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The analysis of cytochrome b nucleotidic sequence for *Carassius gibelio* (Bloch, 1782)

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Abstract. The paper is part of a larger scale study for some genes` (Cytb, ND4L and D-loop) nucleotidic structure identification by sequencing, to distinguish the structural differences and their exact length in base pairs. Research was carried out on individuals of *Carassius gibelio* (Bloch, 1782) (Actinopterygii, Cypriniformes) from two different populations, Iezăreni and Movileni (Iași), from which dorsal muscular tissue was sampled. Mitochondrial DNA (mtDNA) isolation and purification was carried out automatically using Promega's Maxwell 16 (SEV module). Cytochrome b (cytb) was multiplied by a two stage polymerase chain reaction (PCR), using two sets of complementary primers (1 set for each fragment). Direct sequencing of PCR products revealed that the cytochrome b has one sequence of 1140bp. The obtained sequences were subsequently compared with sequences of the same gene from other individuals within this species, towards identifying possible differences in the nucleotidic structure.
Key Words: Carassius, cytocrhome b, mtDNA.

Resumen. El papel es una parte de un estudio más amplio che se propone la identificación de la estructura nucleotidica de unos genes (Cytb, ND4L y D-loop), por la enseñamiento de los diferencias estructurales y a la longitud en parejas de bases. Las recercas se efectuaran con unos bichos de *Carassius gibelio* (Bloch, 1782) (Actinopterygii, Cypriniformes), desde dos diferente populaciones, Iezăreni y Movileni (Iași). Las muestras se han colectado desde el tejido muscular dorsal. La extracción y purificación del ADN mitocondrial estaba automatizada, con la utilización del Maxwell 16 (Promega) con SEV y un kit Promega. El citocromo b estaba ampliado con la utilización de la polimerización de la cadena (PCR), en dos etapas, utilizando dos parejas de primeros complementarios (cada uno por 1 fragmente). La secuenciación directa de los productos del PCR demonstra che el citocromo b, tiene una secuencia de 1140pb. Las secuencias obtenidas se compararon ulterior con las secuencias de las misma gena de otro individuos de la misma especie, por la identificación de los eventuales diferencias en la estructura nucleotidica.
Palabras clave: Carassius, citocromo b, ADNmt.

Rezumat. Lucrarea face parte dintr-un studiu mai amplu ce constă în identificarea prin secvenţiere a structurii nucleotidice a unor gene (Cytb, ND4L şi D-loop), în vederea evidenţierii diferenţelor structurale şi a lungimii exacte în perechi de baze a acestora. Cercetările au fost efectuate pe indivizi de *Carassius gibelio* (Bloch, 1782) (Actinopterygii, Cypriniformes), proveniţi din două populaţii diferite, Iezăreni şi Movileni (Iaşi), de la care au fost prelevate probe de ţesut muscular dorsal. Izolarea şi purificarea ADN mitocondrial (ADNmt) s-a realizat automat, prin utilizarea Maxwell 16 (Promega) cu SEV şi un kit Promega. Citocromul b a fost multiplicat prin polimerizare în lanţ (PCR), în două etape, utilizând două seturi de amorse complementare (câte 1 set pentru 1 fragment). Secvenţierea directă a produşilor PCR a demonstrat că citocromul b, are o secvenţă de 1140pb. Secvenţele obţinute au fost ulterior comparate cu secvenţe ale aceleiaşi gene de la alţi indivizi ai speciei amintite, în vederea identificării eventualelor diferenţe în structura nucleotidică.
Cuvinte cheie: Carassius, citocrom b, ADNmt.

Introduction. The *Cyprinidae* are one of the most successful families of fish, with more than 2000 species grouped in approximately 340 genera (Bănărescu & Coad 1991). Cyprinid fishes have received much attention from evolutionary biologists, as they show a wide distribution around the world and occur in almost every freshwater environment. Earlier classifications of cyprinids were based mainly on external features (e.g. the presence, type and number of barbells), as well as the structure and arrangement of the pharyngeal dentition (Howes 1991). More recently, osteological characters were also used to determine the phylogenetic relationships among different groups of cyprinids (e.g. *Barbus*; Doadrio 1990). Yet, the monophyly and relationships within the *Cyprinidae*

and some of its subfamilies (e.g. *Cyprininae*, *Leuciscinae* and *Rasborinae*) have to be firmly established (Howes 1991).

