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Genetic differentiation and phylogenetic relationships among Greek Chub *Leuciscus cephalus* L. (Pisces, Cyprinidae) populations as revealed by RFLP analysis of mitochondrial DNA

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Abstract

The phylogenetic and systematic relationships among 12 populations of Greek chub (*Leuciscus cephalus* L.) were investigated using RFLP analysis of two PCR-amplified mtDNA segments (D-loop and cytochrome b). Three out of eight, and nine out of ten restriction enzymes were polymorphic for D-loop and cytochrome b, respectively. Twenty-one different haplotypes were detected. Estimated nucleotide sequence divergence among the 21 genotypes ranged from 0.313 to 6.79%; the mean value of sequence divergence among populations was 3.421 $\pm$ 0.00%. The calculated N_{ST} value of 0.94, indicates high interpopulation diversity. Dendrograms based on haplotypes as well as those of populations, support the previous recognition of different subspecies, but these mtDNA data are not concordant with previous allozyme data among these fish. Our results suggest that to clarify phylogenetic relationships multiple genetic systems need to be examined.

Keywords: *Leuciscus cephalus*; mtDNA; RFLPs; Genetic differentiation; Phylogeny; Greece

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1. Introduction

Leuciscus cephalus is found from southern Scotland, eastern Wales and England to the Urals (Cihar, 1976). In Greece (Fig. 1), the species is widely distributed and occurs in most areas except East Peloponnesus, Attiki, and Beotia. Within Greece, four subspecies have been described based on morphometric and meristic characters (Economidis, 1974, 1991): (a) *Leuciscus c. albus* Bonaparte, 1838 found in West Greece (in Aetolia rivers Mornos and Evinos and in Louros river and Ioannina lake of Epirus), and probably, also in the Acheloos, Arachthos and Kalamas rivers (West Greece) (Economidis, 1974); (b) *L. c. macedonicus* Karaman, 1955 found in East

Fig. 1. Sampling sites: AGG, Aggitis; ALF, Alfios; AL1, Aliakmonas 1; AL2, Aliakmonas 2 (Kokkinia-Grevena); AL3, Aliakmonas 3 (Canal 66-Naoussa); ARD, Ardas; AX1, Axios 1; AX2, Axios 2 (Goumenissa); ELA, Elassoniticos; EVI, Evinos; FIL, Filliouris; KOM, Komcatos.

Macedonia, (in Koronia and Volvi lakes and in Strymonas and Nestos rivers) and also in northeastern Greece (in Filliouris and Evros rivers and in Vistonis lake of Thrace) (Economidis, 1974; Economidis and Sinis, 1982); (c) *L. c. peloponnensis* Valenciennes, 1844 found in Peloponnesus (South Greece) in Alfios and Pinios rivers and in Stymphalia lake (Economidis, 1991); and (d) *L. c. vardarensis* Karaman, 1928 found in Central and West Greece (in Aoos river of Epirus and in Pinios river of Thessaly) and also in West and Central Macedonia, in the Aliakmonas, Axios and Gallikos rivers (Economidis, 1974; Economidis and Sinis, 1982).

Molecular studies of fish populations commenced some 35 years ago (Ferguson et al., 1995). While starch gel electrophoresis, coupled with histochemical staining, provided the first simple genetic markers for large-scale studies of natural populations, more recent analysis of mtDNA has proven useful for producing phylogenetically informative characters among closely related taxa (Avise et al., 1987; Moritz et al., 1987). Thus, rapidly evolving regions of the mitochondrial genome are suitable for resolving intraspecific relationships (Zhu et al., 1994). Although phylogenetic studies of site variation in mtDNA have been conducted for many cyprinid species (Richardson and Gold, 1995; Duvernell and Aspinwall, 1995), no comparable studies of genetic structure of chub (*L. cephalus*) populations have been reported.

Previous isozyme analysis of chub populations (Imsiridou et al., 1997) provided partial, but not decisive concordance with the current taxonomy of *L. cephalus*. We examined the genetic structure of Greek chub populations, using RFLP analysis of the mtDNA control region and cytochrome b, in order to assess the validity of the classical systematic assignment of *L. cephalus* populations to four distinct subspecies.

