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Genetic differentiation among Greek lake populations of *Carassius gibelio* and *Cyprinus carpio carpio*

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Abstract The genetic structure of the Western Greece lake populations of *Carassius gibelio* and *Cyprinus carpio carpio* populations was characterized by using a PCR-based RFLP and sequencing analysis of mitochondrial rDNA genes and regions (16S *rDNA*, cytochrome *b* and D-loop). Our analysis was able to detect: (a) two haplotypes in *C. c. carpio* populations and two haplotypes in *C. gibelio* populations (b) a high nucleotide divergence between the two species and (c) two genetically distinct *C. gibelio* populations, one existing in the Amvrakia habitat (AMV1) with a second in Ozeros and Trichonida (OZE1 and TRI1) habitat. The present analysis indicates that genetic diversity observed was limited with a haplotype index between 0.0 and 55.6%, and a nucleotide diversity within and among populations between 0.0 and 1.27%. It also underlines a restricted mtDNA-based evaluation of the phylogenetic relationships among *C. gibelio* and *C. c. carpio* populations. In addition, the present study contributed knowledge on the genetic variation and structure of these populations which is absolutely necessary for any efficient fish management and/or conservation programme.

Keywords *Carassius gibelio* (synonymous *Carassius auratus gibelio*) · *Cyprinus carpio carpio* · Cyt *b* · D-loop · 16S *rDNA* · Genetic diversity · RFLP/sequencing analysis

Abbreviations

cyt *b*	Cytochrome *b*
mtDNA	Mitochondrial DNA
NJ	Neighbor joining
MP	Maximum parsimony
PCR	Polymerase chain reaction
TS	Transitions
TV	Transversions

Introduction

During the last several decades, the optimal management in fish populations has indicated the necessity for the development of efficient and robust techniques for discriminating fish stocks (Allendorf et al. 1987). Protein electrophoresis and allozyme markers have been extensively used for genetic stock identification (Allendorf et al. 1987). However, their value has been constrained due to the fact that many species lack sufficient gene frequency divergence at polymorphic loci to permit population discrimination (Paschos et al. 2001) and problems related to inconsistencies in allozyme nomenclature and other technical issues (Ferguson 1989).

On the other hand, mtDNA-based PCR-RFLP and sequencing analysis has facilitated the characterization of genetic variation of natural populations (Bernatchez et al. 1991, 1992). In most studied vertebrates, mtDNA is

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maternally inherited without recombination and its rate of evolution and differentiation among populations is thought to be approximately four times faster than that of nuclear genes (Birky et al. 1983). Thus, mtDNA has been proven to be an excellent tool for estimating recent and ancient biogeographical events, above or below the species level (Avise 1994), a powerful source of genetic markers useful for discriminating stocks (Billington and Hebert 1991) and a tool for the effective tracing of past isolating events (Taylor et al. 1997). In addition, molecular clock analysis has been used to investigate alternative biogeographic hypothesis (Martin and Palumbi 1993; Taylor and Dodson 1994; Waters and Burridge 1999).

Cyprinid fish belong to the family Cyprinidae (minnows) which is considered the largest freshwater fish family (Economidis 1991; Economidis and Banarescu 1991). Genetic variation at the mtDNA level has been examined among stocks of several species of Cyprinid fish, including *Carassius* spp. (Luo et al. 1999; Murakami et al. 2001; Guo et al. 2004), *Cyprinus* spp. (Zhou et al. 2003; Guo et al. 2004), *Leuciscus cephalus* (Imsiridou et al. 1998), *Silurus* spp. (Triantaphyllidis et al. 1999) and other minnows (Zardoya et al. 1999). In Eurasia, the genera *Carassius* and *Cyprinus* are represented by many species and subspecies including the Asian subspecies *Cyprinus carpio haematopterus* and *Cyprinus carpio rubrofuscus*, and the European subspecies *Carassius carassius*, *C. auratus* and *C. auratus gibelio* (Economidis and Banarescu 1991; Wheeler 2000; Zhou et al. 2003). According to the fish database of Kottelat (1997), the subspecies *Carassius auratus gibelio* has recently been reclassified as Prussian carp *Carassius gibelio* and so this nomenclature is used throughout the present study.

The taxonomic status of many freshwater fish species has been a challenge for study because geographically isolated populations are frequently morphologically diverse and the genetic basis of this variation is often unresolved (McDowall 1972). Although, genetic variation based on mtDNA has been studied for many Cyprinid species (Zardoya et al. 1999), no similar studies have been performed for the Greek lake populations of *C. gibelio* and *C. c. carpio* (var. communis and var. specularis). Nomenclatural problems have also been a challenge because European populations of *C. gibelio* are known with the common name Prussian carp or wild goldfish. In Asia, populations of *C. c. auratus* (or *C. c. gibelio*) are also called goldfish and have phylogenetic affinities with Japanese populations of *C. a. langsdorfi* (Luo et al. 1999; Wheeler 2000; Murakami et al. 2001). Indeed, the classification and the relationships of different populations of *C. auratus* have been obscure (Luo et al. 1999) despite the recent controversial hypothesis that *C. gibelio* originated from other subspecies by a polyploidy event (Buth et al. 1991). Similarly, the origin of the European domestic common carp *Cyprinus carpio carpio* (L., 1758) has not been clearly elucidated (Zhou et al. 2003) despite the fact that this species has been cultivated in European ponds for hundreds of years (Hickling 1962).

