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Genetic Polymorphism and Differentiation Among Chub *Leuciscus cephalus* L. (Pisces, Cyprinidae) Populations of Greece*

ANASTASIA IMSIRIDOU, YIANNIS KARAKOUSIS and COSTAS TRIANTAPHYLLIDIS†

Aristotle University, School of Biology, Department of Genetics, Development and Molecular Biology, GR-54006 Thessaloniki, Macedonia, Greece

Key Word Index—*Leuciscus cephalus*; Cyprinidae; allozymes; genetic polymorphism; genetic differentiation; Greece.

Abstract—To clarify the genetic structure of chub (*Leuciscus cephalus* L.) populations, eleven enzymatic systems corresponding to 20 putative loci were investigated in 15 populations from different rivers throughout Greece and one sample from France. The proportion of polymorphic loci detected ranged from 5 to 45% and the degree of expected heterozygosity from 0.019 to 0.072. Phenograms constructed from the allozyme data were generally concordant with the recognition of four subspecies of *L. cephalus*. Values of genetic distance among the populations, however, do not justify this classification. The possible influence of paleogeographic events on the phylogenetic relationships of the putative subspecies is discussed. © 1997 Elsevier Science Ltd

Introduction

Morphometric and meristic characters of chub (*Leuciscus cephalus* L.) have been well characterized. In addition to information on first year growth and morphological development (Economou *et al.*, 1991), data on hybridization with other species such as *Chalcarburnus chalcoides macedonicus* (Economidis and Sinis, 1988) or *Alburnus albidus* (Bianco, 1982) have been reported. Yet there have been only three studies concerning the genetic structure and differentiation of chub populations (Coelho *et al.*, 1995; Guinand *et al.*, 1996; Tsiggenopoulos and Karakousis, 1996).

Leuciscus cephalus is found from southern Scotland, eastern Wales and England to the Urals, but not in Ireland, Denmark, northern Scandinavia or the Mediterranean islands (Cihar, 1976). In Greece, 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):

(1) *Leuciscus c. albus* Bonaparte, 1838 which is 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);
(2) *L. c. macedonicus* Karaman, 1955 which is found in East Macedonia (in Koronia and Volvi lakes and in Strymonas and Nestos rivers), and also in north-eastern Greece

*This paper is dedicated to the memory of the late Dr Y. Karakousis by both his student (A.I.) and his professor (C.T.).

†Corresponding author (Tel: 031-998309; Fax: 031-998374; E-mail: TRIANT@BIO.AUTH.GR).

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(in Filliouris and Evros rivers and in Vistonis lake of Thrace) (Economidis, 1974; Economidis and Sinis, 1982);

(3) *L. c. peloponnensis* Valenciennes, 1844 which occurs in Peloponnesus (South Greece) in Alfios and Pinios rivers and in Stymphalia lake (Economidis, 1991);

(4) *L. c. vardarensis* Karaman, 1928 which is 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).

We examined the genetic structure of Greek chub populations to assess the validity of the current classification of *L. cephalus* as four distinct subspecies.

Materials and Methods

Samples of *L. cephalus* were collected from 15 different streams (Fig. 1) by electrofishing. To ensure adequate representation of all purported subspecies, samples were taken from all over Greece. One sample from the river Rhône, France, was also included in the analysis. Sample sizes are indicated in Table 1.