2. Materials and methods

2.1. MtDNA analysis

A total of 204 individuals of *L. cephalus*, representing 12 different sampling localities were obtained (Fig. 1) by electrofishing. To ensure adequate representation from all purported subspecies, samples were taken from all over Greece. Sample sizes are indicated in Table 3.

Scales were removed from live specimens and kept in 100% ethanol. DNA was extracted according to Kocher et al. (1989). Mitochondrial DNA was analysed by restriction fragment length polymorphisms (RFLPs) performed on PCR amplified products. The primers used (Briolay et al., in preparation) for D-loop amplification were H355: 5′-CCTGAAATGAGGAACCAGATG-3′; L16473: 5′-CTAAAAGCATC-GGTCTTGTAATCC-3′ and for cytochrome b were H16526: 5′-CTTTGGGA GTTGGGGGTGGGA-3′; L15267: 5′-AATGACTTGAAGAACCACCGT-3′ (“L” and “H” refer to the light and heavy strands, respectively, and the number refers to the position of the 3′ base in *Cyprinus carpio* mtDNA sequence, GenBankX61010).

Double-stranded DNA was amplified in 50 μl reaction volumes containing 2 units of DNA polymerase from *Thermus aquaticus* (Taq polymerase), 5 μl of 10 × reaction buffer (50 mM KCl, 10 mM Tris-HCl), 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.2 μM of each primer and approximately 50–100 ng of DNA.

PCR amplification conditions were as follows: one preliminary denaturation at 94°C (5 min), followed by strand denaturation at 94°C (1 min), annealing at 50°C (45 s) and primer extension at 72°C (1 min) repeated for 35 cycles and a final extension at 72°C (5 min) for the segment of D-loop. For cytochrome b the amplification program was: one preliminary denaturation at 94°C (5 min), followed by strand denaturation at 94°C (1 min), annealing at 55°C (45 s) and primer extension at 72°C (1 min) for 30 cycles and a final extension at 72°C (5 min).

Eighteen restriction endonucleases were used to digest the two amplified segments: *Alu I*, *Ase I*, *Bam HI*, *Ban I*, *Bfa I* (except cytochrome b), *BstUI*, *Bgl II*, *Dde I*, *EcoR I*, *Hae III*, *Hinf I*, *Msp I*, *Not I*, *Nru I*, *Pst I*, *Rsa I*, *Sal I*, *Taqa I*. For each sample 1–4 μl of the PCR reaction containing amplified DNA was digested with the appropriate restriction enzyme. The digested fragments of D-loop segment were electrophoretically separated on 6% polyacrylamide gels (PAGE) while for cytochrome b segment, 8–12% polyacrylamide gels were used.

2.2. Data analysis

Distinct single endonuclease patterns were identified by a specific letter (Tables 1 and 2). Each fish was assigned a multi-letter code that described its composite mtDNA genotype (Table 3). The raw data were fragment profiles, but we inferred site differences among haplotypes from changes in fragment profiles as these could be accounted for by the gain or loss of particular restriction sites. Both distance and character-based analyses were used to define genetic groups and phylogenetic relationships.

A restriction-site presence/absence matrix for individual mtDNA composite genotypes (haplotypes) was constructed using the GENERATE program in the REAP package (McElroy et al., 1991). The restriction site pattern was further analysed using the D and DA programs contained in the same package and the FITCH, MIX, BOOT and NEIGHBOUR programs contained in the PHYLIP 3.4 computer package (Felsenstein, 1992). All autapomorphic and symplesiomorphic characters were removed prior to parsimony analysis using the REDUCE program in REAP. The degree of geographic heterogeneity of mtDNA haplotype distributions was assessed using a χ^2 statistic as described by Roff and Bentzen (1989). The significance level was obtained by 10,000 Monte Carlo randomizations using the MONTE program from the REAP package.

N_{ST} (Lynch and Crease, 1990) was used to estimate the degree of population subdivision at the nucleotide level. The resulting index gives the ratio of the average genetic distance between genes from different populations relative to that among genes in the population. Values of N_{ST} range from 0 (no population subdivision) to 1 (complete population subdivision).

