become the most important marker for molecular diversity studies in animals [13]. The structure and dimensions of the mitochondrial genome varies greatly in the living world. In animals it measures between 15 and 20 kb, consisting of a double-stranded circular DNA, which lacks introns and presents short intergenic sequences ("spacers"). MtDNA contains a set of genes coding the synthesis of 22 RNAt, 2 RNAr and 13

protein (involved in electron transport and oxidative phosphorylation) [10, 26, 35]. MtDNA replication is semiautonome (under nuclear control), and the number of mtDNA copies\cell may vary at different cellular types [35].

MtDNA has a number of features (benefits) that make it an important molecular marker in the analysis of molecular biodiversity (and by default in phylogenesis and phylogeography studies): - it is transmitted (inherited) in mostly uniparental (through the maternal line), being considered non-recombinant; - presents a great variability and a high mutation rate in natural populations; - evolves in a manner almost neutral [9, 16, 26, 35]. The first evidence of uniparental transmission was brought by David and Blackler in 1972 [35].

In a recent work of synthesis, Galtier brings, some arguments that show that mtDNA is not an ideal marker for molecular diversity studies, is not ”immune” it seems neither to recombinations nor to the intermittent evolutionary rate of the positive selection. In spite of the expressed doubts, the authors noted that mitochondrial markers will continue to be used by researchers because of their convenience and the fact that it is the cheapest way to get an idea about the genetic structure of a species [13].

Various regions of the mitochondrial genome are evolving with different rates, so that they can be taken in study the regions convenient to the intended purpose [26]. Within mtDNA, the control region (D-loop) is non-coding and it presents a high rate of variation and evolution, variations consisting often in nucleotide substitutions, small insertions and deletions [10]. At the same time, the mitochondrial gene subunit for cytochrome c-oxidase I (CO I) was chosen as a standard tool in molecular taxonomy and for bioidentification in animals [11, 13].

The main purpose of this paper was to bring molecular evidence of genetic diversity, parentage and evolution within *Carassius* genera, studying individuals from isolated aquatic populations in Romania. In this respect, we will analyse the nucleotide sequences of mitochondrial DNA markers, which help us to make comparisons between individuals belonging to the same population, as well as between individuals belonging to different populations or species. These investigations will provide the identification of new haplotypes specific to the studied areas. We will highlight also some aspects concerning biogeography of subspecies, aiming to migration and origin lineages.

To achieve the main objectives of this study, we will sequence the mitochondrial DNA genes: cytochrome b (Cyt b), mitochondrial control region (D-loop) and cytochrome c oxidase I (COX I) for identifying the differences between individuals of the same population and between individuals belonging to different populations of *Carassius* sp. The results will be the basis of future researches related to survival and dominance of some haplotypes found in these areas during the evolution.

MATERIALS AND METHODS

Biological material

The material used was represented by a number of 160 individuals from the species *Carassius gibelio*, *Carassius auratus* and *Carassius carassius* within *Carassius* genera, sampled from different pools water all over Romania. Thus we analyzed 36 individuals of *C. gibelio* coming from Buzău River (Buzău County, Romania), 47 individuals of *C. gibelio* collected from Sofroneşti Lake (Vaslui County, Romania), 40 individuals of *C. gibelio* sampled from Tăuteşti Lake (Iaşi County, Romania), 26 individuals of *C. gibelio* and *C. carassius* collected from Fortuna Lake (Danube Delta, Romania), 6 individuals of *C. auratus* coming from Băile Felix (Bihor County, Romania) and 5 individuals of *C. gibelio* sampled in Poland (Vistula River, Cracovia County). The samples were collected between 2009-2011 during the summer.

Molecular methods

Our porpose was to obtain the molecular evidence of the genetic diversity within the *Carassius* genera, by studying individuals from isolated aquatic populations in Romania, based on the sequences of three mitochondrial genes: D-loop, Cyt b and COX I.

In this sense, dorsal muscle tissue from all individuals under investigation has been taken and was kept in absolute alcohol 95% at -20C°. The following steps have consisted in: isolation and purification of total DNA, gene amplification through PCR reactions for all individuals and for each analysed gene, spectrophotometric testing of PCR products, amplicons purification, PCR reactions for sequencing, precipitation of samples for sequencing and sequencing itself (for which we used a Beckman Coulter CEQ 8000 sequencer). After obtaining the sequences, they were aligned with the help of Lasergene v.7 and MEGA 5 software and the phylogenetic trees were made based on similarity degree and Neighbor-Joining method [31].

Protocol for isolation and purification of total DNA

Total DNA extraction was performed by two methods: a classical one, involving various buffer solutions and a fast one, based on the use of specialized kits. Classical method was based on the use of phenol, chloroform and isoamyl alcohol (25:24:1) for DNA purification and the second method consisted in using the Wizard SV Genomic DNA purification kit System (Promega) [1].

Gene amplification by polymerase chain reaction (PCR)

For the individuals we studied, three different genes there were amplified. Thus, were amplified: mtDNA mitochondrial control region (D-loop), cytochrome b (Cyt b) and cytochrome c oxidase I (COX I or CO I), we used a series of primers and depending on their alignment temperature were used adequate cycles of temperature, for each gene [16]. (Table 1).

Table 1. Gene amplification primers used in the study

Gene	Primer	Sequence (5'- 3')
Cyt b (first part)	H15149	AAACTGCAGCCCTCAGAATGATATTTGTCCTCA
	L14724	CGAAGCTTGATATGAAAAACCATCGTTG
Cyt b (the entire gene)	15245 F	CCGAGACCAATGACTTGAAGAACAACCG
	16490 R	CTGGGCGCTAGGGAGGAATTTAACC
D-loop	16373 F	TTCGCACTGTTCCTTGTTCTCTTC
	00992 R	GTCGGGACCATGCCTTTGTG
COX I	Fish-F1	TCAACCAACCACAAAGACATTGGCAC
	Fish-R1	TAGACTTCTGGGTGGCCAAAGAATCA

Testing of amplified products by agarose gel electrophoresis

At the end of each amplification reaction a check is required to see whether the amplification occurred, if there were multiple amplification or the detection of potential contamination. The electrophoresis was performed in 1.5% agarose gel [16].

