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Genetic characterization of common carp (*Cyprinus carpio*) populations from Greece using mitochondrial DNA sequences

Anastasia Imsiridou[1], Alexandros Triantafyllidis[2], Athanasios D. Baxevanis[2] & Costas Triantaphyllidis[2]

[1] *Alexander Technological Educational Institute of Thessaloniki, Department of Fisheries and Aquaculture Technology, P.O. Box 157, N. Miltiadi 1, GR-63200 Nea Moudania, Halkidiki, Greece; e-mail: imsiri@otenet.gr*
[2] *Aristotle University, School of Biology, Department of Genetics, Development and Molecular Biology, GR-54124 Thessaloniki, Macedonia, Greece*

Abstract: Wild common carp from two lakes and two rivers in Greece were genetically characterized with sequencing analysis of two mitochondrial DNA segments: *cytochrome b* (1119 bp) and *D-loop* (646 bp). A total of 9 variable singleton sites and 7 unique haplotypes were detected. A common haplotype was found in three out of the four populations examined, which seems to be the ancestral one and represents the European origin of common carp from Greece. This haplotype could be also justified by the introductions reported with individuals belonging to the Central European race, into many natural habitats in Greece. Limited genetic variation – in Evros and Aliakmonas populations – could be due to bottleneck effects and small effective population sizes, whereas the different haplotypes found in Lake Volvi could represent different common carp stocks. Values of sequence divergence among Greek haplotypes ranged from 0.0006 to 0.0023. The Neighbour-Joining (NJ) phylogenetic tree constructed based on the combined sequences, reveals that the populations of common carp from Greece belong to the European group of populations – which is highly divergent from the South East-Asia cluster – and to the subspecies *Cyprinus carpio carpio*.

Key words: *Cyprinus carpio*; mtDNA; sequencing; *cytochrome b*; *D-loop*

Introduction

Common carp (*Cyprinus carpio* L., 1758) has been one of the oldest domesticated species of freshwater fish and it is among the most important cultured fishes worldwide. The natural distribution of wild common carp ranges from Europe throughout Western Asia to China, Japan and South East Asia (Baruš et al. 2001). Although the taxonomic status of different zoogeographic units is still unclear (Baruš et al. 2001), common carp is divided generally into two subspecies, *Cyprinus carpio carpio* L., 1758 from Europe and *Cyprinus carpio haematopterus* Martens, 1876 from Asia. This is also supported by allozyme, microsatellite and Restriction Fragment Length Polymorphism (RFLP) of mitochondrial DNA (mtDNA) markers (Gross et al. 2002; Kohlmann et al. 2003; Memiş & Kohlmann, 2006).

Common carp is native only to a limited number of European countries, namely those of the Danube River drainage system. Present occurrence of wild Danubian carp populations is, however, questionable and probably limited to only a few areas in the drainage system, threatened by anthropogenic effects as well as farm escapees and restocking. The species is considered to be native to Greece (Thessaly, Macedonia and Thrace) (Economidis et al. 2000). However, fingerlings from Italy were initially introduced in lakes Pamvotis and Yliki (Stephanidis 1939) and then to other lakes in the western part of the country. Other introductions and translocations have been made repeatedly since the 1950s in many Greek natural lakes and reservoirs. During the late 1980s and the 1990s further introductions with fry or fingerlings belonging to the Central European race (Hungary) were also made into many natural lakes of Greece (e.g., Koroneia, Volvi) as well as into Greek rivers (e.g., Evros, Aliakmonas) (Economidis et al. 2000).

Techniques using mtDNA have been widely employed for studying the genetic structure, the phylogeny and the origin of common carp populations because this molecule has several useful characteristics including a rapid rate of mutation and stochastic loss of haplotypes, making it effective for establishing genealogical relationships among populations within species (Avise 2000). These studies (based either on sequencing or RFLP analyses) have revealed that European common carp populations show the typical European haplotype (Zhou et al. 2003; Lehoczky et al. 2005), proving that they belong to the Europe/Central Asia phylogenetic group of common carp populations (Memiş & Kohlmann 2006), as well as revealed cases of introduction. (Froufe et al. 2002).