Knowledge of genetic variation and population structure is absolutely necessary for any efficient fish management and/or conservation programme. Populations of *C. gibelio* and *C. c. carpio*, are considered to be native in Greece (Economidis 1991). It is important to note, however, that both species are also cultured in Europe and elsewhere. Also, novel *C. c. carpio* strains have been produced through genetic approaches in several countries making extremely difficult the attempt to identify the ancestor of the European common carp (Hickling 1962; Kottelat 1997; Paschos et al. 2001; Zhou et al. 2003). In addition, many translocation events took place in Greek lakes during the last century as well as in other regions (Mabuchi et al. 2008). It is unclear whether the Greek lake populations of *C. gibelio* and *C. c. carpio* are native or whether they have been introduced from other European countries (Economidis et al. 2000). As a first step towards resolving this problem, we characterized the genetic structure of the Western Greece lake populations of *C. gibelio* and *C. c. carpio* populations by using a PCR-based RFLP and sequencing analysis of mtDNA genes and regions in this study. The mtDNA analysis provided evidence for the different level of genetic variation among populations as well as a restricted mtDNA-based evaluation of the phylogenetic relationships among *C. gibelio* and *C. c. carpio* populations.

Materials and methods

Sample description and location

From 2003 to 2004, a total of 81 *C. gibelio* (Prussian carp or wild goldfish), *C. c. carpio* (var. communis) (scaled carp) and *C. c. carpio* (var. specularis) were sampled from different locations in the Western Greece lakes region (Perfecture: Etoloakarnania), as shown in Fig. 1: (a) samples designated as AMV1 and AMV2 were obtained from Lake

Fig. 1 Map of Western Greece indicating collection sites for *C. gibelio* and *C. c. carpio*. The collection sites are: **a** Lake Amvrakia (*AMV*) **b** Lake Ozeros (*OZE*) **c** Lake Lysimacheia (*LYS*) and **d** Lake Trichonida (*TRI*) [*Source* http://earth.google.com/]

Amvrakia and included specimens of *C. gibelio* ($n = 9$) and *C. c. carpio* (var. specularis $n = 9$) respectively; (b) OZE1 and OZE2 were obtained from Lake Ozeros and included specimens of *C. gibelio* ($n = 9$) and *C. c. carpio* (var. communis $n = 9$), respectively; (c) LYS1, LYS2 and LYS3 were from Lake Lysimacheia and included specimens of *C. gibelio* ($n = 9$), *C. c. carpio* (var. communis $n = 9$) and *C. c. carpio* (var. specularis $n = 9$), respectively and (d) TRI1 and TRI2 were acquired from Lake Trichonida and included specimens of *C. gibelio* ($n = 9$) and *C. c. carpio (var.* communis $n = 9$), respectively. Morphometric and meristic characters were used for preliminary identification of the specimens as reported previously (Kirpichnikov 1981; Economidis and Banarescu 1991).

MtDNA amplification and restriction enzyme analysis

Total DNA was extracted and purified from scales of each individual according to Taggart et al. (1992). The remaining scales from live fish were stored in 100% ethanol for further analysis. Three fragments of the mtDNA genome were amplified by PCR, encompassing the 16S *rDNA*, cyt *b* and D-loop regions respectively, using the following pairs of primers: 16SFCgCcc 5′-CGC CTG TTT ACC AAA AAC AT-3′ and 16SRCgCcc 5′-CCG GTC TGA ACT CAG ATC ACG-3′, cytbF1 5′-ATT AGT ACA TAT ATG TAT TAT CAC C-3′ and cyt *b* R2 5′-CGG GGT TTG ACA AGG ATA ACA G-3′, DloopF2 5′-AAT GAC TTG AAG AAC CAC CGT-3′ and DloopR3 5′-AGT TTA GAA TTC TGG CTT-TG-3′. These primers were designed using the full mitochondrial genome sequence of *Carassius auratus langsdorfi* (GenBank AB006953) and the *Cyprinus carpio* sequence (GenBank X61010). PCR amplifications were performed in a thermal minicycler^{MT} (MJ Research) using protocols reported previously (Bernatchez et al. 1995; Apostolidis et al. 1996), with minor modifications. A reaction mixture (20 µl) containing 10× PCR buffer, 15 mM $MgCl_2$, 0.25 mM each deoxynucleoside triphosphate, the appropriate primers, 0.3 mM each, and 1 U Taq polymerase (Minotech, Greece) was prepared. PCR reactions were performed using an initial step at 94°C for 10 min, followed by 35 cycles of 1 min denaturation at 94°C, 1 min primer annealing at 51°C for cyt *b* and D-loop and 53°C for 16S *rDNA* amplification and 90 s DNA chain extension at 72°C. The PCR was completed by a final extension at 72°C for 10 min. The 16S *rDNA* and cyt *b* amplified DNA fragments were digested with nineteen restrictions enzymes (*Hin*fI, *Sty*I, *Bam*HI, *Bsh*FI, *Alu*I, *Bst*NI, *Bst*UI, *Bgl*I, *Dde*I, *Eco*RI, *Pst*I, *Taq*^{a}I, *Mod*I, *Xba*I, *Hpa*II, *Rsa*II, *Pvu*II, *Hind*III *και* *Eco*RV). Digestions were carried out according to the manufacturer's instructions (Minotech and New England Biolabs Inc.) The sizes of the PCR products and restriction fragments were estimated by comparison with a DNA marker (lambda phage DNA digested with *Hind*III/*Eco*RI or *Pst*I). Restriction fragments were analyzed on 1.5–2% agarose horizontal slab gels, depending on the size of fragments. MtDNA bands were visually identified using ultraviolet light.