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 (Bănărescu 1973). This characteristic distribution has been explained in terms of an ancient isolation of the Iberian Peninsula and southern Greece from the rest of the continent, which would have limited (as they are primary freshwater fish) the number of cyprinid genera able to colonize both regions. 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 (Bănărescu 1989, 1992).

To extract biological information from enormous strings of As, Cs, Ts, and Gs, functional genomics depends on computational analysis of the sequence data. It is unrealistic to expect that every single gene or even a majority of the genes found in the sequenced genomes would ever be studied experimentally. However, using the relatively cheap and fast computational approaches, it is usually possible to reliably predict the protein-coding regions in the DNA sequence with reasonable confidence and to get at least some insight into the possible functions of the encoded proteins. Such an analysis proves valuable for many branches of biology, in large part, because it assists in classification and prioritization of the targets for future experimental research.

Computations on genomes are inexpensive and fast compared to large-scale experimentation. The history of annotation and comparative analysis of the first sequenced genomes convincingly shows that the quality and utility of the final product critically depend on the employed methods and the depth of interpretation of the results obtained by computer methods. Unfortunately, errors produced in the course of computer analysis are propagated just as easily as real discoveries, which make development of reliable protocols and crystallization of the accumulating experience of genome analysis in easily accessible forms particularly important (Koonin & Galperin 2002).

Traditional views on deep evolutionary events have been seriously challenged over the last few years, following the identification of major pitfalls affecting molecular phylogeny reconstruction. Here we describe the principally encountered artifacts, notably long branch attraction, and their causes (i.e., difference in evolutionary rates, mutational saturation, compositional biases). Additional difficulties due to phenomena of biological nature (i.e., lateral gene transfer, recombination and hidden paralogy) are also discussed. Moreover, contrary to common beliefs, we show that the use of rare genomic events can also be misleading and should be treated with the same caution as standard molecular phylogeny. The universal tree of life, as described in most textbooks, is partly affected by tree reconstruction artifacts, e.g. (I) the bacterial rooting of the universal tree of life; (II) the early emergence of amitochondriate lineages in eukaryotic phylogenies; and (III) the position of hyperthermophilic taxa in bacterial phylogenies. We present an alternative view of this tree, based on recent evidence obtained from reanalyses of ancient data sets and from novel analyses of large combination of genes (Gribaldo & Philippe 2002).

Mitochondrial DNA (mtDNA) is DNA that is located in mitochondria. This is in contrast to most DNA of eukaryotic organisms, which is found in the nucleus. Nuclear and mtDNA are thought to be of separate evolutionary origin, with the mtDNA being derived from bacteria that were engulfed by early precursors of eukaryotic cells. Thus in cells in current organisms, the vast majority of proteins found in the mitochondria (~1500 in mammals) are encoded by nuclear DNA: some, if not most, are thought to have been originally of bacterial origin and have since been transferred to the nucleus during evolution. In mammals, 100% of the mtDNA contribution to a zygote is inherited from the mother and this is true for most, but not all, organisms. Currently, human mtDNA is present at 100-10,000 copies per cell, with each circular molecule consisting of 16,569

base pairs with 37 genes, 13 proteins (polypeptides), 22 transfer RNA (tRNAs) and two ribosomal RNAs (rRNAs).

Unlike nuclear DNA in which the genes are rearranged by ~50% each generation (due to the process called recombination), there is usually no change in mtDNA from parent to offspring by this mechanism. Because of this and the fact that its mutation rate is higher than nuclear DNA and easily measured, mtDNA is a powerful tool for tracking matrilineage, and has been used in this role for tracking many species back hundreds of generations. Human mtDNA can also be used to identify individuals; however it is not a failsafe way to discriminate involvement of people at crime scenes and is no longer commonly used in court cases for this purpose.

The primary cause of evolution is the mutational change of genes. A mutant gene or DNA sequence caused by nucleotide substitution, insertion/deletions, recombination, gene conversion and so forth may spread through the population by genetic drift and/or natural selection (Nei 1986; Hartl & Clark 1997) and eventually be fixed in a species. If this mutant gene produces a new morphological or physiological character, this character will be inherited by all the descendant species unless the gene mutates again. Therefore, if we establish a valid phylogenetic tree for a group of species, we are able to identify the lineage of species in which any specific character appeared mutation.

