

Nested Clade and Phylogeographic Analyses of the Chub, *Leuciscus cephalus* (Teleostei, Cyprinidae), in Greece: Implications for Balkan Peninsula Biogeography

Jean Dominique Durand,^{*,1} Alan R. Templeton,[†] Bruno Guinand,^{*} Anastasia Imsiridou,[‡] and Yvette Bouvet^{*}

^{*}CNRS-ESA 5023 Ecologie des Eaux Douces et des Grands Fleuves, Université Claude Bernard Lyon 1, 43 Bd du 11 Novembre 1918, 69622 Villeurbanne Cedex, France; [†]Department of Biology, Washington University, St Louis, Missouri 63130-4899; and [‡]Department of Genetics, Development and Molecular Biology, Aristotle University, GR-540 06 Thessaloniki, Macedonia, Greece

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Phylogenetic relationships among Greek populations of the chub, *Leuciscus cephalus*, were investigated using 600 bp of the cytochrome *b* gene. The aim of this study was to test the assumption that the main difference in ichthyological composition between both sides of the Balkan Peninsula is directly linked to differences in the dispersion mechanisms used by fish in order to extend their distribution range. Phylogenetic and nested clade analyses clearly showed that populations in Greece are significantly differentiated. Greek populations were found to descend from three lineages in three geographical provinces: Western, Central, and Eastern Greece. The chub reached Western Greece at the beginning of the Pleistocene and Eastern Greece during the mid-Pleistocene. Chub dispersion occurred mainly by river confluence due to sea level lowering and river capture in Western Greece and sea dispersal with low-salinity conditions within the Aegean Sea in Eastern Greece. However, in Central Greece, the original mtDNA lineage has presumably been lost owing to a genetic introgression following a second invasion from the Danube during the final stage of the last glaciation. This study provides new elements for a better understanding of the composition of the contemporary ichthyofauna in Greece and highlights possible evolutionary mechanisms responsible for the high endemism rate in the Western Greek biogeographic province. © 1999 Academic Press

Key Words: phylogeography; nested clade analysis; mitochondrial DNA; river capture; sea level changes; genetic introgression; *Leuciscus cephalus*.

INTRODUCTION

Evolution and dispersion of freshwater fishes are closely related to paleogeography and especially to the history of basin connections as a consequence of the geological development of landscapes (e.g., Bianco, 1990; Bermingham and Martin, 1998). Fishes can reach a new river basin mainly through three distinct geological events: (1) by river capture (i.e., new contacts between drainages by some parts of head water courses; Bishop, 1995), (2) by river confluence of downstream courses after sea level lowering, and (3) by sea dispersal when low-salinity conditions are encountered. The evolution of the hydrographic system is the main cause of dispersion, diversification, and speciation of freshwater fishes (Bermingham and Avise, 1986; Strange and Burr, 1997; Bermingham and Martin, 1998). Each of these dispersion modalities has different consequences on the species composition of the biogeographic provinces (Bermingham and Martin, 1998) and may also explain the genetic shaping of the species (Waters *et al.*, 1994). Nowadays, with the use of genetic tools, it is possible to estimate the genetic distance between populations (Avise *et al.*, 1987; Avise, 1994, 1998), to propose a scheme of colonization (e.g., Menozzi *et al.*, 1978; Thorpe *et al.*, 1994; Guinand and Easteal, 1996; Cavalli-Sforza, 1998; Templeton, 1998; and references therein), or, at least, to detect refugia responsible for possible recolonization pathways (e.g., concerning fishes: Taylor and Bentzen, 1993; Lafontaine and Dodson, 1997; Murdoch and Hebert, 1997; Wilson and Hebert, 1997, 1998).

In Southern Europe, the Mediterranean ichthyofauna consists of numerous endemic species with small distribution areas (Crivelli and Maitland, 1995), whereas all other European river systems are deeply influenced by Danubian species with wide distribution ranges. This pattern has been interpreted as a conse-

¹ To whom correspondence should be addressed. Fax: (33) 04 72 43 11 41. E-mail: jddurand@cismsun.univ-lyon1.fr.

quence of the Pleistocene glaciation, which erased species diversity in Northern Europe (Banarescu, 1992).

Several colonization schemes have been proposed in order to explain this pattern. These colonization schemes differ mainly in the dispersion modality used by freshwater fishes. Thus, Bianco (1990) supposed that most fish dispersion around the Aegean Sea during Pleistocene glaciations took place through the confluence of rivers due to sea regressions and by low-salinity conditions in the Aegean Sea (Fig. 2A). These geological events may have allowed the dispersion of endemic species within this area, as supposed for the gobiid *Knipowitschia caucasica* (Ahnelt *et al.*, 1995), as well as the invasion of Danubian species from the Black Sea when it was a fresh or very slightly brackish water lake (Fig. 2A). On the basis of morphological considerations, Bianco (1990) indicated that some Danubian species (*Leuciscus cephalus*, *Rutilus* complex, and *Scardinius erythrophthalmus*) reached Western Greece before the last glaciation, arriving not from Eastern Greece but from the Central Western Balkans (Fig. 2A). This colonization route from Albania to Southern Greece was also suggested for several other species on the basis of different data sets, such as electrophoretic data for *Barbus peloponnesius* (Karakousis *et al.*, 1995) and morphometric data for endemic species of *Cobitis* (Economidis and Nalbant, 1996).

According to Economidis and Banarescu (1991), most freshwater fish dispersion was facilitated by river captures. From their point of view, Central European and Danubian species (Fig. 2B) colonized the northeastern part of the Balkan Peninsula (Ponto-Aegean division; Fig. 1) during the late Pliocene or Pleistocene. These species entered the area by two routes: (1) by the Black Sea and the former Aegeopotamos River, a very large river that discharged the waters of the Black Sea into the Aegean Sea; and (2) directly from the Danube Basin to the River Axios (Fig. 2B) by the capture of a tributary of the River Morava that belonged to the Danube Basin. The presence of Danubian species (*Alburnoides bipunctatus*, *Barbus peloponnesius*, *Gobio gobio*, *Leuciscus cephalus*, and *Chondrostoma nasus*) in Western Greece and Albania was interpreted by Economidis and Banarescu (1991) as a postglacial colonization from the northeastern part of Greece. They considered the River Aous to have been the dispersal route by capture of an Aliakmon River tributary (Fig. 2B). However, regarding the distribution area of chub, *Leuciscus cephalus*, in Western Greece, these authors recognized that the dispersal history of this species is much more complicated than believed at present.

Chub is a wide-ranging European species occurring from the River Ural to Spain and from the south of Sweden to Italy and the Balkans (Berg, 1949). It has the largest distribution area in Greece and can easily be found in every ichthyological province. Its habitat includes lakes as well as both headwater and lowland

courses in the same river basin. This biological feature indicates a very good ability to colonize a river when this is possible either by river capture or by sea level lowering. Consequently, the chub seems to be a suitable species with which to assess at the genetic level the validity of the alternative migration routes proposed by Bianco (1990) and by Economidis and Banarescu (1991) (Figs. 2A' and 2B'). Until now, the phylogeographic history of only *Salmo trutta* has been studied in the Balkans (Apostolidis *et al.*, 1997). Greek chub has been studied with allozymic (Imsiridou *et al.*, 1997; Doadrio and Carmona, 1998) and RFLP analyses (Imsiridou *et al.*, 1998) to test the taxonomic validity of chub subspecies (see Economidis, 1991) but not to test the phylogeographic hypotheses delimited above (see also Figs. 1 and 2). In the studies of both Apostolidis *et al.* (1997) of trout and Imsiridou *et al.* (1997, 1998) of chub, the number of localities sampled was too low to adequately test Bianco's and/or Economidis and Banarescu's phylogeographical hypotheses.