Fish were transported on ice to the laboratory. Samples of white muscle and liver were removed immediately, homogenized in an equal volume of 0.1 M Tris-HCl, 0.0001 M EDTA, 4.8×10^{-5} M NADP buffer, centrifuged at 12,000 rpm for 30 min at 5°C and the supernatant stored at −20°C. Eleven enzymatic systems corresponding to 20 putative loci were investigated using starch gel electrophoresis. Aspartate aminotransferase (AAT, E.C 2.6.1.1), esterases (EST, E.C 3.1.1.2), phosphogluconate dehydrogenase (PGDH, E.C 1.1.1.44) and superoxide dismutase (SOD, E.C 1.15.1.1) have been tested in liver; creatine kinase (CK, E.C 2.7.3.2), glucose-6-phosphate isomerase (GPI, E.C 5.3.1.9), malate dehydrogenase (MDH, E.C 1.1.1.37) and malic enzyme (NADP+) (ME, E.C 1.1.1.40) in muscle, while isocitrate dehydrogenase (NADP+) (IDHP, E.C 1.1.1.42), L-lactate dehydrogenase (LDH, E.C 1.1.1.27) and phosphoglucomutase (PGM, E.C 5.4.4.2) were tested in both tissues. For AAT, IDHP, MDH and PGDH the electrophoretic conditions are: gel buffer: 0.135 M Tris, 0.043 M citric acid, 0.02 M EDTA pH = 7.1, electrode buffer: 0.135 M Tris, 0.04 M citric acid, 0.0013 M EDTA pH = 7.1. For GPI, ME, PGM and SOD the electrophoretic conditions were described by Allendorf *et al.* (1977), for EST by Ashton and Braden (1961) and for CK and LDH by Taggart *et al.* (1981).

Global indices such as allele frequencies, tests for Hardy–Weinberg equilibrium, Nei's genetic distance (Nei, 1978) and hierarchical *F*-statistics were performed using the BIOSYS-1 computer programme (Swofford and Selander, 1989). Based on the genetic distance matrix a dendrogram was constructed, using the NEIGHBOUR programme of the PHYLIP package (Felsenstein, 1989). A consensus tree and bootstrapping estimates on branches were estimated by running 100 bootstrap replicates with the SEQBOOT and CONSENSE programme of PHYLIP. Based on the allele frequency matrix a further dendrogram was constructed according to the maximum likelihood algorithm of the CONTML programme in the software package PHYLIP (Felsenstein, 1989).

Results

Four (*mAAT-1**, *IDHP-3**, *LDH-3** and *SOD-1**) of the 20 loci surveyed (Table 1) were homozygous for the same allele in all of the populations studied. A total of 42 alleles were detected. Guinand *et al.* (1996) examined 28 putative loci in French chub, but found only four to be polymorphic. Twenty of the loci they analysed were included in this work and of these, 23 alleles were found to be the same: the *"100"* allele in all cases and the *IDHP-1* 145*, *EST-1* 80* and *PGM-2* 90* alleles. Most of the genotype frequencies determined for the Greek chub populations examined were in good agreement with Hardy–Weinberg expectation. Only nine out of 87 chi-square tests were found to deviate significantly, all due to a deficiency of heterozygotes, from expectation ($P<0.05$).

The mean number of alleles per locus ranges from 1.0 to 1.5. The percentage of polymorphic loci (*P*) ranges from 5 to 45% (a locus is considered polymorphic if more than one allele is detected). The values of observed heterozygosity (*Ho*) range from 0.015 to 0.068 and those of expected heterozygosity (*He*) from 0.019 to 0.072