This information is useful in understanding the mechanism of evolution of any specific character of interest. Comparisons of the environmental conditions of this lineage of species with those of species lacking the character may suggest whether the character evolved by a particular process of natural selection or by genetic drift. If we can identify the genes involved and study their evolutionary change, we will know what kind of mutational change has generated the particular morphological or physiological character (Nei & Kumar 2000)

The aim of this paper was to determine the complete nucleotide sequence of the mitochondrial cytochrome b (cytb) for 5 individuals of *Carassius gibelio* (Bloch, 1782) (Actinopterygii, Cypriniformes), with the same origin – Iezareni Lake (Iași). The cytochrome b (cytb) was analyzed by gene amplification using polymerase chain reactions (PCR) in two segments, and the products were subsequently used as templates for PCR with 2 sets of fish-versatile primers (1 set for 1 segment) that amplify contiguous, overlapping segments of the cytb. Direct sequencing of the PCR products demonstrated that the cytb has 1140 base pairs (bp).

Material and Method. We used for gene amplification, DNA template which was automatically extracted using Promega Automated DNA purification Maxwell 16 system. The advantages of automated system are the time and consumables saving, and a higher purity, compared to the traditional method using phenol : chloroform : isoamyl alcohol (25 : 24 : 1) (Ausubel et al 1995). The technique was used for DNA extraction from fresh muscular tissues, located under the dorsal fin, preserved in absolute ethanol and stored at 4°C.

The purified DNA was used as template for Cyt b gene amplification. In this way, we used two sets of fish-versatile primers (1 set for 1 segment) that amplify contiguous, overlapping segments of the cytb ($tRNA^{Glu}$/Cytb, Cytb/$tRNA^{Thr}$) (Table 1). For PCR amplification we used Promega Go-taq Green Master, a premix which includes taq polymerase and electrophoresis samples loading and migration buffers.

Table 1

Primers used in cytochrome's b amplification

Fragment	*Primer's code*	*Primer's sequence*
$tRNA^{Glu}$/Cytb	H15149	5`-AAACTGCAGCCCTCAGAATGATATTTGTCCTCA-3`
	L14724	5`-CGAAGCTTGATATGAAAAACCATCGTTG-3`
Cytb/$tRNA^{Thr}$	Thr-R16496	5`-ACCTCCRATCTYCGGAGGACA-3`
	L15138	5`-ATGATGACCGCCTTCGTAGGCTA-3`

Amplifications were performed using total genomic DNA, and the polymerase chain reaction (PCR) (Saiki et al 1988) was done in 25µl of reaction mixture, in gradient research Palm-cycler (Corbett Life Science), with the following step cycles: 5 min at 95°C; then 30 cycles of 1 min at 94°C; 1 min at 45°C; then ramp 72°C for 1 minute with a final extension of 10 min at 72°C. The amplification reactions included negative controls, with water instead of DNA template. PCR amplified segments were loaded into 1.5% agarose gel and run for 40 min at 90V in 1X TBE buffer.

The PCR products were purified using a Promega Wizard SV Gel and PCR Clean-up System kit, in columns. Product quantification was performed by spectrophotometrical analysis, using a ATCGene ASP-3700 nano-drop system.

The sequencing reaction was performed with GenomeLab Methods Development Beckman Coulter kit and the same primers (L14724 and H10720 for the first segment, L15138 and Thr-R16496 for second, but with higher concentrations), following 30 replication cycles: 20 min at 96°C; 20 min at 50°C; then ramp 60°C for 4 minute final extension. For both segments were sequenced 2 DNA chains (forward and reverse). The sequences have been obtained by fragments capillary electrophoresis in 8 capillaries CEQ 8000 Beckman Coulter sequencer and analyzed with CEQ software.

For a clear differentiation of studied species, we compared our sequences with other ones from NCBI Genbank *Carassius cuvieri* Temminck & Schlegel, 1846 (Genbank Ref. no. AB045144), *Carassius auratus auratus* (Linnaeus, 1758) (Genbank Ref. no. NC002079), *Carassius auratus langsdorfii* Temminck & Schlegel, 1846 (Genbank Ref. no. AB006953) and *Carassius carassius* (Linnaeus, 1758) (Genbank Ref. no. AY714387). The sequences were aligned using the Clustal V method (Higgins & Sharp 1989; Higgins 1994) and verified by Clustal W (Thompson et al 1994), obtaining in both cases the same result. LaserGene 7 software was used for sequences analysis regarding to similarity percentage and nucleotide base pair content.