Are the geographic and genetic characteristics of chub a result of vicariant dispersal under a restricted model of gene flow, such as isolation by distance, or a result of range expansion? Additionally, do these characteristics indicate river capture (i.e., Economidis and Banarescu's hypothesis) or river confluence by sea level lowering and dispersal during low-salinity conditions (i.e., Bianco's hypothesis) (Fig. 2)? Our aims were thus to identify and clarify the historical events that have modified the genetic structure of chub and to test the assumption that the absence of Danubian species in the western part of the Balkan Peninsula was strongly determined by historical events which were very different on both sides of the Peninsula. We reanalyzed the mtDNA (cytochrome *b* sequences) of 184 chub from 20 rivers and lakes (31 locations; Fig. 1) used in Durand *et al.* (1999). Nested clade analysis (NCA; Templeton *et al.*, 1995; Templeton, 1998) was used to test geographical association because it identifies the patterns of restricted gene flow and the historical events operating at the population level (e.g., past fragmentation, colonization, or range expansion).

MATERIAL AND METHODS

Populations Sampled

A total of 184 chub were collected from 20 rivers in Greece, Bulgaria, Romania, Slovakia, Slovenia, and Montenegro (Fig. 1). In order to obtain a precise view of genetic diversity, several geographically distant sites were sampled, when possible in the same drainage, e.g., those of the Rivers Axios (two sites), Aliakmon (three sites), Evros (two sites), Danube (five sites), Alfios (two sites), and Strymon (two sites), and the Lakes Prespa (two sites) (Table 1).

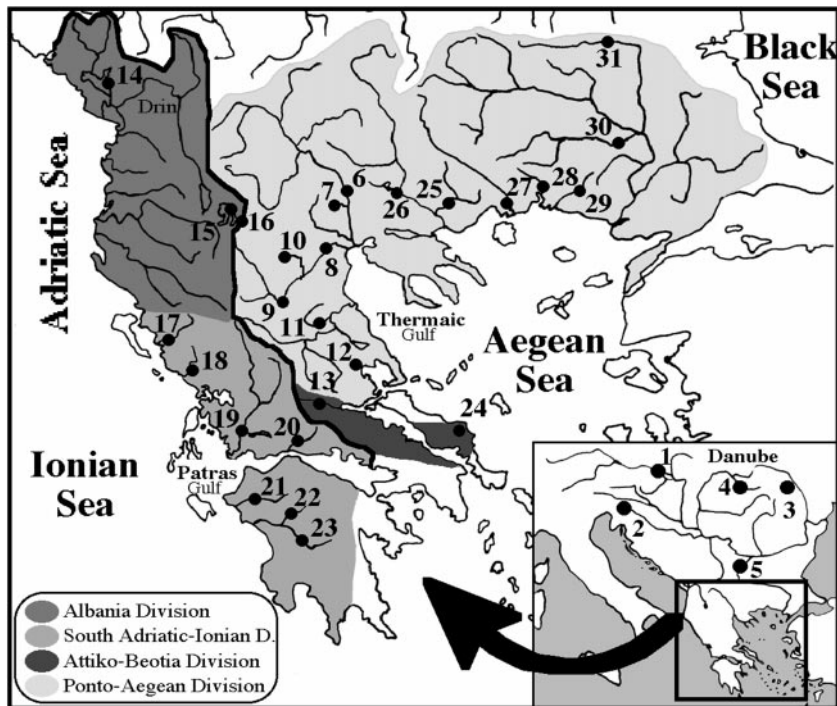


FIG. 1. Ichthyological divisions established in Greece, with our sampling sites. There is general agreement on the subdivision of the Balkan Peninsula into two (eastern and western) parts. Other subdivisions have been proposed by different authors. The Attiko-Beotia division was defined only by Economidis and Banarescu (1991), and the Albanian division was defined only by Bianco (1990). Sampling sites: 1, 2, 3, 4, 5: Danube catchment; 6, 7: Axios; 8, 9, 10: Aliakmon; 11: Pinios (Thessalia); 12: Holorema; 13: Sperchios; 14: Lake Skadar; 15, 16: Lakes Prespa; 17: Thyamis; 18: Aheron; 19: Acheloos; 20: Evinos; 21: Pinios (Peloponnese); 22, 23: Alfios; 24: Manikiotiko; 25, 26: Strymon; 27: Nestos; 28: Filiouris; 29: Kompsatos; 30, 31: Evros.

MtDNA Amplification and Sequencing

Total genomic DNA was extracted from scales following the protocol described by Kocher *et al.* (1989). Amplification conditions for the polymerase chain reaction (PCR) were 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 30 s, and extension at 72°C for 1 min. The PCR was performed in a 50- μ l reaction volume containing 12.5 nM MgCl₂, 10 nM each of the four deoxynucleotides, 1 μ l of each 10 mM primer, 10 \times PCR buffer II (Perkin-Elmer Cetus), and 2 U Amplitaq (Promega) in a programmable thermal cycler (Perkin-Elmer Cetus, Model 9600). A fragment of 650 bp of the 5' cytochrome *b* extremity of all 184 samples was amplified. PCR primers used were L15267 and H15891 (Briolay *et al.*, 1998). Double-stranded PCR products were purified with Qiaquick (QIAGEN) columns.

Direct sequencing was carried out using T7 DNA polymerase kits (Pharmacia). The primers used for sequencing (Briolay *et al.*, 1998) were L15267, H15512, H15833, and L15639 (Durand *et al.*, 1999). Six hundred base pairs were sequenced for all 184 samples. Thirty-four genotypes defined by 86 variable sites were identified (Table 1). Sequences have been deposited in the European Database (EMBL) under Accession Nos. AJ002319 to AJ002352.

Sequence Alignment and Phylogenetic Analysis

Sequences were aligned using the SEA VIEW program (Galtier *et al.*, 1996). Relationships between genotypes were determined both by maximum parsimony (MP) and by distance methods with the PHYLO_WIN program (Galtier *et al.*, 1996). The parsimony algorithm implemented in PHYLO_WIN is that of program DNAPARS from the PHYLIP package (Felsenstein, 1993). Maximum parsimony analysis was also applied using PAUP version 3.1.1 for the Apple Macintosh (Swofford, 1990). Distance trees were estimated according to the neighbour-joining (NJ) method of Saitou and Nei (1987), with Kimura's (1980) two-parameter model and Tajima and Nei's (1984) distance. The bootstrap resampling technique was used to assess the statistical significance of internal nodes. Because of the close relationship between *L. pyrenaicus* and *L. cephalus* (Briolay *et al.*, 1998), three cytochrome *b* sequences of *L. pyrenaicus* (Brito *et al.*, 1997) were used as outgroups to root the trees. Last, a phylogenetic test of the molecular clock (Takezaki *et al.*, 1995) was performed. This test can highlight possible evolutionary rate acceleration within lineages that could bias the colonization scenario reconstruction inferred from the phylogenetic tree topology. Estimation of the relative