Table 1
Fragment size estimates (in base pairs) of all fragment patterns observed on mtDNA D-loop segment among 12 chub populations

Ase I		Bfa I			Dde I				EcoR I		Hinf I		Msp I		Rsa I			Taqa I	
	A		A	B		A	B	C		A		A		A		A	B		A
395	–	475	–		375	–			410	–	455	–	420	–	265	–	–	490	–
80	–	280		–	310		–	–	115	–	70	–	105	–	150	–	–	35	–
55	–	195		–	150	–	–	–							55	–	–		
		50	–	–	65		–								55	–			
					35			–							35		–		
					30			–							20*		–		

Fragment marked with asterisk was not observed but assumed under the criterion of minimum mutational steps involved in fragment changes.

Table 2
Fragment size estimates (in base pairs) of all fragment patterns observed on mtDNA cytochrome b segment among 12 chub populations

Alu I					Ban I			BstU I				Dde I					Hae III		
	A	B	C	D		A	B		A	B	C		A	B	C	D		A	B
1350	–				1350	–		1350	–			720	–	–			1140	–	
1240		–			850		–	770		–	–	480			–	–	720		–
960			–		500		–	580		–		380	–		–	–	420		–
820				–				350			–	305		–			210	–	–
280			–	–				230			–	240			–	–			
140				–								140	–	–	–	–			
110		–	–	–								110	–	–	–				
												90				–			
												75		–		–			
												20							

Hinf I				Msp I					Rsa I						Sal I		Taqa I				
	A	B	C		A	B	C	D		A	B	C	D	E		A		A	B	C	D
770	–	–		700	–	–	–		880			–			1240	–	1180	–			
530	–		–	650	–			–	615	–	–				110	–	990		–	–	–
500			–	490		–	–		465	–			–	–			290				–
280		–		440				–	410					–			190		–		
270			–	260				–	390		–	–	–				130			–	
250		–		160		–			265	–	–		–	–			100	–	–	–	
50	–	–	–	130			–		225				–				70	–	–	–	–
				30			–		205					–			60			–	
									75		–										
									50			–									
									25			–									

Table 3
Composite genotypes (haplotypes), haplotype frequencies, haplotype diversity including standard error, nucleotide diversity (%), and sample sizes (*N*) of the studied populations

Haplotype	Composite genotype	Sample locality											
		AGG	ALF	AL 1	AL 2	AL 3	ARD	AX 1	AX 2	ELA	EVI	FIL	KOM
Type 1	BBACABABBADA	0.077											0.500
Type 2	ABADABABBADA	0.923											
Type 3	BCAABBDBCCBC		0.188										
Type 4	BCAABBCBCCBC		0.812										
Type 5	BABBBABAADED			0.042									
Type 6	BABBBABAADEB			0.958	0.555	0.589		1.000	1.000				
Type 7	BCABBABAADEB				0.445	0.352							
Type 8	BCBBBABAADEB					0.059							
Type 9	BCAAABABCBDA						0.071						
Type 10	BCADABABBADA						0.214						
Type 11	BBADABABBBDA						0.071						
Type 12	BBAAABABCBDA						0.144						
Type 13	BBADABABBADA						0.500						
Type 14	BABBBABAAAEB									0.647			
Type 15	BBABBABAAAEB									0.294			
Type 16	BBBBBABAAAEB									0.059			
Type 17	BBAABBCBCBCC										1.000		
Type 18	BCACABABAAAA											1.000	
Type 19	BBACACABBADA												0.250
Type 20	BBACABABAAAA												0.188
Type 21	BBACABABAADA												0.062
Haplotype diversity		0.154	0.325	0.083	0.523	0.559	0.725	0.000	0.000	0.522	0.000	0.000	0.692
Standard error		±0.126	±0.125	±0.075	±0.048	±0.083	±0.104	±0.000	±0.000	±0.101	±0.000	±0.000	±0.086
Nucleotide diversity (%)		0.114	0.102	0.031	0.574	0.552	1.020	0.000	0.000	0.350	0.000	0.000	0.422
N		13	16	24	18	17	14	20	25	17	15	9	16

Composite genotypes are denoted by capital letters in the following order: D-loop: *Bfa I*, *Dde I*, *Rsa I*. Cytochrome b: *Alu I*, *Ban I*, *BstU I*, *Dde I*, *Hae III*, *Hinf I*, *Msp I*, *Rsa I*, *Taqa I*. For the purposes of illustration common haplotypes were omitted.