Sequencing and data analysis

All fish collected in the present study were used in the sequencing and phylogenetic analysis ($n = 81$ total, nine individuals from each population). For sequencing of the PCR products, PCRs were performed in 50 µl reaction volumes and the amplified products were purified by the Qiagen® Nucleotide Removal Kit. The resulting purified DNA was then precipitated and the appropriate concentration was used for sequencing that was performed by the MACROGEN company. Fragments of 16S *rDNA* (620 bp), cyt *b* (1250 bp) and D-loop (680 bp) were sequenced (double stranded) using the specific primers described above, and two internal primers, cyt *b*1F 5′-CAC GAA ACA GGA TCA AAC AAC-3′ and cyt *b*1R 5′-CCC AAG GAG GTC TTT GTA TG-3′ for the cyt *b* gene. All sequences are available from EMBL/GenBank under accession numbers: (DQ868862-70, EU186829), (DQ868871-79, EU186830) and (EU186831-41) for 16S *rDNA*, cyt *b* and D-loop regions, respectively.

The full length gene sequence of cyt *b* and a partial sequence of the 16S *rDNA* gene and the D-loop mitochondrial region were analyzed separately for genetic distance and character-based variations using the MEGA 3.1 computer package (Kumar et al. 2004) and PAUP (version 4b10) (Swofford 1998), respectively. The obtained sequences were aligned using Clustal X (version 1.83) and phylogenetic distances were calculated according to Kimura (1980) or/and Jukes and Cantor (1969) methods. The resulting genetic distances and character based variations were used to construct phylogenetic trees. The phylogenetic analysis was performed using neighbor-joining and maximum approaches and was based on bootstrap analysis of 10,000 trees (Saitou and Nei 1987; Fitch 1971). In the phylogenetic analysis, all nucleotide sites and substitution classes were weighted equally. In all cases, the phylogenetic trees included the, respective, sequences from *Carassius auratus langsdorfi* (GenBank accession number: AB006953) and *Cyprinus carpio* (GenBank accession number: X61010) for comparative purposes. LRT-tests for clock-like behavior of 16S *rDNA* and cyt*b* gene sequences were performed using PAUP (version 4b10) (Swofford 1998). Genetic diversity was examined by calculating haplotype frequency, haplotype diversity (h) and nucleotide diversity (π) using ARLEQUIN version 3.11 (Excoffier et al. 2005). The same program was used to estimate Fst values. Significance of Fst values for each

locus was determined by bootstrapping over samples. They were estimated using 10,000 permutations and bootstraps for all sample-pairwise comparisons.

Results

RFLP analysis

The sizes of the PCR amplified mitochondrial 16S *rDNA* and cyt*b* gene sequences were 0.62 Kb and 1.25 Kb respectively, for all populations studied. Nineteen restriction enzymes were used for RFLP analysis of the amplified products; however, only ten of them (*Hin*fI, *Sty*I, *Bsh*FI, *Rsa*I, *Bst*NI, *Alu*I, *Taq*^{a}I, BstUI, *Dde*I and *Mbo*I) produced polymorphic sites in the 16S *rDNA* amplified products in all studied populations. Eight restriction enzymes (*Bsh*FI, *Hin*fI, *Sty*I, *Rsa*I, *Bst*NI, *Alu*I, *Dde*I and *Bam*HI) were used in the cyt *b* amplified products. Each unique restriction pattern was denoted by a specific letter as shown in Table 1. The RFLP analysis shown in Table 1 was extremely useful for discrimination of individuals at both inter- and intra generic levels since: (a) the RFLP patterns observed on the amplified cyt*b* gene fragments using the restriction enzymes *Hin*fI, *Sty*I, *Bam*HI, *Bst*NI, *Alu*I, *Dde*I and *Rsa*I can discriminate between the two species, *C. gibelio* and *C. carpio*; (b) the RFLP patterns observed for the amplified cyt*b* gene fragments using the restriction enzymes *Bst*NI, *Alu*I, *Dde*I and *Rsa*I allowed the identification of two distinct haplotypes in the Lysimacheia *C. gibelio* population, LYS1a and LYS1b, as well as the discrimination of the Lake Amvrakia (AMV1) *C. gibelio* population from the Ozeros (OZE1), Trichonida (TRI1) and Lysimacheia (LYS1b) samples, and (c) the RFLP patterns observed for the amplified 16S *rDNA* gene fragments allowed discrimination of the Lake Amvrakia (AMV1) *C. gibelio* population from the Ozeros (OZE1) and Trichonida (TRI1) samples. No diagnostic RFLP pattern was observed for *C. c. carpio* populations (Table 1).