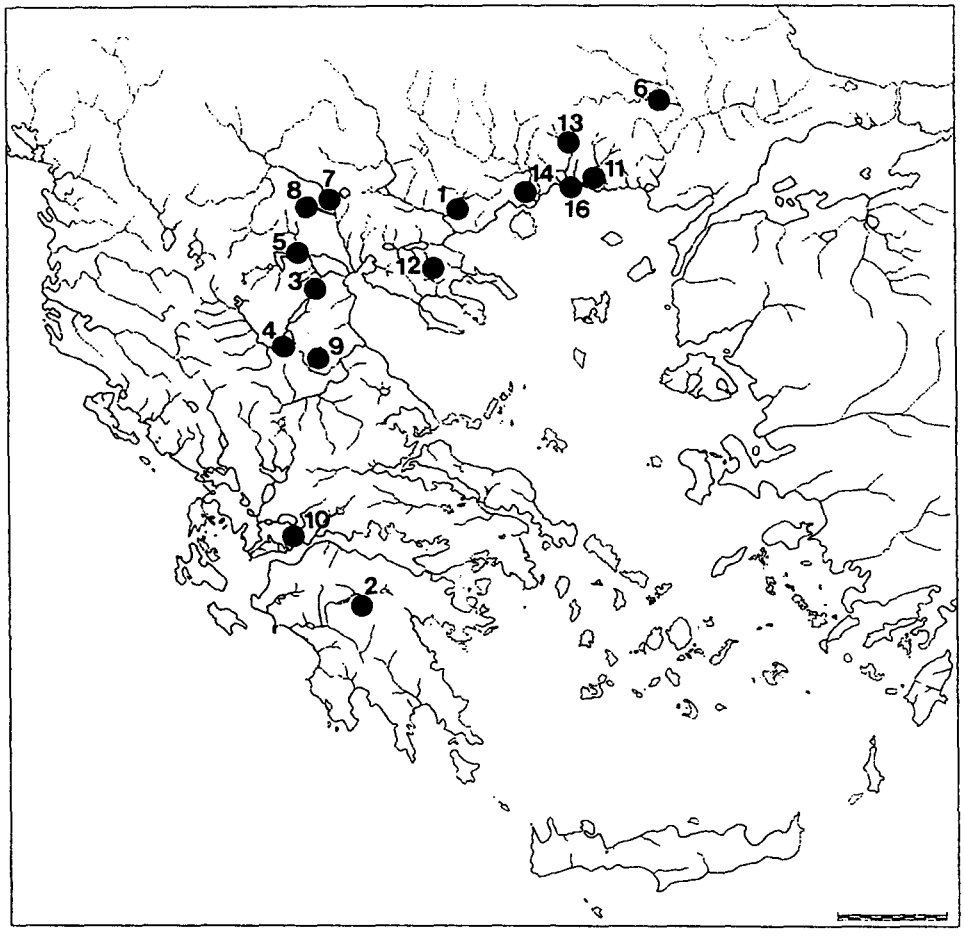

FIG. 1. SAMPLING SITES: 1, Aggitis; 2, Alfios; 3, Aliakmonas 1; 4, Aliakmonas 2 (Kokkinia-Grevena); 5, Aliakmonas 3 (Canal 66-Naoussa); 6, Ardas; 7, Axios 1; 8, Axios 2 (Goumenissa); 9, Elassoniticos; 10, Evinos; 11, Filliouris; 12, Havrias; 13, Komcatos; 14, Nestos; 15, Rhône; 16, Vosvozis. Sample 15 came from the Rhône river, France and it is not indicated in the map.

(Table 1). Nei's genetic distances (Nei, 1978) range from 0.007 to 0.063 between the French and Greek chub populations and from 0.000 to 0.041 between Greek populations (Table 2).

The genetic diversity of the populations examined was further investigated using Wright's hierarchical *F*-statistics (Wright, 1978) (Table 3). We used this method to obtain a clearer picture of genetic variability distribution and especially to test whether the subspecific groupings are differentiated. F_{st} values provide an estimation of the levels of differentiation between populations and values ranging from 0.15 to 0.25 are generally considered to reflect high levels of population differentiation (Wright, 1965). Three out of the seven F_{st} values in Table 3 are higher than 0.15.

A CONTML dendrogram constructed using the allele frequency matrix indicates six major clusters (Fig. 2). The first cluster consists of populations from Havrias, Elassoniticos, Aliakmonas 2, Axios 1 and Aliakmonas 3; the second is the Alfios population;

TABLE 1. POLYMORPHIC LOCI,† ALLELES, ALLELE FREQUENCIES, SAMPLE SIZE, MEAN NUMBER OF ALLELES PER LOCUS (*M.n.a*), PERCENTAGE OF POLYMORPHIC LOCI (*P*), OBSERVED HETEROZYGOSITY (*Ho*) AND EXPECTED HETEROZYGOSITY (*He*) OF THE 16 POPULATIONS INVESTIGATED