3. Results

3.1. Mitochondrial DNA diversity

The amplified segments of D-loop and cytochrome b had an approximate size of 0.525 and 1.350 Kb, respectively. Eight of the 18 restriction enzymes used to screen the chub populations had at least one recognition site on the D-loop segment (Table 1) and ten of the 17 restriction enzymes had at least one recognition site on the cytochrome b segment (Table 2). These enzymes, detected in both amplified segments a total of 41 restriction sites. Fragment patterns generated by each enzyme are presented in Tables 1 and 2. Three (*Bfa I*, *Dde I*, *Rsa I*) out of eight and nine (*Alu I*, *Ban I*, *BstU I*, *Dde I*, *Hae III*, *Hinf I*, *Msp I*, *Rsa I*, *Taqa I*) out of ten restriction enzymes were polymorphic for D-loop and cytochrome b, respectively. Digestion patterns from the restriction enzymes revealed 21 composite mtDNA genotypes (haplotypes) (Table 3). Haplotype frequencies of the sampled populations, haplotype diversity and nucleotide diversity (%) are given in the same table. Estimated nucleotide sequence divergence among the 21 haplotypes ranged from 0.313 to 6.79%. Sequence divergence (based on haplotype frequencies) among pairs of sample localities varied from 0.00 to 6.063% (mean value 3.421 $\pm$ 0.00%) (Table 4). The number of observed haplotypes within populations ranged from 1 to 5 (Table 3). Most of the detected genotypes, 18 out of 21, were private, i.e. observed in one population.

The FITCH phenogram, resulting from the distance matrix among all mtDNA haplotypes using the unconstrained branch-length clustering method of Fitch and Margoliash (1967) is presented in Fig. 2. The overall Wagner parsimony analysis of site changes generated several equally parsimonious trees, each requiring a minimum of 37 steps. Both the FITCH phenogram (Fig. 2) and the majority-rule consensus tree (Fig. 3) revealed four distinct clusters. Haplotype 17, found in Evinos river, was used as an outgroup because it was the most divergent haplotype as revealed by its highest

Table 4
Nucleotide divergence ($\times 10^2$) for mtDNA PCR-RFLP analysis among the 12 chub populations

Pop.	AGG	ALF	AL1	AL2	AL3	ARD	AX1	AX2	ELA	EVI	FIL	KOM
AGG	0.000											
ALF	5.715	0.000										
AL1	5.725	5.879	0.000									
AL2	5.179	4.964	0.201	0.000								
AL3	5.230	5.040	0.154	0.028	0.000							
ARD	0.428	4.133	4.951	4.306	4.364	0.000						
AX1	5.715	5.870	0.000	0.201	0.153	4.943	0.000					
AX2	5.715	5.870	0.000	0.201	0.153	4.943	0.000	0.000				
ELA	4.853	5.041	0.441	0.436	0.416	4.097	0.440	0.440	0.000			
EVI	5.890	1.348	6.063	5.517	5.567	4.509	6.053	6.053	5.200	0.000		
FIL	1.822	3.785	4.538	3.635	3.712	1.098	4.530	4.530	3.683	4.750	0.000	
KOM	0.685	4.688	4.631	4.105	4.154	0.330	4.624	4.624	3.779	4.831	0.857	0.000