There is no previous mtDNA study for common carp populations from Greece, so the first aim of our

work was the genetic characterization of common carp from two lakes and two rivers in Greece. As we included in our statistical analysis individuals from the Volga River (Russia, Europe) and from the Yangtze River (China, South East Asia), a second aim was to study the phylogenetic relationships of common carp from Greece with European and East Asian groups.

Material and methods

Fish samples

In total, 14 individuals of wild common carp were collected and analyzed. The wild status is used here only to indicate that the fish or their parents were caught in lakes and rivers and that they were morphologically similar to wild carps. Five individuals were collected from Lake Volvi (40°41′00″ N, 23°34′00″ E) (Central Macedonia, N Greece), three individuals were collected from Lake Doirani (41°12′43″ N, 22°44′20″ E) (Macedonia, N Greece), three individuals were collected from the Evros River (40°52′48″ N, 26°10′55″ E) (Thrace, N-E Greece) and three individuals were collected from the Aliakmonas River (40°23′53″ N, 21°2′15″ E) (Macedonia, N-W Greece).

DNA extraction and PCR amplification

Total DNA was extracted from muscle according to the CTAB method described by Hillis et al. (1996). Polymerase Chain Reaction (PCR) was applied to amplify a segment of control region (*D-loop*) and the complete sequence of *cytochrome b* (*cyt b*) gene of mitochondrial DNA. The primers for *cyt b* (L14724 and H15915) were described in Xiao et al. (2001), whereas *D-loop* was amplified using the primers described in Zhou et al. (2003).

The PCR reaction and amplification conditions were the same for both segments. Double stranded DNA was amplified in a total reaction volume of 25 μl containing 0.6 units of Taq polymerase (Invitrogen), 2.5 μl of 10× reaction buffer, 2 mM $MgCl_2$, 0.25 mM of each dNTP, 25 pM of each primer and approximately 50–100 ng of DNA. PCR amplification conditions were as follows: one preliminary denaturation step at 94℃ (3 min), followed by strand denaturation at 94℃ (1 min), annealing at 49℃ (1 min) and primer extension at 72℃ (1.5 min) repeated for 33 cycles and a final extension at 72℃ (5 min).

Electrophoresis of 3 μl of the PCR product was performed in 1× TBE buffer for 1 h at 150 V, in 1.5% agarose gel containing 0.5 μg ml^{-1} ethidium bromide. The size of the PCR products was checked against a 100 bp DNA ladder and was approximately 1150 bp for *cyt b* and 930 bp for *D-loop*. After the end of the electrophoresis the resulting DNA fragments were visualized by UV transilumination and photographed.

DNA sequencing and phylogenetic analysis

A sequencing analysis on a 3730 x l DNA Analyzer (Applied Biosystems) was followed using both forward and reverse primers for crosschecking. DNA sequences were deposited in GenBank (accession numbers EU 689059 – EU 689086). The nucleotide sequences of all individuals were aligned using the Clustal X software (Thompson et al. 1997) and the BioEdit software (Hall 1999), set to default parameters and corrected by eye.

The best-fit substitution model (K81uF+Γ) (Kimura 1981) for our dataset was determined by Modeltest 3.7 (Posada & Crandall 1998); the parameters of this model were: unequal base frequencies ($A = 0.31$, $C = 0.25$, $G = 0.14$, $T = 0.30$), number of substitution types Nst = 6, γ-shape parameter $a = 0.276$. Phylogenetic reconstruction for the concatenated *cyt b* and *D-loop* sequences was implemented using MEGA v3.1 program (Kumar et al. 2004). A Neighbour-Joining (NJ) phylogenetic tree was constructed, based on the pairwise genetic distances produced under K81uF+Γ substitution model with PAUP* 4.0b10 (Swofford 1998).

The *cyt b* and *D-loop* sequences of one wild common carp individual from the Yangtze River (China, S-E Asia) (Zhou et al. 2004 – GenBank AY3477291; Zhou et al. 2003 – GenBank AY345331, respectively), as well as the sequence of one wild common carp individual from the Volga River (Russia, Europe) (Zhou et al. 2004 – GenBank AY347294; Zhou et al. 2003 – GenBank AY345340, respectively), were used for the phylogenetic analysis. *Carassius auratus langsdorfii* Temminck et Schlegel, 1846 (Murakami et al. 1998 – GenBank NC-002079) was used as outgroup.