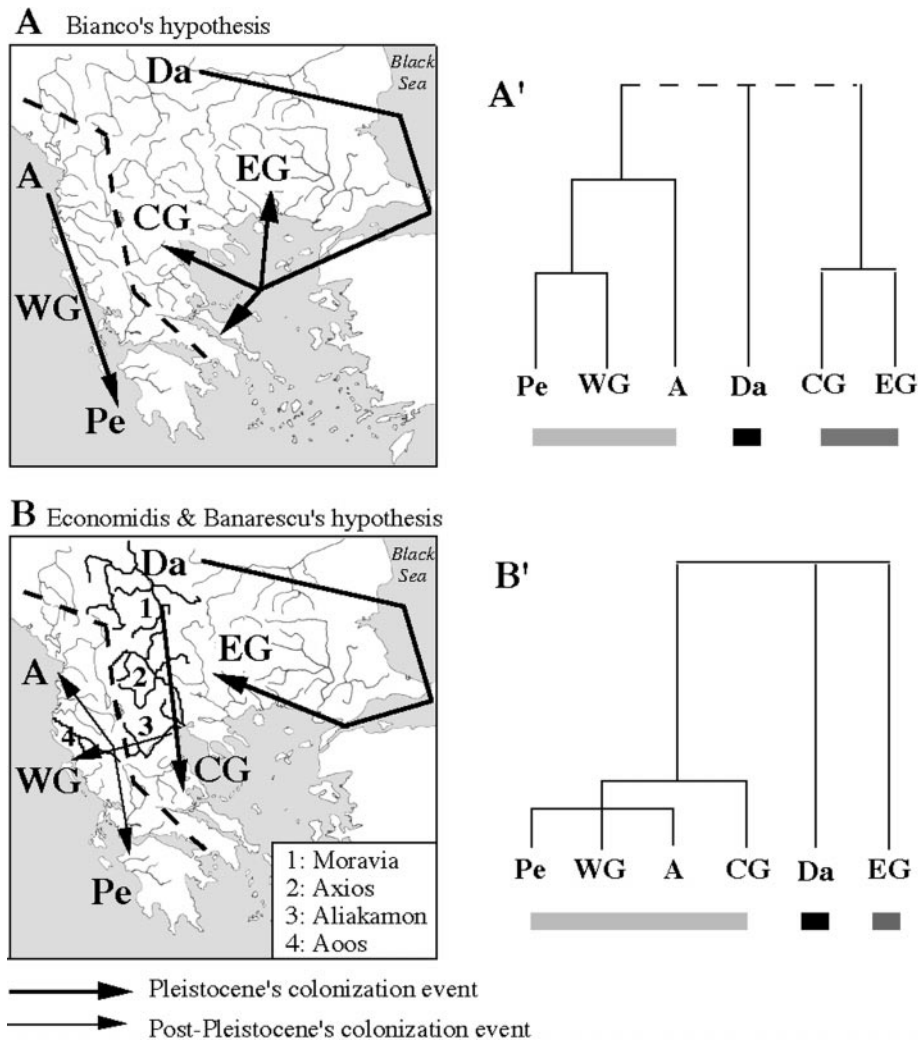


FIG. 2. Schematic representations of general fish colonization schemes in the Balkan Peninsula during Pleistocene and Post-Pleistocene according to (A) Bianco (1990) and (B) Economidis and Banareescu (1991). The biogeographic considerations of these authors are explained in the text. Below each geographical map, expected theoretical topologies (A' and B') of genetic subdivision according to each biogeographic hypothesis are noted (Da, Danube; A, Albania; WG, Western Greece; Pe, Peloponnese; CG, Central Greece; EG, Eastern Greece). Under Bianco's theory, the expected tree topology (A') might oppose Western Greek populations (A, WG, Pe) (shaded rectangles) to Central and Eastern Greek populations (CG, EG). The Danubian populations are opposed to these two groups (note the broken line linking these groups of populations at the top of (A')). Under Economidis and Banareescu's theory, the expected tree topology (B') also implies three distinct lineages (shaded rectangles). However, the main expected genetic difference from Bianco's hypothesis is that Western Greek populations and other populations of the western Balkan Peninsula (A, WG, Pe) would be more closely related to Central Greek populations (CG). Rivers implied as a colonization route in Central and Western Greece, such as those proposed by Economidis and Banareescu, are noted from 1 to 4.

chronology of colonization and diversification events is possible only when nucleotide substitution rates are homogeneous with respect to phylogenetic lineage and time (Page, 1996).

Nested Clade Analysis

The NCA proposed by Templeton *et al.* (1995; see also Templeton, 1997, 1998) is a statistical method that can detect and test (1) evolutionary mechanisms responsible for the spatial distributions of observed genetic patterns and (2) possible deficiencies in a sampling scheme. The first step in this analysis is to convert an

estimated haplotype tree into a series of nested branches (clades) using nesting rules. The polarity of a tree is given by rooting the tree with an appropriate outgroup (Castelloe and Templeton, 1994). These nesting rules are fully described in Templeton *et al.* (1987) and Templeton and Sing (1993), and a brief summary follows. First, starting from the individual haplotypes found on the tips of the tree, one moves toward the interior of the tree by one mutational step and forms the union of all haplotypes that connected to a common interior node by this one-step movement (including the possibility that the interior node itself is a haplotype

found in the current population). These sets of haplotypes formed by this union operation are called "one-step clades." These one-step clades are then pruned off the tree and the process is repeated until all haplotypes and interior nodes are placed into a one-step clade. Next, these one-step clades are treated as the operational taxonomic units in a lower resolution tree and the nested algorithm is repeated to form "two-step clades," and so on until the next higher nesting level would encompass the entire haplotype network.

The second step of the analysis is the quantification of the geographical data by forming two distinct distance statistics (Templeton *et al.*, 1995): (1) the clade distance, D_c , which measures the geographical range of a given clade; and (2) the nested clade distance, D_n , which measures how this clade is geographically distributed relative to its closest evolutionary sister clades (i.e., clades in the same next-higher nested category). Contrasts between clades within and among different nesting levels are used to discriminate between the potential causes of geographical structuring of genetic variation (Templeton *et al.*, 1995; Templeton, 1998). The statistical significance levels of these measures and distance contrasts are determined by random permutation testing. The null hypothesis tested is a random geographical distribution of all clades within a nested clade. The null hypothesis might not be rejected because (1) the sampling scheme is inadequate to detect geographical structuring and/or (2) the population observed is panmictic over the sampled area. In the second case, any haplotype frequency difference would be due only to sampling or to drift effects. There are no means for choosing between these alternatives; so, genetic inference is confined to those cases in which the null hypothesis is rejected. When the null hypothesis is rejected, Templeton *et al.* (1995) considered three distinct cases that may cause significant haplotype-geography associations: (1) recurrent genetic drift coupled with gene flow, especially gene flow restricted to isolation by distance; (2) past fragmentation events; and (3) population range expansion. These distinct types of evolutionary forces and events in the nested pattern of geographical distances are discussed in Templeton *et al.* (1995) and Templeton (1998). An inference key—not reproduced here—is provided in those papers to distinguish between these three types of population evolution as well as to detect possible artifacts due to sampling.

RESULTS

Geographic Distribution of mtDNA Genotypes and Population Differentiation

Among the 184 individuals, 34 cytochrome *b* genotypes were identified. These show a very strong geographic distribution pattern. Of the 34 genotypes, 26 were restricted to one river. The 8 other cytochrome *b*

genotypes were not shared by more than two close rivers, except for haplotype Ax1, which was present in three rivers (Axios 6, 7; Aliakmon 8, 9, 10; and Holorema 12; Fig. 1).

Sequence Divergence and Relationships between Cytochrome b Genotypes

The nucleotide composition of 600 bp of the cytochrome *b* segment sequenced in the 184 specimens was globally G deficient (17.1%), whereas similar frequencies were observed for the other three nucleotides (A: 25.6%; C: 28.7%; T: 28.6%). Such a nucleotidic composition pattern has been reported in several other fish studies (Cantatore *et al.*, 1994; Brito *et al.*, 1997).