Locus	Allele	1 Aggitis	2 Alfios	3 Aliak. 1	4 Aliak. 2	5 Aliak. 3	6 Ardas	7 Axios 1	8 Axios 2	9 Elasson.	10 Evinos	11 Filliour.	12 Havrias	13 Komcat.	14 Nestos	15 Rhône	16 Vosvozis
*sAAT-2**	100	1.000	1.000	1.000	1.000	1.000	0.990	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	130	0.000	0.000	0.000	0.000	0.000	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
*CK-1**	100	1.000	1.000	1.000	1.000	1.000	0.990	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	120	0.000	0.000	0.000	0.000	0.000	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
*EST-1**	100	1.000	0.988	0.802	0.959	0.910	0.970	0.931	0.830	0.913	0.512‡	1.000	1.000	0.944	1.000	0.800	0.981‡
	80	0.000	0.012	0.198	0.041	0.090	0.030	0.069	0.170	0.087	0.450	0.000	0.000	0.056	0.000	0.200	0.019
	125	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.000	0.000	0.000	0.000	0.000
*EST-2**	100	1.000	0.951‡	0.802	0.959	1.000	0.890	1.000	0.904	1.000	0.988	0.909	1.000	0.733	0.500‡	0.750	0.721‡
	90	0.000	0.049	0.198	0.041	0.000	0.110	0.000	0.096	0.000	0.012	0.091	0.000	0.267	0.500	0.250	0.279
*GPI-1**	100	0.933	1.000	0.988	1.000	1.000	0.880	1.000	0.968	1.000	0.890	0.636	1.000	0.589	0.787	1.000	0.692
	70	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.110	0.000	0.000	0.000	0.000	0.000	0.000
	280	0.067	0.000	0.012	0.000	0.000	0.120	0.000	0.032	0.000	0.000	0.364	0.000	0.411	0.213	0.000	0.308
*GPI-2**	100	1.000	0.825‡	0.953	0.986	0.900	1.000	0.903	0.989	1.000	1.000	1.000	1.000	1.000	0.984‡	1.000	1.000
	80	0.000	0.175	0.047	0.014	0.100	0.000	0.097	0.011	0.000	0.000	0.000	0.000	0.000	0.016	0.000	0.000
*IDHP-1**	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.793	1.000	1.000	1.000	1.000	0.950	1.000
	145	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.050	0.000
	175	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.207	0.000	0.000	0.000	0.000	0.000	0.000
*IDHP-2**	100	1.000	0.943	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	90	0.000	0.057	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
*LDH-1**	100	1.000	1.000	1.000	1.000	1.000	1.000	0.986	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	∅	0.000	0.000	0.000	0.000	0.000	0.000	0.014	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

*LDH-2**	100	1.000	0.963	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	Ø	0.000	0.037	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
*mMDH-1**	100	0.983	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	190	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
*sMDH-2**	100	1.000	1.000	0.881	0.851	0.820	1.000	0.917	0.862	0.946	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	70	0.000	0.000	0.119	0.149	0.180	0.000	0.083	0.138	0.054	0.000	0.000	0.000	0.000	0.000	0.000	0.000
*ME-1**	100	1.000	1.000	0.988	1.000	0.970	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.889	0.992	1.000	0.980
	80	0.000	0.000	0.012	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	130	0.000	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011	0.008	0.000	0.020
*PGM-1**	100	1.000	1.000	0.976	1.000	1.000	1.000	0.972	1.000	0.989	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	85	0.000	0.000	0.024	0.000	0.000	0.000	0.028	0.000	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000
*PGM-2**	100	0.800	0.714‡	0.976	0.473	0.550	0.969	0.394	0.936	0.348	0.775	0.977	0.250	0.967	0.943	0.000	0.860‡
	90	0.200	0.286	0.024	0.527	0.430	0.031	0.606	0.064	0.652	0.225	0.023	0.750	0.033	0.057	1.000	0.140
	105	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
*PGD-1**	100	1.000	0.679	0.988	1.000	0.930	0.990	0.900	0.989	1.000	1.000	0.810	1.000	0.988	0.964	1.000	0.863
	70	0.000	0.000	0.012	0.000	0.000	0.000	0.071	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	130	0.000	0.321	0.000	0.000	0.070	0.010	0.029	0.000	0.000	0.000	0.190	0.000	0.012	0.036	0.000	0.137
Sample size		31	41	43	37	50	50	36	47	46	41	44	41	45	62	10	52
M.n.a		1.1	1.4	1.5	1.3	1.4	1.4	1.4	1.4	1.2	1.3	1.2	1.0	1.3	1.3	1.1	1.3
P		15	35	45	25	30	35	35	35	20	25	20	5	30	30	15	30
Ho		0.018	0.039	0.057	0.045	0.068	0.024	0.060	0.048	0.035	0.047	0.046	0.015	0.067	0.041	0.050	0.061
He		0.024	0.072	0.055	0.047	0.068	0.030	0.061	0.046	0.037	0.072	0.050	0.019	0.064	0.053	0.042	0.070