Purification of PCR products

The kit Wizard SV Gel and PCR Clean-Up System (Promega) was used to complete this step, so are purified the PCR products eliminating the excess of nucleotides and primers.

PCR products quantification

Was performed spectrophotometrically and consisted in estimating the quantities of purified product and determined the quantities required for the sequencing reaction.

Sequencing reaction

Sequencing of amplicons was performed using the kits: GenomeLab Methods Development Kit and DTCS Quick Start Kit (Beckman Coulter). The program used had a primers alignment temperature of 50ºC and a total of 30 cycles of replication.

Precipitation samples for sequencing

Was performed in ethanol and on the magnetic plate (Agentcourt SPRIPlate 96R).

Actual sequencing was performed using a Beckman Coulter CEQ 8000 sequencer with eight capillaries.

Sequences analysis

Alignment of all sequences for one gene from different individuals (individuals of the same population), was performed by Clustal W method using the MegAlign module of Lasergene v.7 software [34].

Comparison of sequences, and drawing phylogenetic trees was done using Lasergene v.7 and MEGA 5 software. Phylogenetic trees were constructed based on the similarity degree and through distance based method Neihgbor-Joining (NJ) [31].

RESULTS

Amplification of cytochrome b gene to species of *Carassius* genera

A molecular marker of 100bp was used to determine the length of amplified DNA fragments, positive control (C+) which is a sample of the same species, or other species for which that gene was amplified with the same primers and negative control (C-) which is intended to show any contamination (Fig. 1, 2).

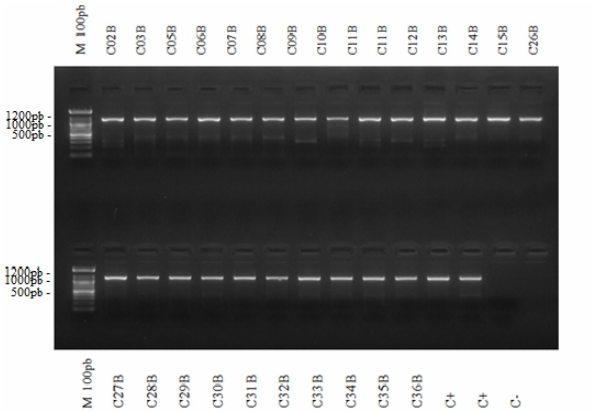

Figure 1. Electrophoresis in agarose gel 1.5% for cyt b from individuals of the species *C. gibelio* (C+ = positive control, C- = negative control, M = molecular marker 100bp

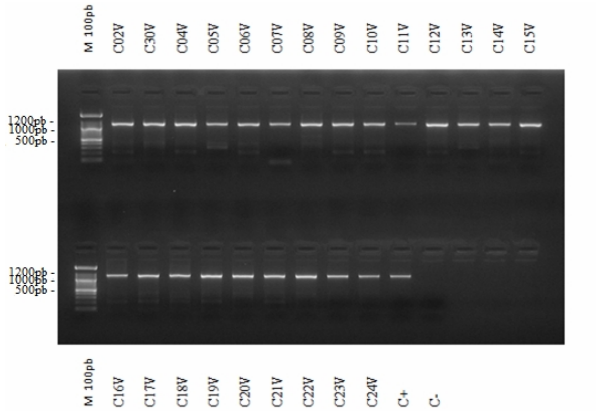

Figure 2. Electrophoresis in agarose gel 1.5% for cyt b from individuals of the species *C. gibelio* (C+ = positive control, C- = negative control, M = molecular marker 100bp)

Amplification of mitochondrial control region, to species of *Carassius* genera

The length of the DNA fragments obtained was relatively measured towards the bands of 100bp marker, in this case amplified fragments were approximately 1200bp (Fig. 3, 4).

Amplification of cytochrome c oxidase subunit I gene, to species of *Carassius* genera

After the amplification of cytochrome c oxidase I gene a segment of approximately 700bp was obtain for the individuals of genera *Carasssius* (Fig. 5, 6).

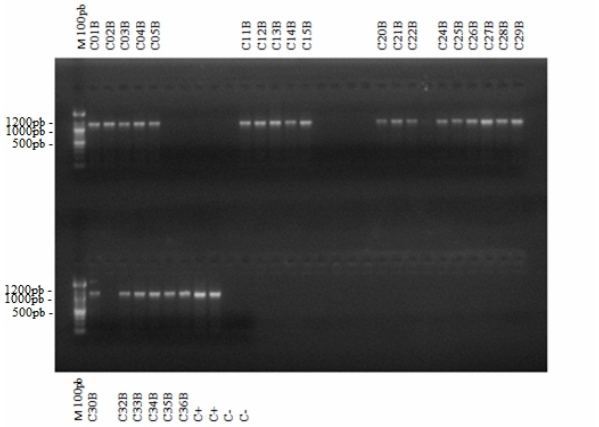

Figure 3. Electrophoresis in agarose gel 1.5% for d-loop from individuals of the species *C. gibelio* (C+ = positive control, C- = negative control, M = molecular marker 100bp)

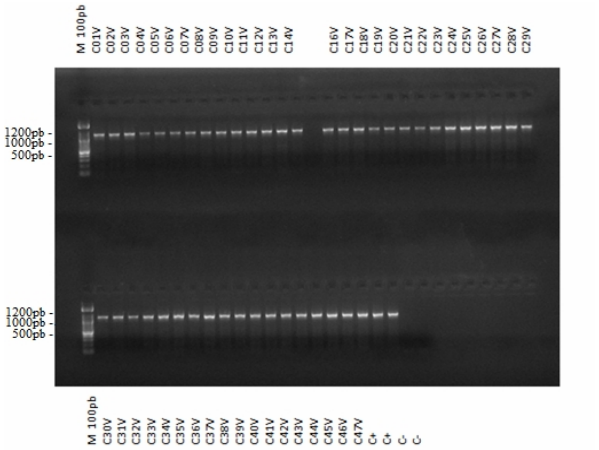

Figure 4. Electrophoresis in agarose gel 1.5% for d-loop from individuals of the species *C. gibelio* (C+ = positive control, C- = negative control, M = molecular marker 100bp)