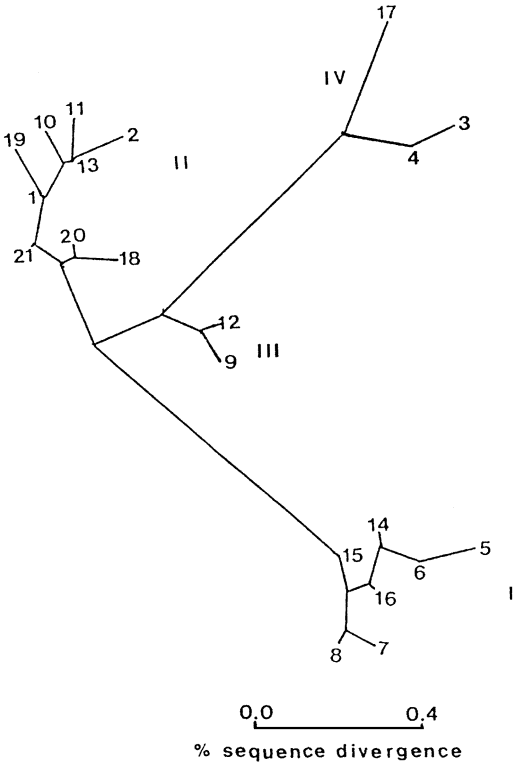

Fig. 2. FITCH phenogram clustering the distance matrix, based on restriction sites, of percentage sequence divergence among 21 mtDNA haplotypes.

value of nucleotide divergence among all the others (mean value = 0.048). The first group (group I) included haplotypes 5, 6, 7, 8, 14, 15 and 16 found in Aliakmonas 1, Aliakmonas 2, Aliakmonas 3, Axios 1, Axios 2 and Elassoniticos rivers. The II group consisted of haplotypes 1, 2, 10, 11, 13, 18, 19, 20 and 21 which were found in Aggitis, Ardas, Filliouris and Komcatos populations. Haplotypes 9 and 12 formed another group (group III) and they were found only in Ardas population. The last group (group IV) included haplotypes 3, 4 and 17, found in Alfios and Evinos populations.

3.2. Population gene diversity

The distance matrix of net interpopulation nucleotide divergence was used to construct a UPGMA tree (unweighted pair-group method using arithmetic averages) relating the 12 populations studied (Fig. 4). The populations clustered into three distinct groups: (A) Aggitis, Ardas, Komcatos, Filliouris; (B) Aliakmonas 1, Axios 1, Axios 2, Aliakmonas 2, Aliakmonas 3, Elassoniticos; (C) Alfios, Evinos.