16S rDNA sequence data analysis

Sequence analysis of the 551 bp long amplified 16S *rDNA* fragments from the 81 individuals sampled showed limited genetic variation. The analysis revealed 19 variable nucleotide sites, 18 of which were informative for parsimony analysis while one of them was an indel. Three haplotypes were detected: (a) the first haplotype represented all *C. c. carpio* populations since no intra- or interpopulation genetic variation was found; (b) the second haplotype represented the AMV1 and LYS1a samples of *C. gibelio* population and (c) the third one included the OZE1, LYS1b, TRI1 samples of *C. gibelio* populations. It should be noted that the transitions—transversions ratio (TS/TV) was 4:1 between *C. gibelio* and *C. c. carpio* populations while the TS/TV ratio was 2:1 between the different populations of *C. gibelio*. In addition, pairwise sequence divergence analysis of the 16S *rDNA* data was in the range of 0.0% up to 0.55% between the different haplotypes of *C. gibelio,* while it varied from 2.97 to 3.15% between the different haplotypes of *C. gibelio* and *C. c. carpio* (Table 2). Phylogenetic analysis of the 16S *rDNA* sequence data using NJ and MP approaches produced identical trees (the NJ tree is shown in Fig. 2a). Two distinct clades were produced representing the *C. gibelio* and *C. c. carpio* samples, respectively. The *C. gibelio* clade was clustered with *C. auratus langsdorfi*, a close relative of *C. gibelio*, into two well-supported groups representing the (AMV1, LYS1a) and the (OZE1, LYS1b, TRI1) haplotypes of *C. gibelio* populations (Fig. 2a).

Cytochrome *b* sequence data analysis

Sequence analysis of the 1,140 bp long amplified cyt *b* fragments from each of the 81 fish showed evidence of genetic variation at least for *C. gibelio* populations. The analysis revealed 156 variable nucleotide sites, 138 of which occurred in the third codon position while the rest

Table 1 Restriction diagnostic enzyme patterns on mtDNA 16S *rDNA* and cyt *b* regions. Letters refer to restriction fragment patterns that occur in studied populations

Population	16S *rDNA*	Cytochrome *b*						
	*Mbo*I	*Hin*fI	*Sty*I	*Bam*HI	*Bst*NI	*Alu*I	*Dde*I	*Rsa*I
Cg (AMV1)	A	A	A	A	A	A	A	A
Cg (LYS1a)	A	A	A	A	A	A	A	A
Cg (LYS1b)	B	A	A	A	B	B	B	B
Cg (OZE1)	B	A	A	A	B	B	B	B
Cg (TRI1)	B	A	A	A	B	B	B	B
Ccs (AMV2)	B	B	B	B	C	C	C	C
Ccc (LYS2)	B	B	B	B	C	C	C	C
Ccc (OZE2)	B	B	B	B	C	C	C	C
Ccc (TRI2)	B	B	B	B	C	C	C	C
Ccs (LYS3)	B	B	B	B	C	C	C	C

The abbreviations of taxa are as follow: *Cg*, *Carassius gibelio*; *Ccc*, *Cyprinus carpio carpio* (var. communis); *Ccs, Cyprinus carpio carpio* (var. specularis)

Table 2 Percentage sequence divergence analysis among *C. gibelio* (AMV1, LYS1a, LYS1b, OZE1, TRI1) and *C. c. carpio* (AMV2, LYS2, OZE2, TRI2, LYS3) 16S *rDNA* (above diagonal) and cyt *b* (below diagonal) sequences