	D-loop							*cyt b*	
		4	4	5	6	6	6		4
	3	6	9	2	0	2	4		8
	1	0	8	3	0	5	5	4	7
H1	**T**	**T**	**T**	**T**	**A**	**T**	**T**	**C**	**A**
H2	.	G	G	.	.	.	.	.	.
H3	G	.	.	.	.	.	.	.	.
H4	.	.	.	.	C	.	G	.	.
H5	.	.	.	G	.	.	.	.	.
H6	.	.	.	.	.	G	.	G	.
H7	.	.	.	.	.	.	.	.	G

Fig. 1. Variable nucleotide positions of the *D-loop* and *cyt b* sequences, among the different haplotypes of common carp populations. The nucleotides in each position are given in comparison to haplotype H1. For all haplotypes variable sites are indicated, while identity is given by dots.

Table 1. Population origin, sample size (n), number of haplotypes (NH) and distribution of the combined *D-loop* and *cyt b* haplotypes, among the four common carp populations investigated.

Population	n	NH	H1	H2	H3	H4	H5	H6	H7
Lake Volvi	5	5	1	1	1	1	1	–	–
Lake Doirani	3	2	2	–	–	–	–	1	–
Evros River	3	1	3	–	–	–	–	–	–
Aliakmonas River	3	1	–	–	–	–	–	–	3

Results

In total 646 bp at the 5 end of the mtDNA *D-loop* region, as well as 1119 bp for the *cyt b* mtDNA region were sequenced. A total of 9 variable singleton sites and 7 unique haplotypes were found from the combined sequences of *D-loop* and *cyt b* (Fig. 1). A common haplotype was shared among one individual from Lake Volvi (H1), two individuals from Lake Doirani and among all three individuals from the Evros River (Table 1). This haplotype was the same also in the sample from the Volga River. The rest six haplotypes were found in four individuals from Lake Volvi (H2, H3, H4, H5), in one

Table 2. Pairwise sequence divergence estimates based on the K81uF+Γ model of nucleotide substitution among the common carp *Cyprinus carpio carpio* (H1–H7), and *Cyprinus carpio haematopterus* (HAE) haplotypes.

	H1	H2	H3	H4	H5	H6	H7
H2	0.0011						
H3	0.0006	0.0017					
H4	0.0011	0.0023	0.0017				
H5	0.0006	0.0017	0.0011	0.0017			
H6	0.0012	0.0023	0.0017	0.0023	0.0017		
H7	0.0006	0.0017	0.0011	0.0017	0.0011	0.0017	
HAE	0.0154	0.0167	0.0161	0.0167	0.0161	0.0168	0.0161

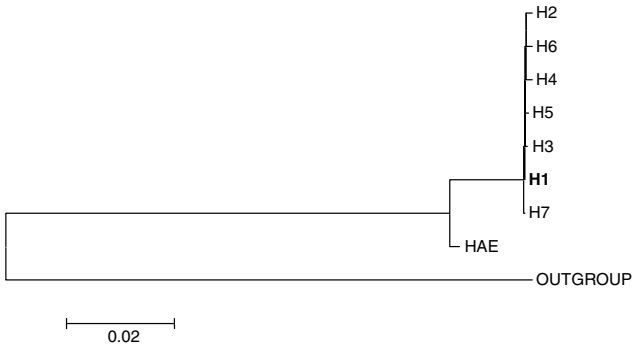

Fig. 2. Neighbour-Joining tree of common carp haplotypes, based on combined *D-loop* and *cyt b* sequences. H1–H7: *Cyprinus carpio carpio*, HAE: *Cyprinus carpio haematopterus* and OUTGROUP: *Carassius auratus langsdorfii.*

individual from Lake Doirani (H6) and another one in all three individuals from the Aliakmonas River (H7) (Table 1). The average nucleotide compositions of C, T, A, and G was 25.09, 29.9, 31.3 and 13.7%, respectively, in the four populations investigated.

Values of sequence divergence among Greek haplotypes ranged from 0.0006 to 0.0023 (mean sequence divergence among H1–H7: 0.00147 ± 0.00053), which denotes that common carp haplotypes from Greece are very similar (Table 2). From the pairwise divergence matrix it is obvious that the Yangtze River haplotype (HAE) (which belongs to the subspecies *C. c. haematopterus*) is the most divergent (sequence divergence between HAE and H1–H7: 0.01627 ± 0.00049) (Table 2).