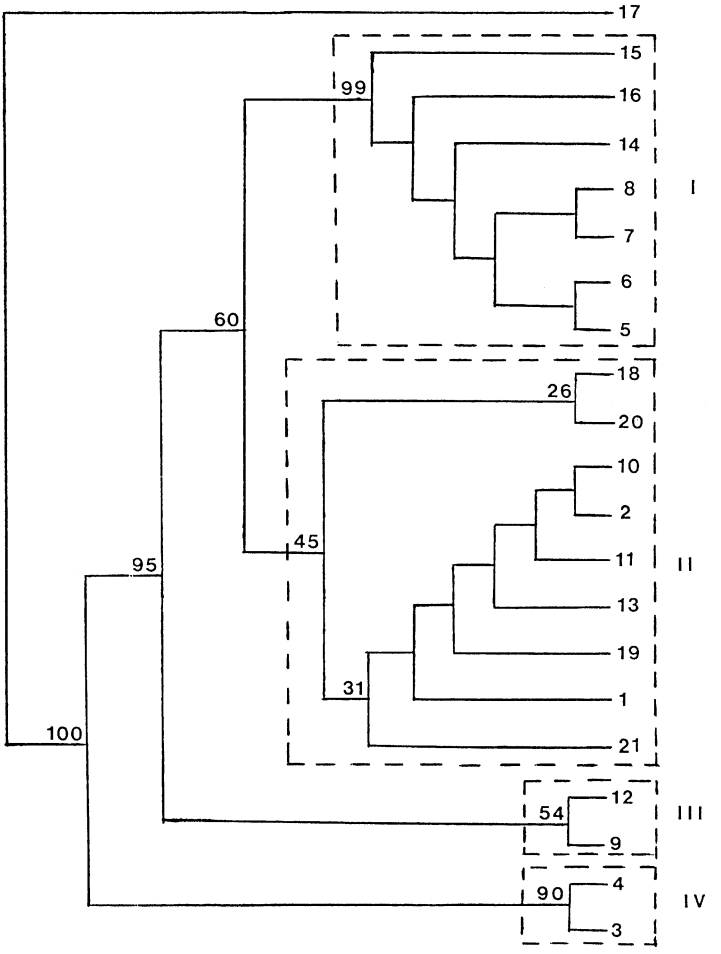

Fig. 3. Majority rule consensus tree clustering the 21 mtDNA haplotypes. Their respective locations are as follows: Type 1, 2: AGG, KOM; Type 3, 4: ALF; Type 5: AL 1; Type 6: AL1, AL2, AL3, AX1,AX2; Type 7: AL2, AL3; Type 8: AL3; Type 9, 10, 11, 12, 13: ARD; Type 14, 15, 16: ELA; Type 17: EVI; Type 18: FIL; Type 19, 20, 21; KOM. Numbers at the forks indicate the number of times the group consisting of the haplotypes located to the right of that fork occurred among 100 bootstrap replicates. The tree was rooted with haplotype 17.

Statistically significant differences in haplotype frequencies among all populations were observed ($\chi^2 = 1483.81$, $P < 0.0001$). All the tests for differences among the four supposed subspecies resulted in significant outcomes too ($P < 0.0001$). Within the purported subspecies *L. c. macedonicus* (cluster A) and *L. c. vardarensis* (cluster B) significant differences among populations were also observed ($\chi^2 = 149.56$, $P < 0.0001$ and $\chi^2 = 169.1$, $P < 0.0001$, respectively). Yet, significant differences were observed between the groups of Aliakmonas and Axios rivers ($\chi^2 = 14.42$, $P < 0.0001$), as well as within the Aliakmonas rivers group ($\chi^2 = 20.92$, $P < 0.0001$).