†*mAAT-1**, *IDHP-3**, *LDH-3** and *SOD-1** were monomorphic in all populations examined.

‡Deviations from a Hardy–Weinberg equilibrium.

TABLE 2. NEI'S GENETIC DISTANCE AMONG THE POPULATIONS EXAMINED

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 Aggitis	0.000															
2 Alfios	0.007	0.000														
3 Aliakmonas 1	0.006	0.013	0.000													
4 Aliakmonas 2	0.007	0.011	0.016	0.000												
5 Aliakmonas 3	0.005	0.007	0.012	0.001	0.000											
6 Ardas	0.002	0.010	0.003	0.015	0.012	0.000										
7 Axios 1	0.010	0.010	0.021	0.001	0.001	0.019	0.000									
8 Axios 2	0.003	0.011	0.000	0.012	0.009	0.002	0.017	0.000								
9 Elassoniticos	0.011	0.014	0.023	0.001	0.003	0.022	0.000	0.019	0.000							
10 Evinos	0.013	0.021	0.011	0.018	0.015	0.015	0.020	0.010	0.020	0.000						
11 Filliouris	0.008	0.013	0.011	0.023	0.020	0.004	0.027	0.010	0.030	0.023	0.000					
12 Havrias	0.015	0.018	0.032	0.003	0.007	0.028	0.002	0.027	0.001	0.028	0.037	0.000				
13 Komcatos	0.011	0.022	0.011	0.026	0.024	0.006	0.032	0.011	0.034	0.024	0.004	0.040	0.000			
14 Nestos	0.015	0.021	0.009	0.026	0.026	0.008	0.033	0.012	0.035	0.030	0.011	0.041	0.005	0.000		
15 Rhône	0.038	0.037	0.051	0.015	0.022	0.052	0.012	0.048	0.009	0.040	0.063	0.007	0.060	0.055	0.000	
16 Vosvozis	0.008	0.012	0.009	0.018	0.016	0.004	0.022	0.009	0.024	0.022	0.002	0.029	0.002	0.003	0.047	0.000

TABLE 3. *F*-STATISTICS COMBINED ACROSS LOCI

Subgroups compared	F_{st} combined across loci
All samples	0.243
Population—locality	0.115
Population—subspecies	0.179
Population—total	0.234
Locality—subspecies	0.072
Locality—total	0.134
Subspecies—total	0.067

