CARASSIUS GENETIC DIVERSITY INVESTIGATION FROM TAUTESTI LAKE (IASI COUNTY)

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Abstact: In this experiment we investigated the genetic diversity of *Carassius gibelio* Bloch, individuals, from Tautesti (Iasi County) pond, using the sequencing of cytochrome b as a codominat-marker. The analysis of cytochrome b first part gene coding region has permitted to establish if there is a wild population or the pond was repopulated in the last years.

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.

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 (Avise, 1994). Such data appear useful because molecular characters are less likely related to adaptative evolution than are morphologic characters.

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.

MtDNA has an important advantage in all phylogenetical 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 comperison 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 synthtesis 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.

The probability to recover mtDNA from very small or degraded biological samples is higher than the one of nuclear DNA, because the mitochondrial DNA molecules exist in thousands of copies per cell, while nuclear DNA has only two copies per cell.

Another charasteristic of mtDNA, generally accepted as a real advantage for the populational 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 cromosomes during the first meiosis. But the genes of organelles coming from diffrent lines of filiation

connot ever recombine because the genom 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).

The principal porpose of this experiment was to identifyed genetic diversity of *Carassius gibelio* Bloch, individuals, from Tautesti (Iasi County) pond, by sequencing the mitochondrial cytochrome b gene. The analysis of cytochrome b first part gene coding region has permitted to establish if there is a wild population or the pond was repopulated in the last years.

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. Tăutești Lake and the sampling point (market with the arrow)

Total cellular DNA was extracted with phenol-clorophorm-isoamylalcohol (25 : 24 : 1) (Ausubel *et al*., 1995). Cellular lyses were 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 minutes, 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 minutes 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 fase 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 ensemble and incubated for 1 minute at room temperature. The SV minicolomns ensemble 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 per cents.

The aligning of all sequences was made by Clustal V metod, similar results (Higgins and Sharp, 1989; Higgins, 1994; Wheeler, 2000) using the MegAlign module within the programme DNA STAR 5, Lasergene.

RESULTS AND DISCUSSIONS

The mitochondrial cyt b gene were amplified by PCR and sequenced in all 40 individuals of *Carassius* genera.

The PCR products were checked by migration in an elecrophoresis gel, and in this way, we observed that the PCR products have the expected lenght – approximately 400 base pairs. For the length determination, it was used a molecular marker (Figure 2, 3, 4) of 100bp and a negative control for eventually contaminations.

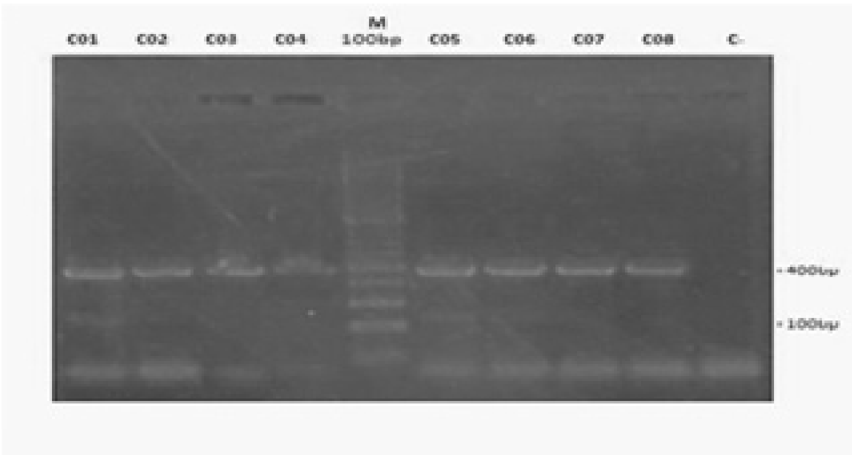

Figure 2. Electrophoresis in agarose gel 1,5% for the cytochrome b first part gene for individuals of *Carassius* genera (C- = negative control, M =100bp molecular marker)

