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

- PhD THESIS SUMMARY -

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CONTENTS

INTRODUCTION

This paper is structured in four chapters, the first two chapters are devoted to literature data, achieving a summary overview of key data regarding the *Cyprinidae*'s family and *Carassius* genera, and in the second part of the work (Chapters 3 and 4), are presented the personal contributions on the analysis of genetic diversity within the *Carassius* genera, based on sequencing mitochondrial markers by studying individuals from isolated aquatic populations in Romania.

Cyprinids is one of the most common fish families, with more than 2000 species grouped in 340 genera (Banarescu and Coad, 1991) and occupies large areas on all continents, can be found in most freshwater ecosystems. This family is an excellent group for biological studies.

However, phylogenetic relationships of the main lines of *Cyprinidae*'s family are still poorly known and even monophyly for the whole family is sometimes questioned (Howes, 1991).

OBJECTIVES

The main purpose of this paper is to bring molecular evidence of genetic diversity, parentage and evolution within *Carassius* genera, studying individuals from isolated aquatic populations in Romania.

The study will add to the scientific information obtained in previous studies, new haplotypes specific to the studied areas.

Will be highlighted aspects of biogeography of subspecies, aiming to migration and origin lines.

Probable evolutionary directions will be suggested within the *Carassius* genera.

It will be the basis of future research studies related to survival and dominance of some haplotypes found in these areas during the evolution.

1. HISTORICAL RESEARCH ON *CYPRINIDAE*'S FAMILY

Regarding cyprinids they represent the largest family of freshwater fish with a very large area (over 2000 species), and therefore have a significant role in freshwater ecosystems.

In this family there is great morphological and genetic variability and phylogenetic relationships of its members are not fully elucidated (Banarescu, 1964).

Thus in recent years efforts were made to clarify the relationships between cyprinid, using molecular biology methods.

2. MOLECULAR PHYLOGENY WITHIN *CARASSIUS* GENERA

The phylogeny represents the study of formation and evolution of living organisms to determine their degree of kinship. Phylogenesis is the term most used to describe a species lineage, a group of species, but also to define a intraspecific level and genealogy between populations and individuals.

MtDNA important feature is that doesn't recombine, the only changes that may occur in mitochondrial DNA is limited exclusively to mutations transmitted over generations, but also within a single generation (Gorgan, 2008).

3. MATERIALS AND METHODS

Biological material

The material used is 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 Buzau River (Buzau County), 47 individuals of *C. gibelio* collected from Sofronesti Lake (Vaslui County), 40 individuals of *C. gibelio* sampled from Tautesti Lake (Iasi County), 26 individuals of *C. gibelio* and *C. Carassius* collected from Fortuna Lake (Danube Delta), 6 individuals of *C. auratus* coming from Baile Felix (Bihor County) and 5 individuals of *C. gibelio* sampled in Poland.

Molecular methods

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 (Ausubel *et al.*, 1995) and the second method consisted in using the Wizard SV Genomic DNA purification kit System (Promega).

Gene amplification by polymerase chain reaction (PCR)

For the studied individuals three different genes 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.

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. Electrophoresis were performed in 1.5% agarose gel.

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 of estimating the quantities of purified product and determinated 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 of 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 (Thompson *et al*., 1994) using the MegAlign module of Lasergene v.7 software.

Comparison of sequences, and drawing phylogenetic trees was done using Lasergene v.7 and MEGA 5 software.

For this study, phylogenetic trees were constructed based on the similarity degree and through distance based method Neihgbor-Joining (NJ).

4. RESULTS AND DISCUSSION

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

A molecular marker of 100bp was used to determined 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.

4.2. 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.

4.3. 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*.

4.4. 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%).

For Tautesti population no differences were found, allowing to establish a single haplotype.

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 alignment 12 differences were found, allowing the establishment of five haplotypes: C01P (20%), C02P (20%), C03P (20%), C04P (20%) and C05P (20%).

Regarding 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 thus, 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%).

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

4.4.1. 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 (Figure 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.

4.5. 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%).

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

Regarding the D-loop sequences, from individuals of the species *Carassius gibelio* and *Carassius carassius*, from Delta population, 56 differences were found and 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 *Carassius 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 *Carassius 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 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%).

Haplotype C09BF (5%) of Buzau population is found in Sofronesti population as haplotype C42VF (2%). Following the same pattern we find that haplotype C10BF (6%) of Buzau population is the same as C15VF (2%) of Sofronesti population respectively C02DLF (15%) of Delta population.

4.5.1. 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, 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.

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

For cytochrome c oxidase I gene were aligned the sequences obtained for each studied population, thus 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 COX I sequences, from individuals of the species *Carassius gibelio*, from Sofronesti population 11 differences were identified and four haplotypes established: C01V (general haplotype, 51.06%), C03V (4.26%), C04V (36.17%) and C19V (8.51%).

From the alignment of COX I sequences, from individuals of the species *Carassius 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%).

On COX I sequences, from individuals of the species *Carassius gibelio* and *Carassius 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 COX I, from individuals of the species *Carassius 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 COX I sequences, from individuals of the species *Carassius 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.

4.6.1. 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 for analyzed sequences was inferred by Neighbor-Joining method, the optimal phylogenetic tree was constructed, total length of branches of 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.

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

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.

CONCLUSIONS:

- Based on the alignment of cytochrome b gene sequences from individuals belonging to *Carassius* genera from analyzed populations, 21 haplotypes have been identified, of these two are found in four of the six examined populations, and one in two of studied populations.

- Regarding the D-loop sequences, originating from individuals of the *Carassius* genera, for the studied populations were identified 20 haplotypes, of which four are found in two or more populations.

- Following COX I sequence alignment, from individuals of the *Carassius* genera, in the six populations studied were identified 22 haplotypes, but only one is 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 the pond for aquaculture.

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

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