Haplotype	Population and sample locality									
	AMV1	LYS1a	LYS1b	OZE1	TRI1	AMV2	LYS2	OZE2	TRI2	LYS3
AMV1	–	0.00	0.55	0.55	0.55	2.97	2.97	2.97	2.97	2.97
LYS1a	0.00	–	0.55	0.55	0.55	2.97	2.97	2.97	2.97	2.97
LYS1b	3.76	3.76	–	0.00	0.00	3.15	3.15	3.15	3.15	3.15
OZE1	3.76	3.76	0.00	–	0.00	3.15	3.15	3.15	3.15	3.15
TRI1	3.76	3.76	0.00	0.00	–	3.15	3.15	3.15	3.15	3.15
AMV2	13.23	13.23	13.12	13.12	13.12	–	0.00	0.00	0.00	0.00
LYS2	13.23	13.23	13.12	13.12	13.12	0.00	–	0.00	0.00	0.00
OZE2	13.23	13.23	13.12	13.12	13.12	0.00	0.00	–	0.00	0.00
TRI2	13.23	13.23	13.12	13.12	13.12	0.00	0.00	0.00	–	0.00
LYS3	13.23	13.23	13.12	13.12	13.12	0.00	0.00	0.00	0.00	–

occurred in the first position. Eleven of these substitutions resulted in amino acid replacements and no indels were observed. The majority of the substitutions were observed in comparisons between the two species (114/156) while the rest (42) were observed between the different populations of *C. gibelio*; no genetic variation was observed between the *C. c. carpio* populations. Similarly to the 16S *rDNA* sequence results, three haplotypes were detected: (a) the first haplotype represented all *C. c. carpio* populations since no intra- or interpopulation genetic variation was found; (b) the second haplotype represented the AMV1 and LYS1a populations of *C. gibelio,* and (c) the third one included the OZE1, LYS1b, TRI1 populations of *C. gibelio*. The TS/TV ratio was about 3.1:1 between *C. gibelio* and *C. c. carpio* populations while it was 20:1 between different populations of *C. gibelio*. In addition, the pairwise sequence divergence analysis of the cyt *b* data ranged from 3.76% between *C. gibelio* haplotypes to 13.23% between *C. gibelio* and *C. c. carpio* haplotypes (Table 2). Phylogenetic analysis of the cyt *b* sequence data using NJ and MP approaches produced nearly identical trees (the NJ tree is shown in Fig. 2b) to that produced by the 16S *rDNA* analysis. Alternative weighting schemes revealed the same topology with only minor differences in bootstrap confidence. Two major clades emerged that were supported by bootstrap analysis. The first clade contained all haplotypes present in the *C. c. carpio* populations while the second one included all haplotypes present in *C. gibelio* populations. In the second clade, the two separate distinct groups are well-supported by bootstrap analysis (Fig. 2b). According to this topology, only the LYS1 population was polyphyletic forming groups with AMV1 or with OZE1 and TRI1 *C. gibelio* populations.

Control region sequence data analysis

Sequence analysis of the 567 bp long amplified D-loop region fragments from each of the 81 fish also showed significant genetic variation. The analysis revealed 67 variable nucleotide sites, 64 of which were informative for parsimony analysis while three of them were indels. This variation allowed the detection of four haplotypes, two for each species. The percentage of the variable sites identified in the D-loop sequence data (11.3% to 64/567) was higher than that detected in the 16S *rDNA* sequence data (3.3% to 18/551) and was lower than that observed in the protein coding gene cyt *b* (13.7% to 156/1140). The TS/TV ratio was about 1:1 between *C. gibelio* and *C. c. carpio* populations while it was 12:1 between different populations of *C. gibelio*. In addition, the pairwise sequence divergence analysis of the D-loop data ranged from 10.25 to 12.09% between *C. gibelio* and *C. c. carpio* haplotypes. Furthermore, the observed genetic distance ranged from 0.0 to 2.15% and from 0.0 to 0.18% among *C. gibelio* and *C. c. carpio* haplotypes, respectively (Table 3). The analysis of the 16S *rDNA* and cyt *b* genes sequences produced nearly completely congruent trees, as expected. Analysis of the 567 bp of D-loop sequence reinforced this congruence (Fig. 2c), which was in turn nearly identical to those trees recovered from 16S *rDNA* and cyt *b* data sets with very high bootstrap values for all nodes. Again, the phylogenetic analysis showed a principal phylogenetic split between *C. gibelio* and *C. c. carpio* populations. All zoogeographic units were recognized as monophyletic groups, except for LYS1a and LYS1b *C. gibelio* haplotypes (Fig. 2c).

Genetic diversity and distribution

Table 4 presents the frequencies of haplotypes, haplotype diversity (h) and nucleotide diversity (π) for each population studied based on the three mitochondrial regions used. The genetic diversity observed was limited since one or two haplotypes were observed in the *C. gibelio* and *C. c. carpio* populations studied. This is reflected in the values of the haplotype diversity index, which varied from 0.0% in most populations to 55.6% in the Lake Ozeros *C. carpio*