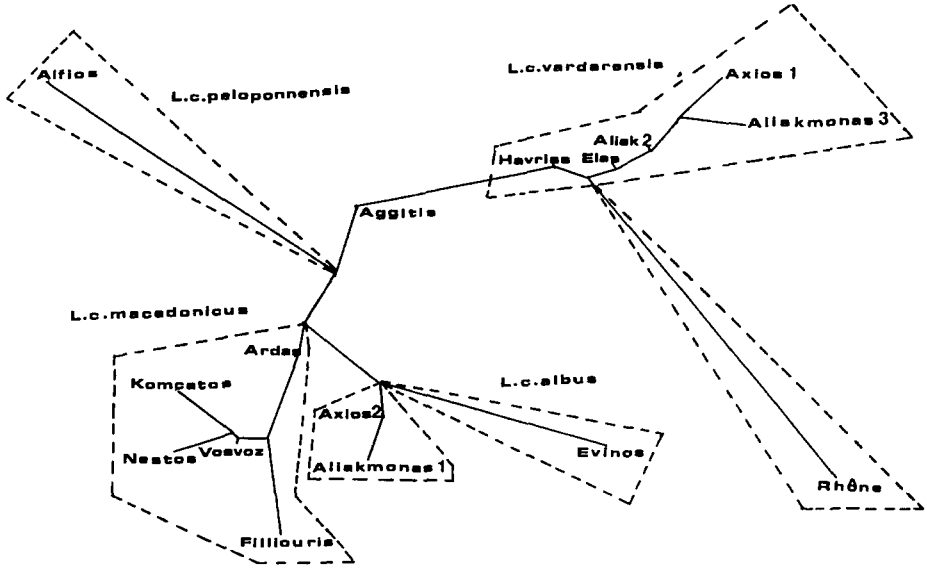

FIG. 2. CONTML DENDROGRAM BASED ON THE ALLELE FREQUENCIES OF THE 16 POPULATIONS EXAMINED, THE FOUR SUPPOSED SUBSPECIES ARE ALSO INDICATED.

the third cluster includes populations from Ardas, Kompsatos, Vosvozis, Nestos and Filliouris; the fourth contains populations from Axios 2 and Aliakmonas 1; the fifth is the population from the Evinos river and the sixth is a population from the Rhône river.

A Neighbour-joining tree was constructed, using the genetic distance values and yielded similar results with the exception of the Rhône population which clustered with the first group containing Havrias, Elassoniticos, Aliakmonas 2, Axios 1 and Aliakmonas 3. Bootstrap values obtained using the SEQBOOT programme are less than 66% and do not give strong support to the subspecific groupings in the tree.

Discussion

Expected heterozygosity (*He*) ranged from 0.019 to 0.072 with a mean value of 0.050, similar to the 0.052 reported for other Cyprinid fishes (Buth *et al.*, 1991). Tsiggenopoulos and Karakousis (1996) reported values of *He* from 0 to 0.062 among

populations of the genus *Leuciscus* from Greece, and Coelho *et al.* (1995) reported values that range from 0 to 0.057 among Portuguese *Leuciscus pyrenaicus* and *L. carolitertii* populations. Moreover the observed mean level of heterozygosity of 4.5% found in this study, is within the 4.3–6.2% range reported by Gyllensten (1985) and Ward *et al.* (1994) for freshwater fishes and similar to the 4.03% reported for the populations of *L. cephalus* in the Rhône river basin (Guinand *et al.*, 1996).

Both of the previous estimates agree with values observed in other freshwater fishes and are based on a sufficiently large number of samples (676 individuals) and loci (20 loci) to be a good estimate of overall gene diversity for this species. The low degree of heterozygosity and the low percentage of polymorphic loci in the population from Havrias stream (Table 1) could be the result of a bottleneck event. This is a small stream which sporadically dries up, consequently fluctuations in population size likely result in a reduction in levels of genetic variability. It is also possible that such populations are threatened with extinction.

Forty-two alleles were found in this investigation (Table 1), 23 of which have already been reported in French chub (Guinand *et al.*, 1996). Nineteen new alleles were described, although Guinand *et al.* (1996) report the *PGM-2* 98* allele in French chub. Sampling in France was done along two main river basins (Rhône and Saône). In the present study populations from all over Greece were examined. These correspond to rivers greatly differing in environmental conditions and lying in various geographical regions. So, perhaps the larger number of private alleles in Greek chub is due to our sampling more environments, than the French study.

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 except for the Havrias stream. The clustering of chub populations—according to the CONTML method—into six distinct clusters is concordant with the previous systematic classification for these populations.

The populations from north-eastern Greece (Ardas, Kompsatos, Nestos, Vosvozis and Filliouris) cluster together as predicted under classical taxonomy, i.e. they all belong to *L. c. macedonicus* (Economidis, 1974). Furthermore, the population from Evinos river representing *L. c. albus* also forms a distinct group, as well as the population from Alfios river representing *L. c. peloponnensis* (Economidis, 1991). The Rhône population forms a separate cluster.