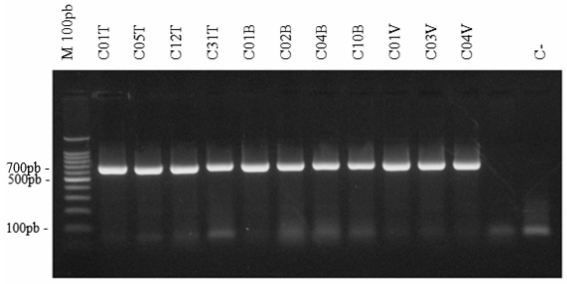

Figure 5. Electrophoresis on 1.5% agarose gel for COX I to individuals of the species *C. gibelio* (C- = negative control, M = molecular marker 100bp)

Comparison of nucleotide sequences of cytochrome b gene, from individuals belonging to *Carassius* genera

After the alignment of the sequences obtained for the cytochrome b gene, we can estimate for Buzau population 4 haplotypes: C01B (general haplotype, 55.56%), C02B (8.33%), C04B (27.78%) and C10B (8.33%), for Sofronesti population four haplotypes were established: C01V (general haplotype, 51.06%), C03V (4.26%), C04V (36.17%) and C15V (8.51%) [24].

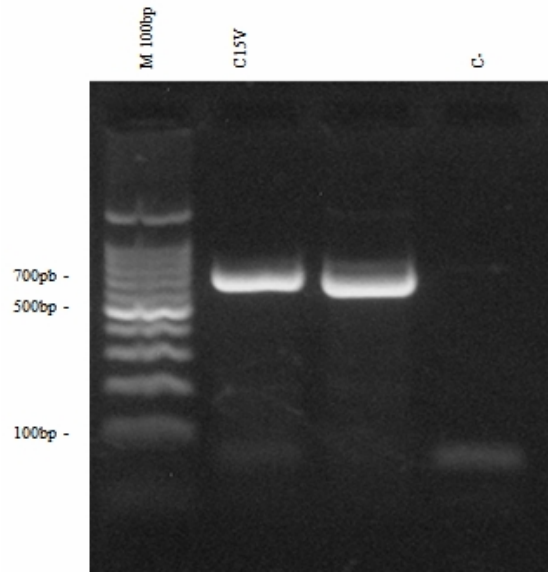

Figure 6. Electrophoresis on 1.5% agarose gel for COX I to individuals of the species *C. gibelio* (C- = negative control, M = molecular marker 100bp)

For Tautesti population no differences were found, allowing to establish a single haplotype [22, 23].

From the alignment of cytochrome b gene, from individuals of *Carassius gibelio* and *Carassius carassius* species, from Delta population 261 differences have been found, identifying seven haplotypes: C02D (12.5%), C02DL (25%), C04D (12.5%), C05DD (12.5%), C12D (12.5%), C13D (12.5%) and C21D (12.25%).

For Baile Felix population 193 differences were found, which led to the identification of six haplotypes: C01C (16.66%), C02C (16.66%), C03C (16.66%), C10C (16, 66%), C11C (16.66%), C12C (16.66%).

With regard to the sequences of cyt b gene, from individuals of the species *Carassius gibelio*, from the population of Poland, after the sequences alignment 12 differences were found, allowing the establishment of five haplotypes: C01P (20%), C02P (20%), C03P (20%), C04P (20%) and C05P (20%).

Regarding to the geographical distribution of the haplotypes identified in the six analyzed populations, for the cytochrome b gene, we can see that general haplotype C01TL (100%) of Tautesti population (Iasi County) is also found in other populations too: in Sofronesti population (Vaslui County) as C04V haplotype (36%), in Buzau population (Buzau County) as C04B haplotype (28%), and Delta population (Fortuna Lake) as haplotype C04D.

We can also see that haplotype C01V (51%) of Sofronesti population is the same with haplotype C01B (56%) of Buzau population, C13D of Delta population and C10C of Baile Felix population and haplotype C15V (9%) of Sofronesti population is found in Buzau population as haplotype C10B (8%), (Fig. 7).

Thus we can deduce that Tautesti Lake population, is a relatively new population, very uniform, which indicates the use of aquatic pool for aquaculture [25].

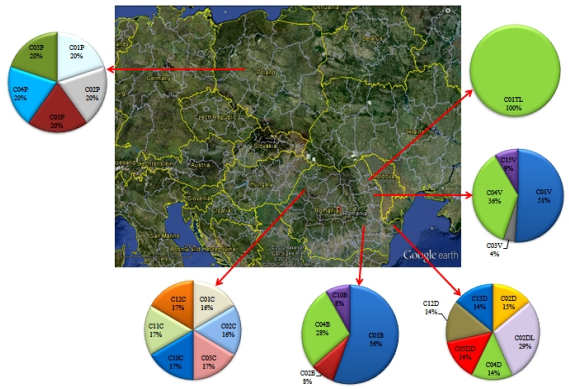

Figure 7. Geographical distribution of haplotypes identified within *Carassius* genera, for cytochrome b

Phylogenetic relationships within *Carassius* genera, based on the differences of cytochrome b gene sequences

For splitting the kinship relations between analyzed haplotypes were used methods based on distances and substitution rates, but which involves mutations as transitions and transversions.

Evolutionary history of analyzed sequences was inferred by Neighbor-Joining method, the optimal phylogenetic tree was constructed (Fig. 8), branches being reported on a scale that uses the same units as those used in determining the evolutionary distances used for the tree, the total length of branches being 0.15178770. Evolutionary distances were determined using the Maximum likelihood method.