Results and Discussion. Electrophoregram (Figure 1) analysis, reveals that the PCR products have: first part of cytochrome b, approximately 480bp and the second part approximately 800bp, which includes a few number of base pairs from $tRNA^{Glu}$ and $tRNA^{Thr}$. This fact indicates us that the entire gene for cytochrome b is about 1141bp, if we exclude all base pairs from adiacent tRNA`s. This result is comparable with other results from literature (see above Genebank references).

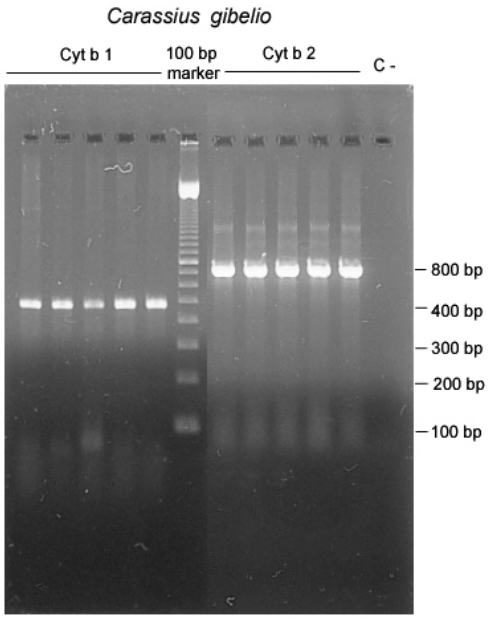

Figure 1. Two different PCR products – Cyt b1 ($tRNA^{Glu}$/cytochrome b first part) and Cyt b 2 (cytochrome b/$tRNA^{Thr}$ second part); C - negative control.

From sequences alignment for 23 individuals of *Carassius* genera and 2 hybrids, 149 differences were recorded. Comparing the sequences from all individuals of both analyzed populations, we noticed the existence of 3 differences (or only one difference, that implies 3 sequences), namely the existence of one transition in 258 position (guanine substitution with adenine), for Cag01I, Cag07I and Cag03M this mutation appears in both populations, but with a particular low frequence.

The highest number of differences (146), appears for the sequences provided by the GenBank (137 transitions and 9 transversions). The sequences that express the highest number of differences compared to the two populations' general haplotype, are the ones of *Carassius cuvieri* species and *Carassius auratus x Cyprinus carpio x Carassius cuvieri* hybrid. The differences consist in 38 transitions and 6 transversions. Most transitions occurred between cytosine – thymine (25) and only 15 transitions were registered between adenine – guanine. Regarding the transversions, two of them occurred between guanine – cytosine, one between adenine – thymine and one between adenine – cytosine.

Carassius auratus langsdörfii, individual is different compared to the two populations' general haplotype through its 38 mutations (37 transitions and 1 transversion).

Carassius carassius species and *Carassius auratus x Cyprinus carpio* hybrid, express 11 differences compared to the general haplotype: 10 transitions and 1 transversions.

In conclusion, two new haplotypes for cytochrome b were identified: a general haplotype (CagIMD) characteristic for sequences that do not present any differences within the 2 populations, and a specific haplotype for sequences which present this mutation in 258 positions Cag137IM.

Based on sequences comparison, a divergence and similarity table was constructed (Table 2) and we concluded that the similarity percentage for the two haplotypes is about 99.9%, because the difference consists in only one nucleotide. We also recorded a high similarity percentage (100%) between *Carassius carassius* and *Carassius auratus x Cyprinus carpio*, *Carassius cuvieri* and *Carassius auratus x Cyprinus carpio x Carassius cuvieri* sequences.

In the first case, based on the high similarity percentage (100%) between *Carassius carassius* species and *Carassius auratus x Cyprinus carpio* hybrid, we can deduce that in the hybrid obtaining crossing, one individual of *Carassius carassius* species participated, and not an *Carassius auratus individual*, as stated. This affirmation, is based on the fact that mtDNA is only transmitted by maternal line, while, in this case, if the female was from *Cyprinus carpio* Linnaeus, 1758 species, than, most certain, the similarity percentage with the other sequences of *Carassius* genera would have been lower. In conclusion, the female appertained to *Carassius*' genera and the male to *Cyprinus carpio* species. By sequences comparing, we observed that the hybrid's sequence corresponds by 100% percents with the sequence of *Carassius carassius* individual so, the participating female at the crossing process appertained to this species. This error has as probable cause the fact that the introduced female in the hybridization process was classified based on morphological characters only, or, the both species are in fact, subspecies of the same species.