Among the 600 bp sequenced, 86 sites, including 72 informative sites, were variable. Sequence variation was due to many transitions ($n = 78$) and to few transversions ($n = 8$). No indels were observed. The number of substitutions between chub genotypes varied from 1 to 42, corresponding to Tajima and Nei's (1984) distance values of 0.2 and 7.5%, respectively. Generally, genotypes within populations and within rivers were genetically the most similar (range: 0.2–0.8%), except for the River Strymon (range: 0.2–2.4%).

The NJ tree revealed two primary subdivisions: one group contained cytochrome *b* genotypes from Eastern Greek drainages and the other group contained genotypes from the River Danube and from Western and Central Greece (Fig. 3). The bootstrap values for these two subdivisions were 100 and 86%, respectively. A second division was observed within the eastern group (see Fig. 1): a Strymon–Nestos group (sites 25 to 27) and an Evros–Filiouris–Kompsatos group (sites 28 to 31), also containing haplotype Man observed in the Manikiotiko (24) population. This division was supported by bootstrap values of 100 and 88%, respectively (Fig. 3). However, genotype St3 was grouped with the Evros sequences. Within the Danubian and Central and Western Greek group, two other clusters were strongly supported: a Western Greek cluster containing all sequences from Western Greek drainages (14 to 23) and a Middle Greek/Danubian cluster. For this last division, the bootstrap values were 90 and 100%, respectively. No clear divisions were evident between Middle Greek (6 to 13) and Danubian sequences. Last, in the western group it can be noted that a southwestern set of sequences (Acheloos, 19; Evinos, 20; Pinios in Peloponnese, 21; Alfios, 22, 23) was supported by a strong bootstrap value (98%) (Fig. 3).

The parsimonious tree revealed with strong bootstrap values the same groups as in the neighbour-joining tree (Fig. 3). Average sequence divergences (Tajima and Nei's distance) between genotypes of the Western Greek and Danubian lineages were $5.3 \pm 0.3\%$; they were $5.43 \pm 0.37\%$ between genotypes of the Eastern Greek and Danubian lineages and $6.24 \pm 0.54\%$ between genotypes of the Western Greek and

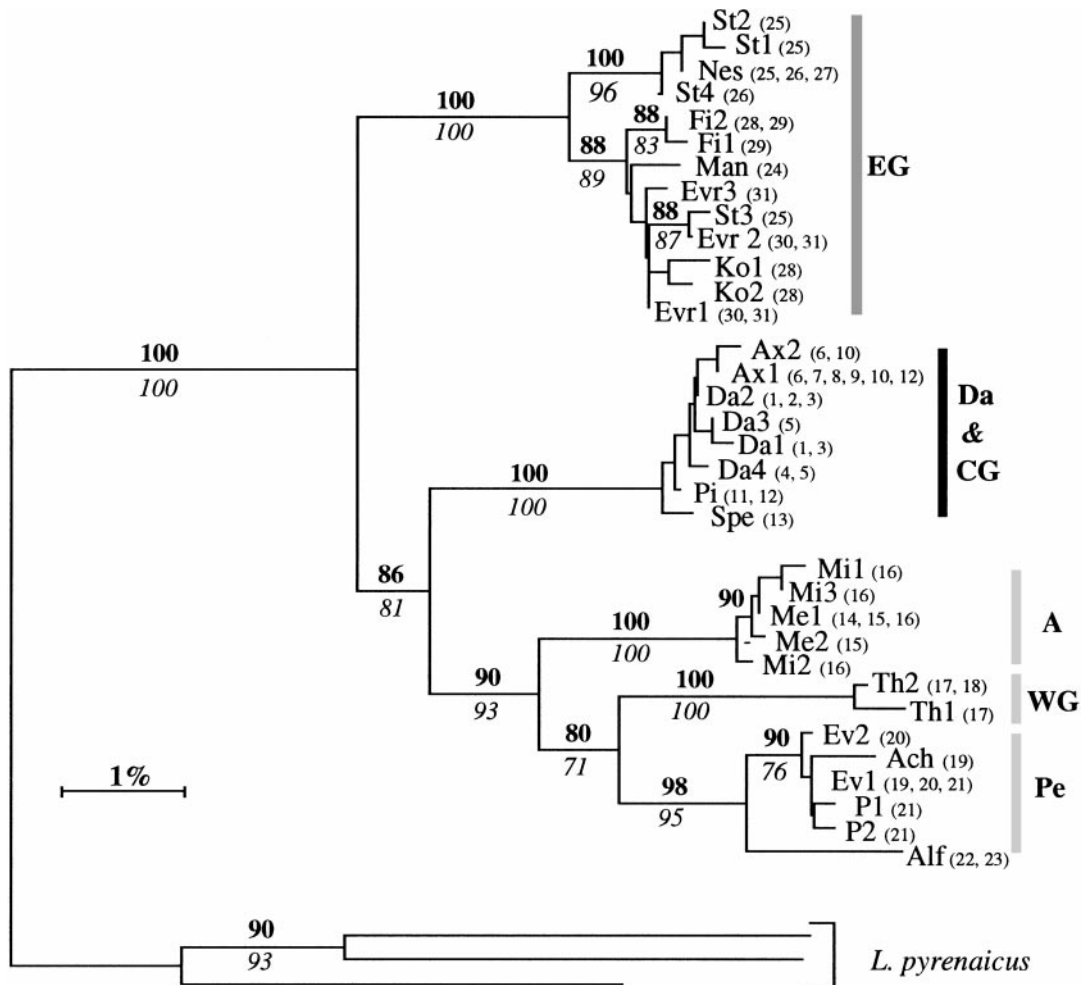


FIG. 3. Neighbour-joining (NJ) tree based on Tajima and Nei distance (1984) and maximum parsimony (MP) tree among 34 cytochrome *b* genotypes. The bold numbers above nodes represent percentage support of each branch in 500 bootstrap replications for the NJ tree (only values above 80% have been noted in order to not overload the figure). The italic numbers under nodes represent percentage support of each branch in 500 bootstrap replications for the MP tree (only values above 70% have been noted). Small numbers following tip (genotype) designation indicate location sampling (in Fig. 1) where the genotype was observed. Genetic subdivisions according to biogeographic assumptions are noted with bold letters according to Fig. 2.

Eastern Greek lineages. Within those lineages, average sequence divergences were $0.39 \pm 0.16\%$ for genotypes of the Danubian–Central Greek lineage, $1.2 \pm 0.68\%$ for genotypes of the Eastern Greek lineage, and $3.1 \pm 1.7\%$ for genotypes of the Western Greek lineage.

A phylogenetic test (Takezaki *et al.*, 1995) showed that the molecular clock can be used since no evolutionary rate differences were significant in our phylogenetic tree ($\chi^2 = 46$, 32 *df*; $P > 30\%$).

Nested Clade Analysis

Figure 4 shows the nested design, using the rules given in Templeton *et al.* (1987) and Templeton and Sing (1993). Three disjoint portions are observed. They agree with previous trees (Fig. 3) characterizing one Eastern Greek lineage, one Western Greek lineage, and one Central Greek and Danubian lineage. Within the

three disjoint portions of the cladogram, there is one topologically ambiguous connection within clade 5-2 and two within clade 5-3 (Fig. 4). A minimum of 28 mutational steps between clades 5-2 and 3-1 and 32 mutational steps between clades 5-2 and 5-3 connect these three haplotype networks.

