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**Molecular phylogenetics within Cyprinidae family inferred by molecular
mitochondrial markers sequencing**

- PhD THESIS SUMMARY -

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The study presents and compares two different mitochondrial gene sequences, cytochrome b gene and the mitochondrial control region. For the DNA isolation, two fish's species were used: *Alburnus alburnus* and *Vimba vimba*, both belonging to the Cyprinidae family. Comparing the gene sequences, we were able to observe the mutational changes and differences that appeared in the gene structure of individuals coming from the same population.

The genetic structure of a population and its evolutionary path can be studied using the molecular investigations. It has been proved that mitochondrial DNA has a greater efficiency in obtaining interesting phylogenetical or phylogeographical results. In the phylogeny studies the most used is mitochondrial DNA because of its great number of nucleotide sequences polymorphisms (Birki, 2001; Yamada et al., 2001; Yhang et al., 2006; Murgia et al., 2002; Nelson et al., 2000; Oleinik et al., 2004; Parson et al., 2000; Pavlov, 2004).

Due to the high reproduction rate and prolificity, essential qualities for the populational genetics studies, fishes represent an important and interesting study material. Family Cyprinidae has many economically important species, that is why it has been chosen for investigations.

The main objective of this study is the sequencing of the cytochrome b gene and the mitochondrial control region, the description of the haplotypes and the comparison between the obtained haplotypes. The genes belong to individuals collected from two biogeographic areas that are little scientifically investigated from the genetical point of view: Suceava and Siret rivers

The species used for the research study are *Alburnus alburnus* Linnaeus 1758 and *Vimba vimba* Linnaeus 1758, belonging to the family Cyprinidae. The *Alburnus alburnus* individuals were collected from Suceava river and the *Vimba vimba* individuals belong to Siret river.

The biological material is represented by a dorsal muscle fragment for each individual, fragment which was kept in ethylic alcohol until the DNA extraction procedure started. The mitochondrial DNA sequences investigation started with total DNA extraction from tissues that were kept in ethanol (Ausubel et al., 1995). The desired sequences were amplified by PCR reaction with its three steps: denaturation of the sequences at high temperatures, binding of the primers at lower temperature and finally the extension with the help of Taq polymerase. The primers used for amplification were universal primers for the mitochondrial control region and for the gene that determines cytochrome b synthesis. The resulting PCR products were tested through agarose gel electrophoresis (for verifying if the amplification was correct), then purified in QIAGEN columns, quantified through electrophoresis and sequenced.

The sequencing process was implemented with an eight capillars Beckman-Coulter sequenator. There have been sequenced three nucleotidic fragments of cytochrome b gene for *Alburnus alburnus* and 2 nucleotidic fragments of the mitochondrial control region for *Vimba vimba*.

The three nucleotidic sequenced fragments of the cytochrome b from *Alburnus alburnus* individuals were aligned using Clustal V method (Higgins and Sharp, 1989).

Each sequence has a number of 1140 nucleotides. The haplotype Aa101Su and Aa102Su are

identical and do not have any mutational modifications.

The third sequence, Aa103Su has a number of nine mutational modifications, all being substitutions of a purinic base with another purine or of a pyrimidinic base with another pyrimidine (transitions), or substitutions of a purine with a pyrimidine or of a pyrimidine with a purine (transversions).

From these nine substitutions six are transitions and three are transversions. Purine transitions took place in locus 135, 426, 883 (thymine was replaced by cytosine), 405, 885 (adenine was replaced by guanine) and 924 (cytosine was replaced by thymine). In two of the three transversions cytosine replaced guanine (in locuses 528 and 711) and in locus 849 adenine replaced thymine.

Comparing the number and the percentage of nucleobases in the three different haplotypes (table 1), it can be noticed that the most frequent base is thymine in the first two haplotypes (28.75%) and cytosine for haplotype Aa103Si. It is interesting the fact that the percentage of pyrimidines is higher than the one of purines. The rarest base is guanine for all the three haplotypes. Regarding the number of complementary bases, the percentage of A+T is higher than that of the G+C. This fact demonstrates that the bounds between the complementary strains are not very strong and the DNA fragment is not very stable.

Table 1. The number and percentage of nucleobases for the studied haplotypes

Analysed sequences	Nucleobases											
	A		G		T		C		A+T		C+G	
	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%
Aa101Su	297	26.03	195	17.09	328	28.75	321	28.13	625	54.78	516	45.22
Aa102Su	297	26.03	195	17.09	328	28.75	321	28.13	625	54.78	516	45.22
Aa103Su	296	25.96	195	17.11	324	28.42	325	28.51	620	54.39	520	45.61

For the individuals of *Vimba vimba*, the mitochondrial control region two haplotypes have been sequenced (Vv101Si and Vv102Si) and compared with a sequence from GenBank (with the code number Dq022090), used as control.

Each fragment has 405 nucleotides. The first haplotype Vv101Si has three mutational modifications; one of these is a transversion in locus 47, where thymine was replaced by guanine. The other two substitutions are transitions and occurred in the same locuses as in the other sequenced fragment Vv102Si: 206 and 365. In these locuses cytosine was replaced by thymine. It can be observed that both sequences belonging to *Vimba vimba* individuals from Siret river have these two locuses with identical mutations compared with the control sequence. This is very important because it means that these two mutations could be fixed inside the genome of Siret river *Vimba vimba* population.

The second haplotype Vv102Si has four substitutions, two in the same locuses as the first one: 206 and 365, one transition in locus 335 where thymine replaced cytosine and a transversion in locus 35 where guanine replaced thymine.

An interesting thing can be noticed in locuses 241 and 242 for both sequences. In these places another type of mutation occurred: deletion of nucleotides. In the control sequence these locuses were

populated with a purine, adenine (locus 241) and a pyrimidine, thymine (locus 242), but both sequenced fragments lack these nucleotides.

Comparing the number and the percentage of nucleobases in the three different haplotypes (table 2), it can be noticed that the most frequent bases are adenine and thymine in almost equal proportions, between 35,24 and 35,82%. The rarest base is guanine for all the three haplotypes. The number of purines is higher than the one of pyrimidines but not with a very large difference.

Regarding the number of complementary bases, the percentage of A+T is much more higher than that of the G+C, comparing with the proportion found in cytochrome b genes. This fact demonstrates that the bounds between the complementary strains are weaker than the ones in analyzed cytochrome b genes.

Table 2. The number and percentage of nucleotides for the studied haplotypes

Analysed sequences	Nucleotides											
	A		G		T		C		A+T		C+G	
	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%
Vv101Si	142	35,24	51	12,66	143	35,48	67	16,63	285	70,72	118	29,28
Vv102Si	142	35,32	51	12,69	144	35,82	65	16,17	286	71,14	116	28,86
DQ022090	143	35,31	50	12,35	143	35,31	69	17,04	286	70,62	119	29,38

Analyzing the cytochrome b gene in three individuals of *Alburnus alburnus* it has been noticed that only one has mutational modifications and this is not a certain proof that these mutations could be fixed in the Suceava river population. This is not the situation with the two haplotypes of mitochondrial control region in *Vimba vimba* population, where the same mutations occurred for both analyzed individuals. This could be a clue that these mutations can fix inside the genome of *Vimba vimba* population.

Both types of genes, cytochrome b gene and mitochondrial control region contain a larger number of A+T nucleotides than G+C, which proves that the DNA fragments is not very stable.