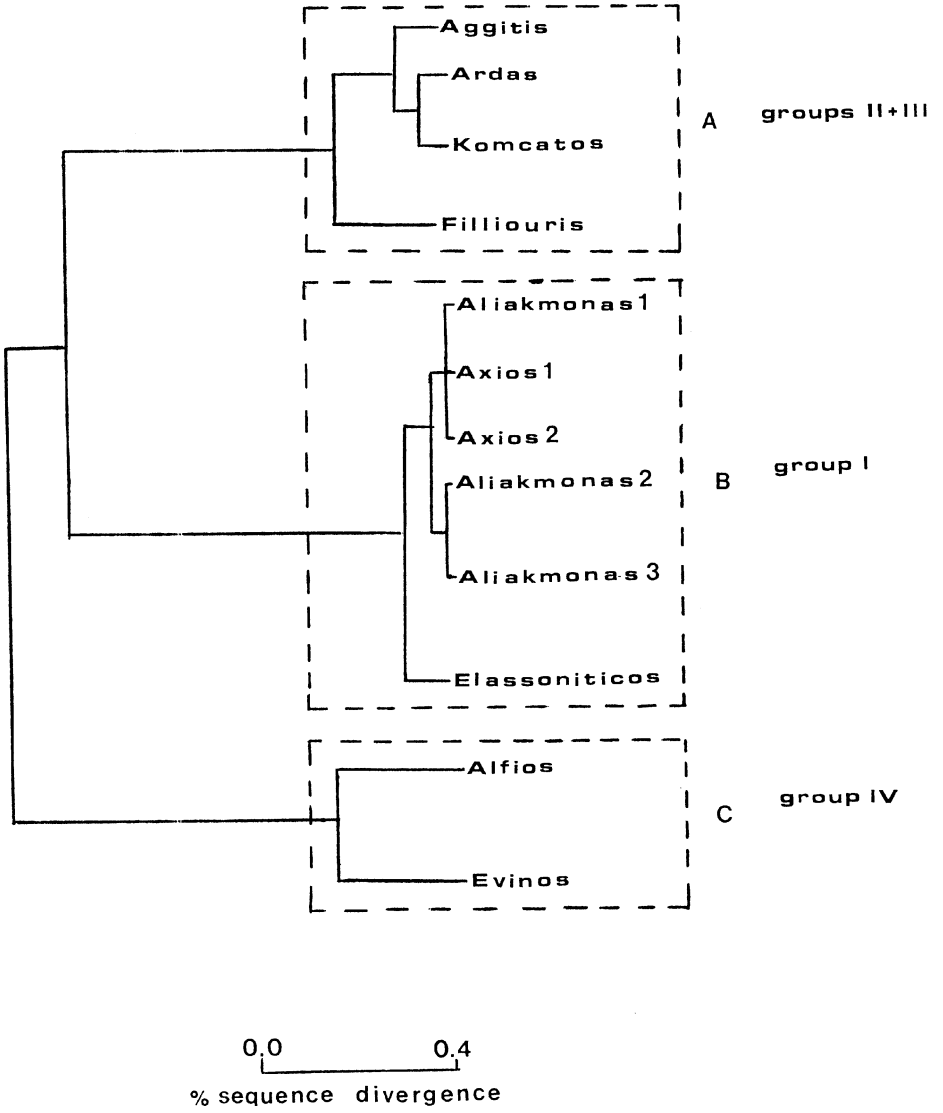

Fig. 4. UPGMA phenogram clustering 12 *L. cephalus* populations according to the distance matrix resulting from the maximum likelihood estimation of the net average number of nucleotide substitutions per site between populations (nucleotide divergence).

Only within the Axios rivers group, were the differences between the two populations found to be non significant ($\chi^2 = 0.000$). Pairwise tests among populations demonstrated significant differences between 61 of 63 tests (data not shown).

The mean value of the interpopulation diversity was $3.684 \pm 0.0007\%$ and the mean intrapopulation diversity was $0.2638 \pm 0.0001\%$. The N_{ST} value of 0.94, shows

that only 6% of the overall genetic diversity observed was within populations as opposed to 94% among populations.

4. Discussion

4.1. Relationships among different haplotypes: Systematic relationships among chub populations

The levels of divergence observed between the 21 mtDNA genotypes studied here range from 0.313 to 6.79% and are consistent with the range 0.14–9.24% observed in a RFLP-mtDNA analysis of *Cyprinella lutrensis* (Cyprinidae) populations (Richardson and Gold, 1995). Furthermore, the levels of divergence found in this investigation are higher than the 0.21–3.42% observed in mtDNA analysis of *Salmo trutta* populations, one of the most structured fish species (Apostolidis et al., 1996).

Eighteen out of 21 detected mtDNA haplotypes in this study, were population-specific. Thus, most of the populations examined had a unique profile without sharing haplotypes with the other populations. This high differentiation among the populations may result from a long time of isolation coupled with probable bottleneck and subsequent genetic drift phenomena. Mitochondrial DNA, with its one quarter effective population size, is more susceptible to these processes (Ferguson et al., 1995).

On the other hand, haplotype 6 shared among Aliakmonas 1, Aliakmonas 2, Aliakmonas 3, Axios 1 and Axios 2 populations, appears not to be strictly localized. The most likely explanation for this pattern involves gene flow among these chub populations. Probably, this is the case, as the sampling sites for these populations are only different positions along the same river basin (for both Axios and Aliakmonas rivers). According to Avise et al. (1987) this haplotype could also be an ancestral one, which was retained in these populations.