Figure 5 shows the results of the NCA of geographical distance for the mtDNA data set of *L. cephalus*. Table 2 presents the results obtained when the key given in Templeton's appendix (1995, 1998) is applied to the statistical results shown in Fig. 5 along with resulting inferences about population structure and history. Very few tests at the one-step level were significant: 11 tests were performed (Fig. 5) but only 3 (haplotypes nested in clades 1-1, 1-2, and 1-11; Table 2) provided information about evolutionary mechanisms that could shape observed genetic variation (Table 2). Six tests were per-

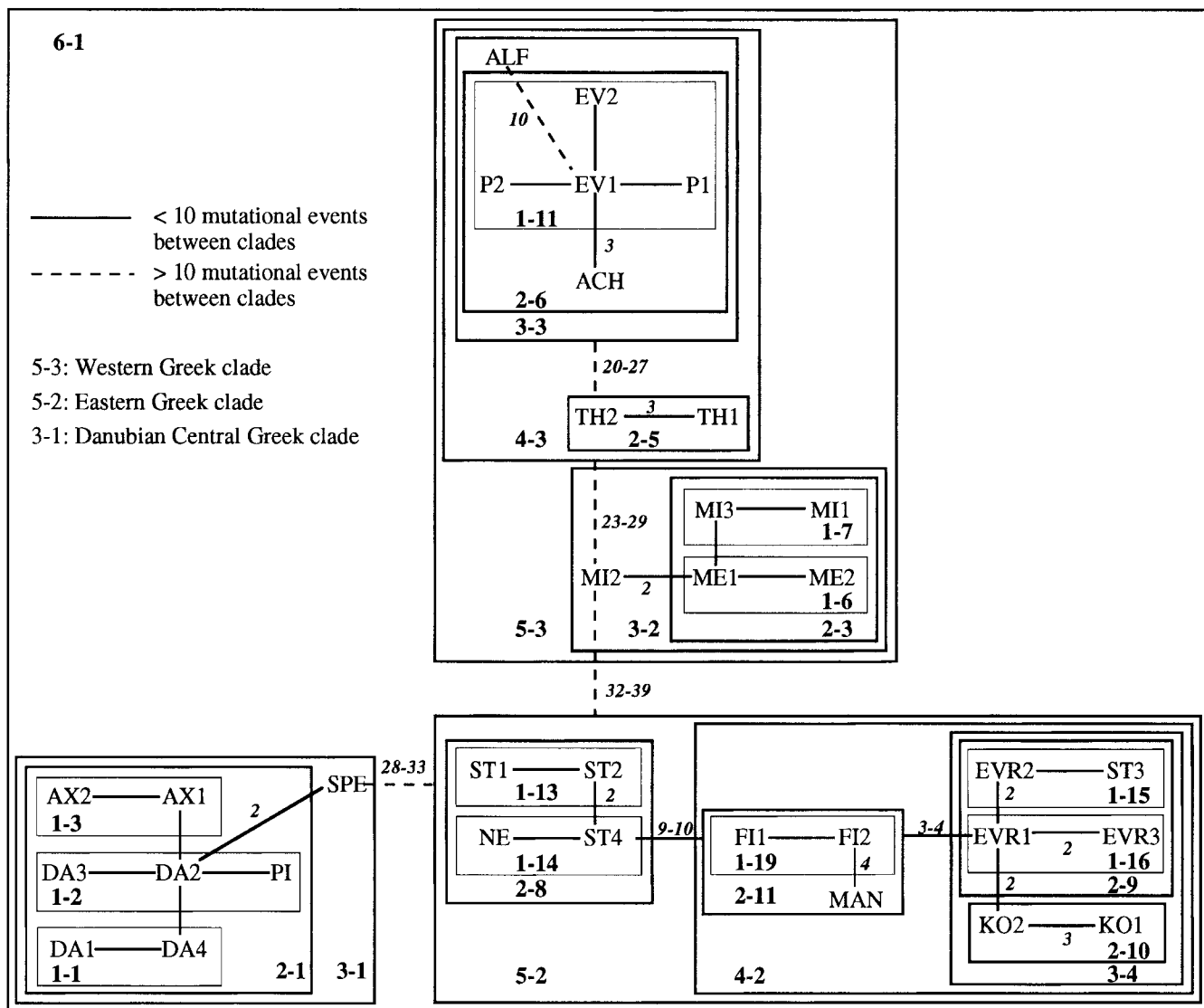


FIG. 4. Chub cytochrome *b* nested cladistic design. Haplotypes are indicated by letter designations, as found in Durand *et al.* (1999). Each solid line without any number represents a single mutational change that interconnects two haplotype states for which parsimony is supported at the 95% level. Each number beside solid and dotted lines is the number of multiple step connections that interconnect two haplotype states.

formed at the two-step level (Fig. 5), and 4 were significant (one-step clades nested in 2-1, 2-5, 2-8, and 2-11; Table 2) and allowed use of the inference key. At the higher levels, most of the nested clades tested lead to inference using the key (three inferential reports for four possibilities at the three-step level, two for three possibilities at the four-step and five-step levels, one for one possibility at the six-step level; Fig. 5 and Table 2). Such a distribution of significant results between haplotype variation and their geographical distribution indicates that a greater phylogeographic interest must be taken in higher-level clades than in lower-level clades.

For lower subdivisions of the Eastern Greek clade (clade 5-2; Fig. 4), the inference chain (Table 2) indi-

cates isolation by distance (IBD) due to restricted gene flow in clades 2-8 and 4-2 and past fragmentation in clades 2-11 and 5-2 (i.e., the complete Eastern Greek clade). Inconclusive inferential results about evolutionary mechanisms shaping the geographical variation of genetic data are observed for clade 3-4 of the Eastern Greek lineage that represent populations 28 to 31 in Fig. 1. It is important to note that IBD and past fragmentation are opposed evolutionary mechanisms that allow gene flow and do not allow gene flow, respectively.

For all significant levels of the Danubian Central Greek clade (clade 3-1, Fig. 4), the inference chain indicates either IBD (clades 1-2 and 2-1; Table 2) or