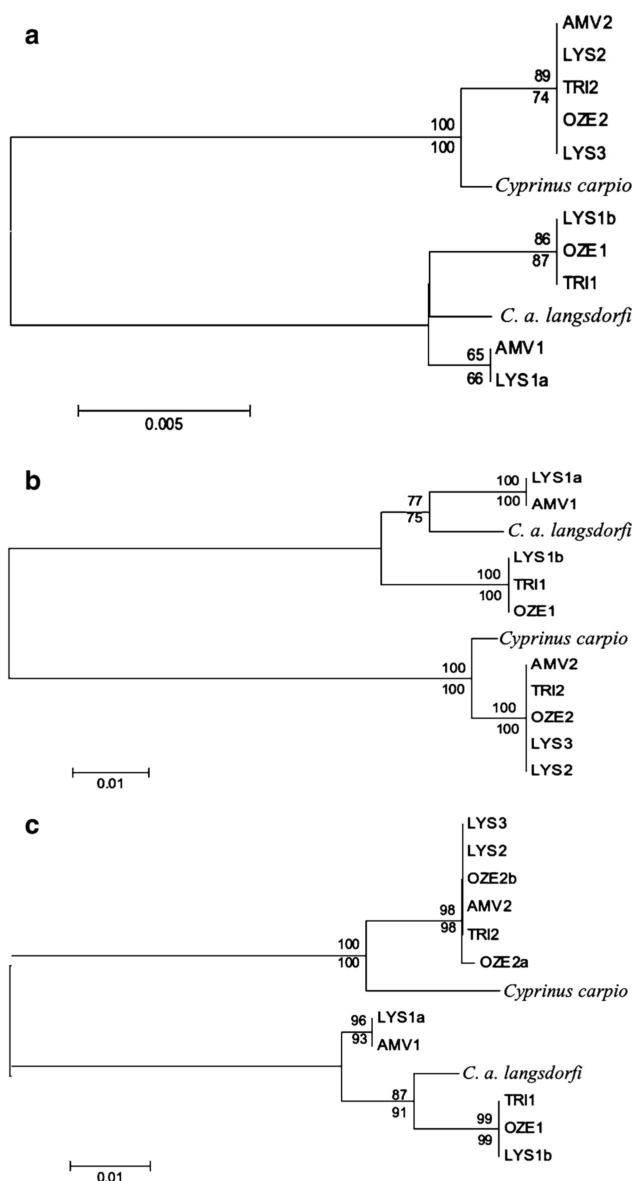

Fig. 2 Neighbor-joining phylograms showing the phylogenetic relation between *C. gibelio* (AMV1, LYS1a, LYS1b, OZE1, TRI1) and *C. c. carpio* (AMV2, LYS2, OZE2a, OZE2b, TRI2, LYS3) haplotypes. The numbers at each node represent bootstrap proportions based on 10,000 replications for both the NJ (above branch) and the maximum-pasrimony (below branch) analysis. The trees represent the analysis of the 16S *rDNA* (**a**), cyt *b* (**b**) and D-loop mtDNA genes (**c**) sequences. *Carassius auratus langsdorfi* (GenBank: AB006953) and *Cyprinus carpio* (GenBank: X61010) sequences were used as outgroups

population. Nucleotide diversity within and among populations ranged from 0.0 to 1.27% for AMV1, OZE1, TRI1 and LYS1 *C. gibelio*, respectively. The intrapopulation genetic diversity appeared to be much lower for *C. c. carpio* (only 0.03% for the OZE2 population) than *C. gibelio*.

Table 5 presents the F_{ST} values for all loci across all lake populations of *C. gibelio* and *C. carpio* studied. Pairwise F_{ST} values for *C. gibelio* ranged from 0.0 (OZE1 and TRI1) to 1.000 (AMV1 and OZE1, AMV1, and TRI1) indicating that AMV1 sample was significantly different from the other lakes ($P < 0.05$). Pairwise F_{ST} values for *C. carpio* ranged from 0.0 (as in AMV2 and LYS2) to 0.375 (as in AMV2 and OZE2) suggesting that there was no significant differentiation between populations ($P < 0.05$).

Molecular clock analysis

Assessing evolutionary rates of the 16S *rDNA* and the cyt*b* gene sequences, with the omission of *C. a. langsdorfi*, indicated that the molecular clock hypothesis could not be rejected for either gene. The estimated timing of haplotype divergence was based on two molecular clock calibrations reported in previously: (a) 0.8–2.8% divergence per million year (mya) for freshwater fish cyt *b*, (402 bp partial gene sequence) and (b) 0.5–0.9% divergence per million years for freshwater fish mtDNA genes (921 bp of combined 16S *rDNA* and cyt *b* partial gene sequences) (Martin and Palumbi 1993; Taylor and Dodson 1994; Waters and Burridge 1999). In each case, the log likelihood of an unrooted and a rooted tree enforcing a molecular clock was calculated using the MODELTEST program (Posada and Crandall 1998). For 16S *rDNA,* an LRT value of 49.69 (8 *df*, $P > 0.01$) and for cyt *b* an LRT value of 470.36 (8 *df*, $P > 0.01$), indicated that the use of molecular clock calibrations to estimate haplotype divergence times was valid in our study.