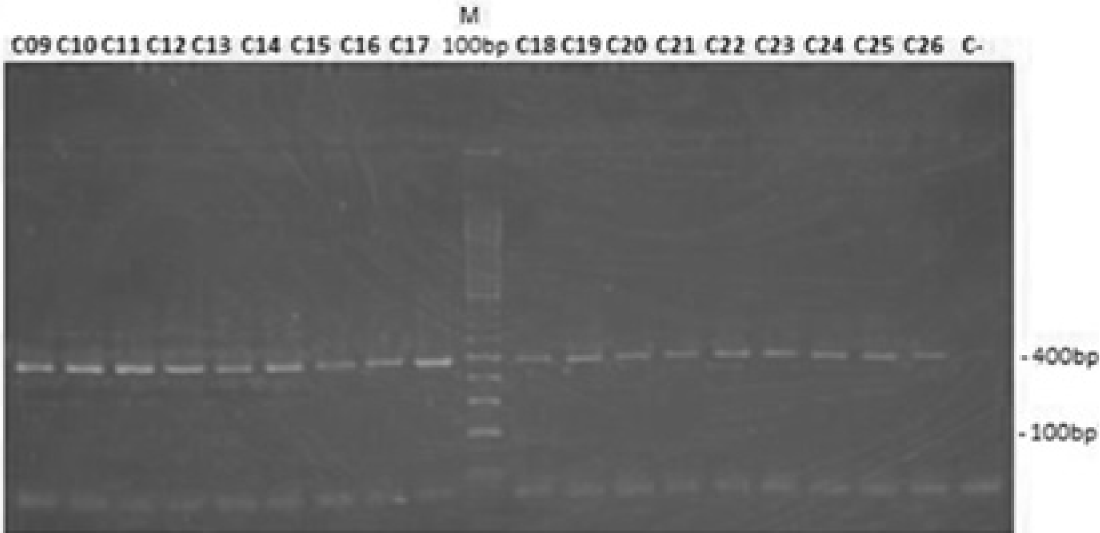

Figure 3. Electrophoresis in agarose gel 1,5% for the cytochrome b first part gene for individuals of *Carassius* genera (C- = negative control, M =100bp molecular marker)

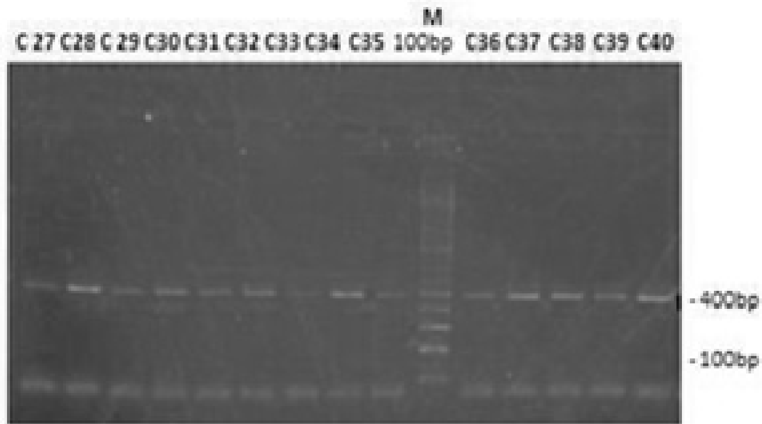

Figure 4. Electrophoresis in agarose gel 1,5% for the cytochrome b first part gene for individuals of *Carassius* genera (M =100bp molecular marker)

A 408bp fragment sequences of cyt b were obtain for 37 individuals (Figure 5, 6, 7, 8). The sequences were aligned using the Megalign Sequences application. After the alignment we observed 142 differences between those individuals, 24 transitions and 85 transversions.