The group consisting of populations Havrias, Elassoniticos, Aliakmonas 2, Axios 1 and Aliakmonas 3 belongs to *L. c. vardarensis* as predicted by classical nomenclature. Only the group including the Axios 2 and Aliakmonas 1 populations do not conform to classical subspecific designations but instead form a separate cluster which could be due to random genetic drift. The position of the Aggitis population between the *L. c. vardarensis* group and the *L. c. macedonicus* group is surprising and may be attributed to its geographically intermediate position.

The NJ tree of the *L. cephalus* populations provided very similar results. The only difference is the placement of the Rhône population which clusters with the *L. c. vardarensis* group and not separately. The *L. c. macedonicus* group (Ardas, Kompsatos, Nestos, Vosvozis and Filliouris) was supported at the 66% level in the majority-rule consensus tree, the group of Axios 2 and Aliakmonas 1 was supported at the 41% level, the Aggitis

population at the 30% level while, the Evinos and Alfios populations were supported at the 60% level in the consensus tree.

As reported, genetic distances ranged from 0 to 0.063. The genetic distance between the Alfios (supposed *L. c. pelopponensis*) and Evinos (supposed *L. c. albus*) populations is 0.021; between the Alfios population and the *L. c. vardarensis* group (Havrias, Elassoniticos, Axios 1, Aliakmonas 2 and Aliakmonas 3) it is 0.01 and it is 0.015 between the *L. c. vardarensis* and the *L. c. macedonicus* groups (Ardas, Kompsatos, Nestos, Vosvozis and Filliouris). These genetic distances are quite low and do not justify separate subspecies designations according to Avise and Smith (1977) who regard a mean genetic distance of 0.17 ± 0.004 necessary in order to recognize different geographic populations as subspecies.

Hierarchical *F*-statistics (Table 3) shows the highest percentage of genetic polymorphism (23.4%) is structured among the chub populations. This suggests little gene flow among separate populations. This fact is predictable as samples have been collected throughout the country and in some cases from rather remote basins (e.g. Alfios, Evinos, Ardas).

On the other hand, the genetic differentiation among the populations of the purported subspecies is lower—$F_{st} = 0.179$—but still large enough to show the subspecific groupings of the species are differentiated. Overall, the mean value of 0.243 for all the samples is similar to the 0.222 reported for freshwater fishes (Ward *et al.*, 1994).

By the late Miocene, the genus *Leuciscus* had appeared in Europe (Cavender, 1991). If we use Vawter *et al.*'s calibration (Vawter *et al.*, 1980) of an electrophoretic molecular clock for fishes we can estimate divergence times between different chub populations. In the last Wurm glacial phase (15,000–18,000 years ago) a maximum lowering of the sea level of about 100 m has been estimated (Bianco, 1990) and Corinth Gulf probably became a freshwater lake, by river discharges allowing freshwater fish dispersion between opposite basins of southern mainland Greece and Peloponnese. According to the above calibration the time of divergence between Alfios and Evinos populations is 400,000 years ago. So the populations of the two purported subspecies could have diverged before this time and much later (15,000 years ago) had the opportunity to exchange individuals because of the transformation of Corinth Gulf in a freshwater lake. So perhaps the low genetic distance (0.021) between the two purported subspecies is due to this probable fish dispersion.

Leuciscus cephalus arrived recently (probably in post glacial times) on the western slope of Greece by means of one or more river captures (Economidis, 1979) which involved Aliakmonas and Aoos river. The Aoos river is believed to have been the dispersal route of the eastern species to the western slope of Balkan peninsula (Economidis *et al.*, 1981). Overall, the dispersal history of the *L. cephalus* complex may be more complicated than at present believed. For this reason, the phylogenetic relationships of chub populations are under investigation using sequences of the mitochondrial DNA as well. Analysis of mtDNA has been shown to be useful for producing phylogenetically informative characters among closely related taxa (Avise *et al.*, 1987). Indeed, our unpublished mtDNA results provide support for the isozyme data and a better resolution of the four supposed *L. cephalus* subspecies.

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