Discussion

The present study used a number of mitochondrial genetic markers (16S *rDNA*, cyt *b* and D-loop) in order to estimate the genetic diversity, structure and differentiation of the Western Greece lake populations of *C. gibelio* and *C. c. carpio*. Our analysis was able to detect: (a) two haplotypes in *C. c. carpio* populations and two haplotypes in *C. gibelio* populations; (b) a high nucleotide divergence between the two species and (c) two *C. gibelio* haplotypes, one existing in the Amvrakia habitat (AMV1) with a second in Ozeros and Trichonida (OZE1 and TRI1) habitat.

One out of two detected mtDNA haplotypes of *C. gibelio* was population specific for Lakes Ozeros and Trichonida (OZE1 and TRI1) but not for Lake Lysimacheia. The other haplotype was fixed for Lake Amvrakia (AMV1), however it was also identified in Lake Lysimacheia (LYS1a). A possible explanation for the above observations is that the Lake Trichonida is connected to Lake Lysimacheia through a controlled canal, while the Acheloos River has an extensive system of side canals which has allowed gene flow between Lake Ozeros and Lysimacheia.

Table 3 Percentage sequence divergence analysis among *C. gibelio* (AMV1, LYS1a, LYS1b, OZE1, TRI1) and *C. c. carpio* (AMV2, LYS2, OZE2a, OZE2b TRI2, LYS3) D-loop sequences

Haplotype	Population and sample locality										
	AMV1	LYS1a	LYS1b	OZE1	TRI1	AMV2	LYS2	OZE2a	OZE2b	TRI2	LYS3
AMV1	–										
LYS1a	0.00	–									
LYS1b	2.15	2.15	–								
OZE1	2.15	2.15	0.00	–							
TRI1	2.15	2.15	0.00	0.00	–						
AMV2	10.25	10.25	11.88	11.88	11.88	–					
LYS2	10.25	10.25	11.88	11.88	11.88	0.00	–				
OZE2a	10.45	10.45	12.09	12.09	12.09	0.18	0.18	–			
OZE2b	10.25	10.25	11.88	11.88	11.88	0.00	0.00	0.18	–		
TRI2	10.25	10.25	11.88	11.88	11.88	0.00	0.00	0.00	0.00	–	
LYS3	10.25	10.25	11.88	11.88	11.88	0.00	0.00	0.00	0.00	0.00	–

Table 4 Haplotype frequencies and genetic diversity estimates for the *C. gibelio* and *C.c. carpio* populations: (*n*) number of individuals, (*h*) haplotype diversity, (π) nucleotide diversity

Haplotypes	*Carassius gibelio*				*Cyprinus carpio carpio*				
	Over 3 loci[e]				Over 3 loci[e]				
	AMV1	LYS1	OZE1	TRI1	AMV2	LYS2	OZE2	TRI2	LYS3
Type 1[a]	1.000	0.333							
Type 2[b]		0.667	1.000	1.000					
Type 3[c]					1.000	1.000	0.556	1.000	1.000
Type 4[d]							0.444		
h (±SD)	0.000	0.500 ± 0.128	0.000	0.000	0.000	0.000	0.556 ± 0.090	0.000	0.000
π (±SD)	0.000	0.0127 ± 0.007	0.000	0.000	0.000	0.000	0.0003 ± 0.0003	0.000	0.000
n	9	9	9	9	9	9	9	9	9

[a] Type 1 haplotype is present in AMV1 and LYS1 populations

[b] Type 2 haplotype is present in LYS1, OZE1, TRI1 populations

[c] Type 3 haplotype is present in AMV2, LYS2, OZE2, TRI2 and LYS3 populations

[d] Type 4 haplotype is present only in OZE2 population

[e] The 3 loci consist of the 16S r*DNA*, cyt*b* and D-loop regions of mt DNA

Table 5 Pairwise F_{ST} for *C. gibelio* and *C. c. carpio* between their respective populations

	Carassius gibelio					*Cyprinus carpio carpio*				
	AMV1	LYS1	OZE1	TRI1		AMV2	LYS2	OZE2	TRI2	LYS3
AMV1	–				AMV2	–				
LYS1	0.625[a]	–			LYS2	0.000	–			
OZE1	1.000[a]	0.250	–		OZE2	0.375	0.375	–		
TRI1	1.000[a]	0.250	0.000	–	TRI2	0.000	0.000	0.375	–	
					LYS3	0.000	0.000	0.375	0.000	–

[a] Significantly different from zero ($P < 0.05$)

Nucleotide divergence between *C. gibelio* and *C. c. carpio* are higher than those reported for congeneric freshwater fish (Billington and Hebert 1991) and slightly higher than those observed (about 12% for intrageneric species) for other fish at the genus level (Johns and Avise 1998). Both NJ and MP analysis of 16S *rDNA*, cyt *b* and D-loop mtDNA genes support a *C. gibelio* clade including the (AMV1, OZE1, LYS1, TRI1) populations and a *C. c. carpio* clade including all of populations sampled (Fig. 2a–c). This clustering is consistent with a previous systematic classification based on morphomeristic characters and on molecular data (Zardoya et al. 1999), although there has not been strong concordance between genetic differentiation revealed by DNA analysis and morphology

diversity among species (Sturmbauer and Meyer 1992; Patterson et al. 1993; Klossa-Kilia et al. 2002). The finding that *C. a. longsdorfi* clusters within *C. gibelio* in all three trees suggests that a more rigorous phylogenetic study is needed in order to determine taxonomic status of *C. gibelio* using more appropriate nuclear genetic markers.