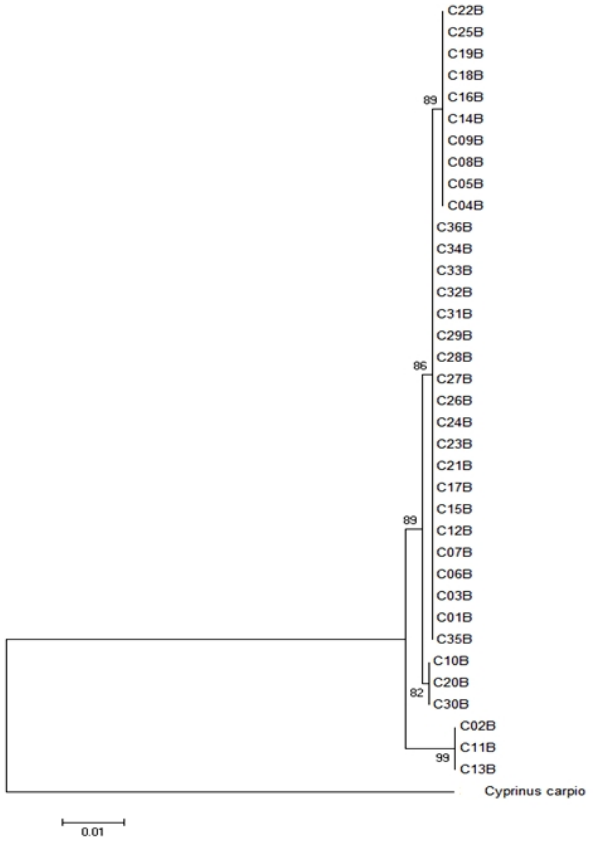

Figure 8. Phylogenetic tree based on the cyt b sequences for the species *C. gibelio* of Buzau population, Neighbor-Joining method

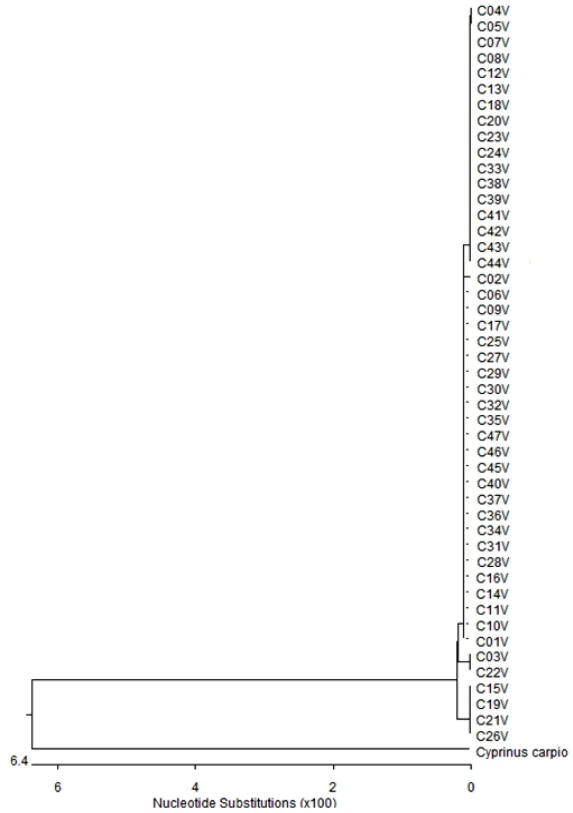

Figure 9. Phylogenetic tree based on the cytochrome b sequences, based on the similarity degree, for individuals of the species *C. gibelio* from Sofronesti population

The evolutionary history of analyzed sequences was inferred by Neighbor-Joining method, the optimal phylogenetic tree was constructed (Fig. 10), branches being reported on a scale that uses the same units as those used in determining the evolutionary distances used for the tree, the total length of branches being 0.12319962. Evolutionary distances were determined using the Maximum likelihood method.

Comparison of nucleotide sequences of the mitochondrial control region, from individuals belonging to *Carassius* genera

The alignment of sequences revealed that for Buzau population 8 haplotypes were identified: C01BF (general haplotype, 50%), C02BF (8.33%), C04BF (19.44%), C09BF (5.56%), C10BF (5.56%), C14BF (2.78%), C21BF (5.56%) and C30BF (2.78%).

Based on sequence alignment of mitochondrial control region, from individuals of the species *Carassius gibelio* of Sofronesti population 52 differences were found, which led to the identification of eleven haplotypes: C01VF (23.91%), C02VF (23.91%), C03VF (4.35%), C04VF (23.91%), C07VF (4.35%), C11VF (8.70%), C12VF (2.17%), C15VF (2.17 %), C42VF (2.17%), C44VF (2.17%) and C47VF (2.17%) [24].

For Tautesti population, the alignment of D-loop sequences, from individuals of the species *C. gibelio*, two haplotypes were identified: C01T (66.67%) and C12T (33.33%) [21].

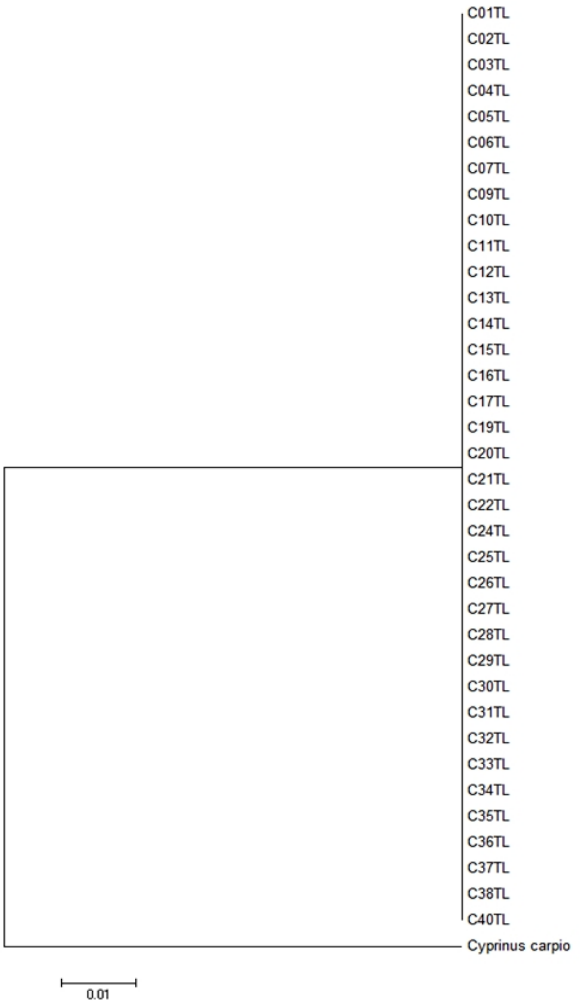

Figure 10. Phylogenetic tree based on the cyt b sequences for the species *C. gibelio* of Tautesti population, Neighbor-Joining method