Neighbour-Joining dendrogram based on the combined sequences of *D-loop* and *cyt b* segments revealed that all Greek haplotypes were clustered together with the Volga River haplotype (H1) in the same group, whereas the haplotype from the Yangtze River (HAE) seems to be the most genetically distinct in a separate branch (Fig. 2).

Discussion

Genetic structure of common carp from Greece

In total, seven unique haplotypes were found in the samples analyzed. In two out of the four Greek populations examined, only one haplotype was found. Similar loss of sequence variation in *D-loop* was found among 21 samples of Austrian and Hungarian common carp (Froufe et al. 2002), and this lack of control region variation in European common carp was explained with a recent common ancestor of all individuals sampled. Additionally, Kohlmann et al. (2003) reported no genetic variation within most of the European common carp populations studied. These authors attributed the loss of genetic variability to genetic drift due to small population sizes or to bottleneck effects, at the beginning or during the culture of populations. Similarly, Thai et al. (2004) reported that a remarkable feature of European common carp samples is the low variation which suggests a history of founder effects and small effective population size, associated with translocation and domestication. However, it must also be stated that limited variation due to bottleneck effects and small effective population sizes is also frequent for a number of Greek freshwater fish populations (Apostolidis et al. 1997; Imsiridou et al. 1997; Triantafyllidis et al. 1999).

Each of the five individuals of the Lake Volvi population had its own haplotype. According to Economidis et al. (2000) many introductions and translocations have been made repeatedly in this lake with individuals from acclimatized and wild populations. The frequent hybridization of different stocks has presumably led to some genetic mixing. So, the different haplotypes found in Lake Volvi, could represent different common carp stocks. However, since those haplotypes only differ by one or two substitutions each, natural genetic variability found in populations cannot be ruled out.

Phylogenetic relationships among common carp populations

According to Balon (1995), common carp originated in the area of the Caspian/Aral seas from where it spread west to the Danube River and East to Asia during postglacial times. Many introductions of common carp are well recorded, but the dating of the spread of *C. carpio* in Europe is unknown, with indications that it reached the British Isles as early as the 14th century (Lever 1996). In our study, the haplotype in the Volga River (H1) was the one that was found in six individuals of common carp from Greece, from three different populations. The most likely explanation of this pattern is that the H1 haplotype could be the ancestral one, which represents the European origin of common carp populations from Greece. On the other hand, this finding could be also justified by the introductions reported with fry

or fingerlings belonging to the Central European race, into many natural habitats in Greece (Economidis et al. 2000). As it is very difficult to know where wild or introduced populations are living, both scenarios could explain the existence of this common haplotype.

The phylogenetic analysis of the *D-loop* and *cyt b* nucleotide sequence data from Greek, European and East-Asian haplotypes reveals that the populations of common carp from Greece belong to the European group of populations and to the subspecies *C. c. carpio*, which is highly divergent from the S-E Asian cluster. In fact, all Greek haplotypes were clustered together with the Volga haplotype (European group), whereas the haplotype from the Yangtze River (S-E Asian group) formed a distinct clade.

Our results are in agreement with the RFLP data from mtDNA ND-3/4 and ND-5/6 genes of different strains of common carp (Gross et al. 2002) which indicated highly divergent groups of haplotypes, the European and the Asian groups suggesting an ancient separation. Kohlmann et al. (2003) using the same mitochondrial DNA markers supported the previous clustering as these genetic markers revealed two haplotype groups: European/Central Asian (*C. c. carpio*) and East/South-East Asian (*C. c. haematopterus*). This has also been supported by subsequent sequence analysis of *cyt b* and *D-loop* of mtDNA, which showed at least two distinct subspecies *C. c. carpio* and *C. c. haematopterus* (Zhou et al. 2004). Our results are therefore in general agreement with previous reconstructions.

As there is no previous DNA study for common carp from Greece, our results provide initial data for possible homogenization and some genetic mixing in common carp populations. It is clear that further DNA studies on common carp from the whole area of Greece are required, as understanding of the genetic resources available within the species could prove useful for designing future breeding programs.

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