Our results also revealed the presence of two *C. gibelio* haplotypes, one existing in the Amvrakia habitat (AMV1) with a second in Ozeros and Trichonida (OZE1 and TRI1) habitat which are united with bootstrap support (Fig. 2a–c), while the Lake Lysimacheia population consists of two haplotypes (LYS1a and LYS1b). The low genetic diversity observed in the *C. gibelio* populations studied might be explained by introductions from Europe during the last century (Economidis et al. 2000). Indeed, the introduction of non-native domesticated species and the translocation of native species from one drainage system into another are widely accepted methods for enhancement of many natural waters with poor and/or low fish productivity around the world. Some species, such as *C. c. carpio* and *Oncorhynchus mykiss*, have been successfully introduced into new habitats. In other cases, transfer of exotic fish or unknown species during stocking have had considerable negative impacts to native species, especially when they are endemic, as in the case of *Silurus glanis* versus *Silurus aristotelis* in Lake Volvi, Greece (Cowx 1998; Economidis et al. 2000). Nevertheless, it is difficult to determine the geographical origin of the haplotypes observed in the present study because of stocking activities that have been reported or due to a polyploid lineage of *C. gibelio*. Further studies are required to elucidate the biogeography of the species, as in cases of the origination of Japanese *C. auratus* (Murakami et al. 2001), of European *C. carpio* (Zhou et al. 2003) and of Japanese *C. carpio* (Mabuchi et al. 2008), as well as whether the two haplotypes observed in the present study.

Low levels of genetic variation observed in *C. c. carpio* could be due to selection favoring single haplotypes, founder effects in new colonizing populations, or a combination of these processes (Duvernell and Aspinwall 1995). Additionally, low sample sizes could lead to an underestimation of genetic variability since mtDNA is more susceptible to the effects of genetic drift (Ferguson et al. 1995). However, this effect alone cannot explain the observed diversity between the two species since both samples were of the same size. Local environmental conditions may also have played an important role in shaping this genetic difference between the two species. Additionally, the lack of genetic diversity in *C. c. carpio* population studied may due to a founder effect at the introduction of the non-native common carp and/or loss of genetic diversity during hatchery rearing and breeding (Mabuchi et al. 2008).

Genetic stock identification, the use of genetic markers for identifying the contributing populations and the proportions of different populations in mixed stock fisheries, are powerful and valuable tools for the management of fisheries. Molecular analysis of genetic variation in natural and mixed populations when combined with data from biogeographical information can identify important trends in population dynamics and can assist in setting priorities for aquaculture and conservation programmes (Avise 1994). The haplotypes of the two species can serve as "genetic tags" for stock identification in future aquaculture programmes and therefore warrant protection. The results of this study clearly suggest that protection measures and a management programme of the two Cyprinid species should be undertaken, for both financial and ecological reasons. Firstly, a number of specific haplotypes were revealed. Based on our analysis, the AMV1 population of *C. gibelio* inhabiting the Lake Amvrakia is different from the populations of the Ozeros (OZE1) and Trichonida (TRI1) lake systems, while the LYS1 population was represented by two distinct haplotypes (LYS1a and LYS1b); LYS1a is very closely related to AMV1 while LYS1b is very closely related to OZE1 and TRI1. The above results suggest that distinct management plans may be necessary to be developed for these populations. Also, an accurate assessment of *C. gibelio* biodiversity in other Greek lakes will aid in taking the appropriate decisions about the management of the populations of this species. Secondly, low levels of mtDNA diversity, especially in the *C. c. carpio* populations, were found. The lack of genetic diversity in *C. c. carpio* (except the OZE2 population) could lead in the near future to a threat in the survival of the species. The haplotypes of *C. c. carpio* need to be protected since they contain unique portions of the total variation of the species which may be essential for the adaptation and the survival possibilities of the species as a whole. Stocking in any of the lakes mentioned could have disastrous results for the native populations. Even a small number of fish can produce hybrids which could result in the breakdown of the reproductive isolation. For this reason, any future stocking of these particular lakes must be carefully planned. Also, any future conservation plans should consider: (a) the careful management of each one of the populations of the different observed lineages, (b) the *C. c. carpio* population as a separate management unit and (c) the fact that the population of *C. gibelio* gradually increases in other Greek lakes, such as Pamvotis (NW Greece), while that of other fish, such as *C. c. carpio*, declines (Paschos et al. 2004).

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