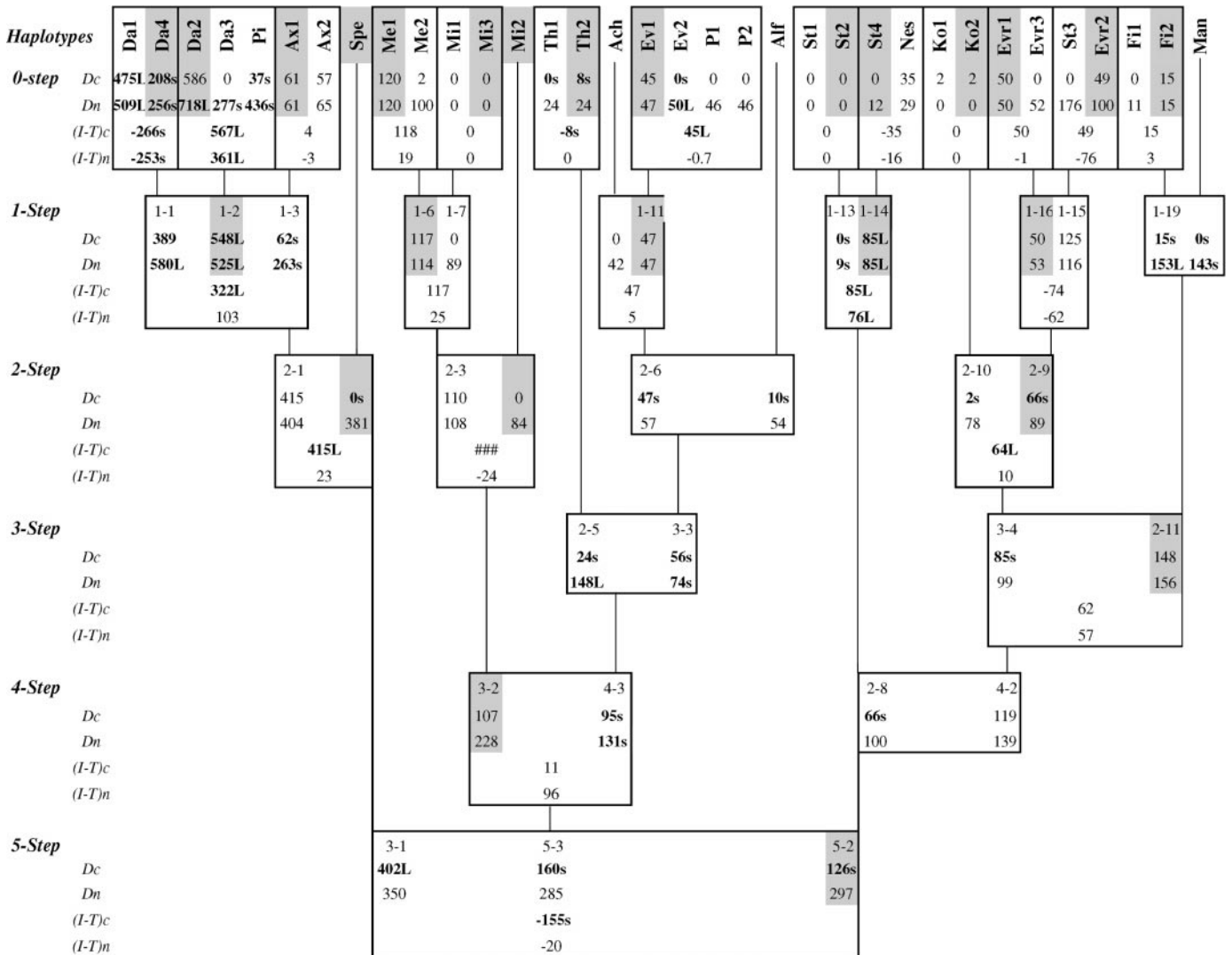


FIG. 5. Results of the nested geographic analysis of the chub cytochrome *b* haplotypes. The nested design is given in Fig. 4, as are the haplotype and clade designations. Following the number of any given clade are the clade (Dc) and nested clade (Dn) great circle distances. The oldest clade within the nested group is indicated by shading; higher level clade designations are given as one moves down the figure, with boxed groupings indicating the nesting structure. An “s” indicates that the distance is significantly small at the 5% level, and “L” indicates that it is significantly large. At the bottom of the boxes that indicate the nested groups containing both tip and interior clades, the lines indicated by the symbols “(I-T)c” and “(I-T)n” give the average differences of the distances between interior clades and tip clades within the nested group for clade distances and nested clade distances, respectively.

continuous range expansion (clades 1-1 and 3-1, Table 2). In this case, these evolutionary mechanisms are not opposed because both indicate an extension of the area containing the observed haplotypes in their ancestral and/or derived forms.

In the Western Greek clade (clade 5-3, Fig. 4), inferential results concerning higher clades (clades 4-3 and 5-3, Fig. 4) do not distinguish whether genetic variation in populations was due to fragmentation (i.e., isolation, independent evolution of haplotypes in each population) or IBD with restricted gene flow. However, at lower levels in this western clade (clades 1-11 and 3-3), gene flow with long-range dispersal and past fragmentation, respectively, are observed (Table 2). For

this Western Greek clade, note that haplotypes nested in clade 3-2 (Fig. 4, corresponding to populations 1 to 16 in Fig. 1) did not produce any significant results.

Finally, the inference for the three major clades (clades 3-1, 5-2, and 5-3; Fig. 4)—corresponding to the Danubian Central Greek, Eastern Greek, and Western Greek clades, respectively—indicate that continuous range expansion shaped genetic variation in the overall Balkan Peninsula (Table 2).

DISCUSSION

The biogeographic history and dispersion patterns of the Greek chub examined here can be inferred from the

TABLE 2

Inference Chain for the Results of the Chub Phylogeography Given in Fig. 4

Clade	Chain of inference	Inference
Western Greek lineage		
Haplotype nested in 1-11	1-2-3-5-6-7-Yes	Restricted gene flow with long distance dispersal
One step nested in 2-5	1-2-11	Inconclusive
Two step nested in 3-3	1-2-3-4-9-No	Past fragmentation
Three step nested in 4-3	1-2-3-5-15-16-18-Yes	Fragmentation or isolation by distance
Four step nested in 5-3	1-2-3-4-9-10-No	Fragmentation or isolation by distance
Eastern Greek lineage		
One step nested in 2-8	1-2-3-4-No	Restricted gene flow with isolation by distance
One step nested in 2-11	1-2-3-5-9-No	Past fragmentation
Two step nested in 3-4	1-2	Inconclusive
Three step nested in 4-2	1-2-3-4-No	Restricted gene flow with isolation by distance
Four step nested in 5-2	1-2-3-4-9-No	Past fragmentation
Danubian and Central Greek lineages		
Haplotypes nested in 1-1	1-2-11-12-No	Contiguous range expansion
Haplotypes nested in 1-2	1-2-3-4-9-10-No	Fragmentation or isolation by distance
One step nested in 2-1	1-2-3-4-9-10-No	Fragmentation or isolation by distance
Two step nested in 3-1	1-2-11-12-No	Contiguous range expansion
Overall lineages		
Five step nested in 6-1	1-2-11-12-No	Contiguous range expansion

Note. The chain uses the key in the appendix of Templeton (1998) on the results given in Fig. 5. Only those clades that resulted in a rejection of the null hypothesis (i.e., no significant relationships between genetic variation and respective geographical locations of haplotypes) are included in this table. Results are presented for each of the three main lineages (Fig. 3) and for the overall observed genetic variation.

comparison of the phylogenetic tree with the geographic distribution of chub haplotypes in conjunction with geological data. Moreover, the present study uses population genetic models in order to give a statistical basis for the geographic structuring of chub haplotypes and identifies the possible causes of significant association between haplotypes and geography. This approach is also used to test our central assumption that the main difference in ichthyological composition between both sides of the Balkan Peninsula is directly linked to difference in the means of dispersion used by fish for extending their range.

Relevance of the Nested Clade Analysis

Phylogenetic trees are classical tools in phylogeographic analysis but criticisms exist in the literature about trees which can be “fallible friends” (e.g., Cavalli-Sforza, 1998; Smouse, 1998) because of, for instance, (1) the algorithms generating their construction (e.g., Felsenstein, 1978, 1981; Goldman, 1990; DeBry, 1992), (2) the difficulty of rooting intraspecific trees (Maddison *et al.*, 1992; Castelloe and Templeton, 1994), and (3) the entities shared by different kinds of trees (genes/haplotypes or populations; see Hey, 1998). Such cases often occurred in fishes (see Wilson and Hebert, 1998 for a recent example). However, in their broadest generality, gene trees and related methods, such as the coalescent process (e.g., Kingman, 1982; for a review see Hudson 1998), are essential to give a temporal basis—at least partial, see below about molecular clock—for the observed genetic variation in haplotypes, whereas population trees are essential to give a geographical basis for this variation. Thus, chub trees (Fig. 3), as well as the haplotype network, despite topological imprecisions (Fig. 4), clearly indicate that the Western Greek lineage is more distinct than the Danubian/Central Greek and Eastern Greek lineages. Moreover, the NJ tree (Fig. 3) provides an opportunity to get an estimation of sequence divergence which is coherent here with the molecular clock hypothesis (Takezaki *et al.* (1995) test) but the geographical basis of sequence variation and the evolutionary mechanisms responsible for this variation are not explicitly addressed by such approaches. The reconciliation of these two approaches in order to reveal how geography reflects the history and/or the succession of temporal events (e.g., Felsenstein, 1982; Barton and Wilson, 1995) is a challenge. The NCA proposed by Templeton *et al.* (1995) is one of the solutions to such a problem.