Regarding the D-loop sequences, from individuals of the species *Carassius gibelio* and *Carassius carassius*, from Delta population, 56 differences were found and were established seven haplotypes: C02DF (14.29%), C02DLF (14.29 %), C03DF (14.29%), C04DF (14.29%), C05DDF (14.29%), C12DF (14.29%) and C13DF (14.29%).

Based on alignment of sequences from individuals of the species *C. auratus*, from Baile Felix population, 56 differences were found, which led to the identification of five haplotypes: C01CF (20%), C02CF (20%), C10CF (20 %), C11CF (20%) and C12CF (20%).

For the D-loop sequences, from individuals of the species *C. gibelio*, from Poland population, 7 differences were established, identifying three haplotypes: C01PF (25%), C02PF (25%) and C03PF (50%).

Regarding the geographical distribution of haplotypes identified in analyzed populations for mitochondrial control region, we can see that general haplotype C01BF (50%) of Buzau population (Buzau River, Buzau County) is found in Sofronesti population as C02VF haplotype (24%) and in Delta population as C13DF haplotype (14%).

We can also notice that C04BF haplotype (19%) of Buzau population is the same with C04VF (24%) of Sofronesti population respectively C04DF (14%) of Delta population, but it is also found in Baile Felix population as haplotype C10CF (20%).

The haplotype C09BF (5%) of Buzau population is found in Sofronesti population as haplotype C42VF (2%). Following the same pattern we found that haplotype C10BF (6%) of Buzau population is the same as C15VF (2%) of Sofronesti population, respectively C02DLF (15%) of Delta population (Fig. 11) [25].

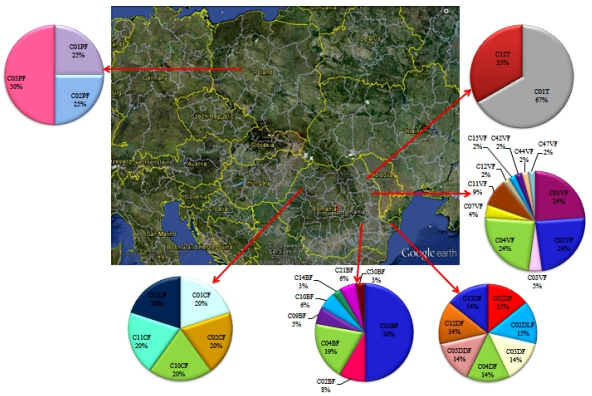

Figure 11. Geographical distribution of haplotypes identified within *Carassius* genera, for the mitochondrial control region

Phylogenetic relationships within *Carassius* genera, based on the differences of d-loop sequences

The methods used in phylogeny and in the construction of phylogenetic trees used in this case, are those that include transitions and transversions because analyzed haplotypes, present both types of mutations.

The evolutionary history of analyzed sequences, it was inferred by Neighbor-Joining method, making the optimal phylogenetic tree (Fig. 13), branches are reported on a scale that uses the same units as those used in determining the evolutionary distances used for the tree, the total length of branches being 0.15155252.

Comparison of nucleotide sequences of cytochrome c oxidase I gene, from individuals belonging to *Carassius* genera

For cytochrome c oxidase I gene, we were aligned the sequences obtained for each studied population. From the alignment of sequences for Buzau population 3 haplotypes were identified: C01B (general haplotype, 83.33%), C02B (8.33%) and C10B (8.33%).

On CO I sequences, from individuals of the species *Carassius gibelio*, from Sofronesti population 11 differences were identified and four haplotypes were established: C01V (general haplotype, 51.06%), C03V (4.26%), C04V (36.17%) and C19V (8.51%).

From the alignment of CO I sequences, from individuals of the species *C. gibelio* of Tautesti population 4 differences were found, which led to the identification of four haplotypes: C01T (25%), C05T (25%), C12T (25%) and C31T (25%).

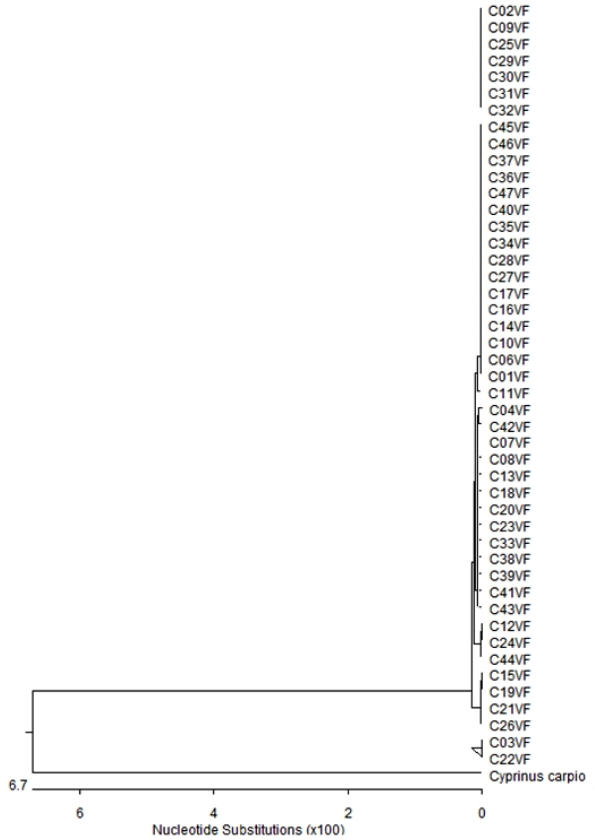

Figure 12. Phylogenetic tree based on the d-loop sequences, based on the similarity degree for individuals *C. gibelio* species of Sofronesti population