As indicated earlier, four subspecies of *L. cephalus* have been described in Greece: *L. c. albus*, *L. c. macedonicus*, *L. c. peloponnensis* and *L. c. vardarensis* based on morphological characters. We assume that since each sample comes from a different area it represents a particular subspecies. All of these sample sites are recorded as having these subspecies. Both the FITCH phenogram (Fig. 2) and the majority-rule consensus tree (Fig. 3) revealed four clusters, and this clustering is almost concordant with the systematic classification of the populations which correspond to these haplotypes, as distinct subspecies. Indeed, group I includes haplotypes found in Aliakmonas 1, Aliakmonas 2, Aliakmonas 3, Axios 1, Axios 2 and Elassoniticos populations (supposed *L. c. vardarensis* subspecies). The II group contains haplotypes found in Aggitis, Ardas, Filliouris and Komcatos populations (supposed *L. c. macedonicus* subspecies). Haplotypes 3, 4 and 17 are clustered separately (group IV) and were found in Alfios population (haplotypes 3, 4-supposed *L. c. peloponnensis* subspecies) and in Evinos population (haplotype 17-purported *L. c. albus* subspecies). However, the bootstrap values for groups II and III are too weak to support strongly the subspecific groupings in the tree (in contrast with the bootstrap values for groups I and IV). Haplotype 17 was used as an outgroup in the Wagner parsimony analysis.

Haplotype 9 was observed in one individual of Ardas population whereas haplotype 12 was found in two individuals of the same population. These two haplotypes form a distinct group (group III) in both the FITCH phenogram and the majority-rule consensus tree (where is supported in 54% of the bootstrap estimates). The separate clustering of these haplotypes can be interpreted as an ancestral polymorphism retained in the Ardas population. Another possible explanation could be the stochastic lineage extinction, which is in many cases responsible for the elimination of mtDNA genotypes (Avise et al., 1987; Moritz et al., 1987).

The clustering of chub populations according to the UPGMA method (Fig. 4) is almost concordant with the previous systematic classification of these populations into different subspecies. The grouping of Alfios and Evinos populations in the same cluster (group C) – as well as the same clustering of haplotypes 3, 4 and 17 in the FITCH phenogram – can be interpreted to be the consequence of biogeographic and geological events (Imsiridou et al., 1997). Further mtDNA sequence studies of Greek chub populations suggest the same clustering (Durand et al., in prep.).

4.2. Congruence between allozyme and mtDNA variation

Given the rapid rate of change of vertebrate mtDNA, relative to that revealed by allozyme studies, mtDNA comparisons should provide better phylogenetic resolution among closely related taxa (Kessler and Avise, 1985). The mtDNA results of this study suggest that this is indeed the case. The clustering of chub populations according to the sequence divergence among them indicates three distinct groups (Fig. 4) which support the previous systematic taxonomy, in contrast to the allozyme grouping (Imsiridou et al., 1997) where the systematic relationships are not very clear (Axios 2 and Aliakmonas 1 populations were clustered separately whereas Aggitis population was not clustered in any group).

Furthermore, the results obtained from the analysis of mtDNA and allozyme variation, indicate discrepancies between the two methods, in the extent of the intrapopulation levels of genetic variation. Thus no mtDNA (haplotype) diversity was found in Axios 1, Axios 2, Evinos and Filliouris populations (Table 3) which showed high values of nuclear heterozygosity (the highest value of $He = 0.072$ observed in Evinos population; Imsiridou et al., 1997). Reciprocally, the highest value of haplotype diversity ($h = 0.725$, Table 3) was detected in Ardas population, where nuclear heterozygosity found to be very low ($He = 0.030$; Imsiridou et al., 1997).

Neigel and Avise (1985) have demonstrated the potential importance of ancestral polymorphism and stochastic lineage extinction in explaining inconsistencies between phylogenies generated from mtDNA and nuclear DNA-based data. Furthermore, lack of congruence between the two phylogenies can result from the different modes of transmission and evolution of these genetic systems. Similar discrepancies between the two methodologies have frequently been reported in the literature (Dowling and Brown, 1989; Bernatchez et al., 1992; Apostolidis et al., 1996). The low levels of intrapopulation mtDNA variation observed may have resulted from selection favoring single haplotypes, the founder effect in new colonizing populations, bottleneck events or a combination of these processes (Duvernell and Aspinwall, 1995). In

conclusion, our results, while reinforcing the utility of mtDNA data for the determination of the different *L. cephalus* subspecies, emphasize also the importance of considering a number of genetic systems and possibly techniques when addressing such questions. Thus, further mtDNA as well as nuclear DNA analyses will be necessary for determining the phylogenetic relationships among the *L. cephalus* groups.

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