In the present case, processing the NCA on the whole chub data set is justified because the hierarchy of the haplotype network (Fig. 4) clearly reproduces the trees’ topologies (Fig. 3). The NCA provides an opportunity to test some evolutionary processes shaping the observed geographic variation and this is not possible with classical phylogeographical trees. The inspection of how geography overlays a haplotype tree is classical in intraspecific phylogeography (e.g., Avise, 1994). However, using the NCA, Templeton and Georgiadis (1996) have shown that such an approach may be misleading in the inference of evolutionary forces shaping genetic variation. Inferring evolutionary processes at the intra- or interspecific level from the shapes of phylogenetic trees is just beginning to develop (but without geographical consideration; see Mooers and Heard, 1997 for a review of literature). Moreover, distinguishing distinct evolutionary processes from the geographical distribution of genetic variation—the basis of the NCA—is still limited in the framework of coalescent

theory (see Slatkin and Maddison, 1989; Takahata and Slatkin, 1990; Wakeley, 1996). Combinations of NCA and other approaches are rare. As far as we know, only Hammer *et al.* (1998) combined NCA and other analyses implying coalescence. The application of NCA to the chub data set in the Balkan Peninsula is also interesting because the nested levels are numerous (six, Fig. 5). No study using NCA has previously considered such a hierarchy (Templeton *et al.*, 1995; Templeton, 1993, 1997, 1998; Hammer *et al.*, 1998).

Finally, it is important to note that NCA does not require that the major evolutionary patterns shaping geographic variation (i.e., restricted gene flow due to isolation by distance, fragmentation, and continuous range expansion) are mutually exclusive; instead, it searches for multiple and overlaying patterns within the same data set (i.e., at each nested level).

Chub Colonization Scheme

The interpretation of these trees (Fig. 3) as a succession of several colonization events is possible here since these trees have a strong geographical basis, as highlighted by the network topology of haplotypes at the origin of the NCA (Fig. 4) and by the results of the statistical test in the NCA (Table 2; see previous section). Furthermore, the absence of sequences evolving fast or slowly in our data set allows us to include a temporal approach in our interpretation. However, the use of evolutionary rates to calibrate colonization timing was used as little as possible because of the many ambiguities stressed by Hillis and Moritz (1990). The high degree of haplotype differentiation indicates extensive subdivision of the ancestral *L. cephalus* stock. If the evolutionary rate obtained for cytochrome *b* in sticklebacks (Pisces, Teleostei) by Ortí *et al.* (1994) (2.8% sequence divergence/My) is used, clade divergence probably occurred before the Pleistocene. This rate was used very cautiously but it leads to interpretations sufficiently interesting to be explicitly cited here. For instance, clade divergence occurring before the Pleistocene is rather in accordance with the biogeographical hypothesis proposed by Bianco (1990) for Western Greece (Fig. 2A; see Introduction).

The strong genetic structure (Fig. 4) of chub populations from Western Greece and the level of sequence divergence within this area ($3.1 \pm 1.7\%$) suggest that the colonization events are ancient on this side of the Balkan Peninsula. The pattern of divergence in sequence trees (Fig. 3) suggests that colonization of Western Greece probably proceeded during the mid-Pleistocene from the north of Albania (from River Drin, which is connected with Lake Skadar, 14, Fig. 1) southwards (to Peloponnese) (Figs. 6A and 6B). This conclusion is based on the basal position of sequences from Lakes Skadar (14) and Prespa (15, 16), from which those of Rivers Aheron (18) and Thyamis (17) diverge (Fig. 3). Sequences from Southern Greece (sites 19 to

23) are more closely related to each other and were the last to diverge (Fig. 3). This route of colonization in the western part of the Balkan Peninsula confirms the colonization scheme of Bianco (1990) (Fig. 2A). No western haplotypes phylogenetically close to eastern haplotypes were found (Fig. 3), which is in disagreement with the colonization scheme of Economidis and Banarescu (1991) (Figs. 2B and 2B'). However, we have not analyzed any samples from the River Aaos, which was assumed by these latter authors to be the colonization route between Central and Western Greece (Fig. 2B). Thus, we cannot reject the possibility of a limited contact between the Rivers Aliakmon and Aaos, especially if we consider the allozymic results of Doadrio and Carmona (1998), which clearly indicated a strong genetic link between chub populations of the Aaos and Central Greek Rivers.

According to Bianco (1990), freshwater fish dispersion in Western Greece was achieved owing mainly to several river confluences with sea level regressions. Sea level regression during the last glaciation and subsequent sea level rising are certainly too recent phenomena to have allowed a genetic fragmentation. The case—observed using the NCA—of restricted gene flow with long-distance colonization (clade 1-11, Table 2) for chub populations located in rivers of the Patras Gulf (Evinos, 20; Acheloos, 19; Pinios, 21) agrees well with Bianco's model. These rivers are known to have been influenced by sea level regression during the Pleistocene glaciations (Chronis *et al.*, 1991). However, if there is strong evidence for river confluences in Western Greece (especially in the Patras Gulf) during the last glaciation, their importance in older dispersion events must have been limited since very few (only two) glaciations implied as great a sea level regression as the last glaciation (Shackleton, 1987). Thus, as stressed by Economidis and Banarescu (1991), river capture may have been an important dispersal mechanism as a complement to confluence events, especially for neighboring rivers for which there was no obvious possibility of confluence. Under this assumption, ancient river capture represents a strong vicariant event which might succeed a pattern of past fragmentation (as in the NCA) and large genetic divergence (as in the NJ and MP trees). This assumption is consistent with the NCA results between rivers Alfios (22, 23) and other Peloponnese Rivers (sites 19 to 21) (clade 3-3, Table 2). However, it was not possible to definitively link the degree of genetic divergence (Figs. 3 and 4) between clades 2-5 and 3-3 and between clades 3-2 and 4-3 to a past fragmentation event (Table 2) because chub are found in intermediate geographical rivers that were not sampled. Chub are found in two rivers between Aheron (18) and Acheloos (19) (clades 2-5 and 3-3) and four rivers between the Skadar–Drin system (14) and Thyamis (17) (clades 3-2 and 4-3). This suggests that under an IBD model, two to four intermediate haplotypes may