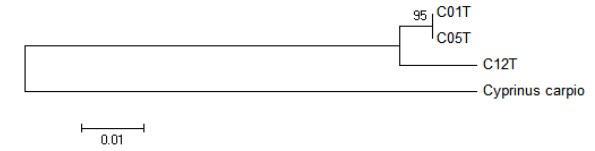

Figure 13. Phylogenetic tree based on the d-loop sequences, of *C. gibelio* from Tautesti population by Neighbor-Joining method

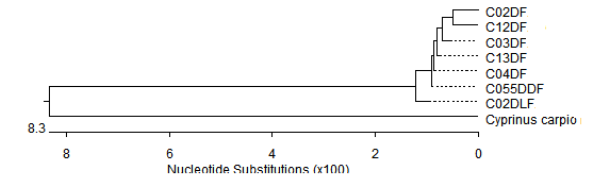

Figure 14. Phylogenetic tree based on the d-loop sequences, based on the similarity degree for individuals *C. gibelio* and *C. carassius* species of Delta population

On CO I sequences, from individuals of the species *C. gibelio* and *C. carassius*, of Delta population 102 differences were found, which led to the establishment of eight haplotypes: C02D (3.85%), C02DL (3.85%), C03D (3.85%), C04D (73.08%), C05DD (3.85%), C12D (3.85%), C13D (3.85%) and C21D (3.85%).

Based on sequence alignment for CO I, from individuals of the species *C. auratus*, from Baile Felix population 66 differences were found and five haplotypes identified: C01C (16.67%), C02C (16.67%), C03C (16.67%), C10C (16.67%) and C11C (33.33%).

From the alignment of CO I sequences, from individuals of the species *C. gibelio*, from Poland population 88 differences were found, which led to the identification of four haplotypes: C01P (40%), C02P (20%), C03P (20%) and C05P (20%).

In matters of biogeography of identified haplotypes in the six analyzed populations for cytochrome c oxidase I, we see that general haplotype C01B (83.33%) of Buzau population (Buzau River, Buzau County) is found in Tautesti population as haplotype C01T (25%), in Baile Felix and Delta populations as haplotype C10C, respectively haplotype C04D (Fig.15), [25].

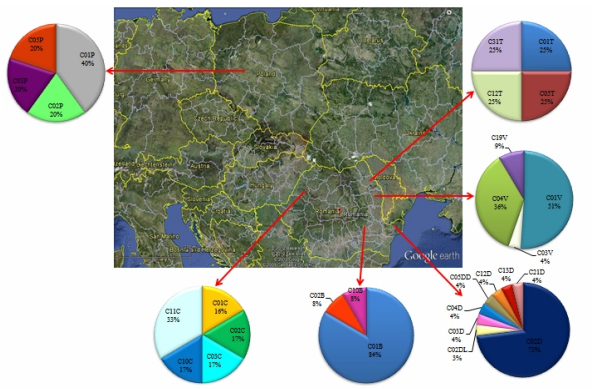

Figure 15. Geographical distribution of haplotypes identified within *Carassius* genera, for cytochrome c oxidase I

Phylogenetic relationships within *Carassius* genera, based on the differences of cytochrome c oxidase I gene sequences

For the phylogenetic trees constructions were used methods based on distances and substitution rates, but which involves also mutations as transitions and transversions.

Evolutionary history of analyzed sequences was inferred by Neighbor-Joining method, the optimal phylogenetic tree was constructed (Fig. 16), branches being reported on a scale that uses the same units as those used in determining the evolutionary distances used for the tree, the total length of branches being 0.18450024. Evolutionary distances were determined using the Maximum likelihood method.

This phylogenetic tree was made MEGA 5 software (Molecular Evolutionary Genetic Analysis, Center for Evolutionary Medicine and Informatics, Tempe, USA).

Evolutionary history for analyzed sequences was inferred by Neighbor-Joining method, the optimal phylogenetic tree was constructed (Fig. 18), total length of branches was 0.16664520. Evolutionary distances were determined using the Maximum likelihood method.

Identifying migration routes of the species *Carassius gibelio* in Europe was based on cytochrome b and cytochrome c oxidase I sequences (Fig. 19).

Analyzes have suggested that the invasion of under study species was facilitated by anthropogenic activities.

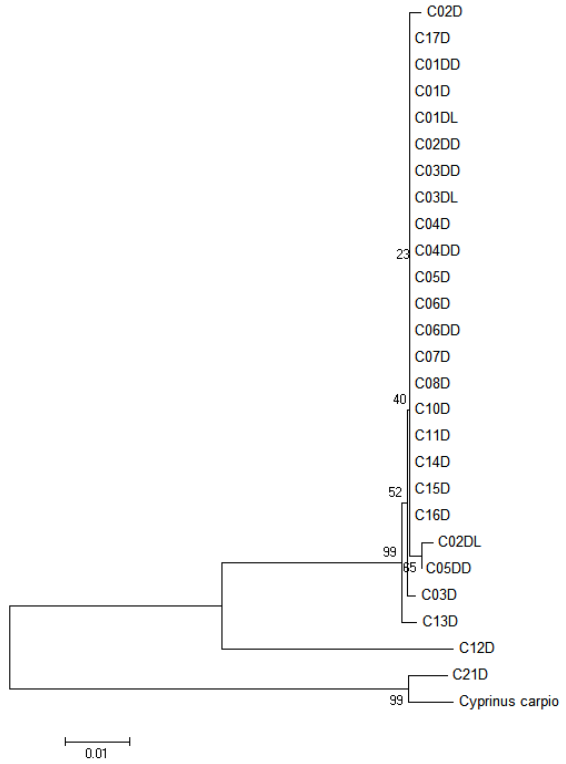

Figure 16. Phylogenetic tree based on the cytochrome c oxidase I sequences for individuals of the species *C. gibelio* and *C. carassius*, Delta population, Neighbor-Joining method