In the second case, of the similarity situation, between *Carassius cuvieri* and *Carassius auratus x Cyprinus carpio x Carassius cuvieri* hybrid sequences, taking into account the fact that mtDNA is only transmitted by maternal line, the most probable situation, in this case, is the one in which the obtained hybrid from the crossing process, *Carassius auratus x Cyprinus carpio*, follows the breeding process with a *Carassius cuvieri* species female. In this case only the female's DNA shall be transmitted.

For *Cyprinidae* in general and for some *Carassius's* genera individuals, gynogenesis phenomenon is frequent, and in these cases there is a possibility of their being false hybrids, meaning gynogenetic individuals. In this case, *Cyprinus carpio* species, has not contribute with its genetic information at the descendant's forming process, but the seminal material took part only in cellular division intiation.

These two examples show once again the importance of molecular phylogeny and genetic studies in hybrids determination, their genitors as well as genitors' gender.

Furthermore, towards establishing a more accurate differentiation between all new haplotypes, the nucleotides' number and percentage were analyzed for each of them (Table 3).

Table 2

Similarity and divergence percents for the analyzed haplotypes

		Similarity percent							
		CagIMY	Cag137IM	Cacuv	Caulan	CaxCy	CaxCyx Ccuv	Ccaras	
Divergence percent	CagIMY		99.9	93.6	94.3	98.2	93.6	98.2	CagIMY
	Cag137IM	0.1		93.7	94.5	98.4	93.7	98.4	Cag137IM
	Cacuv	6.8	6.7		93.7	93.9	100.0	93.9	Cacuv
	Caulan	6.0	5.9	6.7		94.6	93.7	94.6	Caulan
	CaxCy	1.8	1.6	6.5	5.7		93.9	100.0	CaxCy
	CaxCyx Ccuv	6.8	6.7	0.0	6.7	6.5		93.9	CaxCyx Ccuv
	Ccaras	1.8	1.6	6.5	5.7	0.0	6.5		Ccaras
		CagIMY	Cag137IM	Cacuv	Caulan	CaxCy	CaxCyx Ccuv	Ccaras	

Table 3

Nucleotide percent for the established haplotypes

Nucleotides		*Analyzed haplotypes*						
		CagIMY	Cag137IM	Ccaras	Ccuv	Caulan	CaxCy	CaxCyxCcuv
A	Nr.	198	199	202	194	198	202	194
	%	28.95	29.09	29.53	28.36	28.95	29.53	28.36
G	Nr.	100	99	97	101	101	97	101
	%	14.62	14.47	14.18	14.77	14.77	14.18	14.77
T	Nr.	203	203	200	205	198	200	205
	%	29.68	29.68	29.24	29.97	28.95	29.24	29.97
C	Nr.	183	183	185	184	187	185	184
	%	26.75	26.75	27.05	26.90	27.34	27.05	26.90
A+T	Nr.	401	402	402	399	396	402	399
	%	58.63	58.77	58.77	58.33	57.89	58.77	58.33
C+G	Nr.	283	282	282	285	288	282	285
	%	41.37	41.23	41.23	41.67	42.11	41.23	41.67

Conclusions. From sequences alignment for 23 individuals of *Carassius* genera and 2 hybrids, 149 differences were recorded.

The comparison of the sequences from all individuals of the 2 analyzed populations shows the existence of 3 differences: 1 transition in 258 position (where guanine was substituted with adenine), for Cag01I, Cag07I and Cag03M sequences.

One mutation was detected for both populations, but with a quite low frequency.

The highest number of differences (146) occurs for sequences taken from the GenBank, comparing to the ones characteristic for the studied populations, showing the existence of 137 transitions and 9 transversions.

Two new haplotypes for cytochome b were found: one general haplotype and a specific haplotype for sequences with a mutation in 258 position Cag137IM.

The similarity percents for the 2 studied haplotypes are of 99.9% because the difference consists of one nucleotide.

Molecular phylogeny and genetic analyzes are needful for hybrids determination, hybrids' genitors and gender establishment in selection and amelioration studies.

We can conclude that for the both new haplotypes from the analyzed populations, the adenine concentration is 28.95% for the general haplotype (CagIMY) and 29.09% for the second one (Cag137IM).

Guanine quantity fluctuates between 14.62% for the general haplotype of both populations and 14.47% for the second haplotype.

Thymine and cytosine have the same values for both haplotypes (29.68% for thymine and 26.75% for cytosine).

A+T percent is 58.63% for the first haplotype and 58.77% for the second one, while, C+G concentration is 41.37% for general haplotype and 41.23% for the second.

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