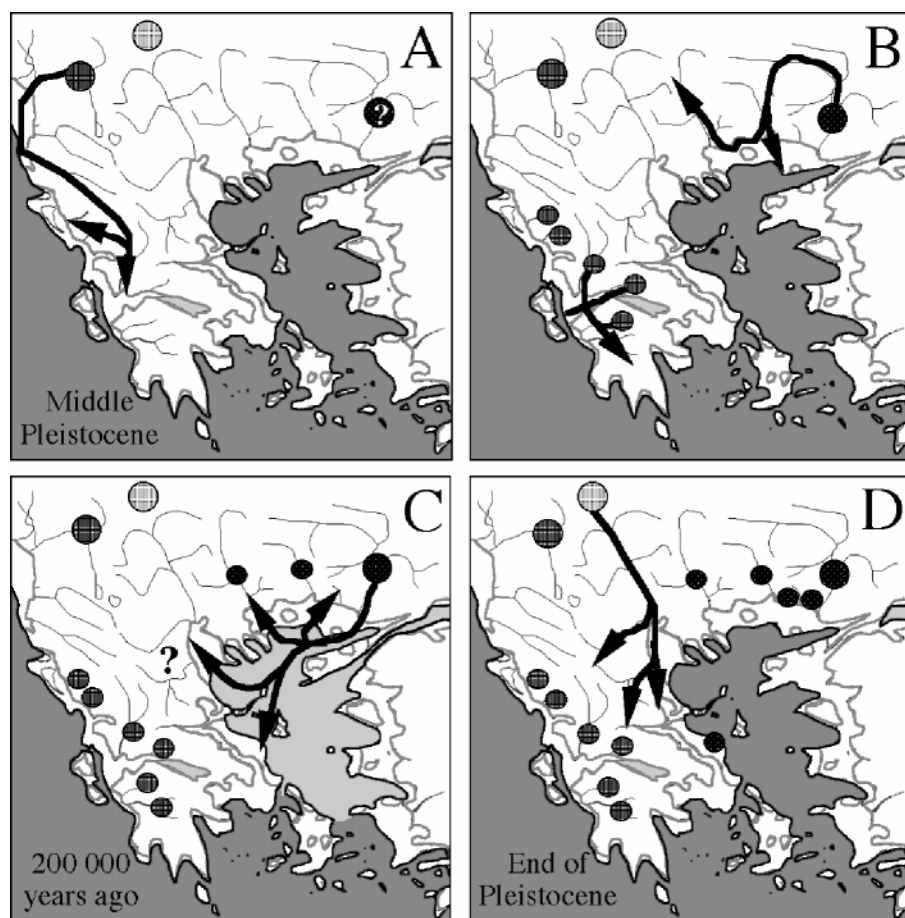


FIG. 6. Colonization scheme proposed to explain the observed genetic structure. The question marks indicate uncertainty of the presence of the lineage. In each figure the black coastline has been drawn according to a sea regression of 130 m, as was estimated for the last glaciation (Van Andel and Shackleton, 1982). The grey lines mark the present coast, black circles the eastern lineage, hatched circles the western lineage, and grey circles the Danubian lineage. The low salinity condition of the Aegean Sea is indicated in C by a lighter shade than in the other figures. Contact between the Black Sea and the Aegean Sea is a schematic representation (not based on any geological data) of a potential freshwater inflow from the fresh or oligohaline Black Sea. See the text for more explanations.

be observed, respectively. However, if we consider the genetic distance that separates these populations (Fig. 4), this genetic structure could have arisen from a succession of fragmentation events. Sampling more populations in this area might lead to better NCA results (see Templeton, 1998) and to a clearer colonization scenario.

Colonization of the eastern part of the Balkan Peninsula is much more complicated than that of the western part. None of the colonization schemes (presented in the Introduction) perfectly match our results, although several features of both theories must be used in order to interpret chub genetic structure in this part of Greece. Two different lineages colonized the Aegean rivers: the Danubian–Central Greek and the Eastern Greek lineages. This last lineage must have been present earlier than the Danubian–Central Greek lineage since the oldest historical event is a fragmentation event between clades 2–8 and 4–2 (clade 5–2, Table 2), a

consequence of capture between the Evros and the Nestos–Strymon rivers (Fig. 6B) (Psilovikos, 1987). Conversely, the second evolutionary process also implies that the Eastern Greek lineage is an IBD event between populations of clades 2–11 and 3–4 (clade 4–2, Table 2). Regarding the distant geographic locations of populations included in clade 2–11 (sites 24 and 28, Fig. 1) versus the similar and homogeneous genetic distances separating haplotypes of populations 24 and 28 to 31 ($0.63 \pm 0.2\%$, including haplotype St3 in the Strymon, 25), this result is quite surprising and does not seem compatible with a pattern of IBD. The only scenario consistent with this result is a colonization, at the same time, of the Manikiotiko river on Euboea Island (Fig. 1) on the southwestern side of the Aegean Sea and those in Eastern Greece. This can only have been possible owing to an important geologic event, such as a great decrease in salinity in the Aegean Sea, as supposed by Bianco (1990) (Fig 6C). Even if the

estimated salinity in the Aegean Sea (Aksu *et al.*, 1995) was not tolerable for freshwater fishes, this assumption is consistent with the observation of high abundance of salinity-sensitive planktonic Foraminifera in interglacial stage 7 (200,000 years ago) sediment layers of the Aegean Sea cores (Thunell and Williams, 1983). Assuming a constant substitution rate of 2.8% per My (Ortí *et al.*, 1994), the degree of estimated sequence divergence is congruent with this interglacial stage 7.

No haplotypes from the eastern lineage were observed in the central part of Greece, which could be expected if we suppose the dispersion of a freshwater fish through the Aegean Sea (Fig. 2A). However, two allozymic studies on Greek chub (Imsiridou *et al.*, 1997; Doadrio and Carmona, 1998) have shown that the Aegean populations belong to the same clade, whereas the Danubian population belongs to a different clade. The absence of the eastern mtDNA lineage in Central Greece may be the consequence of genetic introgression following a second invasion by Danubian populations (Fig. 6D). This assumption is consistent with the capture of a Danubian tributary by the Axios river as supposed by Economidis and Banarescu (1991) (Fig. 2B) and a fast dispersion through several river confluences (Axios, Aliakmon, and Pinios) in the Thermaic Gulf (Fig. 1) that emerged during the last glaciation (Cramp *et al.*, 1988) (Fig. 6). The genetic pattern of the Danubian–Central Greek lineage is consistent with this scenario (Fig. 6D) since the oldest event is a contiguous range expansion (from site 5) (clade 3-1, Table 2). The present distribution of mtDNA in Aegean drainages might be explained by mtDNA lineage extinction (Avise *et al.*, 1987) and loss of the original mtDNA in the introgressed population. The likelihood of such a hypothesized genetic event is demonstrated by the occurrence of similar mtDNA lineage replacements in other organisms (Dowling and Hoeh, 1991; Echelle and Dowling, 1992; Duvernell and Aspinwall, 1995).

CONCLUSION

Chub history in the Balkan Peninsula provides new elements for a better understanding of the composition of present Greek ichthyofauna. Trout phylogeography in Greece (Apostolidis *et al.*, 1997) shows several points that converge with chub phylogeography (three highly differentiated lineages, Danubian haplotypes located in the Aliakmon river in Central Greece). However, the trout phylogeographic pattern differs from the chub phylogeographic pattern since no geographical structure corresponds with trout lineages (category II, *sensu* Avise *et al.*, 1987). Chub cytochrome *b* variation observed in this study is explained by some elements of both theories: Bianco's (1990) for Western Greece colonization and a rapid colonization of the Aegean Sea (Fig. 2A) and Economidis and Banarescu's (1991) for the importance of river capture shaping a part of the

genetic variation in the Eastern Greek lineage and the colonization of Central Greek rivers by Danubian-related haplotypes. Patterns of sequence divergence obtained with trees as well as tree topologies have been important in drawing such a conclusion but the definition of evolutionary mechanisms based upon NCA was certainly the main complementary methodological approach in validating and detailing the role of various biogeographical hypotheses that shape genetic variation.

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