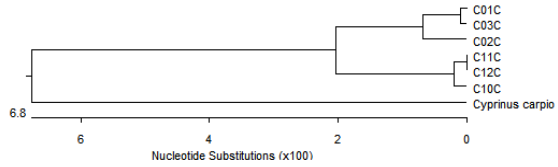

Figure 17. Phylogenetic tree based on the cytochrome c oxidase I sequences, based on similarity degree, for individuals of the species *C. auratus*, from Baile Felix population

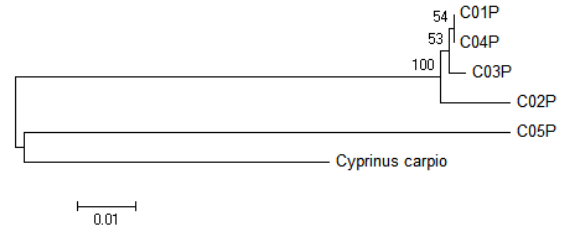

Figure 18. Phylogenetic tree based on the cytochrome c oxidase I sequences for individuals of the species *C. gibelio*, Poland population, Neighbor-Joining method

Phylogenetic and biogeographic analyzes showed that the species *Carassius gibelio* has two migration routes to Europe from East to West through the basins of Ukraine and Turkey, which is a consequence of accidental or deliberate introduction of the species from intensive aquaculture systems [12].

Regarding the species *Carassius carassius*, was noticed that it comes from an ancestral node, from which come also the common branches of the other sequences. This species, that in the past was dominant in our country, is found today only in isolated

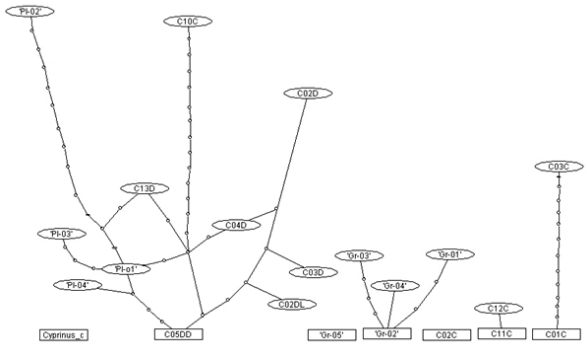

Figure 19. Graphical representation of mutational processes and identification of haplotypes within *Carassius* genera

population, in restricted areas limited only to some aquatic ecosystems, because it was replaced by the species *Carassius gibelio*. The fact that it was replaced proves a better adaptation of the species *Carassius gibelio* at current conditions and that it is situated on a higher evolutionary step as the species *Carassius carassius*, fact confirmed also by this analysis.

With regard to the species *Carassius auratus*, based on the analysis of the phylogenetic trees it was observed that all individuals come from the same species of origin and from the same common ancestral node. *C. auratus* is a popular freshwater fish in temperate and subtropical climates, for this reason in our country, the species was identified in Baile Felix (Bihor County) where there is a moderate-continental climate with oceanic influences from the West, with mild winters and summers with moderate temperature and Mediterranean influences.

DISCUSSION

To build a comprehensive phylogeny of *Cyprinidae* is complicated, 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 [8, 14, 15, 37].

Given the small size, the maternal inheritance and a rapid rate of evolution, the mitochondrial DNA is often used as a genetic marker in phylogeny and evolution studies in vertebrates (including fish) [27]. 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 [2]. Such data appear useful because molecular characters are less likely related to adaptative evolution than are morphologic characters. These studies have allowed the development of some hypotheses about the geographical origin of the species and establish phylogenetic links between them [30, 33, 36].

The main aim of this work was an intra- and interspecific study within the *Carassius* genera, by bringing some molecular evidence of genetic diversity, evolution within this genera, using individuals from

some aquatic populations of Romania. We also obtained the nucleotide sequences of the mitochondrial DNA markers, which help us to make comparisons between individuals belonging to the same population, as well as between individuals belonging to different populations or species from *Carassius* genera.

The data we obtained regarding the genetic diversity, are in according to those suggested by other researchers [7, 17, 18, 29]. In terms of biogeographic aspects, our results are concordant with the results of some authors, thus the species *Carassius carassius* was replaced by the species *Carassius gibelio* because of the better adaptation of the species *Carassius gibelio* at current conditions and that it is situated on a higher evolutionary step as the species *Carassius carassius* [28].

The taxonomic status of the species *C. gibelio* is controversial. As mentioned Hanfling and Harley is not yet clear whether it is a distinct species, a subspecies of *C. auratus* or has hybrid origin [19]. According to profile information, the *Carassius* genera would include two species namely: *C. carassius* (widespread in Europe and Xinjiang region of China) and the species *C. auratus* (common in Europe, Japan and China), or 3 species, the two mentioned adding *C. cuvieri* [27, 33]. The main controversy within *Carassius* genera is actually rised by the species *C. auratus*, a species with enormous morphological variability and different ploidy level [32]. Some authors consider that within *C. auratus* there would be two subspecies, namely *C. auratus auratus* and *C. a. gibelio*, others have marked a number of six, being added to the already presented *C. a. cuvieri*, *C. a. grandoculis*, *C. a. burgers* and *C. a. langsdorfii* [27, 32].

It seems that a clear delimitation between species/subspecies of carp is not an easy task at all, because genetic analysis performed by Harley and Hanfling on a total of 24 individuals (from 3 British population), established after morphological criteria as belonging to *C. a gibelio*, showed that they were grouped into two genotypes: I - polyploid individuals with exclusive alleles of *C. auratus* and II - diploid F1 hybrids *C. auratus* x *C. carassius*. The authors find it difficult to say which of the two categories are in fact *C. a. gibelio* pure, or whether *C. a. gibelio* is a set of lineages with different origin [19].

In this paper, based on the the results obtained, we considered *Carassius gibelio* as a valid species alongside *C. carassius* and *C. auratus*.