The analises of cytochrome b sequences from individuals of *Carassius* genera

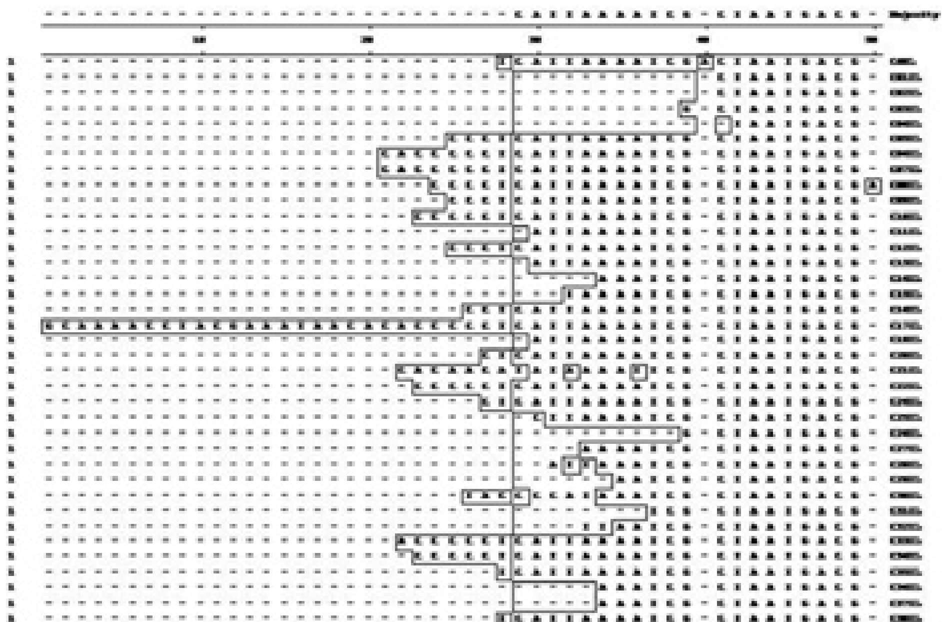

Figure 5. Clustal V fragment alignment of mitochondrial cyt b gene

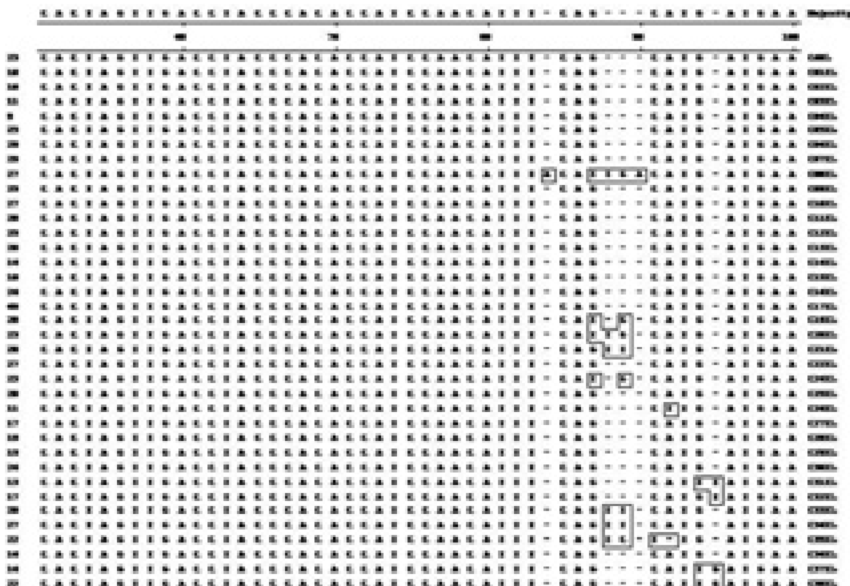

Figure 6. Clustal V fragment alignment of mitochondrial cyt b gene

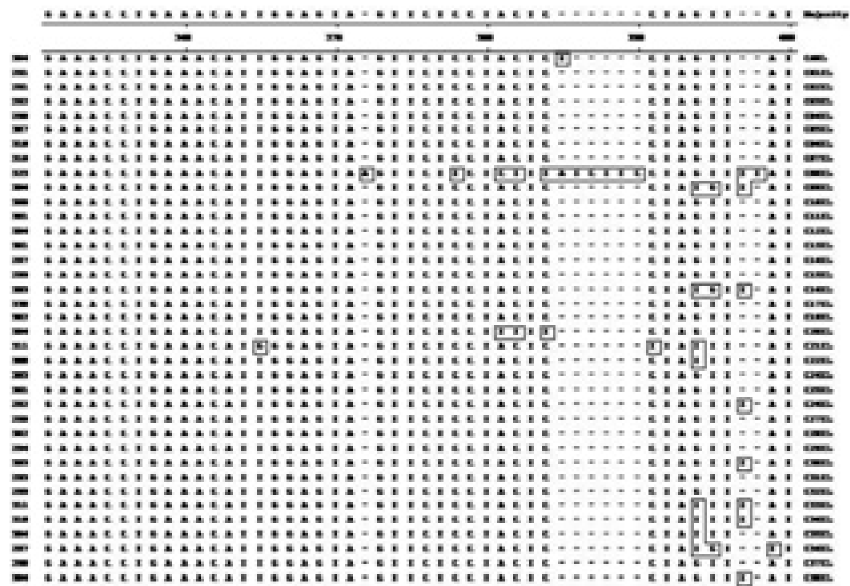

Figure 7. Clustal V fragment alignment of mitochondrial cyt b gene

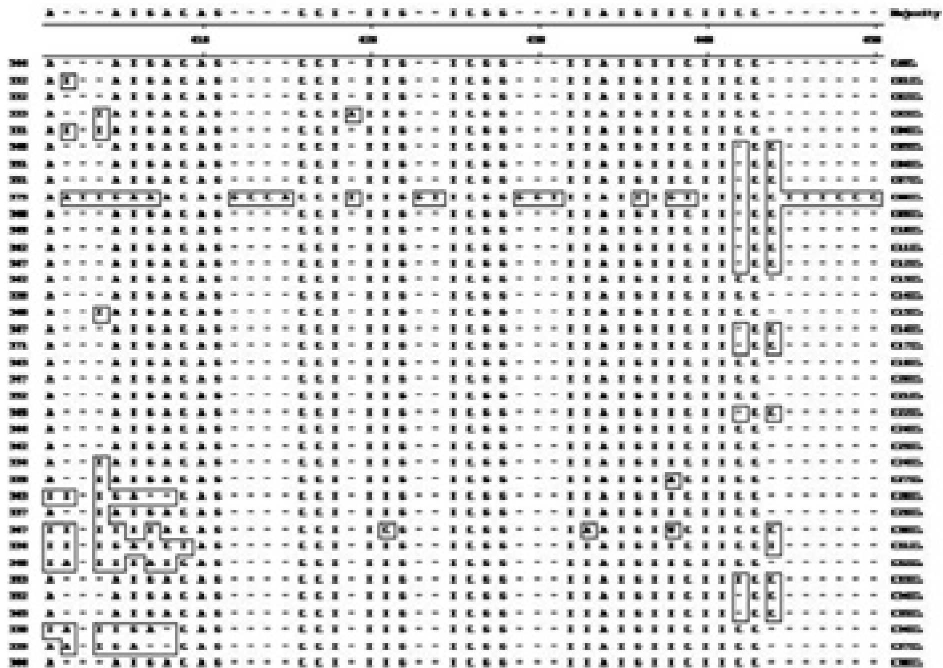

Figure 8. Clustal V fragment alignment of mitochondrial cyt b gene

CONCLUSIONS

Analyzing the cytochrome b gene in 40 individuals of *Carassius gibelio* Bloch., it has been noticed that the lenght for the first part of cytochrome b gene is about 400bp.

After the sequencing process, were obtain 37 sequences, with a lenght of 408bp.

The alignment show that exist differences (mutational modifications) between those individuals which confirm the genetic diversity from Tautesti Lake and that the pond was repopulated in the last years.

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