Another objective of the paper was represented by highlighting aspects of biogeography of subspecies, aiming to migration and origin lines. The results of our investigations showed the followings:

- The alignment of cytochrome b gene sequences from individuals of the species *Carassius gibelio* of Buzau population evinced 71 differences, and allowed us to identify four haplotypes: C01B (general haplotype, 55.56%), C02B (8.33%), C04B (27.78%) and C10B (8.33%). The same analyses for Sofronesti population of *C. gibelio* revealed 52 differences between individuals and led us to establish four haplotypes: C01V (general haplotype, 51.06%), C03V (4.26%), C04V (36.17%) and C15V (8.51%). After the alignement of the *cyt b* gene sequences for the individuals of *C. gibelio* from Tautesti population no differences were found and we established only a single haplotype;

- From the alignment of *cyt b* gene, from individuals of the species *Carassius gibelio* and *Carassius carassius* from Delta population, 261 differences have been found, and we identified seven haplotypes: C02D (12.5%), C02DL (25%), C04D (12.5%), C05DD (12.5%), C12D (12.5%), C13D (12.5%) and C21D (12.25%). Between sequences of *cyt b* gene of *C. auratus* individuals from Baile Felix population, 193 differences there were found, and led to the identification of six haplotypes: C01C (16.66%), C02C (16.66%), C03C (16.66%), C10C (16.66%), C11C (16.66%), C12C (16.66%);

- After the alignment of *cyt b* gene sequences from individuals of the species *C. gibelio*, from the Poland population, 12 differences were found, allowing the establishment of five haplotypes: C01P (20%), C02P (20%), C03P (20%), C04P (20%) and C05P (20%);

- For the D-loop sequences from individuals of the species *C. gibelio*, of Buzau population, 136 differences were found and 8 haplotypes were identified: C01BF (general haplotype, 50%), C02BF (8.33%), C04BF (19.44%), C09BF (5.56%), C10BF (5.56%), C14BF (2.78%), C21BF (5.56%) and C30BF (2.78%). The same analyses in the Sofronesti population of *C. gibelio* revealed 52 differences between individuals, which led to the identification of eleven haplotypes: C01VF (23.91%), C02VF (23.91%), C03VF (4.35%), C04VF (23.91%), C07VF (4.35%), C11VF (8.70%), C12VF (2.17%), C15VF (2.17 %), C42VF (2.17%), C44VF (2.17%) and C47VF (2.17%). For the individuals of Tautesti population of *C. gibelio* 9 differences were found between D-loop sequences and two haplotypes were identified: C01T (66.67%) and C12T (33.33%);

- Analysing the D-loop sequences of the individuals of *C. gibelio* and *C. carassius* from Delta population we found 56 differences and we identified seven haplotypes: C02DF (14.29%), C02DLF (14.29 %), C03DF (14.29%), C04DF (14.29%), C05DDF (14.29%), C12DF (14.29%) and C13DF (14.29%). The same analyses of the Baile Felix population of *C. auratus* evinced 56 differences between the sequences of D-loop region and the identification of five haplotypes: C01CF (20%), C02CF (20%), C10CF (20%), C11CF (20%) and C12CF (20%). The individuals of *C. gibelio* from Poland population evinced 7 differences of this gene sequences, which allowed the identification of 3 haplotypes: C01PF (25%), C02PF (25%) and C03PF (50%);

- In terms of cytochrome c oxidase I (COX I) gene sequences from individuals of the species *C. gibelio*, from Buzau population, 9 differences have been found, which led to the establishment of three haplotypes: C01B (general haplotype, 83.33%), C02B (8.33%) and

C10B (8.33%). The analysis of COX I sequences of the *C. gibelio* individuals from Sofronesti population found 11 differences and the identification of 4 haplotypes: C01V (general haplotype, 51.06%), C03V (4.26%), C04V (36.17%) and C19V (8.51%). The individuals of *C. gibelio* from Tautesti population revealed 4 differences between sequences of COX I gene, and the identification of 4 haplotypes: C01T (25%), C05T (25%), C12T (25%) and C31T (25%);

- We found 102 differences of COX I sequences, between individuals from Delta population of *C. gibelio* and *C. carassius*, which led us to establish eight haplotypes: C02D (3.85%), C02DL (3, 85%), C03D (3.85%), C04D (73.08%), C05DD (3.85%), C12D (3.85%), C13D (3.85%) and C21D (3.85%). The alignment of COX I sequences of the individuals of *C. auratus* from Baile Felix population evinced 66 differences, which allowed the identification of 5 haplotypes identified: C01C (16.67%), C02C (16.67%), C03C (16.67%), C10C (16.67%) and C11C (33.33%). The alignment of COX I sequences of the individuals of *C. gibelio* from Poland population, evinced 88 differences, which led to the identification of 4 haplotypes: C01P (40%), C02P (20%), C03P (20%) and C05P (20%);

- Through the alignment of *cyt b* gene sequences, of the individuals belonging to *Carassius* genera from analyzed populations, 21 haplotypes have been identified: two of these were found in four of the six examined populations, and one in two of the investigated populations. The alignment of D-loop sequences of *Carassius* genera individuals for the investigated populations allowed us to identify 20 haplotypes, of which four were found in two or more populations. Following COX I sequence alignment, from individuals of the *Carassius* genera, in the six populations, there were identified 22 haplotypes, but only one was found in four of the analyzed populations;

- From the analysis of all sequences, it was found that the rate of occurrence of transitions is greater than the occurrence of transversions;

- In terms of spatial distribution for the analyzed haplotypes, we can say that Tautesti Lake population, has a very high uniformity, which indicates the use of this pond for aquaculture;

- Phylogeographic aspects of the D-loop gene showed that there are common haplotypes for Buzau, Sofronesti, Delta and Baile Felix populations, and for COX I gene for Buzau, Tautesti, Delta and Baile Felix populations.

However, our study brought new information regarding the genetic diversity, parentage and the evolution within the *Carassius* genera studying individuals from isolated aquatic populations from Romania and Poland. Moreover, this results can be the foundations of future research related to the study of survival and dominance of some haplotypes discovered in these areas throughout evolution.

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